EPR Analysis of a Two-State Conformational Equilibrium in an *N. pharaonis* HAMP Domain - Activation/Deactivation of a Signaling Unit?
EPR Analysis of a Two-State Conformational Equilibrium in an *N. pharaonis* HAMP Domain
- Activation/Deactivation of a Signaling Unit?

A thesis submitted for the degree of
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by

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from Westoverledingen

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The scientist does not study nature because it is useful; he studies it because he delights in it, and he delights in it because it is beautiful. If nature were not beautiful, it would not be worth knowing, and if nature were not worth knowing, life would not be worth living. Jules Henri Poincaré (1854-1912), French mathematician. All of physics is either impossible or trivial. It is impossible until you understand it, and then it becomes trivial. Ernest Rutherford (1871-1937), English physicist. The Paradox of Life: A bit beyond perception’s reach I sometimes believe I see that Life is two locked boxes, each containing the other’s key. Piet Hein, Danish mathematician, physicist, philosopher, writer and creator of puzzles and games. Now, my own suspicion is that the universe is not only queerer than we suppose, but queerer than we can suppose.

I have read and heard many attempts at a systematic account of it, from materialism and theosophy to the Christian system or that of Kant, and I have always felt that they were much too simple. I suspect that there are more things in heaven and earth that are dreamed of, or can be dreamed of, in any philosophy. That is the reason why I have no philosophy myself, and must be my excuse for dreaming. John Burden Sanderson Haldane (1892-1964), English geneticist. The mind likes a strange idea as little as the body likes a strange protein and resists it with similar energy. It would not perhaps be too fanciful to say that a new idea is the most quickly acting antigen known to science. If we watch ourselves honestly we shall often find that we have begun to argue against a new idea even before it has been completely stated. Wilfred Batten Lewis Trotter (1872-1939), English surgeon. We know very little, and yet it is astonishing that we know so much, and still more astonishing that so little knowledge can give us so much power. Bertrand Russell (1872-1970), English philosopher, mathematician. I do not like it, and I am sorry I ever had anything to do with it. Erwin Schrödinger (1887-1961), Austrian physicist. Speaking of quantum mechanics. To say that a man is made up of certain chemical elements is a satisfactory description only for those who intend to use him as a fertilizer. Hermann Joseph Muller (1890-1967), U. S. geneticist. Every honest researcher I know admits he’s just a professional amateur. He’s doing whatever he’s doing for the first time. That makes him an amateur. He has sense enough to know that he’s going to have a lot of trouble, so that makes him a professional. Charles Franklin Kettering (1876-1958), U. S. engineer and inventor. I have deep faith that the principle of the universe will be beautiful and simple. Albert Einstein (1879-1955) U. S. physicist, born in Germany. The scientist is a practical man and his are practical (i.e., practically attainable) aims. He does not seek the ultimate but the proximate. He does not speak of the last analysis but rather of the next approximation. His are not those beautiful structures so delicately designed that a single flaw may cause the collapse of the whole. The scientist builds slowly and with a gross but solid kind of masonry. If dissatisfied with any of his work, even if it be near the very foundations, he can replace that part without damage to the remainder. On the whole he is satisfied with his work, for while science may never be wholly right it certainly is never wholly wrong; and it seems to be improving from decade to decade. G. N. Lewis. Quoted in Stoichiometry by Leonard K. Nash. Addison-Wesley 1966. p. vii.
LIST OF PUBLICATIONS
CORRESPONDING TO THIS THESIS

Parts of this thesis were already published


# Contents

1 Introduction 13
   1.1 Introduction .................................... 13
   1.2 Motivation .................................... 16

2 Background Information 19
   2.1 The HAMP domain in extremely haloalkaliphilic archaea ............ 19
   2.2 Preparational constraints ..................................... 27
   2.3 Site-directed spin labeling ..................................... 30

3 Theoretical Basis 33
   3.1 Electron paramagnetic resonance ........................... 33
      3.1.1 Electron spin and magnetic momentum ..................... 33
      3.1.2 Spin Hamiltonian .................................... 36
         3.1.2.1 Electron Zeeman interaction and the g-tensor ........ 36
         3.1.2.2 Nuclear Zeeman interaction .......................... 38
         3.1.2.3 Hyperfine interaction and the A-tensor ............. 39
         3.1.2.4 Electron spin-spin interactions ....................... 41
         3.1.2.5 The total Spin Hamiltonian .......................... 43
      3.1.3 The cw EPR spectrum .................................. 43
         3.1.3.1 Macroscopic magnetization and resonance phenomenon .. 45
         3.1.3.2 Relaxation and Bloch equations ......................... 49
         3.1.3.3 Energy level populations and density matrix .......... 50
         3.1.3.4 Line broadenings .................................. 51
         3.1.3.5 Rotational correlation time and "mobility" .......... 52
         3.1.3.6 Power saturation and accessibilities for quencher reagents 56
      3.1.4 Cw EPR spectra simulation ................................ 59
         3.1.4.1 Freedfit ........................................ 59
         3.1.4.2 Dipfit ........................................ 62
      3.1.5 Double resonance in pulse EPR ........................... 69
         3.1.5.1 Pulse ELDOR ..................................... 70
         3.1.5.2 Pulse ENDOR ..................................... 81
   3.2 Thermodynamics ..................................... 84
## Material & Methods

### 4.1 Sample preparation

- **Bacterial strains and plasmids**
- **Protein expression**
- **Cell disruption and protein purification**
- **Spin labeling of cysteine mutants**
- **Extraction of polar lipids and bilayer composition**
- **Reconstitution into purple membrane lipids (PML)**
- **Denaturing gel electrophoresis**

### 4.2 EPR technique and performance

- **cw EPR**
- **Pulse EPR**
- **Pulse ENDOR**
- **Amendatory equipment and experimental procedure**

### 4.3 Data handling

- **Fast and slow motion cw EPR spectra**
- **Calculation of exchange rates $W_{ex}$**
- **cw EPR powder spectra**
- **Pulse EPR data**
- **Pulse ENDOR data**

## Results

### 5.1 Side chain dynamics subject to environmental inputs

- **Effects of high salt concentrations and high lipid contents**
- **Effects of acidic pH and different environmental agents**
- **Micelle vs. lipid bilayer**

### 5.2 Temperature-driven dynamics

### 5.3 Side chain accessibilities for quencher reagents

- **Effects of high salt concentrations and high lipid content**
- **Micelle vs. lipid bilayer**

### 5.4 Environmental polarity

- **Effects of high salt concentrations and high lipid content**
- **Micelle vs. lipid bilayer**

### 5.5 Structural properties of NpSRII/NpHtrII in membranes

### 5.6 Non-exchangeable environmental protons

### 5.7 Inter-spin distance analysis

- **Salt- and lipid-induced effects on singly labeled NpHtrH157 mutants**
- **Doubly labeled NpHtrH157 mutants**
- **Receptor-transducer interactions**
5.7.4  Micelle vs. lipid bilayer .............................. 186

6  DISCUSSION .......................... 189
   6.1  cHAMP - the compact HAMP conformation ............... 193
   6.2  dHAMP - the dynamic HAMP conformation ................ 197
   6.3  A "two-state" equilibrium - physiological relevance? ... 197
   6.4  Outlook ..................................................... 206

7  SYNOPSIS ............................ 209

A  SUPPLEMENTARY DATA .................. 213

BIBLIOGRAPHY .......................... 1
### Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CrOx</td>
<td>Chromium oxalate</td>
</tr>
<tr>
<td>DDM</td>
<td>N-Dodecyl-β-D-maltoside</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPPH</td>
<td>Diphenyldipicrylhydrazide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>HsHtrII</td>
<td><em>Halobacterium salinarum</em> transducer of sensory rhodospin II</td>
</tr>
<tr>
<td>HsSRII</td>
<td><em>Halobacterium salinarum</em> sensory rhodopsin II</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranosid</td>
</tr>
<tr>
<td>LT</td>
<td>Low temperature</td>
</tr>
<tr>
<td>MTSSL</td>
<td>(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate spin label</td>
</tr>
<tr>
<td>mw</td>
<td>Microwave</td>
</tr>
<tr>
<td>NaPi</td>
<td>Na$_2$HPO$_4$/NaH$_2$PO$_4$</td>
</tr>
<tr>
<td>Ni$^{2+}$-NTA</td>
<td>Nickel-Nitrilotriacetic acid</td>
</tr>
<tr>
<td>NiEDDA</td>
<td>Ni(II)ethylenediamine diacetate</td>
</tr>
<tr>
<td>NpHtrII</td>
<td><em>Natronomonas pharaonis</em> transducer of sensory rhodospin II</td>
</tr>
<tr>
<td>NpSRII</td>
<td><em>Natronomonas pharaonis</em> sensory rhodopsin II</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>rf</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEMPOL</td>
<td>4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl</td>
</tr>
<tr>
<td>Tris</td>
<td>Trishydroxymethylaminomethane</td>
</tr>
<tr>
<td>(v/v)</td>
<td>volume per volume</td>
</tr>
<tr>
<td>(w/v)</td>
<td>weight per volume</td>
</tr>
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</table>
CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Despite the fact that membrane proteins account for approximately 30% of all proteins expressed in nature, their structural and functional elucidation offers one of the greatest challenges in structural biology. Only less than 1% of all protein structures revealed so far refer to membrane-spanning entities, reflecting the difficulties in determining their structural features [Spencer et al. 2001, Smith et al. 2001]. Combination of knowledge from natural sciences such as physics, chemistry and biology in terms of interdisciplinary research is needed to progress in this field. Particularly in the case of membrane proteins such progress is not solely demanded in terms of inventing new technologies, illuminating more and more the mystery of life. The focus is rather on understanding cellular mechanisms on a molecular level to boost human cure since more than 50% of all present and future drug targets are membrane proteins. They perform important cellular tasks such as e.g. energy transduction/conversion, pH or cell volume regulation, and most notably cell signaling. Functional inability, thus, implicates diseases as cancer, neurological disorders, Parkinson’s or Alzheimer’s disease, to mention only a few of them. However, essentially understanding the proteins’ biological functions requires information on the respective biochemical processes taking place, and foremost structural details [Hemminga and Berliner 2007]. In this regard, most of the biophysical techniques available nowadays are not satisfactory. X-ray crystallography, NMR (nuclear magnetic resonance) spectroscopy and EM (electron microscopy) provide a fundamental basis in the research field of 3-dimensional protein structure and function in general. However, each of these techniques bears major problems in conjunction with membrane proteins. X-ray experiments are mostly limited by the fact that it is generally difficult to crystallize membrane proteins from detergent solutions (see for a recent review [Fernández and Wüthrich 2003]), not to mention the impossibility of investigating the protein in the native membrane. Moreover, although crystallization of dynamic protein regions can be successful, structural information is then usually sparse due to the weakly defined electron densities for these domains (e.g. see figure 2.3). NMR, the second high-resolution method, has also shown to be insufficient
in studying complex protein-lipid assemblies. This is largely due to the very heterogeneous character of the protein environment, including the bulk water, the hydrocarbon core of the lipid bilayer and the interfacial regions between these two phases. NMR is furthermore restricted with respect to the size of the biomacromolecules that can be studied. Besides the mentioned difficulties by virtue of the lipid bilayer, electron microscopy studies actually gaining high-resolution images of the protein structures require fixed objects. Consequently, dynamics of signal transducing units as part of membrane anchored proteins also exclude EM as adequate technique. In order to investigate membrane proteins in their native environment, namely the membrane, different techniques are needed. Although the structural resolution of methods like \textit{FRET (fluorescence/Förster resonance energy transfer)}, \textit{FTIR (Fourier transform infrared)} spectroscopy, and \textit{EPR (electron paramagnetic resonance)} spectroscopy is much lower compared to NMR and X-ray crystallography, the investigation of membrane proteins under native conditions is possible (for recent publications see [Chattopadhyay and Raghuraman 2004, Vigano et al. 2000, Bordignon and Steinhoff 2007]). Hence, the techniques mentioned above can be used complementary to characterize structure and function of membrane proteins and membrane protein complexes. Since particularly the \textit{site-directed spin labeling (SDSL)} [Altenbach et al. 1990] (see sections 2.11 and 4.1.4) EPR method used in this study provides detailed information on structure and dynamics, enables measurement at different temperatures and under various environmental conditions, and is applicable to molecular complexes independent of their size, it has become one of the most powerful tools to analyze membrane proteins.

In general, EPR can be regarded as the highly sensitive\textsuperscript{*} little sister of NMR since it is a spectroscopic method exploiting the quantum mechanical electron spin instead of the nuclear spin as in NMR. Due to the fact that only spins of unpaired electrons can contribute to the EPR signal, the species under investigation has to be of paramagnetic nature. This applies amongst others for certain transition metal ions, organic radicals, and free electron centers but usually not for proteins. The site-specific incorporation of a paramagnetic nitroxide spin label in the amino acid sequence, i.e. site-directed spin labeling (SDSL), is a tremendously useful method to overcome this problem.

Interactions of the spin label side chain with its microenvironment reflected in the EPR spectrum provide a wealth of information. Since the nitroxide side chain can be attached to almost each desired position in the amino acid sequence, global information on structural elements as well as real-time functional elucidation can be achieved. In this study, different methodologies of SDSL EPR are applied in order to obtain information on

\textsuperscript{*}Much lower concentrations and volumina are needed compared to NMR.
1. spin label dynamics illuminating structural features of the local environment as well as backbone dynamics, conformational changes and motion of the nitroxide itself on a nanosecond time scale from EPR line shape analysis;

2. spin label side chain accessibilities for specific paramagnetic quencher molecules yielding secondary structure elements as well as nitroxide orientations within the lipid bilayer, in the protein interior and in the water phase from the determination of collision frequencies with the respective reagent;

3. inter-spin distances ranging from 0.8 up to approximately 8 nm yielding structural features from dipolar interactions between the single spins using continuous wave as well as pulse EPR methods;

4. the local polarity of the nitroxide characterizing the environment (water phase, lipid phase) and structural features (nearby charged residues etc.) from electrical field changes at the nitroxide;

5. electron nuclear double resonance (ENDOR) measurements to distinguish between exchangeable and non-exchangeable protons in the nitroxide microenvironment from the detection of nuclear transitions in an EPR experiment.

Since in an EPR spectrometer the protein sample absorbs microwave energy dependent on a static magnetic field applied and due to the fact that according to Planck’s law electromagnetic radiation can be absorbed at various frequencies as long as \( \Delta E = h \cdot \nu \) (\( E \): energy, \( h \): Planck constant, \( \nu \): frequency of radiation field) is fulfilled, experiments can be performed at each frequency or magnetic field. However, due to sensitivity and technical reasons only few regions have shown to be working (see e.g. table 1.1) [Hemminga and Berliner 2007]. In this study, EPR measurements have solely been performed in X-band.

<table>
<thead>
<tr>
<th>notation</th>
<th>frequency (GHz)</th>
<th>wavelength (mm)</th>
<th>magnetic field (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-band</td>
<td>9.5</td>
<td>31.6</td>
<td>0.34</td>
</tr>
<tr>
<td>K-band</td>
<td>24</td>
<td>12.5</td>
<td>0.86</td>
</tr>
<tr>
<td>Q-band</td>
<td>35</td>
<td>8.6</td>
<td>1.25</td>
</tr>
<tr>
<td>W-band</td>
<td>95</td>
<td>3.2</td>
<td>3.39</td>
</tr>
<tr>
<td>D-band</td>
<td>130</td>
<td>2.3</td>
<td>4.65</td>
</tr>
</tbody>
</table>

Table 1.1: Selected microwave bands and the corresponding magnetic fields for nitroxide spin labels in EPR spectroscopy.
Methodologies that have not been used in the present study but are worth mentioning due to their increasing relevance in EPR measurements are

1. time-resolved EPR characterizing protein kinetics from EPR transient traces [Wegener et al. 2000];

2. high-field EPR (higher than X-band) yielding information on the proticity (affinity to form hydrogen bonds) of the spin label microenvironment, providing increased signal-to-noise ratio as well as improved spectral resolution by enhanced g-factor resolution, and being more sensitive to faster molecular motions compared to conventional EPR [Steinhoff et al. 2000];

3. multi-frequency EPR elucidating the origin of different dynamics present in the nitroxide spectrum [Liang et al. 2004].

1.2 Motivation

During evolution all living organisms had to develop efficient mechanisms to recognize attractants (e.g. nutrients) and repellents (e.g. toxins) in their environments in order to survive. For precise adaptation to external conditions various mechanisms are used by cells. In (extremely) halophilic organisms, the salt concentration of the solvent surrounding the functioning units has shown to be decisive for structural and functional integrity (for one of the first reviews see [Lanyi 1974]). In this regard, the term “stability” becomes of utmost importance, particularly since the consequences of destabilized structural elements on the cellular functioning of certain organisms are not fully predictable by now. Due to the fact that moreover various studies suggest that conformational states of different dynamics rather than a steady-state create sophisticated signaling mechanisms in the halophilic branch of archaea (amongst others see [Kim 1994, Borrok et al. 2008]), one may conclude that minor deviations in structure already cause major dysfunctions. To what extent the degrees of dynamicity can be represented by folding/unfolding processes, stabilization/destabilization of structural elements, or the exchange between different protein conformations remains to be determined.

To solve this issue, the photosensitive sensory rhodopsin II/transducer complex from the extremely halophilic archaean *Natronomonas pharaonis* was investigated by means of SDSL EPR spectroscopy in the present study. Two major aspects are the purpose of this communication: i) the structural elucidation of the cytoplasmic, membrane-proximal region of the signal transducing unit, the so-called HAMP domain, and ii) the examination of conformational changes of the protein region under investigation occurring upon changes in the solvent’s salt concentration, temperature, pH, or the addition of compounds as polyethylene glycol (PEG), paramagnetic quenchers, or reducing agents. The experimental data reveals the presence of two HAMP conformations possessing different degrees of dynamics,
a compact conformation, where the HAMP domain is found to be arranged in a parallel four-helix bundle, and a highly dynamic conformation. These two protein conformations can be explained in terms of a “two-state” equilibrium existing between them.
CHAPTER 2

BACKGROUND INFORMATION

2.1 The HAMP domain in extremely haloalkaliphilic archaea

In the following paragraphs the signal transduction domain from Natronomonas pharaonis (Np) and most notably one part of it, the HAMP domain, will be of interest. In order to define the system under investigation, a brief introduction to a model organism for the halophilic branch of archaea, i.e. Halobacterium salinarum, is given, and the information available on structural and functional aspects of the investigated complex is presented to provide an insight into the current knowledge.

As other archaea, the rod-shaped, motile Halobacterium salinarum (see figure 2.1) constitutes a complex living under extreme conditions. Besides archaea living at very high temperatures or pressures, halobacterial systems prefer environments exhibiting salt concentrations of 4 M and higher. In order to lead the bacteria to environmental conditions favorable for energy conversion, halobacterial archaea use a family of four photoactive rhodopsins (see figure 2.2) that are structurally and functionally related to the visual pigments of higher organisms.Structurally, each of the rhodopsins exhibits seven transmembrane helices (A-G) and a retinal chromophore bound to a lysine residue on helix G via a protonated Schiff base. The chromophore’s interaction with the protein determines the position of the absorption maximum [SHIMONO et al. 2001], i.e. $\lambda_{max}(BR) = 568 \text{ nm}$, $\lambda_{max}(HR) = 578 \text{ nm}$, $\lambda_{max}(SRI) = 580 \text{ nm}$ and $\lambda_{max}(SRII) = 490/500 \text{ nm}$.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{halobacterium_salinarum.png}
\caption{Electronmicrograph of a Halobacterium salinarum cell with bipolar flagellation. Taken from [ALAM and OESTERHIELT 1984].}
\end{figure}
Figure 2.2: Photoactive rhodopsins from *H. salinarum*, the ion pumps BR (bacteriorhodopsin) and HR (halorhodopsin), and the sensors SRI and SRII (sensory rhodopsin I and II, respectively). The orientation and color of the arrows describe the direction of and the species (either $H^+$ or $Cl^-$) involved in the ion transport. Dashed arrows indicate a lower ion pump efficiency. The picture has been modified slightly from [Engelhard et al. 2003].

Functionally, the four rhodopsins can be split in two groups. Bacteriorhodopsin (BR), one of the most intensively studied proteins, and halorhodopsin (HR) are ion pumps responsible for the efficient export of protons and import of chloride ions, respectively. BR and HR are consequently acting as energy converters. SRI and SRII are phototaxis proteins influencing the swimming behavior of the cell in order to lead the cell to environments optimal for the function of bacteriorhodopsin and halorhodopsin. In case the cell is located in environments offering minor amounts of oxygen, the three rhodopsins BR, HR and SRI are active, where SRI displays a dual function. Irradiation with orange light leads to a positive phototaxis (swimming behavior of the cell is kept), whereas additional ultraviolet light results in a photophobic response (swimming behavior/direction is changed). BR can export protons and thereby create an electrochemical gradient across the lipid bilayer, in which the proton-motive force is used for the synthesis of ATP\(^a\). In case the bacterium meets both high oxygen pressure and high luminacy, that could cause a photo-oxidative damage to the cell, only SRII (also known as phoborhodopsin) is active. In a repellent response, SRII directs the cell away from the unfavorable light conditions. Under these conditions the respiratory chain (also: oxidative phosphorylation) is responsible for energy conversion, where also ATP is synthesized.

First structural information on SRII was obtained via X-ray crystallography [Luecke et al. 2001, Royant et al. 2001] from the extremely haloalkaliphilic archaeon *Natronomonas pharaonis* (NpSRII) due to its higher stability compared to the SRII from the organism *Halobacterium salinarum* (HsSRII). By virtue of the substantial similarities between HsSRII and NpSRII [Scharf et al. 1992], most of the information obtained so far as well as those described in this study have been derived from *N. pharaonis*,

\(^a\)ATP $\rightarrow$ ADP + phosphate + free energy and *vice versa*
2.1 The HAMP domain in extremely haloalkaliphilic archaea

a bacterium living in salt saturated lakes [Soliman and Truper 1982] possessing pH values around 11 [Falb et al. 2005] and temperatures in the range from 318 to 323 K [Rodriguez-Valera 1993].

In contrast to the ion pumps BR and HR, SRII (as well as SRI) is tightly bound to a transducer molecule, HtrII (HtrI), that is also an integral membrane protein but extends far into the cytoplasm of the cell. Structural data has been obtained first via X-ray crystallography [Gordeliy et al. 2002]. Due to predictable crystallization problems by virtue of the complexity of the full length transducer, the transmembrane part of the NpSRII/NpHtrII complex with a shorter transducer construct comprising only residues 1-114 (NpHtrII1–114) has been used. In figure 2.3a the crystal structure of the transmembrane part of NpHtrII in complex with NpSRII is depicted in ribbon diagrams showing the 2:2 stoichiometry (two receptors and two transducers) that is found in the membrane. Each receptor molecule exhibits seven transmembrane helices, each transducer molecule two. That the shortened transducer can be used as adequate source of information for the full-length counterpart, is due to the fact that the slight ability of SRII to export protons during its photocycle is blocked when HtrII is bound to SRII [Schmies et al. 2001, Spudich 1994]. This effect is preserved in the presence of the shorter NpHTRII1–114 variant [Hippler-Mreyen et al. 2003]. Studies involving the binding of another truncated transducer variant, NpHtrII159 (comprising residues 1-159), to the receptor using isothermal calorimetry experiments and blue native gel electrophoresis have also shown the same binding properties to and the same influence on SRII [Wegener et al. 2000]. However, due to lacking electron density, the X-ray structure shown here could only be resolved for residues 24 to 82.

The structure of the transducer is assumed to be quite similar to that of chemoreceptors (see figure 2.3b, left) [Spudich 1994, Falke and Hazelbauer 2001, Trivedi and Spudich 2003]. Although the signal in case of the transducer HtrII is - contrary to chemoreceptors - not sensed by an extracellular ligand binding domain but by SRII, HtrII from H. salinarum exhibits this sensor domain. However, this does not apply to the transducer molecule from N. pharaonis investigated here. Since furthermore the huge cytoplasmic domain of NpHtrII has structurally not been resolved so far, and due to the high homologies of this region with that of chemoreceptors, their structural features are considered to be consistent. A model, where the transmembrane part of the complex resolved by X-ray experiments is attached to the resolved cytoplasmic domain of the chemoreceptor Tsr from E. coli [Kim et al. 1999] is depicted in figure 2.3b, right. Analogous to the affinity of chemoreceptors to form dimers (that aggregate to higher-order complexes) [Bray et al. 1998, Vaknin and Berg 2007], the transducer crystal structure as well shows dimer formation, thereby putatively arranging the two long, rod-shaped struc-

---

1Halobacterial transducer of rhodopsin.
2Shortened from about 550 to 114 amino acids.
Figure 2.3: Structural features of the NpSRII/NpHtrII complex in its 2:2 stoichiometry. a) Ribbon diagram representation of the transmembrane part (Protein Data Bank (PDB) 1H2S [Gordeliy et al. 2002]). Receptor helices are depicted in red, transducer helices in green. The bound retinal is shown in yellow. Upper part: View from the cytoplasmic side with labeled NpSRII (A-G) and NpHtrII (TM1, TM2) helices. Symmetrically related helices in the 2:2 complex are marked by a prime. Lower part: Side view of the complex, where the dotted lines describe the limits of the hydrophobic part of the protein (CS: cytoplasmic side, ES: extracellular side). The figure was taken from [Klare et al. 2004]. b) Left: Model of a chemoreceptor showing the X-ray structure of the four-helical bundle of the ligand binding domain (from the S. typhimurium aspartate receptor Tar [Yeh et al. 1996]) as well as of the long cytoplasmic domain (from the E. coli serine receptor Tsr [Kim et al. 1999]). The monomeric subunits of the dimer are depicted in different colors (cyan, gray). The figure was taken from [Oprian 2003]. Right: Model of the NpSRII/NpHtrII complex in full-length combining the crystal structure of the transmembrane part with the cytoplasmic domain from the modeled E. coli chemoreceptor Tsr. The figure is adapted from [Klare et al. 2004]. Colors are chosen according to part a. The not yet characterized linker region (HAMP) is highlighted by the red question mark.

structures of the two cytoplasmic domains in a four-helix bundle. It is worth mentioning that the resulting superhelical twist of Tsr and HtrII exhibit the difference that its direction in Tsr is left-handed and in HtrII right-handed [Oprian 2003].

In order to facilitate the response on external conditions, SRII can be photoactivated. Here, the absorption of a photon leads to the isomerization of the bound retinal from the all-trans to the 13-cis form. This process is reverted while the protein passes different
intermediates of thermal relaxation in its photocycle (see figure 2.4) eventually returning to the ground state. The intermediates can be distinguished from each other spectroscopically by virtue of their different absorption maxima. Analogous to the bacteriorhodopsin photocycle, the intermediate states have been denoted as $K_{510}$, $L_{495}$, $M_{400}$, $N_{485}$ and $O_{535}$, where the subscript numbers describe the absorption maxima in nm. However, the fact that ion pumps as BR and HR exhibit photocycles typically faster than 20 ms, whereas that of sensors exceeds typically 300 ms [Jung et al. 2003], defines a striking difference.

The external signal from SRII is forwarded via protein-protein interactions with the tightly bound transducer molecule propagating the signal through a linker region comprising two HAMP domains (highly conserved signal transduction modules acting as putative signal transducing units in protein families as histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases; will be discussed in more detail later on) [Appleman et al. 2003, Appleman and Stewart 2003, Aravind and Ponting 1999] to its cytoplasmic tip. There, in analogy to the bacterial chemotaxis system, an intracellular signaling cascade, the “two-component” signaling pathway (see figure 2.5 for the SRII/HtrII complex), is activated (for reviews see amongst others [Spudich 1994, Spudich 1998, Engelhard et al. 2003]) that in turn influences the flagellar motor to control the cell’s swimming behavior. In the absence of external stimuli this mechanism results in a random walk behavior of the cell, whereas a biased random walk is performed in the photophobic response.

Concerning the SRII photocycle a spectrally silent irreversible transition between two $M$-states, i.e. $M_1$ and $M_2$, with a time constant of 2 ms has been revealed [Chizhov et al. 1998]. Amongst others, a time-resolved EPR study performed on site-directed spin labeled NpSRII variants revealed a mobilization of the receptor F helix.

Figure 2.4: Model of SRII’s photocycle showing the different intermediates. This picture was taken from [Klare 2002] according to [Chizhov et al. 1998].
Archaebacterial signal transduction, the two-component signaling pathway. The external signal is mediated by SRH to its bound transducer HtrII that transfers the signal to its cytoplasmic tip. In analogy to bacterial chemotaxis, via the adapter protein CheW the homodimeric histidine kinase CheA is bound, activating the autophosphorylation of CheA. In turn, the phosphate is transferred to the response regulator CheY that is thereby activated for the interaction with the flagellar motor. In case phosphorylated CheY is bound to the flagellar motor, the frequency of switching the motor’s rotational direction from counterclockwise (forward direction) to clockwise (backward direction in \textit{H. salinarum}, randomly chosen direction in \textit{E. coli}) changes. The adaptation process is exhibited by CheB (methylesterase) and CheR (methyltransferase). For detailed information see [Falke et al. 1997, Falke and Hazelbauer 2001]. The figure was taken from [Gordeliy et al. 2002].

Contrarily to information from studies on BR, a movement of the receptor helix G could not be observed [Wegener et al. 2000] (recently confirmed by [Bordignon et al. 2007]), indicating minor differences between the photoactivation of the two rhodopsins. Since the main interactions of helix F and G are van der Waals contacts with the two transmembrane transducer helices TM1 and TM2, the conformational change (an outward movement) of helix F is assumed to trigger a rotation of helix TM2, what has been confirmed by EPR spectroscopy [Wegener et al. 2001] and X-ray crystallography [Moukhametzianov et al. 2006]. This action is regarded as the starting event of the signaling pathway. However, it is not known in detail how the rotation of TM2 is coupled to a downward propagation of the signal to the cytoplasmic tip of the transducer NpHtrII.

In order to determine the complete signal pathway from the receptor activation to the initiation of the two-component signaling cascade at the cytoplasmic tip of the transducer molecule, distinct structural and functional information has to exist on the whole transducer construct at first. Via the described X-ray structural analyses detailed information
already exists for the transmembrane as well as on the cytoplasmic domain. However, these analyses did not yield data on the membrane adjacent region linking the transmembrane transducer helix TM2 to the cytoplasmic domain, namely the HAMP domain, that is of crucial importance for signaling.

Figure 2.6 shows the sequence alignment of HAMP domains from different photo- and chemoreceptors. Secondary structure analysis for HAMP domains predicts two helical regions, i.e. the amphipathic sequences 1 (AS1, residues G83-M100 in NpHtrII) and 2 (AS2, residues I117-E136 in NpHtrII), separated by a short connector of undefined structure. The amphipathic sequences are furthermore characterized by amino acid motifs typical for \( \alpha \)-helical coiled coil structures. Coiled coils, consisting of two to five amphipathic \( \alpha \)-helices forming a supercoil, describe one of the principal subunit motifs for protein oligomerization. Heptad repeat patterns (see figure 2.7) as found in HAMP domains define sequences of parallel left-handed coiled coils. The positions of the heptad repeat are denoted by the lower case letters a to g in figure 2.6 and 2.7 defining the seven-residue periodicity. Hydrophobic residues occupy positions a (1) and d (4), whereas polar residues are found at the remaining positions [Burkhard et al. 2001]. A so-called "knobs-into-holes" packing of the hydrophobic side chains into the hydrophobic core (see figure 2.7) is the decisive factor for the coiled coil’s stability [Crick 1953].

Though the amphipathic helices play different roles in signal transduction, their functions are conserved throughout different HAMP domains according to [Appleman et al. 2003] and [Appleman and Stewart 2003]. These studies furthermore support the hypothesis that specific interactions within the HAMP domain are of major importance, i.e. that rather minor differences in the linker may have significant effects on its function. A further characteristic feature of the HAMP domain is the augmented occurrence of charged residues in the connector region.

Especially in recent years, information from several studies analyzing the structural and functional aspects of the HAMP domain became available. A breakthrough in the mech-
anistic picture of the HAMP was the solution NMR structure of the isolated HAMP domain (residues T278-E331) of unknown function from the hyperthermophile archaean Archaeoglobus fulgidus (Af1503) showing a homodimeric, four-helical, parallel coiled coil (see figure 2.8) [Hulko et al. 2006]. In agreement with the secondary structure analysis mentioned above, two alpha-helical regions are linked by the connector that is characterized by higher flexibility compared to the helical core. This study furthermore suggested a model for signal propagation, in which “the HAMP domain alternates between the observed conformation and a canonical coiled coil” via concerted helix rotations. Subsequent chemical reactivity studies on the full-length, membrane-bound E. coli aspartate receptor Tar in
order to test the archaeal HAMP domain from Af1503 yielded high correlations in terms of solvent exposure between the two tested species [Swain and Falke 2007]. In the same study, disulfide mapping revealed the consistency of the two HAMP domain arrangements, thereby fully supporting the solution NMR structure. Moreover, Swain and Falke concluded from experiments using disulfide bonds in order to trap the chemoreceptor’s active state that only minor structural rearrangements are thought to occur upon on-off switching.

In vivo cross-linking experiments performed on residues 210 to 290 in the HAMP and proximal domains in the E. coli aerotaxis receptor Aer also confirmed the presence of two alpha-helical regions linked by a structured loop in the HAMP [Watts et al. 2008]. The proximal signaling domain was found to exhibit two alpha-helical domains connected by a short undefined structure. Further data obtained from these cross-linking studies supported once more the validity of the solution NMR structure of the isolated HAMP from Af1503, also for the Aer receptor.

Contrary to these clear evidences for defined structural elements, further recent studies demonstrated the existence of unstable HAMP domain structures. For instance, the electron paramagnetic resonance analysis of the HAMP structure from the extremely halophilic N. pharaonis yielded a molten-globule-like state under low salt conditions (150 mM KCl) [Bordignon et al. 2005, Doebber 2005]. Although the main features of the AS1-connector-AS2 pattern was considered to be present, an extremely dynamic structure was observed. One peculiarity in this model is the fact that no fixed structure could be observed for the upper part (cytoplasmic end) of the AS1.

Moreover, fusion studies on the HAMP domain from the osmosensing transmembrane histidine kinase EnvZ in E. coli documented via circular dichroism (CD) experiments have shown the flexible/unstable folding of the isolated construct. The EnvZ HAMP domain by itself is therefore not able to form a stable structure [Kishii et al. 2007]. The linkage with another domain resulted in significant increase in secondary structure. The authors pointed out that the fragility of the investigated domain may be important for its role in signal transduction.

Although the solution NMR structure of Af1503 has been supported by several studies and besides the fact that this HAMP domain from A. fulgidus retains the ability to trigger the two-component signaling pathway in non hyperthermophile organisms as demonstrated in case of the generated fusion constructs with the aspartate receptor Tar [Hulko et al. 2006], environmental constraints have to be taken into account carfully in order to describe do-mains that are thought to sensitively react on external conditions.

2.2 Preparational constraints

Expression and purification of sufficiently high amounts of protein needed for biophysical techniques pose two of the challenges in investigating protein structures and functions. In
the majority of cases homologous expression\(^d\) only results in sparse protein portions due to minor protein occurrence in the native cell. This problem can be overcome by homologous overexpression. However, since that in the case of the investigated *N. pharaonis* would need much more time due to the moderate growth of the cells compared to heterologous\(^e\) expression in *Escherichia coli* (*E. coli*), the latter procedure is used instead. Thus, many copies of the desired protein can be synthesized in the cell. The well understood and commonly used bacterium *E. coli* was furthermore chosen due to the easier performance of molecular biological applications as cloning and mutations compared to *N. pharaonis*.

Protein purification generally needs the protein under investigation to be in solution, since chromatographic techniques (and also several biophysical and biochemical methods) are based on this property. Membrane proteins embedded in the lipid-bilayer of the membrane do not fulfill this condition. A complete removal of the lipid-bilayer would result in aggregation of the membrane proteins due to hydrophobic interactions between the hydrophobic parts of the proteins. However, synthetically manufactured detergents as DDM are able to remove the proteins from the membrane bilayer and solubilize them in aqueous environment. Detergents, as well as the bilayer composing phospholipids, exhibit polar headgroups forming hydrogen bonds with water molecules, and hydrophobic carbon chains (tails) surrounding the hydrophobic regions of the proteins. Unlike phospholipids, that contain two hydrophobic tails and therefore arrange in form of bilayers, in which the tails by virtue of hydrophobic interactions face each other while the headgroups are pointing towards the aqueous medium (see figure 2.9 right), detergents form spherical structures (micelles) due to the existence of solely one tail (see figure 2.9 left) [BHAIRI 2001]. Consequently, in order to purify the membrane protein under investigation efficiently, solubilization is used as an intermediate step.

![Figure 2.9](image_url)

**Figure 2.9:** Cartoon representation of a detergent-solubilized (left) and phospholipid-reconstituted (right) Bacteriorhodopsin (gray ribbon presentation from PDB file 1FBB).

In general, polar lipids used in this study for reconstitution of the protein complexes into the more native lipid-bilayer environment compared to the solubilized form were extracted from the purple membrane from *Halobacterium salinarum* due to problems in isolating those from *Natronomonas pharaonis*. Though the two types of lipid membranes

\(^d\)Expression in the natural host.

\(^e\)Gene of one organism is expressed in another organism easy to cultivate.
are quite similar in terms of lipid bilayer composition, they are not the same. Different groups of halobacteria contain their own lipid compositions [Kates et al. 1982, Corcelli et al. 2000, Xu et al. 1999]. The most common polar lipids in haloalkaliphilic bacteria, PG (M_r: 807 g/mol) and PGP (M_r: 930 g/mol) (see figure 2.10) are found in each of those groups. Beyond the high occurrence of polar lipids (≈ 90% of total lipid content), neutral lipids only amount for neglectable degrees (≈ 10% of total lipid content). Hence, only polar lipids will be discussed in the following. Although not all lipid compo-

![Figure 2.10: Overview of phospholipids (PGP(-Me), PG and PGS; left panel) and glycolipids (S-TGD-1 and GLS, right panel). Hydrophilic headgroups are fully specified whereas hydrophobic tails (alkyl chains) are abbreviated with R (light green circles). The tail’s architecture is shown at the bottom of the figure. In all compounds the alkyl group R is 3,7,11,15-tetramethylhexadecyl. Differences in the phospholipids are highlighted by the gray ellipsoids. Various diether lipids, occurring in minor amounts in the lipid bilayer, vary in the design of the R2 (light pink) side chain. Drawings presented are rebuilt and modified from [Kates et al. 1982, Corcelli et al. 2000].

\[
\begin{align*}
\text{Sulfated triglycosyl diether-I (S-TGD-1)} & : \text{R2} = \text{O-CH}_3, \text{HO-CH}_3, \text{R2} = 3\text{SO}_3-\beta-\text{galp} \\
\text{Glycolipid sulfate (GLS)} & = \text{TGD} + \text{H}_2\text{SO}_4 \\
\text{Phosphatidyl-glycerol (PG)} & : \text{R} = \text{CH}_3 - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \\
\text{Phosphatidyl-glycerol sulfate (PGS)} & : \text{R} = \text{CH}_3 - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \\
\text{Phosphatidyl-glycercophosphate (PGP(-Me))} & : \text{R} = \text{CH}_3 - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \text{(CH}_2)_{15} - \text{CH} - \\
\end{align*}
\]

nents of the purple membrane are know so far, the lipid bilayer extracted from \textit{H. salinarum} is investigated extensively. It essentially comprises the methylated phospholipid PGP-Me (≈ 60 mol % in polar lipids) and the glycolipid S-TGD-1 (15 mol % in polar lipids, M_r: 1,219 g/mol). The remaining fraction mainly consists of PG, PGS (M_r: 887 g/mol), GLS (M_r: 1,240 g/mol), so far unknown constituents and two further lipids (one glycolipid with M_r: 1,521 g/mol, one phospholipid with M_r: 1,933 g/mol) exhibiting bulkier headgroups, as described previously [Corcelli et al. 2000]. On the other hand, much less is known about the lipids extracted from \textit{N. pharaonis}, generally build up by the phospholipids PGP and PG together with minor amounts of unidentified lipids. These two lipid compositions also differ in the average lengths of their alkyl chains. While the purple membrane mostly exhibits C_{20} tails, C_{25} chains are common in the \textit{N. pharaonis} membrane.
2.3 Site-directed spin labeling

The method of **site-directed spin labeling (SDSL)** based on the spin-labeling technique introduced by H. McConnell in the early 1960s, was developed by W. Hubbell and coworkers in the 1990s [Todd et al. 1989, Altenbach et al. 1990, Hubbel and Altenbach 1994]. Combining **site-directed mutagenesis** with spin labeling and EPR makes it a powerful tool to investigate the microenvironment of almost each desired amino acid in a protein sequence. How the spin label used here is introduced at a specific site and what opportunities SDSL EPR provides is briefly explained in the following paragraphs.

The most common procedure to insert a spin label species at a specific amino acid site in the protein is by means of chemical bonding to the already expressed and purified protein. To do so, *via* a hydrocarbon chain the compound carrying the unpaired electron is attached to a reactive group that covalently binds to specific amino acids. The MTS spin label selectively binds to sulfhydryl groups, i.e. to reduced disulfides in cysteine residues.

Dependent on the type of experiment to be performed, one or more spin labeled side chains are needed. In the case the primary sequence of the protein under investigation exactly exhibits one cysteine, the binding site for the spin label is unambiguous. However, attachment of the spin marker to a position not possessing a wild-type cysteine or deletion of unwanted cysteine residues in the sequence requires site-directed mutagenesis, where by virtue of molecular biological methods a mutation in the genetic code is carried out. After expression, instead of the wild-type amino acid the protein exhibits a cysteine where wanted (see figure 2.11) and in case of the deletion of unwanted cysteines alanines or serines are introduced (for further details see section 4.1.4).

![Figure 2.11: Site-directed spin labeling method. Via site-directed mutagenesis the native amino acid (left) is substituted by a cysteine (center) that can be spin labeled with methanethiosulfonate (MTSSL, top). The figure was adapted from Wegener 2002.](image)

In almost all SDSL studies a spin label exhibiting a nitroxide group (**N⁺-O⁻**) (here (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTS); molecular volume similar to phenylalanine or tryptophane; in the following abbreviated by **R1**), is used.
2.3 Site-directed spin labeling

In the $\pi$-orbital between the nitrogen and the oxygen of the nitroxide an unpaired electron is located defining the paramagnetic character of the spin probe. In case of the MTSSL used here the nitrogen is part of a five-membered ring (see figure 2.12).

![Figure 2.12: Schematic representation of the MTS spin label attached to a cysteine in the amino acid sequence of a protein. $C_\alpha$ and $C_\beta$ atoms are labeled accordingly. In order to agree with figure 3.2, the molecular frame axes are designated with (-x, -y, -z). The five dihedral angles ($\chi_1 - \chi_5$) defining the chain conformation/geometry, and the 4-H-atom on the nitroxide ring are indicated.](image)

Since unpaired electrons are highly reactive, they have to be shielded sterically from their environment in order to prevent the nitroxide’s reduction. Therefore, each of the two carbon atoms directly neighboring the nitrogen carries two methyl groups (CH$_3$) to remain the spin label’s paramagnetic nature over months even at room temperature. However, reduction of the nitroxide can be achieved using redox agents as ascorbic acid, dithiothreitol$^f$ (DTT) and others.

One disadvantage of the SDSL technique compared to e.g. non-perturbing isotope labeling methods as used in FTIR (Fourier transform infrared) and NMR (Nuclear magnetic resonance) spectroscopy is the possibility of structural perturbation upon amino acid substitution, particularly when this includes a bulky headgroup like that of the nitroxide spin label. Although the past has shown that for the majority of cases spin labeling of protein side chains, including those in the protein interior, does not result in perturbing structural and functional changes, the activity of the mutated protein ought to be checked always, in the ideal case by a functional assay.

$^f$Also known as Cleland’s reagent.
3.1 Electron paramagnetic resonance

In the following chapter a theoretical introduction to EPR spectroscopy from continuous wave (cw) to pulse methods is given on the basis of selected literature [Schweiger and Jeschke 2001, Jeschke 1998, Galla 1988]. Due to the involvement of quantum objects on a microscopic level, EPR theory has to be based on quantum mechanics. However, at least some of the basic aspects can be understood in the classical frame.

3.1.1 Electron spin and magnetic momentum

It is well known that electrons, besides their mass $m_e$ and charge $e$, possess an intrinsic angular momentum $s$, the so-called electron spin. Note that $s$ should not be associated with the electron rotation about itself but understood as a quantum mechanical angular momentum without classical counterpart. Together with the orbital angular momentum $\mathbf{l}$, the total angular momentum $\mathbf{j}$ (describing the spin-orbit coupling) of the electron arises:

$$\mathbf{j} = \mathbf{l} + \mathbf{s},$$

where the momentum quantum numbers $l$, $s$ and $j$ together with $\hbar$, the reduced Planck constant, set up the respective angular momentum vectors:

$$|j| = \sqrt{j(j+1)}\hbar,$$

$$|l| = \sqrt{l(l+1)}\hbar,$$

$$|s| = \sqrt{s(s+1)}\hbar.$$

\[\text{In the following scalars are designated in normal type face, first rank and higher tensors in bold face type (except greek symbols that are designated by superscript arrows), and operators by the "hat".}\]
Closely connected with the angular momenta are the particular magnetic moments. The total magnetic moment thus results in
\[
\vec{\mu} = \vec{\mu}_l + \vec{\mu}_s. \tag{3.3}
\]

The quantitative relationship between \(\vec{\mu}\) and \(j\) is
\[
\vec{\mu} = \gamma_e j, \tag{3.4}
\]
where the proportionality factor \(\gamma\) (here with index \(e\) for the electron) gives the gyromagnetic ratio describing the ratio of a particle’s magnetic dipole moment and its angular momentum. It can furthermore be written in terms of a molecule specific Landé g-factor \(g_e\) and the Bohr magneton \(\beta_e\) of the electron:
\[
\gamma_e = \frac{g_e \beta_e}{\hbar} = \frac{-e}{2m_e} g_e, \tag{3.5}
\]
with
\[
g_e = 1 + \frac{j(j + 1) - l(l + 1) + s(s + 1)}{2j(j + 1)}. \tag{3.6}
\]

The shown relations can be reduced for many applications to the exclusive consideration of the electron spin \(s\) since the orbital angular momentum \(l\) in organic radicals is of neglectable magnitude at first. With an electron spin \(s = \frac{1}{2}, l = 0\) and a small relativistic correction not being described here, one obtains \(g_e = 2.002319315\) for a free electron. For the electron’s magnetic moment follows furthermore
\[
|\vec{\mu}_S| = -\frac{g_e \beta_e}{\hbar}|s| = -g_e \beta_e \sqrt{s(s + 1)}. \tag{3.7}
\]

If we further consider the electron in an homogeneous magnetic field of magnetic induction \(B_0\)\(^b\), the vector of the electron spin aligns parallel or anti-parallel to the magnetic field direction. Considering the z-axis (direction of applied magnetic field) as preferential direction, the spin projection quantum number \(m_s\) can reach the values \(\pm \frac{1}{2}\) due to the quantum mechanical quantisation orientation of an angular momentum (see figure 3.1, right). Note that due to the spatial quantisation, each single spin orientation comprises a certain angle between the \(B_0\) and the spin vector. According to the \(m_s\) states, two discrete values for the magnetic moment are possible:
\[
\mu_{S,z} = \pm \frac{1}{2} g_e \beta_e. \tag{3.8}
\]

\(^b\)In general, the magnetic field strength \(H\) is used. Here, the magnetic induction \(B\) is referred as the magnetic field since it takes into account bulk susceptibility effects on the actual field experienced by the spin often neglected in experimental work.
Since a magnetic dipole $\vec{\mu}$ in a magnetic field $\mathbf{B}_0 = (0, 0, B_0)$ exhibits the energy
\[ E = -\vec{\mu} \mathbf{B}_0 = -\mu_z B_0, \] (3.9)
two energy levels are available for the electron (see figure 3.1, left)
\[ E_\alpha = +\frac{1}{2} g_e \beta_e B_0, \] (3.10a)
\[ E_\beta = -\frac{1}{2} g_e \beta_e B_0, \] (3.10b)
with
\[ \Delta E = E_\alpha - E_\beta = g_e \beta_e B_0 \] (3.11)
as the magnetic field dependent difference between them. This splitting of degenerated energy-levels was named according to its discoverer Pieter Zeeman, a Dutch physicist.

In order to initiate a so-called electron spin flipping from one energy level to another one, in EPR spectroscopy electromagnetic radiation of the energy $h\nu = \Delta E$ is applied to the sample. In other words, the energy of an applied radiation field (of frequency $\nu$) exhibiting the magnetic field $\mathbf{B}_1$, perpendicular to $\mathbf{B}_0$, has to fit the energy gap between the two levels (resonance condition). For standard field strengths $B_0$ of 0.1 to 1 T the frequency $\nu$ is in the order of microwaves.
3.1.2 Spin Hamiltonian

In quantum mechanics energy levels and the time evolution of a system are obtained from the Hamilton operator $\hat{H}$. In order to prepare for such considerations the following sections give a brief introduction to the static Hamiltonian describing the elementary properties and interactions in the system under investigation. Additional information, e.g., on the oscillatory Hamiltonian, can amongst others be found in [Abragam 1961, Slichter 1996, Banci et al. 1991, Gamliel and Levanon 1995, Schweiger and Jeschke 2001].

The Spin Hamiltonian of a system with an electron spin $S$ interacting with the external magnetic field, magnetic moments from nuclear spins as well as from other electron spins can be described as the sum of different contributors:

$$\hat{H}_S = \hat{H}_{EZ} + \hat{H}_{NZ} + \hat{H}_{HF} + \hat{H}_{ZFS} + \hat{H}_{EXCH} + \hat{H}_{DD} + \hat{H}_{NQ} + \hat{H}_{NN},$$  \hspace{1cm} (3.12)

with $\hat{H}_{EZ}$ describing the electron Zeeman interaction, $\hat{H}_{NZ}$ the nuclear Zeeman interactions, $\hat{H}_{HF}$ the hyperfine couplings between the electron and nuclear spins, $\hat{H}_{ZFS}$ the zero-field splitting for spin systems with effective spin $S > \frac{1}{2}$, $\hat{H}_{EXCH}$ and $\hat{H}_{DD}$ the Heisenberg exchange couplings and the dipole-dipole couplings, respectively, between the electron and other electrons in the system, $\hat{H}_{NQ}$ the nuclear quadrupole interactions for spins with nuclear spin quantum numbers $I \geq 1$ and $\hat{H}_{NN}$ the spin-spin interactions between pairs of nuclear spins.

The interactions described by $\hat{H}_{ZFS}$, $\hat{H}_{NQ}$ and $\hat{H}_{NN}$ are only of minor contribution in the system under investigation ($S = \frac{1}{2}$, $I = 1$ ($^{14}$N)) and remain consequently unconsidered. Furthermore will the energies described here be given in angular frequency units.

3.1.2.1 Electron Zeeman interaction and the g-tensor

The interaction between the electron spin and the externally applied magnetic field $B_0$ is described by the electron Zeeman term that, according to the correspondence principle and equations 3.8 and 3.9, gives

$$\hat{H}_{EZ} = \frac{\beta_e}{\hbar} g B_0 \hat{S},$$  \hspace{1cm} (3.13)

where $g$ describes the g-tensor and $\hat{S}$ the Spin Operator. In nuclear physics an unpaired electron in the s-state does not possess an orbital angular momentum. Even though the orbital state describing the electron considered here is described by p-orbitals, the expectation value for the orbital angular momentum $l$ vanishes due to the molecular binding, an effect known as ”quenching of orbital angular momentum”. However, the presence of a magnetic field partly cancels out this behavior; the resulting effective spin is not a pure spin state anymore but contains also orbital components. The value of the magnetic mo-
Electron paramagnetic resonance becomes orientation dependent. This anisotropy can be accounted for by the use of a g-tensor instead of the isotropic g-value:

\[
g = \begin{pmatrix}
g_{xx} & g_{xy} & g_{xz} \\
g_{yx} & g_{yy} & g_{yz} \\
g_{zx} & g_{zy} & g_{zz}
\end{pmatrix}.
\] (3.14)

As described in the cited literature, a symmetric g can be assumed, where \( g_{yx} = g_{xy}, \ g_{zx} = g_{xz} \) and \( g_{zy} = g_{yz} \). In the principle axis system (PAS) \( g \) is diagonal. Here, the PAS is characterized by the axes described by the MTSSL with the z-axis oriented parallel to the \( 2p_z \)-orbital (nitrogen as well as oxygen), the x-axis oriented parallel to the N-O bond and the y-axis perpendicular to them \cite{Griffith1965} (for visualization see figure 3.2).

In the diagonalized form we have:

\[
g = \begin{pmatrix}
g_{xx} & 0 & 0 \\
0 & g_{yy} & 0 \\
0 & 0 & g_{zz}
\end{pmatrix}.
\] (3.15)

In a simplified model of the electronic structure of the nitroxide bond (see figure 3.2) the essential contributions to the g-tensor can be described via \cite{Plato2002}:

\[
g_{xx} \approx g_e + \frac{2\zeta(O)\rho^O_n c_n^2}{\Delta E_n \rightarrow \pi}, \quad (3.16a)
\]

\[
g_{yy} \approx g_e + \frac{2\zeta(O)\rho^O_n c_n^2}{\Delta E_n \rightarrow \pi}, \quad (3.16b)
\]

\[
g_{zz} \approx g_e, \quad (3.16c)
\]

with \( \zeta(O) = 151 \text{ cm}^{-1} \) \cite{Carrington1969} as spin-orbit coupling parameter, \( \rho^O_n \) as spin density on the oxygen \( 2p_z \) atomic orbital, \( c_{nx} \) and \( c_{ny} \) as molecular orbital coefficients of the \( 2p_x \) and \( 2p_y \) atomic orbitals contributing to \( \Psi_n \) (briefly n), the oxygen lone pair orbital (see figure 3.2), and \( \Delta E_n \rightarrow \pi \) as the excitation energy for a transition between an orbital of the non-bonding oxygen electron pairs and the N-O bond \( \pi \)-orbital \cite{Plato2002}. However, for MTSSL in aqueous environments possessing low viscosities the g-tensor anisotropy averages out due to fast molecular motions. The spectrum consequently becomes orientation independent and the isotropic g value \( g_{iso} \) can be used:

\[
g_{iso} = \frac{1}{3} (g_{xx} + g_{yy} + g_{zz}) \approx g_{xx} \approx g_{yy} \approx g_{zz}.
\] (3.17)
Figure 3.2: Schematic overview of the electronic structure of the N-O bond with an external electric field $E_x$ (from surrounding polar regions) and with one H-bond formation in the molecular frame (x, y, z). The superposition of oxygen 2$s$, 2$p_x$ and 2$p_y$ orbitals results in the non-bonding lone pair orbitals $\Psi_n$. (Figure taken from [PLATO et al. 2002].)

Furthermore, the B-field intensities used in this study ($\approx 0.3$ T) are not sufficient to resolve the g-tensor anisotropy in the EPR spectra. An experimental setup providing magnetic fields in the order of 3 T (W-band) and higher would be needed.

3.1.2.2 Nuclear Zeeman interaction

Analogous to the electron Zeeman interaction, the nuclear Zeeman interaction describes the coupling between a nuclear spin $I$ and the external magnetic field $B_0$. The respective Hamiltonian has the form

$$\hat{H}_{NZ} = -\frac{\beta_n}{\hbar} g_n B_0 \hat{I},$$

(3.18)

where $\beta_n = \frac{e\hbar}{2m_p}$ is the nuclear magneton. $\hat{I}$ and $g_n$ are the nuclear spin quantum number and the nuclear g-factor, respectively, inherent properties of a nucleus. The latter one is displayed in form of a scalar according to the minor anisotropic character of the $g_n$-tensor compared to the resolution accessible with EPR. As well as the electron spin, the nuclear spin possesses different energy states when exposed to a static magnetic field ($B_0$).

Even though the nuclear Zeeman interaction is insignificant in most EPR experiments, it may be of major importance in double resonance measurements (e.g. DEER or ENDOR), where nuclear spin spectra are measured via electron spins. This particularly holds true when the nuclear Zeeman interaction becomes of the order of the hyperfine interaction described in the following section. Compared to the electron Zeeman interaction, e.g. the nuclear Zeeman interaction for protons is 658 times smaller [SCHWEIGER and JESCHKE 2001].
3.1 Electron paramagnetic resonance

3.1.2.3 Hyperfine interaction and the A-tensor

Figure 3.3: Schematic demonstration of the effect of the hyperfine interaction on a system described by $S = \frac{1}{2}$ and $I = 1$ ($^{14}$N). Two energy levels resulting from the electron Zeeman interaction additionally split in a total number of six energy levels. Together with the quantum mechanical selection rules for allowed transitions ($\Delta m_I = 0$ and $\Delta m_S = \pm 1$), three EPR transitions can be measured with the resulting absorption signals (here: first derivative) at the bottom. The apparent hyperfine splitting is designated by $a_0$.

One of the most important sources of information in EPR is given by the interaction between an electron spin and a nuclear spin, the hyperfine interaction. This interaction leads to a splitting of the EPR resonance lines according to the magnetic quantum numbers of the nucleus $m_I$ (see figure 3.3) and is described by the Hamiltonian

$$\hat{H}_{HF} = \hat{S} \hat{A} \hat{I},$$

(3.19)

with the hyperfine tensor $\hat{A}$ characterizing the orientation dependence and magnitude of the hyperfine splitting [Abragam and Bleaney 1986]. The expression can be split in two parts: the isotropic Fermi contact interaction described by $\hat{H}_{contact}$ and the anisotropic dipolar interaction described by $\hat{H}_{dip}$:

$$\hat{H}_{HF} = \hat{H}_{contact} + \hat{H}_{dip}$$

(3.20)

$$= a_{iso} \hat{S} \hat{I} + \hat{S} \hat{A}_{dip} \hat{I}.$$  

(3.21)
The isotropic hyperfine interaction originates from the finite spatial probability density of the electron at the nucleus. Since only electrons in s-orbitals characterized by spherical symmetry possess this property, the Fermi contact interaction describes an isotropic interaction. Accordingly, the hyperfine tensor $A$ shown in equation 3.19 is orientation independent and becomes isotropic ($a_{iso}$):

$$a_{iso} = \frac{2\mu_0}{3\hbar} g_e \beta_e g_n \beta_n |\psi(0)|^2,$$

with $|\psi(0)|^2$ as electron spin density at the nucleus. In case of the used MTS spin label, the principal axis system of $A$ coincides with that of the mentioned $g$-tensor [Plato et al. 2002]. Hence, analogous to equation 3.17 follows for the isotropic hyperfine contribution:

$$a_{iso} = \frac{1}{3} (A_{xx} + A_{yy} + A_{zz}) \approx A_{xx} \approx A_{yy} \approx A_{zz}.$$  

The second term, the anisotropic hyperfine interaction, describes the dipole-dipole interaction between the electron and the nucleus, where $A_{dip}$ is the dipolar coupling tensor. In its principal axis system $A_{dip}$ can be written in the diagonalized tensor-form [Schweiger and Jeschke 2001] (a more detailed derivation of dipole-dipole interactions between electrons is given in section 3.1.2.4):

$$A_{dip} = \frac{\mu_0}{4\pi\hbar} \frac{g_e \beta_e g_n \beta_n}{r_{SI}^3} \begin{pmatrix} -1 & 0 & 0 \\ 0 & -1 & 0 \\ 0 & 0 & 2 \end{pmatrix},$$

with $\mu_0$ as vacuum permeability and $r_{SI}$ as distance between the electron and the nuclear spin. Note that this expression generally applies only for an isotropic electron Zeeman interaction but nevertheless is often used in an approximative way in case of small $g$ anisotropies.

Since the dominant hyperfine splitting in EPR using $^{14}$N spin labels is observed along the $g$-tensor principal $z$-axis that coincides with that of the $A$-tensor, the $A_{zz}$ value will furthermore be specified in terms of the spin densities at the O- and N-atom, respectively, in the following part. The splitting by virtue of the isotropic and anisotropic interactions gives the contributors:

$$A_{zz} = a_{iso} + A_{dip,zz}.$$  

According to [Beveridge and McIver(JR.) 1971, Lemaire and Rassat 1964], in an approximation the spin density $\rho_O^2$ of the unpaired electron on the neighboring O-atom is for both contributors of neglectable magnitude. Therefore, the contributors will further on be designated by a superscript $N$. The isotropic term consequently only considers the spin polarization in the $\sigma$-orbital through $\pi - \sigma$-coupling and is thus directly proportional to the
spin density $\rho_{\pi}^N$ in the $2p_z^N$-orbital. Nevertheless, non-planar N-O-systems with perturbed $sp^2 - p_z$-hybridization of the N-atom may lead to a non-vanishing spin density $\rho_S^N$ from the $2s^N$-orbital contributing to $a_{iso}^N$:

$$a_{iso}^N = Q_{\pi - \sigma}^N \rho_{\pi}^N + Q_s^N \rho_s^N. \tag{3.26}$$

Eventually, in combination with $A_{dip,zz}$, determined entirely by the spin density $\rho_{\pi}^N$ [PLATO et al. 2002, BEVERIDGE and McIVER (JR.) 1971], it follows:

$$A_{zz}^N = Q_{tot}^N \rho_{\pi}^N + Q_s^N \rho_s^N, \tag{3.27}$$

with the proportionality constants $Q_{tot}^N = 7.3 \ mT$ [PLATO et al. 2002] and $Q_s^N = 23.2 \ mT$ [PLATO et al. 1991].

In order to obtain information from EPR data through these expressions, it is noteworthy that the $\pi$ spin density at the nitrogen atom $\rho_{\pi}^N$ is dependent on the electric field component $E_x$ being oriented along the N-O-bond of the MTSSL [GRIFFITH et al. 1974]. Since the average local electric field in this region reflects the polarity effects originating from various fields, possible structural changes of the label as well as of the whole complex are traceable. It has been shown that non-polar environments are characterized by an $A_{zz}^N$ of approximately $3.36 \ mT$, highly polar environments of approximately $3.64 \ mT$ [PLATO et al. 2002]. However, predictions on the number of H-bonds to the nitroxide spin label can not be done solely through the knowledge of the $A_{zz}$ value.

3.1.2.4 Electron spin-spin interactions

So far, systems exhibiting only one unpaired electron have been regarded in the construction of the Spin Hamiltonian. Considering moreover the presence of two or more unpaired electrons in the system under investigation, interactions between them have to be accounted for. In the case two unpaired electrons are located in the same or directly neighboring atoms, they are strongly interacting and the system has to be described by a group spin $S > \frac{1}{2}$. This phenomenon is defined as zero-field interaction and will be neglected in this theoretical background since the EPR samples investigated here do not possess unpaired electrons located in so close proximity. However, this is not the only electron spin-spin interaction detectable in EPR spectroscopy. Weaker interactions between two electrons can be characterized by their individual spins $S_1$ and $S_2$, an exchange coupling parameter $J$ and a dipole-dipole coupling tensor $D$. As can be derived from the relevant tensors and analogous to the electron-nuclear interactions already discussed, the inter-spin distance dependent isotropic exchange and anisotropic dipolar interactions have to be taken into account. The

\footnote{Though the exchange interaction also exhibits an anisotropic portion, it cannot be distinguished from the isotropic one in an experiment. However, the anisotropic part is caused by the spin-orbit coupling that can usually be neglected in case of organic radicals.}
former one only plays a decisive role when two spins are closer than approximately 10 Å, what leads to the at least partial overlap of their orbitals and consequently to the exchange of their spin functions. This may define a weak binding with the spins aligned antiparallel according to the Pauli exclusion principle or a weak anti-binding due to coulombic interaction with parallel spins [Jeschke 1998]. The effective spin in the first case would become zero and no signal would be detected any more.

Although EPR samples investigated in this study do not possess distances below the mentioned limit of 10 Å, the exchange coupling Hamiltonian $\hat{H}_{\text{EXCH}}$ will be given briefly due to its relevance in power saturation measurements (see section 3.1.3.6). Spin label accessibilities to different media are therein defined by the use of rapidly relaxing paramagnetic species exchanging with the unpaired electrons from the attached spin labels.

The Hamiltonian describing the Heisenberg exchange interaction has the form:

$$\hat{H}_{\text{EXCH}} = J \hat{S}_1 \hat{S}_2,$$

with $J$ giving information on the character of the electronic couplings and energetics of the system (anti-ferromagnetic, binding interaction: $J < 0$; ferromagnetic, anti-binding interaction: $J > 0$). In contrary to the cubic distance dependence of the subsequent dipolar interaction, the Heisenberg exchange interaction is generally less far detectable. In solutions containing radicals the exchange interaction is supposed to fall approximately exponentially [Jeschke 1998]. Further information can be collected from the literature, e.g. [Jeschke 1998, Schweiger and Jeschke 2001].

In the anisotropic dipolar interaction between two electrons the spins can be considered as two magnetic point dipoles $\vec{\mu}_1$ and $\vec{\mu}_2$ since their orbitals are too far away from each other to overlap. Classically, the energy $E$ between two interacting dipoles can be described via:

$$E = -\vec{\mu}_1 \mathbf{B}_2(\mathbf{r}_{12}) = -\vec{\mu}_2 \mathbf{B}_1(\mathbf{r}_{21}),$$

with the field $\mathbf{B}_2$ (of dipole 2) at dipole 1 being dependent on the distance vector $\mathbf{r}_{12}$ connecting dipole 1 and 2, and vice versa. From the equation describing the spatial field distribution of a magnetic dipole (not shown) and by the use of the correspondence principal, the Hamiltonian describing the dipole-dipole interaction follows as

$$\hat{H}_{\text{DD}} = \frac{\mu_0}{4\pi} \left( \frac{\vec{\mu}_1 \vec{\mu}_2}{r_{12}^3} - 3 \frac{\left( \vec{\mu}_1 \mathbf{r}_{12} \right) \left( \vec{\mu}_2 \mathbf{r}_{12} \right) \mathbf{r}_{12}}{r_{12}^5} \right),$$

and in terms of the two spin operators $\hat{S}_1$ and $\hat{S}_2$ (through equation 3.7) and the sum of them $\hat{S}_1 + \hat{S}_2 = \hat{S}$

$$\hat{H}_{\text{DD}} = \frac{\mu_0}{4\pi \hbar} \frac{g_1 g_2 \beta_c^2}{r_{12}^3} \left( \hat{S}_1 \hat{S}_2 - \frac{1}{r_{12}^2} \left( \hat{S}_1 \mathbf{r}_{12} \right) \left( \hat{S}_2 \mathbf{r}_{12} \right) \right) = \hat{S} \mathbf{D} \hat{S},$$
where \( g_1 \) and \( g_2 \) are the g-values of the two electron spins, \( r_{12} = |\mathbf{r}_{12}| \) and \( \mathbf{D} \) the already mentioned dipole-dipole coupling tensor. In case of neglectable \( g \)-anisotropy and in the high-field approximation, \( \mathbf{D} \) can be written in its principal axis system:

\[
\mathbf{D} = \frac{\mu_0}{4\pi\hbar} \frac{g_1 g_2 \beta_e^2}{r_{12}^3} \begin{pmatrix}
-1 & 0 & 0 \\
0 & -1 & 0 \\
0 & 0 & 2
\end{pmatrix}.
\]

(3.32)

The dipolar interaction between the two electrons is proportional to \( \frac{1}{r_{12}^3} \), thereby being predestined for the inter-spin-distance determination in spin labeling EPR.

3.1.2.5 The total Spin Hamiltonian

Inclusion of all the relevant particular Hamiltonians described above leads to the total Spin Hamiltonian for \( i = 2 \) electron spins \( S_i \) and \( k \) coupled nuclear spins \( I_k \):

\[
\hat{H}_S = \hat{H}_{\varepsilon Z} + \hat{H}_{N Z} + \hat{H}_{HF} + \hat{H}_{\varepsilon CH} + \hat{H}_{DD}
\]

(3.33)

\[
= \sum_i \frac{\beta_i}{\hbar} g B_0 \hat{S}_i - \sum_k \frac{\beta_n}{\hbar} g_n B_0 \hat{I}_k + \sum_{i,k} \hat{S}_i \hat{A}_k \hat{I}_k + J \hat{S}_1 \hat{S}_2 + \hat{S} \hat{D} \hat{S}.
\]

(3.34)

It is worth mentioning that the coordinate systems of the electron spins described here were assumed to be similar with the exception of minor deviations that are to be neglected here [RADZWILL 2001]. The relative contribution of each term in equation 3.34 depends on the sample and type of experiment performed. For instance, for the samples used here (in aqueous solution) in continous wave experiments the \( \hat{H}_{N Z} \) term can be neglected, for single electron spin systems \( \hat{H}_{\varepsilon CH} \) and in some cases also \( \hat{H}_{DD} \) may be neglected.

3.1.3 The cw EPR spectrum

In order to characterize the nitroxide EPR spectrum, the Spin Hamiltonian has to be solved with regard to its eigenvalues. To do so, the coordinate systems for the relevant parameters have to be consistent. Since the \( A \)- and \( g \)-tensor so far were described in their principal axis systems coinciding with the molecular frame and the external magnetic field \( B_0 = (0, 0, B_0) \) in the laboratory frame, a transformation from the molecular into the laboratory coordinate system has to be done according to figure 3.4.

Transformation of \( g \) and \( A \) into the laboratory frame \( (g^L, A^L) \) can be achieved by the application of the rotary matrix \( L \). It follows:

\[
g^L = LgL^{-1},
\]

(3.35)

\[
A^L = LAL^{-1},
\]

(3.36)
For a transformation from the molecular (nitroxide z-axis) into the laboratory frame (B₀ z-axis) a counterclockwise rotation by the angle \( \varphi \) (here 0°) about the nitroxide z-axis has to be followed by a clockwise rotation by the angle \( \vartheta \) about the new nitroxide y-axis. (The picture was taken and slightly changed from \([\text{Abé} 2006]\)).

with

\[
L = \begin{pmatrix}
\cos \vartheta \cos \varphi & \cos \vartheta \sin \varphi & -\sin \vartheta \\
-\sin \varphi & \cos \varphi & 0 \\
\sin \vartheta \cos \varphi & \sin \vartheta \sin \varphi & \cos \vartheta
\end{pmatrix}.
\] (3.37)

Considering now the simplified case of one oriented nitroxide (%(S = \frac{1}{2}%; one nitrogen nucleus with \( I = 1 \)) and the application of the high-field approximation\(^d\) due to the major influence of the electron Zeeman interaction compared to all the other interactions, only the electron Zeeman and the hyperfine interaction have to be accounted for, but all terms involving the elements \( S_x \) or \( S_y \) can be neglected. According to \([\text{Libertini and Griffith 1970}]\) the Spin Hamiltonian in the laboratory frame

\[
\hat{\mathcal{H}}^L = \frac{\beta_e}{\hbar} g^L B_0 \hat{S} + \hat{S} A^L \hat{I}
\] (3.38)

leads to the eigenvalues

\[
E_{m_S,m_I} = m_S \beta_e g(\vartheta, \varphi) B_0 + m_S m_I A(\vartheta, \varphi),
\] (3.39)

with

\[
g(\vartheta, \varphi) = g^{L}_{zz} = g_{xx} \sin^2 \vartheta \cos^2 \varphi + g_{yy} \sin^2 \vartheta \sin^2 \varphi + g_{zz} \cos^2 \vartheta,
\] (3.40)

\[
A(\vartheta, \varphi) = A^{L}_{zz} = \sqrt{A_{xx} \sin^2 \vartheta \cos^2 \varphi + A_{yy} \sin^2 \vartheta \sin^2 \varphi + A_{zz} \cos^2 \vartheta}.
\] (3.41)

\(^d\)Applicable for radicals in solution in X-band and higher fields.
With the nitroxide system properties \((S = \frac{1}{2}, I = 1)\) and the quantum mechanical selection rules \((\Delta m_S = \pm 1, \Delta m_I = 0)\) the allowed EPR transitions (see also figure 3.3) in terms of the resonance magnetic fields follow to be

\[
B_{0(m_I=+1)} = \frac{\hbar \omega - A(\vartheta, \varphi)}{\beta_e g(\vartheta, \varphi)},
\]

\[
B_{0(m_I=0)} = \frac{\hbar \omega}{\beta_e g(\vartheta, \varphi)},
\]

\[
B_{0(m_I=-1)} = \frac{\hbar \omega + A(\vartheta, \varphi)}{\beta_e g(\vartheta, \varphi)}.
\]

(3.42a)

(3.42b)

(3.42c)

Since an experimental EPR spectrum consists of the resonance lines from various nitroxides not being oriented crystal-like but randomly, and due to spin dynamics and spin relaxation effects not mentioned so far, line broadenings have major influence on the spectral shape. In sections 3.1.3.2 and 3.1.3.4 these effects are derived and discussed briefly.

### 3.1.3.1 Macroscopic magnetization and resonance phenomenon

In order to explain the characteristic features of an experimental EPR spectrum, first, the overall macroscopic magnetization \(M\) is introduced and the resonance phenomenon is explained in its frame. The examination of the macroscopic magnetization instead of the microscopic counterpart is convenient particularly with regard to the pulse EPR methods described later.

Considering a typical EPR sample volume containing \(10^{15}\) and more spin systems of same type, statistics have to be used to describe the populations of the two energy levels \(E_\alpha\) and \(E_\beta\) mentioned in equations 3.10a and 3.10b since the transition probabilities are equal for both directions (emission or absorption induced). In a system of an ensemble of \(N\) spins \(s = \frac{1}{2}\) being in thermal equilibrium, the populations \(n_\alpha\) and \(n_\beta\) with \(N = n_\alpha + n_\beta\) are described by the Boltzmann distribution:

\[
\frac{n_\beta}{n_\alpha} = \exp\left(\frac{\Delta E}{kT}\right) = \exp\left(-\frac{g_e \beta_e B_0}{kT}\right).
\]

(3.43)

Hence, the upper energy level \(E_\alpha\) is occupied to a lesser extent than the lower one \(E_\beta\) describing the fact that absorption is the prevailing process in such a system. In the so-called high temperature approximation (in X-band applicable for \(T > 1.5 K\)) the expression can further be simplified by the use of \(\exp(-x) \approx 1 - x\) applying for small exponents:

\[
\frac{n_\beta}{n_\alpha} = 1 - \frac{g_e \beta_e B_0}{kT}.
\]

(3.44)
The difference in population $\Delta N = n_\beta - n_\alpha$ with respect to the total spin number $N$ accounts for

$$\Delta N/N = n_\beta \left[ 1 - \exp \left( -\frac{g_e \beta_e B_0}{kT} \right) \right] \approx \frac{g_e \beta_e B_0}{2kT} \approx \frac{g_e \beta_e B_0}{2kT}, \quad (3.45)$$

yielding the tininess of the EPR involved number of spins (less than one percent) compared to the whole ensemble.

This population difference (also known as polarization) furthermore leads to the presence of a macroscopic magnetic moment, the so-called (macroscopic) magnetization $M$, described by the net magnetic moment per unit volume $V$. At thermal equilibrium it is given by

$$M_0 = \frac{1}{V} \sum_{i=1}^{N} \vec{\mu}_i, \quad (3.46)$$

with $\vec{\mu}_i$ as all individual magnetic moments in the ensemble of electrons present.

As long as relaxation effects are neglected, the time dependence of the magnetization vector $M$ can be treated in an analogous way to that of a single magnetic moment. The state of minimum energy is obtained at thermal equilibrium, where $M_0$ is aligned parallel to the external magnetic field $B_0$. As already noted, the $B_0$ direction was chosen as the $z$-axis of the laboratory frame $z^L$.

The resonance phenomenon in the classical picture can now be understood through the Lamor frequency in angular frequency units

$$\omega_s = \frac{g_e \beta_e B_0}{\hbar} \quad (3.47)$$

describing the precession frequency of the magnetization on a cone about $B_0$ (see figure 3.5a) by virtue of their interaction. Analogous to the single electron system described earlier, the angle $\theta$ between the macroscopic magnetization $M$ and the $z^L$-axis is present as a consequence of the spatial quantisation. $\theta$ as well as the length of the magnetization vector are invariant. In continuous wave EPR, the application of microwave radiation (of frequency $\omega_{mw}$) to the system and the property of electromagnetic radiation to exhibit an electric and a magnetic field component, is used to produce a linearly polarized magnetic field $B_1$ perpendicular to the $B_0$-axis. Here, the direction of $B_1$ is chosen to coincide with the $x^L$-axis:

$$B_{1xL}(t) = 2B_1 \cos (\omega_{mw} t), \quad (3.48a)$$

$$B_{1yL}(t) = 0, \quad (3.48b)$$

$$B_{1zL}(t) = 0. \quad (3.48c)$$
This linearly polarized field can be considered as the superposition of a right-hand \( (\mathbf{B}_1^r) \) and a left-hand \( (\mathbf{B}_1^l) \) rotating circularly polarized field given by

\[
\begin{align*}
B_{1x}^r(t) &= B_{1x}^l(t) = B_1 \cos(\omega_{mw}t), \\
B_{1y}^r(t) &= -B_{1y}^l(t) = B_1 \sin(\omega_{mw}t), \\
B_{1z}^r(t) &= B_{1z}^l(t) = 0.
\end{align*}
\]

Since a frame exhibiting a time-dependent \( \mathbf{B}_1 \) complicates the further description of the magnetization’s motion, a shift to the rotating frame is convenient. This frame described by \( (x, y, z) \) rotates clockwise with the microwave frequency \( \omega_{mw} \) about the \( z^L \)-axis of the laboratory frame used so far. A transformation of the circularly polarized fields to the rotating frame results in

\[
\begin{align*}
B_{1x}^r &= B_1, \\
B_{1y}^r &= B_{1z}^l = 0, \\
B_{1x}^l &= B_1 \cos(2\omega_{mw}t), \\
B_{1y}^l &= B_1 \sin(2\omega_{mw}t), \\
B_{1z}^l &= 0.
\end{align*}
\]
Hence, the left-hand rotating field is off-resonant and only the right-hand polarized field $B_1^r$ can be resonant with the precession frequency of the electron spins in our system. The $B_1$ time-dependency is thereby eliminated. The motion of $\mathbf{M}$ in the rotating frame (see figure 3.5b) can accordingly be described via

\[
\frac{dM_x}{dt} = -(\omega_s - \omega_{mw})M_y = -\Omega_s M_y, \tag{3.51a}
\]

\[
\frac{dM_y}{dt} = (\omega_s - \omega_{mw})M_x - \left(\frac{g_e \beta_e B_1}{\hbar}\right) M_z = \Omega_s M_x - \omega_1 M_z, \tag{3.51b}
\]

\[
\frac{dM_z}{dt} = \left(\frac{g_e \beta_e B_1}{\hbar}\right) M_y = \omega_1 M_y, \tag{3.51c}
\]

with the resonance offset frequency $\Omega_s = \omega_s - \omega_{mw}$ and $\omega_1 = \frac{g_e \beta_e B_1}{\hbar}$ describing the frequency of the additional precession about the microwave field direction taking place in the presence of off-resonant microwave irradiation. The superposition of the described precessions leads to a nutation about an effective field which is the vector sum of the off-resonance contribution $\Omega_s$ and the microwave field $\omega_1$ (see figure 3.5c). The effective field-axis is inclined by the angle $\theta$ with respect to the $B_0$-field direction ($z$) and the nutation exhibits the frequency $\omega_{eff}$ (see equations 3.52a and 3.52b, respectively). In case of the system being in resonance ($\omega_{mw} = \omega_s$), $\Omega_s$ becomes 0 and the magnetization vector can be flipped by a certain angle about the $x$-axis depending on the duration and phase of the microwave field application.

\[
\theta = \arctan\left(\frac{\omega_1}{\Omega_s}\right), \tag{3.52a}
\]

\[
\omega_{eff} = \sqrt{\Omega_s^2 + \omega_1^2}. \tag{3.52b}
\]

It is noteworthy that the magnetization $\mathbf{M}$ is generally characterized by two components, a longitudinal ($M_z$) and a transverse ($M_x$ and $M_y$) one that describe the superposition of the various spin states present. In case of the system being in thermal equilibrium, the magnetization is aligned along the $z$-axis, only the longitudinal component is present. This state is in an approximation described by pure spin polarization. Disequilibrating the system, as is done by microwave irradiation, entails the presence of the transverse component (see also equations 3.51a to 3.51c). This transverse magnetization reflects the fixed phase relationship (known as coherence) between the different spin states via its precession with the Lamor frequency and can be detected by EPR. Both transversal magnetizations $M_x$ and $M_y$ can be detected simultaneously (quadrature detection) in an EPR experiment since they couple (slightly) to the resonator [SCHWEIGER and JESCHKE 2001].
3.1.3.2 Relaxation and Bloch equations

In the systems considered so far only interactions between the spins and the magnetic fields $B_0$ and $B_1$ but no relaxation effects have been regarded. In the absence of relaxation processes, excitation of EPR transitions could only be performed up to the equilibration of the population differences due to the Boltzmann distribution (saturation). Recording an EPR spectrum would not be possible. However, knowledge about different relaxation effects (detailed descriptions of the different relaxation processes can amongst others be found in [Slichter 1996, Gamliel and Levanon 1995, Banci et al. 1991, Muus and Atkins 1972]) that are responsible for the radiationless return of the excited system back to thermal equilibrium after a certain time, enables either to avoid the occurrence of saturation or to exploit this phenomenon in selected EPR methods.

The relaxation effects originate from interaction of the spins with their environments, namely the lattice and other spins. Relaxation due to interactions with the spin-lattice is defined by the longitudinal relaxation time $T_1$ (also known as spin-lattice relaxation time). This type of relaxation brings along a change in the magnetic quantum number $m_s$, consequently in the energy of the spin system. Since spontaneous emission would take too long in the EPR measurements applied here, thermal motion of the environment plays a major role. In solids, the energy transfer in longitudinal relaxation is mediated through the fluctuations of local magnetic fields caused by lattice vibration. In liquids these fluctuations are induced by molecular motions, molecular collisions, intramolecular motions, with the major contributor given by the stochastic Brownian motion.

On the other hand, relaxation due to interactions with other spins is characterized by the transverse relaxation time $T_2$ (also known as spin-spin relaxation time). In contrast to the longitudinal, the transverse relaxation does not require an exchange of energy with the environment, but some kind of interaction, as e.g. in an energy conserving spin flip-flop process of two spins. Note that the polarization of the spin system in a single spin flip process remains unchanged, whereas the fixed phase relation between the flipping spin and the others gets lost. In case of spin flip-flop rates of $\frac{1}{T_2'}$ it follows:

$$\frac{1}{T_2} = \frac{1}{2T_1} + \frac{1}{T_2'}.$$  \hspace{1cm} (3.53)

To further distinguish liquid from solid samples, different thermal motions have to be taken into account, since particularly in solid state measurements the assumption of a well-defined transverse relaxation time may bear major problems. This will not be explained in detail here but can be found amongst others in [Abragam 1961]. Characteristic decay times as the phase memory time $T_m$ necessary to describe relaxation effects in solid state, are described in section 3.1.5.1.

A phenomenological description of these relaxation effects can be done by the use of the Bloch equations considering a simple two-level system within the framework of the macro-
scopic magnetization $M$ introduced earlier. Assuming the relaxation of the longitudinal magnetization towards its thermal equilibrium value $M_0$ in an exponential way leads to

$$\frac{dM_z}{dt} = -\frac{M_z - M_0}{T_1},$$

(3.54)

and the time dependent, exponentially decaying relaxation of the transverse magnetization to

$$\frac{dM_{x,y}}{dt} = -\frac{M_{x,y}}{T_2}.$$  (3.55)

Together with the rotating frame motions of the magnetization vector components described in equations 3.51a to 3.51c the rotating frame Bloch equations arise that fully describe the spin dynamics in a two-level system.

$$\frac{dM_x}{dt} = -\Omega_s M_y - \frac{M_x}{T_2},$$

(3.56a)

$$\frac{dM_y}{dt} = \Omega_s M_x - \omega_1 M_z - \frac{M_y}{T_2},$$

(3.56b)

$$\frac{dM_z}{dt} = \omega_1 M_y - \frac{M_z - M_0}{T_1}.$$  (3.56c)

In equilibrium state in case of continuous wave irradiation with constant microwave power relaxation and excitation processes are balanced, therefore $\frac{dM_x}{dt} = \frac{dM_y}{dt} = \frac{dM_z}{dt} = 0$. Insertion in the rotating frame Bloch equations 3.56a to 3.56c leads to the description of the three magnetization components, with $M_x$ defining the dispersion signal being dephased by 90° with respect to $M_y$ (absorption signal). Since in cw EPR, with the exception of some applications, only the absorption signal\footnote{One reason for the dispersion disregard is its phase balance with the microwave, therefore being strongly influenced by the m.w. source noise.} is detected, solely $M_y$ will be regarded in the following [SCHWEIGER and JESCHKE 2001]:

$$M_y = M_0 \frac{\omega_1 T_2}{1 + \Omega_s^2 T_2^2 + \omega_1^2 T_1 T_2},$$

(3.57)

with the precession frequency $\omega_1 = \frac{g_e \beta e B_1}{\hbar}$.

3.1.3.3 Energy level populations and density matrix

The description of a spin system in terms of transition frequencies and probabilities increases in complexity with the consideration of increasing systems. For instance, the calculations for spectra with and without state mixtures (e.g. radical in solution and solid state, respectively) strongly differ from the simple case presented since the electron and nuclear
magnetic moments interact in different ways. The corresponding transition energies and probabilities do consequently not coincide. An example of the calculation of transition energies and probabilities for a system accounting for interactions between one electron spin $S = \frac{1}{2}$ and one nuclear spin $I = \frac{1}{2}$ in solution as well as in solid state is briefly explained in section 3.1.5.1. For larger systems exceeding the number of eigenstates used for simplified calculations, the so-called density matrix $\sigma$ describes statistically the energy state populations and coherences. Hence, the classical macroscopic magnetization detected in experiments on very large spin ensembles can be predicted with high accuracy by these quantum mechanical expectation values. Detailed information on the derivation and further aspects as time-evolution of the density matrix during measurements can be obtained from [Schweiger and Jeschke 2001]. Exemplificative, the form of a density matrix in a simple four-level system of two coupled spins ($S = \frac{1}{2}, I = \frac{1}{2}$) is shown in figure 3.6.

![Figure 3.6: Density matrix elements and transitions on the basis of a four-level system with the eigenstates $|\alpha\alpha\rangle$, $|\alpha\beta\rangle$, $|\beta\alpha\rangle$ and $|\beta\beta\rangle$ for two coupled spins ($S = \frac{1}{2}, I = \frac{1}{2}$). Left: Density matrix with the diagonal elements $P_{jk}$ representing the population of the eigenstate $|jk\rangle$ and the off-diagonal elements (ZQ: zero-quantum coherence, SQ$^{(S)}$ and SQ$^{(I)}$: single-quantum coherence of spin $S$ and $I$, respectively, DQ: double-quantum coherence) quantifying coherences. The difference between the diagonal elements describes polarization and is therefore proportional to the longitudinal magnetization $M_z$. The magnitude of the off-diagonal elements is proportional to the magnitude of the transverse magnetization. Right: Energy level diagram for the system considered on the left. Full lines describe allowed single quantum transitions, whereas the forbidden transitions (in the left part of the figure also highlighted by gray boxes) are denoted via dashed lines. (The figure was reconstructed from [Schweiger and Jeschke 2001]).

3.1.3.4 Line broadenings

As shown via equations 3.42a to 3.42c, one characteristic feature of an EPR spectrum is given by the magnetic field positions of its resonance lines. Due to the energy-time uncertainty principle $\Delta E \Delta t \geq \hbar$ the widths of the particular lines cannot become infinite narrow but possess a finite width defined as natural linewidth. Moreover, relaxation processes due to the spin-lattice and spin-spin interactions lead to homogeneous line broadenings. The $^{15}N$ MTSSL variant was used instead of $I = 1$ due the importance of the calculations for the performed ENDOR measurements (see section 3.1.5.2), where a $^{15}N$ MTSSL variant was used.
absorption signal of the resonance line in this case is characterized by a Lorentzian lineshape
with a FWHM of $\frac{2}{T_2}$.

In order to describe the effects that further broaden the homogeneous lines, it is convenient
to consider whole ensembles of spins experiencing the same local fields, i.e. spin packets.
A single homogeneous line can be described by one spin packet, whereas inhomogeneously
broadened lines are composed of signals from different spin packets. For instance, the
distribution of the spin orientations in a solid state sample leads to an inhomogeneous
line broadening due to the presence of different spin ensembles, each of them oriented
in a different direction and accordingly contributing one homogeneous line to the total,
inhomogeneously broadened line. If the extent of the inhomogeneous broadening is large
compared to the linewidth of the single spin packets, the resulting line exhibits a gaussian
distribution.

There are many other sources for inhomogeneous line broadenings as e.g. an inhomoge-
neous external magnetic field $B_0$ or unresolved hyperfine couplings in case of the interaction
of the unpaired electron with several nuclei.

Although a separation of the different inhomogeneous effects present is not simple, the
distinction of completely homogeneously and inhomogeneously broadened lines is straight-
forward. A strong microwave radiation applied to a certain position in the homogeneous
line broadens (saturates) the whole line due to the comparable interactions of all spins with
the radiation field. On the contrary, the application of the same field to an inhomogeneously
broadened line leads only to the broadening of the homogeneous line, whose spin ensemble
frequency matches that one of the applied field. Thereby a spectral hole is burnt into the
line.

The behavior of homogeneous lines upon the increase of irradiated microwave power is
exploited in the so-called power saturation measurements described in section 3.1.3.6.

3.1.3.5 Rotational correlation time and "mobility"

We have seen that the relaxation rates depend on the extent of the spin lattice vibra-
tions/ field fluctuations induced by molecular motions. Correlation times can be used to
characterize the dynamics of such motions.

The autocorrelation function $G(\tau)$ quantifies the correlation between stochastical orien-
tations at time $t$ with that at time $t + \tau$ [Slichter 1963]. For times $\tau$ less than the critical
time $\tau'_c$, namely the correlation time, the two correlations are strongly correlated and the
stochastic motion can be neglected. For times larger than $\tau'_c$, the state described by the
time $t + \tau$ becomes progressively less correlated to that at time $t$ and finally completely
looses the correlation.

The fourier transformation of $G(\tau)$ yields the spectral density function $J(\omega)$, describ-
ing the frequency distribution in this stochastic process. As described above, $\frac{1}{T_1}$ behaves
proportionally to $J(\omega)$ having its maximum at $\tau_c = \frac{1}{\omega}$ (with phonon frequency $\omega$) in the \textit{Redfield limit} [Schweiger and Jeschke 2001, Jeschke 1998, Slichter 1963].

For (nearly) spherical molecules in liquid solutions the correlation time $\tau'_c$ corresponds to the \textit{rotational} correlation time $\tau_c$, known from the \textit{Stokes-Einstein relation}:

$$\tau_c = \frac{4\pi \eta a^3}{3kT} = \frac{V\eta}{kT}, \tag{3.58}$$

with $a$ as rotationally effective radius of the molecule and $\eta$ as solvent viscosity. Since the molecule’s volume $V$ is approximately proportional to its molar mass $M$, $\tau$ is proportional to $M$, too. A separated spin label consequently has a 100 to 1000 times smaller rotational correlation time than a protein.

To express the rotational correlation time in terms of the relaxation times $T_1$ and $T_2$, equation 3.53 can be simplified to $T_2 \leq T_1$ in case of a liquid sample by using $T'_2 \leq 2T_1$ [Schweiger and Jeschke 2001]. The consideration of a sample exhibiting low viscosity leads further to very short rotational correlation times and identical mean square deviations of the fluctuating field components ($B^2_x = B^2_y = B^2_z = \overline{B^2}$) [Schweiger and Jeschke 2001]. It follows the expression describing the \textit{motional narrowing}, i.e. the narrowing of EPR lines and relaxation slow down with shorter correlation times:

$$\frac{1}{T_1} = \frac{1}{T_2} = 2 \left( \frac{g\beta_e}{\hbar} \right)^2 \overline{B^2} \tau_c. \tag{3.59}$$

With regard to cw X-band EPR spectra, typical time scales for distinguishable reorientational correlation times range from approximately 0.1 to 300 ns. Since this corresponds quite well to reorientational correlation times of a protein bound spin label, analysis of the correlation time is often used to define protein conformations and to trace conformational changes. In figure 3.7 cw X-band EPR spectra are displayed to exemplify correlation time dependent spectral shapes. These spectra can be divided into three subgroups. The first describes the case of free diffusion (see figure 3.7 top), where all $g$ and $A$ anisotropies are averaged out by the fast reorientational motion of the spin label side chain. The apparent hyperfine splitting and the line widths are small. The corresponding correlation times are small and the term \textit{fast motional regime} is commonly used for this kind of spectra. The second group describes the opposite extreme, namely the spectra accounting for anisotropies due to the superposition of spectra statistically oriented over all possible spatial directions with respect to the external magnetic field $B_0$ (for further information see section 3.1.4.2). Such a static spectrum (see figure 3.7 bottom) is characterized by correlation times above 300 ns, and is called \textit{powder spectrum}. Respective hyperfine splittings and line widths are dramatically increased compared to the isotropic case. The $A_{zz}$-component of the hyper-

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*Redfield limit is attained for motions fast enough to average partially the anisotropic interactions. Applies for times $\tau \leq T_2$. 


Figure 3.7: Cw X-band EPR nitroxide spectra exhibiting different rotational correlation times. Isotropic spectra as in case of free diffusion with small $\tau_c$ values possess three distinct lines (top), whereas larger $\tau_c$ values are accompanied by spectral broadenings visualizing $g$ and $A$ anisotropies. Correlation times above 300 ns characterize the case of complete spin label immobilization, i.e. the so-called powder spectrum. (The figure was taken from [RADZWILL 2001] and slightly changed).

Fine tensor sensitive to the environment’s polarity (see equation 3.25) in a powder spectrum is given by half the distance between the low ($m_I = +1$) field maximum and the high ($m_I = -1$) field minimum in units of the applied field (here $mT$). The third subgroup called slow motional regime exhibits correlation times between the two other subgroups and possesses spectra with broadened lines and different degrees of averaging of $g$ and $A$ anisotropies as can be seen in figure 3.7. The spectra in this regime are very sensitive to any changes in motion. Note that the correlation times given here are only valid for cw EPR spectroscopy performed in X-band, since spectral lineshapes obtained at higher microwave frequencies and magnetic fields differ from the ones showed here.

It is furthermore worth mentioning that in case of a nitroxide selectively attached to a suitable protein site, several aspects might influence the rotational correlation time. The most important fact is that the nitroxide group is not attached to the protein in a rigid way since then nearly no averaging of the $g$ and $A$ anisotropies would be possible. Since the linker between nitroxide and protein remains flexible, $\tau$ is dependent on spin label as well as on protein aspects. Spin label architecture, tertiary structure interactions, internal backbone dynamics, other spatial interactions as well as several “external” influences like thermal
conditions and solute properties have to be accounted for when analyzing such spectra. For instance, it has been shown in [Mchaourab et al. 1996] that the main contribution to the R1 side chain motion of a spin label attached to a helix surface does not originate from interaction with the nearest residue neighbors but with those at position $i + 4$ (with $i$ describing the R1 side chain residue). Recently published data on the contrary also includes the residues $i + 1$ [Guo et al. 2008] or $i - 1$ [Sezer et al. 2008]. More information on the influencing parameters can amongst others be obtained from [Altenbach et al. 1989a, Mick et al. 1991, Hubbel and Altenbach 1994, Mchaourab et al. 1996].

Though both parameters describe the spin label motion, the term mobility is often used instead of rotational correlation time since the former can be calculated directly from a cw EPR spectrum. However, the expression mobility is used in a more general sense regarding all effects mentioned above. Evaluation of this expression leads to the semi-empirical mobility parameter $\Delta H_{pp}^{-1}$, the inverse of the central resonance line ($m_I = 0$) width [Hubbel and Altenbach 1994]. Weak interactions as in case of sites on the protein surface or loop regions corresponding to fast correlation times (see figure 3.7 top), result in a high spin label mobility. Contrary, strong interactions as found in case of buried sites or in a pocket on the protein surface corresponding to long correlation times (see figure 3.7 bottom), yield low mobility values. In order to get a fingerprint of the secondary and tertiary interactions present in an amino acid sequence, a commonly used method is to plot the mobility parameter $\Delta H_{pp}^{-1}$ against the respective residue number. Periodic patterns, very low or high mobility regions then provide an insight into the structural contraints. A classification (see figure 3.8) to assign R1 side chain mobilities to a certain protein structure established by Mchaourab et al. [Mchaourab et al. 1996] might well be helpful in this context.

![Figure 3.8: Classification of side chain localizations by plotting the reciprocal spectral second moment $<H^2>^{-1}$ versus the reciprocal central line width $\Delta H_{pp}^{-1}$ calculated from cw X-band EPR spectra. Though the second moment values have not been determined in this study, the classification is useful in combination with solely the "mobility" values. (The figure was created according to [Mchaourab et al. 1996]).](image-url)
3.1.3.6 Power saturation and accessibilities for quencher reagents

Beyond the information obtainable from the spectral shape of a nitroxide cw EPR spectrum about side chain mobilities and environmental polarity, a commonly used data source is the behavior of the longitudinal relaxation time $T_1$ upon the addition of fast-relaxing paramagnetic quencher molecules. Obtaining $T_1$ while the spin system is led to saturation in the presence and absence of the quencher reagents NiEDDA or molecular oxygen (see section 4.2.4), allows the determination of the spin label accessibilities to the different reagents. The specific quenchers possess characteristic access to the different phases of the sample (bulk water, lipid phase, protein interior) and offer there an additional relaxation path, thereby changing $T_1$ (see also sections 4.3.1 and 4.3.2). A distinction whether the spin label sites are located in the protein interior, the water or the lipid phase can be done.

In times of various pulse EPR techniques being available, the saturation recovery method using saturating microwave pulses, directly yields $T_1$ of the nitroxide and the exchange rate with the quencher [Percival and Hyde 1975, Yin et al. 1987, Altenbach et al. 1989a, Altenbach et al. 1989b]. Although the pulse method has shown to be more accurate [Hyde and Subczynski 1989], in this study the continuous wave analogon was used, namely the power saturation method [Poole and Farach 1971, Subczynski and Hyde 1981, Altenbach et al. 1989a] that can be carried out on a conventional cw EPR spectrometer (see section 4.2.4) under room temperature conditions.

In order to obtain an expression for a saturation curve, the already introduced solution of the rotating frame Bloch equations in equilibrium state (see equation 3.57) can be taken into account:

$$M_y = M_0 \frac{\omega_1 T_2}{1 + \Omega^2 T_2^2 + \omega_1^2 T_1 T_2},$$

where the precession frequency $\omega_1 = \frac{g_e \beta_e B_1}{\hbar}$ describes the strength of the applied microwave field $B_1$ ($B_1 = \Lambda \sqrt{P}$, where $\Lambda$ is a constant describing the resonator efficiency).
As long as $\omega_1^2 T_1 T_2 \ll 1$, the absorption has a lorentzian line shape and the signal amplitude increases linearly with the square root of the microwave power. In case this condition does not apply any more, the saturation process starts. The signal amplitude increases less than linear with $\sqrt{P}$. With further microwave power increase does $\omega_1^2 T_1 T_2$ (denominator) initially compensate and eventually overcompensate $\omega_1 T_2$ (numerator), so that the signal amplitude starts to fall until no signal can be detected any more.

For the first derivative peak-to-peak amplitude $Y'_{pp}$ of the central resonance line of the EPR spectrum in case of resonance ($\Omega_s = \omega_s - \omega_1 = 0$) and in terms of the microwave power $P$, results a quite similar expression (a complete derivation can be found in [KÜHN 2003]):

$$Y'_{pp}(B_1) = \frac{A \Lambda \sqrt{P}}{(1 + \gamma^2 A^2 PT_1 T_2)} \epsilon,$$

(3.60)

where $P = \left(\frac{\omega_1}{\Lambda}\right)^2$ with $\gamma = \frac{g_e \beta_e \bar{\hbar}}{h}$ and $\epsilon$ accounting for the homogeneity of the saturation. The values for $\epsilon$ are to range between 0.5 and 1.5, the two extremes for fully inhomogeneous and homogeneous saturation, respectively [ALTMANN et al. 2005].

We consider now an aqueous sample containing the spin labeled species and paramagnetic quencher molecules, where the relaxation rate $\frac{1}{T_{1q}}$ of the latter is much higher than the nitroxide’s one. Spatial proximity of the two species in the extent that the orbitals of the two unpaired electrons overlap might lead to an exchange of their spin states, i.e. Heisenberg exchange takes place [MOLIN et al. 1980]. Though dipolar interactions between spins in solution may also partition in spin-lattice relaxation, Heisenberg exchange is regarded as the dominating process [HYDE and SARNA 1978]. The nitroxide system thereby gains a new way to relax with a shorter longitudinal relaxation time $T_1$, the saturation of the system is retarded proportional to the exchange frequency $W_{ex}$ of bimolecular encounters. Since the relaxation time of the paramagnetic quencher is too fast to be resolved in the EPR spectrum, the exchange with the reagent is indistinguishable from other spin-lattice relaxation events. The paramagnetic quencher can be assumed to be part of the lattice. Additional knowledge of the spatial probability distributions of the particular exchange reagent in the different sample environments enables us to determine the nitroxide location. NiEDDA is a reagent highly soluble in the water phase, whereas molecular oxygen is known to have high probability densities in the lipid phase and only lower ones in the water phase. Both reagent are excluded from the protein interior.

Correspondingly, a high exchange frequency $W_{ex}$ between the nitroxide and a water-soluble reagent reveals the nitroxide’s water accessibility, whereas the same spin labeled site is expected not to exhibit major encounters with a lipid-phase-soluble reagent. The effective longitudinal relaxation rate in the presence of a paramagnetic quencher species, assuming only Heisenberg exchange to take place, is given by:

$$\frac{1}{T_1} = \frac{1}{T_1} + f P_{max} W_{ex} = \frac{1}{T_1} + \beta C_{PQ},$$

(3.61)
with $T_1^0$ as spin-lattice relaxation time in the absence of a paramagnetic quencher species, $f$ as statistical correction depending on the spin states of the colliding species [Subczynski and Hyde 1981], $P_{max}$ as maximum exchange efficiency ($P_{max} = 1$ in the strong exchange limit assumed here), $C_{PQ}$ as the quencher’s concentration and $\beta$ as a constant describing the nitroxide accessibility [Altenbach et al. 1989b]. Hence, the saturation curve described by equation 3.60 has a characteristic shape dependent on $\beta$ and $C_{PQ}$.

An empirical parameter commonly used to describe the saturation behavior is $P_{1/2}$, giving the incident microwave power where the first derivative amplitude $Y^{\prime}_{pp}$ is reduced to half of its unsaturated value (see figure 3.9).

![Figure 3.9: Plotting the course of the first derivative amplitudes $Y^{\prime}_{pp}$ of the central resonance line (black filled circles) versus the square root of the incident microwave power gives a particular saturation curve (black line). The parameter $P_{1/2}$ characterizes the microwave power where $Y^{\prime}_{pp}$ is reduced to half of its unsaturated (blue line) value.](image)

$P_{1/2}$ can be determined via the expression [Altenbach et al. 2005]

$$P_{1/2} = \frac{2^{1/\epsilon} - 1}{\Lambda^2 \gamma^2 T_1 T_2}. \quad (3.62)$$

Under the assumption that the transverse relaxation time $T_2$ of the nitroxide remains constant in the common case $W_{ex} \ll \frac{1}{T_2}$, the difference between $P_{1/2}$ and $P_{1/2}^0$ in the presence and absence of the exchange reagent, respectively, results in the following expression:

$$\Delta P_{1/2} = P_{1/2} - P_{1/2}^0 = \frac{(2^{1/\epsilon} - 1)}{\Lambda^2 \gamma^2 T_2} \left( \frac{1}{T_1} - \frac{1}{T_1^0} \right), \quad (3.63)$$

which by the use of equation 3.61 and the assumption of $f = 1$ shows to be proportional to the exchange frequency $W_{ex}$ in the way:

$$\Delta P_{1/2} = \frac{(2^{1/\epsilon} - 1)}{\Lambda^2 \gamma^2 T_2} W_{ex}, \quad (3.64)$$
3.1 Electron paramagnetic resonance

To eliminate further the $\Delta P_{1/2}$ dependence on the nitroxide transverse relaxation time $T_2$ and the resonator efficiency $\Lambda$, the dimensionless quantity $\Pi_1$, the so-called accessibility parameter for species 1, is used [Farahbakhsh et al. 1992, Oh et al. 2000]:

$$\Pi_1 = \left( \frac{P_{1/2}^1}{\Delta H_{pp}^1} - \frac{P_{1/2}^0}{\Delta H_{pp}^0} \right) \cdot \frac{\Delta H_{DPPH}^{PPH}}{P_{1/2}^{DPPH}} , \tag{3.65}$$

with $\Delta H_{pp}$, the peak-to-peak line width of the central resonance line in the saturation-free first derivative EPR spectrum, normalizing for the nitroxide transverse relaxation time $T_2$, and $\frac{\Delta H_{DPPH}^{PPH}}{P_{1/2}^{DPPH}}$, the corresponding values for the reference substance DPPH, normalizing for the resonator efficiency.

Finally, due to the fact that normalization with the reference substance DPPH has shown to bear uncertainties, a direct calculation of the exchange frequencies $W_{ex}$ (see section 4.2.4 and 4.3.2) is recommended to keep errors as small as possible.

3.1.4 Cw EPR spectra simulation

In order to evaluate the data obtained from the variety of investigation methods available in EPR spectroscopy, different fit programs have been developed. A brief theoretical background is given here only on for two programs frequently used in this study, namely Freedfit (by R. Fiege and H.-J. Steinhoff according to [Freed 1976]) and dipfit (by H.-J. Steinhoff and coworkers [Steinhoff et al. 1997]) which simulate cw EPR spectra in the slow motional regime and powder spectra, respectively.

3.1.4.1 Freedfit

To describe EPR spectra in the slow motional regime it is not sufficient to mimic the spectrum by the superposition of several Lorentzian lines as could be done in the fast motional regime and in case of powder spectra, assuming that only homogeneous line broadening occurs. Especially for nitroxides attached to macromolecules and for those located in a viscous medium, EPR measurements at ambient temperature yield rotational correlation times in the slow motional regime, i.e. $1 \text{ ns} \leq \tau_c \leq 300 \text{ ns}$. Since the spectra are neither characterized by the rigid-limit extreme nor by motionally narrowed spectra with averaged $g$ and $A$ tensors, their shape is affected by molecular motions and the magnetic spin interactions. Due to the stochastic character of molecular diffusion processes, Liouville’s theorem

$$\frac{\partial}{\partial t} \rho = -\frac{i}{\hbar} \{ \hat{H}(t), \rho \} \tag{3.66}$$
is not sufficient to describe the time dependent behavior of the already introduced density matrix $\rho$ (see section 3.1.3.3). But according to [Kubo 1969, Freed et al. 1971, Freed 1976] an average $\overline{\rho}$ can be considered instead:

$$\overline{\rho} (\Omega, t) = \int \rho \ p (\rho, \Omega, t) \ d\rho,$$

(3.67)

where $p (\rho, \Omega, t)$ is the probability of finding a system in a certain orientation described by $\Omega$ at time $t$, and $\Omega$ describes the Euler angles specifying the transformation between the molecular and the laboratory frame. The time dependence of $\overline{\rho}$ can then be expressed by the *stochastic Liouville equation of motion*. It provides a quantum mechanical basis to account for the mentioned effects and thereby to properly simulate the cw EPR spectra obtained [Kubo 1969, Freed et al. 1971, Freed 1976]:

$$\frac{\partial}{\partial t} \overline{\rho} (\Omega, t) = - \frac{i}{\hbar} \left[ \hat{\mathcal{H}} (\Omega), \overline{\rho} (\Omega, t) \right] - \Gamma_{\Omega} \overline{\rho} (\Omega, t).$$

(3.68)

$\hat{\mathcal{H}} (\Omega)$ is derived from the time dependent Hamiltonian $\hat{\mathcal{H}} (t)$. The reorientational motion modulating the Hamiltonian is described in terms of $\Omega$ and in equation 3.68 it is assumed [Kubo 1969] that the $\Omega$ time dependence is subject to a stationary *Markov process*, i.e. the probability of the system to be in a certain state $\Omega_2$ at time $t_2$ is only dependent on the time $\Delta t$ elapsed since a second known state $\Omega_1$. $\Gamma_{\Omega}$ is a Markovian evolution operator given by the time evolution of $p (\Omega, t)$:

$$\frac{\partial}{\partial t} p (\Omega, t) = - \Gamma_{\Omega} p (\Omega, t).$$

(3.69)

Due to the stationary character of the Markov process, $\Gamma_{\Omega}$ describes a time independent parameter and the process’s unique equilibrium distribution $p_0 (\Omega)$ is given by

$$\Gamma_{\Omega} p_0 (\Omega) = 0.$$

(3.70)

In order to find a numerical solution, i.e. to facilitate the simulation of EPR spectra in the slow motional region, $\hat{\mathcal{H}} (\Omega)$ can be split in three parts

$$\hat{\mathcal{H}} (\Omega) = \hat{\mathcal{H}}_0 + \hat{\mathcal{H}}_1 (\Omega) + \epsilon (t),$$

(3.71)

where $\hat{\mathcal{H}}_0$ describes the Hamiltonian for the unperturbed state including the electron Zeeman, the nuclear Zeeman and the Hyperfine interaction (see section 3.1.2.5) in the high-field approximation, characterizing the isotropic contribution of the spin Hamiltonian. $\hat{\mathcal{H}}_1 (\Omega)$ on the other hand describes the rotationally dependent perturbation that is subject to the Euler angle orientations described by $\Omega$. Hence, $\hat{\mathcal{H}}_1 (\Omega)$ describes the anisotropic contribu-

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\[b\]Describes a random process whose future probabilities are determined by its most recent values.
3.1 Electron paramagnetic resonance

tion to $\hat{H}(\Omega)$. The effect of the applied alternating microwave field $\mathbf{B}_1$ is described by $\epsilon(t)$ [Freed 1976]

$$\epsilon(t) = \frac{1}{2} \gamma_e B_1 \left( \hat{S}_+ \exp(-i\omega_{mw} t) + \hat{S}_- \exp(i\omega_{mw} t) \right),$$

(3.72)

with $\hat{S}_+$ and $\hat{S}_-$ as creation and annihilation operators and $\omega_{mw}$ as frequency of the applied microwave field.

It has furthermore been shown (amongst others in [Freed 1976]) that for an ensemble average (designated by the superscript horizontal line) and for the average microwave power $P_i$ absorbed by the sample per cycle follows:

$$P = \frac{\partial}{\partial t} \epsilon(t) = \overline{\epsilon(t)} = Tr (\dot{\epsilon}(t) \rho).$$

(3.73)

Freed, Bruno and Polnaszek [Freed et al. 1971, Freed 1976] use this relation to calculate the EPR absorption spectrum by considering the deviation of a certain state from the equilibrium density matrix $\overline{\rho}$

$$(\overline{\rho} - \rho_0)_{\lambda_j} \equiv \chi_{\lambda_j},$$

(3.74)

with the steady-state solution

$$\chi_{\lambda_j} = \sum_{n=-\infty}^{\infty} \exp(in\omega t) Z_{\lambda_j}^{(n)},$$

(3.75)

and the time-independent coefficients of $n$th harmonic from the Fourier series used in equation 3.75

$$Z_{\lambda_j}^{(n)} = (Z_{\lambda_j}^{(n)})' + i (Z_{\lambda_j}^{(n)})''.$$

(3.76)

According to [Freed et al. 1971], the total absorption is then obtained as the equilibrium average of the single hyperfine line contributions over all orientations $\Omega$.

Eventually, the considered Brownian model of isotropic\(^1\) reorientational motion describes now a Markov process and can be expressed through the rotational diffusion equation [Freed et al. 1971]

$$\frac{\partial}{\partial t} p(\Omega, t) = R \nabla^2_{\Omega} p(\Omega, t),$$

(3.77)

and according to equation 3.69

$$\Gamma_{\Omega} = -R \nabla^2_{\Omega}.$$  

(3.78)

\(^1\)In the classical picture $P$ can be described by the macroscopic magnetization $\mathbf{M}$ on the basis of $P = -\mathbf{M} \frac{\partial \mathbf{B}}{\partial t}$.

\(^2\)Anisotropic rotational motions are neglectable here.
with $\nabla_2^2$ as rotational diffusion operator (also Laplacian operator) and $R$ as diffusion coefficient. In case of molecules exhibiting nearly spherical shape with radius $a$ in a liquid of viscosity $\eta$, $R$ can be approximated via a rotational Stokes-Einstein relationship:

$$R = \frac{kT}{8\pi a^3 \eta}. \tag{3.79}$$

3.1.4.2 Dipfit

In order to interpret cw EPR spectra in the fast motional region or at the rigid limit that are not characterized by any inhomogeneous broadening effects, a superposition of the single Lorentzian lines supplies the need, as already mentioned in the previous section. Contrary to powder spectra analysis, the simulation of the motionally narrowed counterparts as obtainable in the fast motional regime are not to be discussed here due to their unimportance in this study. Although the basis used before also establishes a way to simulate powder spectra, the solution developed by H.-J. Steinhoff and coworkers [Steinhoff et al. 1997] including the determination of inter-spin distances of two or more nitroxide spin labels attached to molecules was used here. Only the theory of this method will be explained.

Equation 3.32 showed that the dipolar interaction present in an EPR spectrum is proportional to $\frac{1}{r_{12}^3}$ ($r_{12}$: distance between the two unpaired electrons). In contrast to Heisenberg exchange interaction between two unpaired electrons that is visible in a room temperature cw EPR spectrum, the effect of dipolar interactions under those conditions is not recognizable due to the effect of motional averaging. Since diffusive residual motions are strongly restricted at temperatures below $200 \, K$, samples are frozen to this temperature region for interpin-distance determination measurements. There, proteins exhibit glass-like features, rotational correlation times of the nitroxide attached to a protein specific site exceed 100 ns [Steinhoff et al. 1989], and vibrational motions averging out the $g$ and $A$ anisotropies are of neglectable extent. The cw EPR spectra are then composed of the lines from species with different relative nitroxide orientations with respect to the external magnetic field. Figure 3.10 shows the distinctive EPR spectra characterized by particular $g_{ii}$ and $A_{ii}$ values in the monocrystal\textsuperscript{k} case for different orientations of the $B_0$-field in the nitroxide’s coordinate system. The indices $ii$ can easily be understood by application of the respective angles $\vartheta$ and $\varphi$ describing the actually considered system in equations 3.40 and 3.41.

In order to obtain now the particular resonance lines according to equations 3.42a to 3.42c for all nitroxides statistically distributed in space, a spherical surface is divided into intervals $d\vartheta$ and $d\varphi$ (for angle definition see figure 3.4), each comprising a $3^\circ$ sector for both $\vartheta$ and $\varphi$ [Steinhoff et al. 1997]. Due to symmetry reasons, the consideration of one octant is sufficient to describe the whole nitroxide distribution. The three resonance lines are calculated for each interval. Since the area of an interval near the equator (see figure 3.4)

\textsuperscript{k} All units in a monocrystal are oriented in the same way.
3.1 Electron paramagnetic resonance

Figure 3.10: Orientation dependence of monocrystal EPR spectra for $B_0$-fields aligned along the axes (x,y,z) of the nitroxide’s coordinate system (see figure 3.4) as designated at the high field end of the spectra. Specific g- and hyperfine values characterizing the anisotropy of the nitroxide radical spectra were taken from [Galla 1988].

is larger than that of an interval near the pole (see figure 3.4) the number of molecules pointing with their z molecular axis into the former one is much bigger. Since furthermore the intensity of the resulting powder spectrum is dependent on this number of molecules, the single intensities calculated for the various intervals have to be weighted according to their occurrence, i.e. with $\sin \vartheta$. The addition of all lines obtained for an interval $\Delta B$ leads to the resonance line intensity distribution, namely the stick spectrum (e.g. see figure 3.11).

To account for line-broadening mechanisms in the simulated EPR spectrum, the shown stick spectrum has to be convoluted with a line broadening function $f(B - \overline{B})$ composed of a superposition of a Gaussian (including inhomogeneous effects) and a Lorentzian (including homogeneous effects), a \textit{Pseudo-Voigt profile}:

$$f(B - \overline{B}) = p_L \cdot f_L + (1 - p_L) \cdot f_G,$$

$$= p_L \cdot \frac{1}{1 + \sigma_L (B - \overline{B})^2} + (1 - p_L) \cdot \exp \left( -\frac{(B - \overline{B})^2}{\sigma_G} \right),$$

with $B$ describing the $B_0$-field value and $\overline{B}$ the expectation value of the distribution, $p_L$ as portion of the Lorentzian compared to the whole distribution, $f_L$ and $f_G$ as Lorentz- and Gauss-functions, respectively, with areas normalized to unity. The line widths of the two functions are described by $\sigma_L$ and $\sigma_G$. 
In the case an EPR spectrum without dipolar broadening effects has to be simulated, the experimental spectrum can be fitted with a convolution of the stick spectrum and the line broadening function. In this fitting procedure the initially chosen values for the parameters of the $g$- and $A$-tensor ($g_{xx}$, $g_{yy}$, $g_{zz}$, $A_{xx}$, $A_{yy}$, $A_{zz}$) as well as the Gaussian and the Lorentzian line width and the fraction of the Lorentzian are varied iteratively until the root mean square function

$$Q = \sum_B (F(B_0) - Y(B_0))^2$$

(3.82)

reaches its minimum. Since $F(B_0)$ describes the single signal amplitude at the corresponding magnetic field value $B_0$ and $Y(B_0)$ describes the amplitude values of the simulated spectrum, minimization of $Q$ is synonymous with the minimization of the squared difference between the experimental and the simulated spectrum.

However, in the case of dipolar interactions present, this approach will not yield adequate results. An additional broadening function, the dipolar line shape function, has to be convoluted with the non-interacting spectrum. This function is based on the fact that the resonance lines of a pair of spins are shifted by $\Delta B$ from the non-interacting B-field positions when dipole-dipole interactions are present between them. Since the extent of the dipolar interaction and thereby the extent of the $\Delta B$ shift is, besides its distance dependence, strongly dependent on the orientations of the considered spins with respect to the external magnetic field, first, a qualitative description of this phenomenon in terms of the pake pattern is done.

Starting from a two-spin system in the externally applied magnetic field $B_0$, we know that spin up and spin down states are allowed. Considering furthermore the first spin $S_1$ located in the center of a sphere aligned along the $B_0$-field direction, and the second
one $S_2$ on the sphere’s equator aligned either parallel or anti-parallel to $B_0$ (see black arrows in figure 3.12). Their spatial proximity can be described via the distance vector $r$ connecting them. Top right of figure 3.12 both spins are aligned parallel to $B_0$, whereby the magnetic field of spin $S_2$ at $S_1$ is oppositely oriented to $B_0$. $S_1$’s experienced magnetic field and consequently the energy level splitting is decreased, i.e. excitation is shifted to higher external magnetic field values. Contrary, the presence of a spin species $S_3$ in the same picture aligned parallel to $B_0$ but located at the pole position of the sphere leads to the shift of the spin excitation to lower $B_0$ values. This is the logical consequence since the magnetic fields oriented in the same way add up and the corresponding magnetic field experienced by $S_1$ increases. Equal distribution of several spins $S_i$ on the sphere’s surface then results in the magnetic field shift distribution shown down right in figure 3.12. The striking fact that most of the transitions in such a frame take place at higher magnetic fields can easily be understood through the sphere’s nature, since under the assumption of a statistical distribution of all spins present (except the one in the center) much more spins are located at the equator than at the poles.

Analogous to the just explained distribution, the inversion of the spin orientations on the sphere’s surface leads to the inversion of the transition distribution as shown down left in figure 3.12.

Superposition of the two distributions eventually yields the Pake pattern shown in the center of the lower panel of figure 3.12.

![Figure 3.12: Clarification of Pake pattern. Top) Alignment of spins in the externally applied magnetic field $B_0$ and indicated dipolar interactions between them. Bottom) Shifts of the resonance lines according to the cases shown above and the resulting Pake pattern (center) as superposition of the two cases [HOLT 2003].](image)
The mathematical derivation of the B-field shift $\Delta B$ starts from the spin Hamiltonian describing the fine structure. From the total spin Hamiltonian in the molecular coordinates derived earlier (see equation 3.34) only the contributions from the electron Zeeman, the exchange and the dipole-dipole interaction have to be accounted for:

$$\hat{H} = \hat{H}_{\text{EZ}} + \hat{H}_{\text{EXCH}} + \hat{H}_{\text{DD}},$$

$$= \frac{\beta_e}{\hbar} g_{0} \hat{S}_i + J \hat{S}_1 \hat{S}_2 + \hat{S} \hat{D} \hat{S},$$

it follows:

$$\hat{H} = \frac{\beta_e}{\hbar} (B_x, B_y, B_z) \cdot \begin{pmatrix} g_{xx} & 0 & 0 \\ 0 & g_{yy} & 0 \\ 0 & 0 & g_{zz} \end{pmatrix} \cdot \begin{pmatrix} \hat{S}_x \\ \hat{S}_y \\ \hat{S}_z \end{pmatrix}$$

$$+ J \left( \hat{S}_{1x}, \hat{S}_{1y}, \hat{S}_{1z} \right) \cdot \begin{pmatrix} \hat{S}_{2x} \\ \hat{S}_{2y} \\ \hat{S}_{2z} \end{pmatrix}$$

$$+ \left( \hat{S}_x, \hat{S}_y, \hat{S}_z \right) \cdot \begin{pmatrix} D_{xx} & 0 & 0 \\ 0 & D_{yy} & 0 \\ 0 & 0 & D_{zz} \end{pmatrix} \cdot \begin{pmatrix} \hat{S}_x \\ \hat{S}_y \\ \hat{S}_z \end{pmatrix}$$

As shown in equation 3.32 the trace of the dipole-dipole tensor $D$ is zero. It is therefore convenient to rewrite $D$ only in the dependence of two parameters $D$ and $E$ [Schweiger and Jeschke 2001]:

$$\hat{H} = \frac{\beta_e}{\hbar} (B_x, B_y, B_z) \cdot \begin{pmatrix} g_{xx} & 0 & 0 \\ 0 & g_{yy} & 0 \\ 0 & 0 & g_{zz} \end{pmatrix} \cdot \begin{pmatrix} \hat{S}_x \\ \hat{S}_y \\ \hat{S}_z \end{pmatrix}$$

$$+ J \left( \hat{S}_{1x}, \hat{S}_{1y}, \hat{S}_{1z} \right) \cdot \begin{pmatrix} \hat{S}_{2x} \\ \hat{S}_{2y} \\ \hat{S}_{2z} \end{pmatrix}$$

$$+ \left( \hat{S}_x, \hat{S}_y, \hat{S}_z \right) \cdot \begin{pmatrix} -\frac{1}{3}D + E & 0 & 0 \\ 0 & -\frac{1}{3}D - E & 0 \\ 0 & 0 & \frac{2}{3}D \end{pmatrix} \cdot \begin{pmatrix} \hat{S}_x \\ \hat{S}_y \\ \hat{S}_z \end{pmatrix}$$
Using equation 3.35 with the rotary matrix \( L \) given in equation 3.37 and the analogous transformation for the dipole-dipole term, the components of the two tensors and hence the Hamiltonian can be expressed in the laboratory system \((x^L, y^L, z^L)\) with the magnetic field \( B \) oriented in the \( z^L \)-axis direction via [CIECERSKA-TWOREK et al. 1973]:

\[
\hat{H}^L = B_x \hat{S}_x^L + B_y \hat{S}_y^L + B_z \hat{S}_z^L + J \left( \hat{S}_{1x}^L \hat{S}_{2x}^L + \hat{S}_{1y}^L \hat{S}_{2y}^L + \hat{S}_{1z}^L \hat{S}_{2z}^L \right)
\]

\[
+ D_{xx}^L \hat{S}_x^L + D_{yy}^L \hat{S}_y^L + D_{zz}^L \hat{S}_z^L + D_{xy}^L \left( \hat{S}_x^L \hat{S}_y^L + \hat{S}_y^L \hat{S}_x^L \right)
\]

\[
+ D_{xz}^L \left( \hat{S}_x^L \hat{S}_z^L + \hat{S}_z^L \hat{S}_x^L \right) + D_{yz}^L \left( \hat{S}_y^L \hat{S}_z^L + \hat{S}_z^L \hat{S}_y^L \right),
\]

\[ (3.87) \]

with

\[
B_x = \frac{\beta_e}{\hbar} g_{xx}^L B_0,
\]

\[
B_y = \frac{\beta_e}{\hbar} g_{yy}^L B_0,
\]

\[
B_z = \frac{\beta_e}{\hbar} g_{zz}^L B_0,
\]

\[ (3.88) \]

\[
g_{xx}^L = (g_{xx} \cos^2 \varphi + g_{yy} \sin^2 \varphi - g_{zz}) \sin \vartheta \cos \vartheta,
\]

\[
g_{yy}^L = (-g_{xx} + g_{yy}) \sin \vartheta \sin \varphi \cos \varphi,
\]

\[
g_{zz}^L = (g_{xx} \cos^2 \varphi + g_{yy} \sin^2 \varphi) \sin^2 \vartheta + g_{zz} \cos^2 \vartheta,
\]

\[ (3.89) \]

\[
D_{xx}^L = \frac{1}{3} D \left( 3 \sin^2 \vartheta - 1 \right) + E \cos^2 \vartheta \cos (2\varphi),
\]

\[
D_{yy}^L = -\frac{1}{3} D - E \cos (2\varphi),
\]

\[
D_{zz}^L = \frac{1}{3} D \left( 3 \cos^2 \vartheta - 1 \right) + E \sin^2 \vartheta \cos (2\varphi),
\]

\[ (3.90) \]

\[
D_{xy}^L = -E \cos \vartheta \sin (2\varphi),
\]

\[
D_{xz}^L = (-D + E \cos (2\varphi)) \sin \vartheta \cos \vartheta
\]

\[
D_{yz}^L = -E \sin \vartheta \sin (2\varphi).
\]

\[ (3.91) \]
In the approximate solution using first order perturbation theory according to [Cieciierska-Tworek et al. 1973], the energy levels resulting from this Hamiltonian are the following:

\[
E_{+1} = B_z + J_4 + \frac{1}{2} (D_{xx}^L + D_{yy}^L) + D_{zz}^L,
\]

\[
E_0 = B_z + J_4 + D_{xx}^L + D_{yy}^L,
\]

\[
E_{-1} = -B_z + J_4 + \frac{1}{2} (D_{xx}^L + D_{yy}^L) + D_{zz}^L,
\]

and consequently two allowed transitions in this system are separated by

\[
d \equiv (E_{+1} - E_0) - (E_0 - E_{-1}),
\]

\[
= 2D_{zz}^L - D_{xx}^L - D_{yy}^L,
\]

\[
= 3D_{zz}^L - (D_{xx}^L + D_{yy}^L + D_{zz}^L),
\]

\[
= 3D_{zz}^L.
\]

According to the set of equations given in 3.90, it follows

\[
d(\vartheta, \varphi) = D \left(3 \cos^2 \vartheta - 1\right) + E \left(3 \sin^2 \vartheta \cos(2\varphi)\right),
\]

and assuming that \( D \gg E \) maintains only the first term on the right side

\[
d(\vartheta, \varphi) = D \left(3 \cos^2 \vartheta - 1\right).
\]

Since the center of the two transitions is given by \( h\omega = g(\vartheta, \varphi)\beta_e B_0 \), with \( g(\vartheta, \varphi) \) already introduced in equation 3.40, the splitting according to the dipolar interaction is given by

\[
h\omega = g(\vartheta, \varphi)\beta_e B_0 \pm \frac{d(\vartheta, \varphi)}{2}.
\]

In terms of the \( B \)-field deviation and using equation 3.32 finally leads to

\[
\Delta B = \frac{\mu_0}{4\pi} \frac{3g\beta_e}{4r_1^2} \left(3 \cos^2 \vartheta - 1\right).
\]

To transfer these calculations to a dipolar broadened powder spectrum, the dipolar line shape function described by \( \Delta B \) is calculated using increments of 3° for \( \vartheta \), the angle between the magnetic field \( B_0 \) and the distance vector \( \mathbf{r} \). Since in a frozen solution the orientation of \( \mathbf{r} \) may be assumed to be isotropically distributed, the line intensities in each interval are once more weighted with \( \sin \vartheta \). The resulting distribution looks like the final one of the Pake pattern explained in the context of figure 3.12. However, since the spin labeled proteins as well as the spin label side chains investigated are not assumed to be rigid but
are regarded as objects exhibiting a distribution of conformations, a Gaussian distribution of the distances with a mean distance $r$ and a standard deviation $\sigma$ is furthermore applied [Steinhoff et al. 1997]. A broadened dipolar line shape function and analogous a broadened Pake pattern result.

A convolution of the non-interacting spectrum (stick spectrum convoluted with line broadening function) with the broadened dipolar line shape function finally yields the cw EPR powder spectrum exhibiting dipolar broadening due to dipole-dipole interactions between the sterically proximal electron spins.

It is worth mentioning that this method should not be used to estimate inter-spin distances where Heisenberg exchange coupling is comparable to or larger than the dipole-dipole coupling. This applies for inter-spin distances shorter than 0.8 nm.

3.1.5 Double resonance in pulse EPR

Beyond the various applications of cw EPR and the outcoming information sources, additional knowledge about the system under investigation can be obtained by correlating different transitions of one spin species. Such electron-electron double resonance (ELDOR) experiments can be achieved by the application of microwave pulses instead of a continuous microwave irradiation. Sequences of mw pulses are applied to the spin system and the following time-evolution is observed. In this study most notably the pulse ELDOR method of four-pulse double electron electron resonance (4-pulse DEER) will be described in the following paragraphs in terms of inter-spin distance determination in the range from 1.5 to 8 nm. Here, in order to excite all considered spins of two different EPR transitions, two microwave pulses of different frequency are applied to irradiate the two transitions\(^1\). As in the cw EPR section, the strength of the dipolar coupling is determined to obtain the separation of the involved unpaired electrons.

Although hyperfine couplings between nuclear and electron spins can be specified by one of the methods that will be described in the course of ELDOR applications, it is more convenient to determine the hyperfine interactions in terms of pulse electron nuclear double resonance (ENDOR) yielding increased resolution. Here, nuclear magnetic resonance (NMR) transitions are monitored in an EPR experiment to combine spectral simplification with high resolution. The spectral simplification originates from the fact that in considering nuclear transitions (with $n_n$ as number of allowed transitions, $I_k$ as spin quan-

\(^1\)A single mw pulse possessing a bandwidth large enough to bridge the frequency gap between the two transitions is technically not realizable since the shortest pulse possible is limited by the quality factor $Q$ (see section 4.2.2) of the resonator.
tum number and \( n \) as number of coupled nuclear spins) the number of allowed transitions only increases additively, i.e.

\[
n_n = 2(2S + 1) \sum_{k=1}^{n} I_k,
\]

(3.97)

since each nuclear spin is assumed to significantly couple to solely one electron of spin \( S \). Contrarily leads the observation of electron spin transitions to a multiplicative increase of transitions

\[
n_e = \prod_{k=1}^{n} (2I_k + 1)
\]

(3.98)

that are visible in the EPR spectrum, since one electron is considered to significantly couple to several nuclei.

As is shown in the following sections, one of the biggest advantages in pulse methods is the fact that relaxation times can be determined directly unlike in cw techniques.

3.1.5.1 Pulse ELDOR

In order to describe the final pulse sequence necessary for inter-spin distance determination, a few fundamental aspects are to be introduced here.

The macroscopic magnetization \( \mathbf{M} \) described in detail in sections 3.1.3.1 and 3.1.3.2 has to be regarded to understand the effect of a mw pulse on the electron spin system under investigation. Considering a resonant \((\Omega_s = 0)\) microwave irradiation applied along the rotating-frame x-axis and a pulse length \( t_p \) created by turning the field on at time \( t = 0 \) and off at time \( t_p \), \( \mathbf{M} \) precesses about the x-axis \((\theta = 90^\circ \) in figure 3.5\) during this time. When relaxation is assumed to be negligible, integration of equations 3.51a to 3.51c for a system at thermal equilibrium \((M_x = M_y = 0, M_z = M_0)\) at time \( t = 0 \) leads to the following components at time \( t_p \):

\[
M_x = 0,
\]

(3.99)

\[
M_y = -M_0 \sin(\omega_1 t_p),
\]

(3.100)

\[
M_z = M_0 \cos(\omega_1 t_p),
\]

(3.101)

with \( \omega_1 t_p = \beta \) describing the flip angle about the x-axis. Due to the fact that furthermore a phase shift of a linearly polarized mw field by an angle \( \phi \) changes its rotating frame direction by \( \phi \) [SCHWEIGER and JESCHKE 2001], any rotation of the macroscopic magnetization can be carried out by use of the appropriate mw pulse phase and time when the system is in resonance. In a good approximation this is also valid for small resonance offsets \( \Omega_s \ll \)
\( \omega_1 \). This is a quite important fact, since \( \mathbf{M} \) generally can not be regarded as indivisible magnetic moment, but rather as an ensemble of spins that all behave independently. The spins have slightly different, fluctuating resonance offsets \( \Omega_s \), as they are e.g. interacting differently with their particular lattices. The resulting phenomenon is known as \textit{fan-out} of the transverse magnetization in the x-y-plane (see figure 3.13).

![Figure 3.13: Macroscopic magnetization averaging by virtue of its fan-out in the x-y-plane of the rotating frame. Left: Directly after a \( \beta = \frac{\pi}{2} \) pulse along the x-axis (\( t_p \ll T_1, T_2 \)) the magnetization vector \( \mathbf{M} \) is aligned along the y-axis. Right: Immediately after the situation shown on the left, \( \mathbf{M} \) starts to precess about the z-axis (\( B_0 \)) with the frequency \( \Omega_s \). Here, also the defocusing/dephasing of the transverse magnetization starts (highlighted by the dark gray to white fan) due to the different resonance offsets present. According to their \( \Omega_s \) values, the spin species fan out clockwise and counterclockwise (indicated by the bended arrows), respectively. The resulting magnetization \( \mathbf{M} \) diminished with respect to the former situation.

The detectable magnetization vectors are described by the averages of all spin contributions. Observing the spin system under these conditions and taking into account relaxation effects, the rotating frame Bloch equations 3.56a to 3.56c for the application of a single pulse yield the following transverse magnetization components:

\[
M_x(t) = M_0 \sin \beta \sin (\Omega_s t) \exp \left( -\frac{t}{T_2} \right),
\]

\[
M_y(t) = -M_0 \sin \beta \cos (\Omega_s t) \exp \left( -\frac{t}{T_2} \right).
\]

A \( \frac{\pi}{2} \)-pulse can still flip \( \mathbf{M} \) to the x-y-plane, but relaxational losses will already occur during the application of the pulse. The experiment can be repeated as soon as \( \mathbf{M} \) has reached the equilibrium state.
Since the detection of the signals in pulse EPR measurements is performed using a “quadrature-detection scheme”, the signal $V(t)$ can be expressed in complex numbers proportional to $M_y - iM_x$, i.e. both $M_x$ and $M_y$ can be detected separately and simultaneously. The resulting signal is known as free induction decay (FID):

$$V(t) = \exp(i\Omega_s t) \exp \left( -\frac{t}{T_2} \right).$$  \hfill (3.104)

The flip angle $\beta$, the equilibrium magnetization $M_0$, the resonance offsets $\Omega_s$ and, due to the knowledge of $\omega_{mw}$ and $\Omega_s$, the resonance frequency $\omega_s$ can be derived.

Since the signal detected describes a time-domain signal, a Fourier transformation (FT) has to be applied in order to generate a frequency-domain signal allowing the characterization of the interactions under investigation in a more convenient way. The complex character of the signal $V(t)$ requires the application of the complex Fourier transformation to obtain the frequency-domain signal $S(\omega)$:

$$S(\omega) = \int_0^\infty V(t) \exp(-i\omega t) dt.$$  \hfill (3.105)

The resulting real and imaginary parts eventually yield an absorption and a dispersion signal, respectively, where the absorption signal describes a Lorentzian centered at $\nu = \Omega_s / 2\pi$ with a FWHM characterized by the transverse relaxation time $T_2$.

**Electron spin echo**

In order to describe the nature of an electron spin echo (ESE), the elementary Hahn echo experiment [Hahn 1950] provides an adequate starting point. Top of figure 3.14 the corresponding pulse sequence ($\pi/2 - \tau^* - \pi - \tau^* - \text{echo}$) is depicted, whereas in the lower part the time evolution of the magnetization vector $\mathbf{M}$ is shown.

Before the pulse sequence is applied to the system, it is assumed to be in thermal equilibrium, i.e. the equilibrium magnetization $M_0$ is aligned along the z-axis. Immediately after the first ($\pi/2$) pulse along the x-axis, all magnetization vectors ($\mathbf{M}$) are aligned along the y-axis. The dephasing starts. In figure 3.14 an equal distribution of the magnetization components is assumed. After the time $\tau^*$ has passed, the situation is reversed by the application of a $\pi$-pulse ($\beta = 180^\circ$) along the x-axis, i.e. the single magnetization vectors are mirrored with respect to the x-axis. Since the circulation direction of the magnetization vectors despite the applied $180^\circ$-pulse remains the same, consequently a fan-in takes place. All components are combined again aligned along the -y-axis after the time $\tau^*$ has elapsed. This situation already describes the maximum of the echo. The rise and fall of the

---

mA phase-sensitive detector mixes the output of the resonator with a reference signal from the microwave source, and yields the frequency component $\Omega_s$. Applying two reference signals phase shifted by 90°, both transverse magnetization components can be measured at once.
3.1 Electron paramagnetic resonance

Figure 3.14: Hahn echo experiment. Upper part: $\pi/2 - \tau' - \pi - \tau' - \text{echo}$ pulse sequence with the FID (decaying during dead time $t_d$) depicted after the first pulse. The echo is shown for the sequence described below (solid line, negative amplitude) and for a 90° phase-shifted $\pi$-pulse (dotted line, positive amplitude) Lower part: Time evolution of the macroscopic magnetization $M$ and the corresponding spin species (fan-out) in the rotating frame. The course of the time evolution is indicated by the arrows carrying the information of the action performed between the respective frames. No relaxational effects are taken into account.

Echo amplitude are defined by a distinct time window that is dependent on the frequency distribution of the magnetization vectors, and during that the rephasing before and the dephasing after the maximum echo situation contribute to the signal. Therefore, a spin echo can be regarded as the composition of two FIDs being assembled back-to-back.

It is worth mentioning that the negative amplitude (solid line) of the echo described in figure 3.14 can be inverted to be positive (dotted line) since an adequate phase shift of the applied $\pi_x$-pulse results in a $\pi_y$-pulse that leads to a rephasing of the magnetization vectors along the +x-axis.

The time evolution of the echo intensity $E$ of such a Hahn echo sequence is characterized by a decrease with the so-called phase memory time $T_m$, the time constant that comprises all effects causing echo dephasing, with the transverse relaxation time $T_2$ as one of its con-
tributors [Jeschke 1998, Schweiger and Jeschke 2001]. In case of a mono-exponential decay, \( T_m \) equals \( T_2 \). For the determination of \( T_m \), the Hahn sequence with increasing interpulse delay times \( \tau^* = \tau^*_0 + n \cdot \tau^*_{\text{step}} \) (with \( n \) describing real numbers) can be applied. The resulting course of the echo intensity can then be fitted to the function:

\[
E(\tau^*) = E_0 \exp \left( -\frac{2\tau^*}{T_m} \right),
\]

where \( E_0 \) describes the maximum echo intensity. In case several paramagnetic species are present in the sample under investigation as well as for the presence of additional relaxation processes (e.g. due to interaction of spins with different frequencies), the echo intensity decrease can no longer be described by a single exponential [Rowan et al. 1965].

**Two-pulse ESEEM**

Beyond the non-mono-exponential behavior of the echo intensity decrease mentioned, it has been found [Rowan et al. 1965] that a modulation exhibiting frequencies of the coupled nuclear spins is superimposed to the exponential decay. Thus, the Hahn sequence also facilitates the measurement of hyperfine interactions. The two-pulse electron spin echo envelope modulation (ESEEM) uses the Hahn-sequence with a less selective second (\( \pi \)) pulse for this purpose.

To analyze the coupled nuclei’s influence on the coherence observed in our experiments, the spin Hamiltonian (see section 3.1.2.5) can be reduced to the electron Zeeman, the nuclear Zeeman and the hyperfine interaction terms [Kurreck et al. 1988] to appropriately describe a system of one electron and one nucleus with spins \( S = \frac{1}{2} \) and \( I = \frac{1}{2} \), respectively. In case of only isotropic interactions present, and with the external magnetic field \( B_0 \) aligned along the z-axis, we obtain:

\[
\hat{H}_S = \hat{H}_{\text{EZ}} + \hat{H}_{\text{NZ}} + \hat{H}_{\text{HF}},
\]

\[
= \frac{\beta_e}{\hbar} g_e B_0 \hat{S}_z - \frac{\beta_n}{\hbar} g_n B_0 \hat{I}_z + a_{\text{iso}} \hat{S} \hat{I}.
\]

In the high-field approximation, where the hyperfine interaction is much smaller than the electron Zeeman interaction, the energy levels for the considered system are described by

\[
E_{m_s, m_I} = m_s \beta_e g_e B_0 - m_I \beta_n g_n B_0 + a_{\text{iso}} m_s m_I,
\]

where \( m_s \) and \( m_I \) denote the respective spin quantum numbers with \( m_s, m_I = \pm \frac{1}{2} \).

---

\*\*An important feature of the pulse is its excitation bandwidth, thus its length and power. A distinction is drawn between selective (minor microwave power and major length \( \rightarrow \) low excitation bandwidth) and non-selective (major microwave power and minor length \( \rightarrow \) high excitation bandwidth) pulses.

\*\*I = \( \frac{1}{2} \) is used in the following calculations due to their relevance in the pulsed ENDOR measurements described in section 3.1.5.2 that were performed using a \( ^{15}N \) MTSSL variant.
With the electron and nuclear Zeeman terms in angular frequency units, $\omega_s = \beta_e g_e B_0 / \hbar$ and $\omega_I = \beta_n g_n B_0 / \hbar$, respectively, the four resulting energy levels are

$$\begin{align*}
E_1 / \hbar &= \frac{1}{2} \omega_s + \frac{1}{2} \omega_I - \frac{1}{4} a_{iso}, \\
E_2 / \hbar &= \frac{1}{2} \omega_s - \frac{1}{2} \omega_I + \frac{1}{4} a_{iso}, \\
E_3 / \hbar &= -\frac{1}{2} \omega_s + \frac{1}{2} \omega_I + \frac{1}{4} a_{iso}, \\
E_4 / \hbar &= -\frac{1}{2} \omega_s - \frac{1}{2} \omega_I - \frac{1}{4} a_{iso}.
\end{align*}$$

Figure 3.15 shows the resulting energy level splitting, with the four energy levels labeled by the product functions $|m_s, m_I\rangle$ with the symbols $|\alpha\rangle$ and $|\beta\rangle$ describing the signs of the functions $|+\rangle$ and $|-\rangle$, respectively. Note that here $g_n, a_{iso} > 0$ and the case of weak coupling with $\omega_I > |a_{iso}/2|$ is assumed. The invalidity of one of these conditions would lead to a changed energy level diagram.

\begin{figure}[h]
\centering
\includegraphics[width=0.6\textwidth]{figure3.15.png}
\caption{Energy level diagram including the electron Zeeman, the nuclear Zeeman and the hyperfine splittings. The energy levels are designated by the respective spin quantum numbers $m_s$ and $m_I$. Free electron ($\omega_s$) and free nuclear ($\omega_I$) angular Lamor frequencies are indicated for the isotropic $g$-factor of the nucleus $g_n > 0$. The isotropic hyperfine coupling constant $a_{iso}$ is positive and we assume the case of weak coupling $\omega_I > |a_{iso}/2|$. The two possible EPR transitions are presented by solid lines and the NMR counterparts by dotted lines. The four energy levels are labeled by $|m_s, m_I\rangle$ and numbers (1, 2, 3, 4) to simplify identification of the corresponding energies given in equations 3.110 to 3.113.}
\end{figure}
With the selection rules for electron spin transitions ($\Delta m_s = \pm 1, \Delta m_I = 0$), the two allowed EPR transitions ($1 \rightarrow 3$ and $2 \rightarrow 4$ in figure 3.15) are characterized by the resonance angular frequencies:

$$\omega_{epr} = \omega_s \pm \frac{a_{iso}}{2}. \quad (3.114)$$

Analogously, the angular frequencies for the two nuclear spin transitions ($1 \rightarrow 2$ and $3 \rightarrow 4$ in figure 3.15) can be obtained by the application of the corresponding selection rules ($\Delta m_I = \pm 1, \Delta m_s = 0$):

$$\omega_{nmr} = \left| \omega_I \pm \frac{a_{iso}}{2} \right|. \quad (3.115)$$

However, as can be seen from the upper part of figure 3.16, the simple knowledge of the determined angular frequencies does not allow to calculate any nuclear angular frequencies from such an EPR experiment. Indeed, the above derived case only holds true in the high-field approximation where the nuclei do not exhibit too large hyperfine couplings. In X-band for solid state samples, second-order effects have to be regarded in the anisotropic hyperfine term e.g. for coupled protons. The field at the nucleus and thus the NMR angular frequencies change according to

$$\omega_{12} = \omega_\alpha = \sqrt{\left(\omega_I + \frac{A}{2}\right)^2 + \left(\frac{B}{2}\right)^2}, \quad (3.116)$$

$$\omega_{34} = \omega_\beta = \sqrt{\left(\omega_I - \frac{A}{2}\right)^2 + \left(\frac{B}{2}\right)^2}, \quad (3.117)$$

with $A$ and $B$ in the rotating frame [Schweiger and Jeschke 2001]:

$$A = a_{iso} + (3 \cos^2 \vartheta - 1) A_{dip}, \quad (3.118)$$

$$B = 3 A_{dip} \sin \vartheta \cos \vartheta, \quad (3.119)$$

with $A_{dip}$ already introduced in equation 3.24.

The second-order effect on the nuclear transition angular frequencies affects the EPR transitions in that way that the transition probabilities of the allowed transitions $\omega_{13}$ and $\omega_{24}$ (see figure 3.16, upper part) decrease slightly and the ones of the forbidden transitions $\omega_{14}$ and $\omega_{23}$ become allowed with a minor transition probability [Jeschke 1998, Schweiger and Jeschke 2001]. The height of the solid vertical lines in the lower part of figure 3.16 indicates now the transition probabilities for the particular EPR transition. Most notably does this effect eventually enable us to calculate the NMR angular frequencies given in equations 3.116 and 3.117 by observing the EPR transitions (see figure 3.16). Hence, a hyperfine splitting $a' = |\omega_{12} - \omega_{34}|$ and a corrected nuclear Zeeman frequency $\omega'_I = |\omega_{12} + \omega_{34}|/2$ can be calculated.
3.1 Electron paramagnetic resonance

Figure 3.16: Schematic representation of the allowed (solid vertical lines) and forbidden (dashed vertical lines) EPR transitions in the system \( S = \frac{1}{2}, I = \frac{1}{2} \) under investigation. The EPR angular frequencies are designated by the numbers (see figure 3.15) of the energy levels involved in the respective transition. Upper part: In case of isotropic hyperfine interaction \( a_{iso} \), the EPR transitions for \( \Delta m_s = 1 \) and \( \Delta m_I = 1 \) are strictly forbidden. The NMR angular frequency \( \omega_I \) can not be obtained in an EPR experiment. Lower part: Second-order effect on the EPR transition probabilities and angular frequencies due to anisotropic hyperfine contributions present in the solid state for the considered system. The previous (upper part) forbidden EPR transitions become allowed with minor transition probabilities. Second-order effected nuclear angular frequencies \( \omega_\alpha \) and \( \omega_\beta \) can be determined via the measurement of the corresponding EPR transitions.

In this study the two-pulse ESEEM scheme was solely used to determine the phase memory time \( T_m \) in order to optimize the setup for the four-pulse DEER experiment. The distance determination to coupled nuclei on the basis of pulse ENDOR measurements is described in section 3.1.5.2. Therefore, the second-order effect is not described in detail here. A complete derivation can amongst others be found in [SCHWEIGER and JESCHKE 2001].

Echo detected field sweep

A field-swept electron spin echo (FS ESE) is the pulse analogon to a conventional cw EPR spectrum. It was developed for EPR spectra with very broad lines, since no modulation has to be used in pulse methods and thus the absorption signal is measured directly. Due to the fact that the derivative of a very broad absorption line would be very small, the sensitivity for this kind of lines in cw EPR is much lower.

For the FS ESE experiment once more the Hahn sequence (see figure 3.14) is used. But instead of incrementing the inter-pulse delay stepwise, \( \tau^* \) is fixed, while the external magnetic field \( B_0 \) is swept through the whole spectrum. To obtain the absorption spectrum, the echo intensity has to be integrated for each magnetic field step unlike in a two-pulse ESEEM experiment, where solely the maximum echo intensities are of interest. From the
resulting field-swept EPR spectrum, the $B_0$-field value exhibiting the maximum intensity can be obtained and used further for the optimization of the four-pulse DEER experiment.

Beyond the simple detection of the absorption spectrum, a FS ESE can give insight into the existence of more than one spin species with different $T_m$ since FS ESEs measured with different inter-pulse delay times would differ in shape. On the other hand, can the same effect be achieved by changing the time between the single echo integrations. Short repetition times might saturate one of the species present and therefore also change the absorption spectrum.

**Inversion recovery**

An inversion recovery experiment enables the determination of the longitudinal relaxation time $T_1$ by virtue of the disequilibration of the spin polarization and the observation of its recovery back to equilibrium. The pulse sequence ($\pi - T - \frac{\pi}{2} - \tau^* - \pi - T - \tau^* - \text{echo}$) is depicted in the upper part of figure 3.17.

![Inversion Recovery Experiment](image)

**Figure 3.17:** Schematic representation of an inversion recovery experiment. Upper part: Pulse sequence showing a negative echo. Lower part: Time evolution of an echo signal’s intensity for a longitudinal relaxation time $T_1 = 20\mu s$ (the lower part is taken from [Jeschke 1998]).

A properly adjusted first $\pi$-pulse should lead the system to the case of complete polarization inversion (negative echo with maximum amplitude). During the following evolution time $T$, the inversed magnetization recovers back towards the equilibrium state that will be reached after the longitudinal relaxation time $T_1$ has elapsed. The extent of the recovery can be monitored by the subsequent application of the Hahn sequence. In order to obtain the time evolution of the echo signal recovery, the time $T$ is incremented stepwise ($T = T_0 + n \cdot T_{\text{step}}$) since with increasing delay time between the first ($\pi$) and the second
Electron paramagnetic resonance

(\frac{\pi}{2}) pulse, the negative echo intensity first decreases, vanishes and then increases until the maximum amplitude is reached again in thermal equilibrium (see lower part of figure 3.17).

Fitting the function

\[ E(T) = E_0 \left( 1 - 2 \exp \left( -\frac{T}{T_1} \right) \right) \]  

(3.120)

to the course of the echo intensity versus time, i.e. the recovery curve, the longitudinal relaxation time \( T_1 \) can be determined directly. Also in this kind of pulse experiment, the superposition of different spin species leads to a multiexponential behavior of the recovery and will hence be noticed.

With regard to the application of several pulses on short time scales, the phenomenon of echo crossings is worth mentioning. Here, the pulses interact differently with the remaining transverse magnetization and are thereby able to create, dependent on the inter-pulse delay times, additional echoes that might distort the signal. Such echoes are not solely generated by the interaction of a set of two pulses but can amongst others be the result of a refocused echo. The phase-cycling method has been developed, where by an appropriate summation of experiments possessing phase-shifted pulses provides the opportunity to cancel out these unwanted echoes. In [FAUTH et al. 1986] the method is described in detail in terms of a three-pulse experiment.

**Four-pulse DEER**

In the cw EPR section described earlier we have seen that inter-spin distance measurements on biomacromolecules using a continuous wave EPR spectrometer is limited to a distance of about 2 nm. Pulsed ELDOR methods on the other hand are able to determine distances in the range from 1.6 to 6 nm, in very favourable cases even up to 8 nm [JESCHKE and POLYHACH 2007]. In the following paragraphs the four-pulse DEER method is explained in order to extract this distance information from the dipole-dipole interactions between two electron spin species \( A \) (\( S_A = \frac{1}{2} \)) and \( B \) (\( S_B = \frac{1}{2} \)).

The four-pulse DEER sequence developed by Pannier et al. [PANNIER et al. 2000] is shown in figure 3.18. The mentioned spin species \( A \) is considered to possess the resonant microwave frequency of the observer microwave and the spin species \( B \) that of the pump microwave. In the pulse sequence at the observer microwave a refocused Hahn-echo sequence is applied. The first pulse of the Hahn sequence (\( \frac{\pi}{2} \)) flips the magnetization of the \( A \) spins in the x-y-plane of the rotating frame. The spins immediately start to evolve under the dipole-dipole interaction with the second spin species and the resonance offsets \( \Omega_s \) present. When the time \( t_1^* \) has passed, the following \( \pi \)-pulse leads to the creation of the known negative Hahn echo (dotted line in figure 3.18) that will not be observed in our measurements. After time \( t_2^* \), the final \( \pi \)-pulse in this channel refocuses the negative echo to create the final echo at time \( t_2^* \) after the last pulse. Additionally, a \( \pi \)-pulse at the pump microwave (spin
species \( B \)) is applied at a variable time \( t' \) after the first observer \( \pi \)-pulse. It inveres the polarization of the \( B \) spins, what indirectly leads to the change of the magnetic field at the \( A \) spin species. The refocused electron spin echo in the observer channel is consequently a coherence transfer echo. Due to the variable character of the time \( t \) before the \( B \) spins are flipped by 180°, the final echo oscillates with the frequency of the dipolar coupling.

In order to analyze four-pulse DEER spectra in terms of distance information, one has to separate the wanted dipolar interactions from other couplings and broadenings occurring in the EPR spectrum. For instance, the presence of proton modulation from couplings with the matrix protons describe such an unwanted effect. Although the nuclear modulation can not be eliminated completely, it can be suppressed through the variation of otherwise fixed inter-pulse delay times and addition of all echoes for the different delays. The spin Hamiltonian describing the coupling between the two spin species \( A \) and \( B \), under the assumption of negligible nuclear modulation, is given by the contributions from the dipole-dipole and the exchange interaction (see section 3.1.2.5):

\[
\hat{H}_{AB} = \hat{H}_{\text{EXCH}} + \hat{H}_{\text{DD}} = J \hat{S}_A \hat{S}_B + \hat{S}_A \hat{D} \hat{S}_B.
\]  

Under the assumption of isotropic exchange interaction, parallel alignment of the electron spins to the external magnetic field \( B_0 \), and the electron spin localized at the centre of the \( N-O \) bond, the dipole-dipole tensor \( \mathbf{D} \) can be described by the point-dipole approximation,
3.1 Electron paramagnetic resonance

where it is axial with the axis given by $\mathbf{r}_{AB}$, the spin-to-spin vector. With these assumptions, the spin Hamiltonian is described by the sum of a secular (equation 3.123) and a pseudo-secular (equation 3.124) term, where $\vartheta$ describes the angle between $\mathbf{r}_{AB}$ and the magnetic field direction [Jeschke and Polyhach 2007]:

$$\hat{H}_{AB,\text{SEC}} = (J + \omega_{dip} (1 - 3 \cos^2 \vartheta)) \hat{S}_{A,z} \hat{S}_{B,z},$$  
(3.123)

$$\hat{H}_{AB,\text{PSEC}} = \left( J - \frac{1}{2} \omega_{dip} (1 - 3 \cos^2 \vartheta) \right) \left( \hat{S}_{A,z} \hat{S}_{B,z} + \hat{S}_{A,y} \hat{S}_{B,y} \right),$$  
(3.124)

with

$$\omega_{dip} = \frac{1}{r_{AB}^3} \frac{\mu_0}{4\pi h} g_A g_B \mu_B^2.$$  
(3.125)

The g values for nitroxides can furthermore be approximated by the isotropic value $g_A = g_B = 2.0026$, since the distance deviation due to this assumption is less than 3% of the measured distance [Jeschke and Polyhach 2007]. According to [Jeschke 2002], in the kind of nitrooxide systems under investigation here, the exchange coupling interaction is about one order of magnitude smaller than the dipole-dipole interaction. The negligence of the exchange term accordingly solely gives an error of less than 0.05 nm. Eventually, also the pseudo-secular term can be neglected for distances longer than 1.5 nm for the system considered here [Jeschke and Polyhach 2007]. However, below 1.5 nm distance determination is not reliable any more.

Since major deviations in the distance determination are known to arise from unequally distributed spin label orientations with respect to the external magnetic field $B_0$, such deviations have to be treated carefully. Here, the orientations of the spin-to-spin vectors $\mathbf{r}_{AB}$ are assumed to be equally distributed in space.

3.1.5.2 Pulse ENDOR

The observation of nuclear transitions in an EPR experiment helps to partially overcome one of the drawbacks of conventional EPR experiments, i.e. spectral crowding due to the presence of various lines. Electron nuclear double resonance (ENDOR) spectroscopy exploits this fact to tremendously reduce the number of lines and in order to characterize nuclei coupled to the unpaired electron present.

Figure 3.19 shows a schematic representation of the energy levels in the two-spin system ($S = \frac{1}{2}$ and $I = \frac{1}{2}$ ($^{15}$N)) considered so far. In cw ENDOR experiments, one EPR transition (e.g. $2 \rightarrow 4$) is saturated by high power microwave irradiation. According to section 3.1.3.6 this transition’s EPR signal amplitude consequently decreases and finally vanishes. Simulta-neous application of a radio frequency field inducing a transition of a coupled nucleus (e.g.

---

$vi$Corresponding to dipolar coupling frequencies smaller than 15.5 MHz
Figure 3.19: Schematic overview of the four-level energy system in the considered case \((S = \frac{1}{2}, I = \frac{1}{2} (^{15}N))\). The four levels indicated by rectangles are designated by the numbers according to figure 3.15. For simplification, the particular rectangles are filled according to the polarization of the EPR transitions in thermal equilibrium (Boltzmann distribution). Nuclear polarizations are not demonstrated. Allowed EPR and NMR transitions are depicted via solid lines, whereas forbidden cross-relaxational paths are highlighted by dashed lines.

1 \(\rightarrow\) 2) leads to the at least partial desaturation of the observed EPR transition, namely the reemergence of the EPR signal amplitude. Additional sweeping of the radio frequency field through a certain field range yields the cw ENDOR spectrum showing the EPR signal at the frequencies of the nuclear transitions. Furthermore, it has to be considered that according to equations 3.116 and 3.117 each nucleus exhibits two transition frequencies. In case of weak coupling \((\omega_I > |A/2|)\), the two resonances are centered symmetrically around the nuclear Zeeman frequency \(\omega_I\) with a doublet distance deviating slightly from \(A\) due to the second-order effect. The stronger the coupling the larger the splitting between the two lines. In the extreme case of very strong coupling \((\omega_I < |A/2|)\) the doublet is centered around \(A/2\) with a splitting slightly deviating from \(2\omega_I\) according to the second-order effect. Nevertheless, to generalize, each group of equivalent nuclei contributes only two lines to the ENDOR spectrum, regardless of the number of nuclei and their spin numbers.

It is worth mentioning that the minor transition probabilities of the intrinsically forbidden cross-relaxation paths (see figure 3.19 transitions 1 \(\rightarrow\) 4 and 2 \(\rightarrow\) 3) described in section 3.1.5.1 are of major importance in cw ENDOR. Since cw ENDOR measurements have not been used in this study, the reader is referred to the literature [Jeschke 1998, Schweiger and Jeschke 2001].

Due to the fact that cw ENDOR signal intensities strongly depend on the balance between electron and nuclear spin-spin as well as spin-lattice relaxation rates, this method is often restricted to a narrow temperature range. This is one of the main reasons for the common application of pulse ENDOR techniques. Here, much larger temperature ranges can be used since, besides the restriction by the phase memory time of the spin system, the lengths of the applied pulse sequence can usually be adjusted to time scales preventing unwanted relaxation effects. Although a series of pulse ENDOR methods is available, only the so-called Davies-type ENDOR based on the polarization transfer between electron and nuclear transitions was used here. The corresponding pulse sequence is shown in the upper part of figure 3.20. As indicated by the division in three subsequences, a Davies-ENDOR
3.1 Electron paramagnetic resonance

Figure 3.20: Davies-ENDOR experiment. Upper part: Pulse sequence and interpulse delay times for the applied microwave and radio frequency fields in the preparation, mixing and detection period. Lower part: Schematic representation of the energy level populations in a two-spin system \((S = \frac{1}{2}, I = \frac{1}{2}, (^{15}N))\) according to figure 3.15. The rectangles are filled corresponding to the electron spin polarization in terms of the Boltzmann distribution (black: higher occupied level, white: lower occupied level). Thermal nuclear polarization can be neglected in first approximation. Three states of the spin system are depicted as indicated by the gray dotted arrows. In thermal equilibrium (a) selective detection (application of Hahn sequence) on the \(24\)-transition leads to a positive echo (bottom). A resonant first mw \(\pi\)-pulse on the \(24\)-transition (indicated by bold black line in b) inverts the echo’s amplitude. In case of a subsequent non-resonant rf \(\pi\)-pulse (b), the echo remains unchanged. A resonant rf \(\pi\)-pulse on transition \(12\) (c) results in echo deletion.

The experiment comprises a preparation period creating nuclear polarization via the first microwave \(\pi\)-pulse, a mixing period (here: first rf \(\pi\)-pulse) to change the nuclear polarization, and eventually the detection period, where the transfer of the nuclear coherence to electron coherence is indirectly observed through an echo of the electron spins by the application of the known Hahn sequence. It is noteworthy that in Davies-type ENDOR measurements the selective character of the first microwave pulse is of major importance. Such a selective pulse has to exhibit a smaller excitation bandwidth than the transition frequency difference \(|\omega_{13} - \omega_{24}|\) in order to invert solely one EPR transition and thereby to create nuclear polarization on both nuclear transitions. In case the microwave field excites an inhomogeneously broadened line, only part of it will be in resonance and consequently only this part will be affected. In the example given in figure 3.20, the \(24\)-transition is inverted by the applica-
tion of this first pulse and leads to an echo inversion (see figure 3.20b) when the detection period would be applied subsequently. Due to the fact that the electron spin-lattice relaxation time $T_1$ in frozen solutions\(^a\) is typically in the region of 100 $\mu s$ and higher, time scales are long enough to invert the nuclear polarization on one of the nuclear transitions (see figure 3.20, transitions 12 and 34) via the application of a selective radiofrequency $\pi$-pulse. In figure 3.20c, the rf pulse hits the 12-transition and leads to the deletion of the population difference of the formerly excited EPR transition (24), and thereby to the disappearance of the echo. Is the applied rf field not resonant with one of the existing nuclear transitions, the echo amplitude as shown in figure 3.20b remains unchanged. For the consideration of the second EPR- or NMR-transition in the assumed system, the situation can be described analogously.

In order to detect the nuclear frequencies of all coupled nuclei in the system under investigation, in a Davies-type ENDOR experiment the echo amplitude of the inverted echo (case described in figure 3.20b) is detected at a fixed external magnetic field $B_0$ and fixed inter-pulse delay times, while the frequency of the applied radio field is changed. Analogous to the two-pulse ESEEM experiment described in section 3.1.5.1, from the resulting ENDOR spectrum the hyperfine coupling can be obtained through $|\omega_{12} - \omega_{34}|$ and a corrected nuclear Zeeman frequency $\omega_I'$ through $|\omega_{12} + \omega_{34}|/2$.

Eventually, it is worth mentioning that due to the finite excitation bandwidth of the radio frequency pulse, weakly coupled protons existing in the environment of the unpaired electron cause a sharp ENDOR signal centered at the proton frequency that is characterized by a single line due to the unresolved hyperfine splittings. This signal is known as matrix line and forms one of the major spectral disturbances in Davies-ENDOR spectra.

3.2 Thermodynamics

Beyond the observation of the static picture of protein structure, insight into its dynamics can be obtained via monitoring snap-shots of the system’s behavior upon changes in a state variable. Since temperature changes are technically quite easy to realize, in this study processes/chemical reactions taking place over a certain temperature range are traced and quantified by the thermodynamic parameters noted below. The EPR parameters analyzed in this regard are the reorientational correlation time $\tau_c$ of the attached nitroxide (see section 3.1.3.5), which has been obtained for two components present in the cw EPR spectra (see section 4.3.1), and the fractions of the two components, which were found to represent a thermal equilibrium of two conformational states.

\(^a\)In this study Davies-ENDOR experiments were performed at 80 K.
The equation for the Gibbs energy describes the key criterion in such thermodynamic approaches:

\[
G = U + pV - TS, \tag{3.126}
\]

\[
= H - TS, \tag{3.127}
\]

with \(U\) as the system’s internal energy, \(p\) as pressure, \(V\) as volume, \(T\) as actual temperature, and \(S\) and \(H\) as entropy and enthalpy, respectively.

We consider a simple reaction between two protein states \(A \rightleftharpoons B\), in which \(A\) and \(B\) can be interconverted. In equilibrium this reaction has no capacity to do work, but whenever the reaction is disequilibrated the two reactants change their concentrations in order to regain equilibrium values since every system seeks to achieve a minimum of free energy. The force constituted during this reaction can be quantified by the changes in entropy and enthalpy, and the resulting changes in the Gibbs energy according to:

\[
\Delta G = \Delta H - T \Delta S, \tag{3.128}
\]

with \(T\) as a constant. \(\Delta G\) tells us about the spontaneity of the reaction with \(\Delta G < 0\) describing a favored, spontaneous reaction, \(\Delta G = 0\) neither describing a forward nor a reverse reaction (equilibrium state), and \(\Delta G > 0\) describing a disfavored, non-spontaneous reaction [Mortimer 2001]. Furthermore, the changes in Gibbs energy can be written in terms of the effective concentrations of the reactant \(c_A\) and product \(c_B\), respectively:

\[
\Delta G = \Delta G^o + RT \ln \frac{c_B}{c_A}, \tag{3.129}
\]

where \(\Delta G^o\) describes the standard Gibbs energy and \(R\) the (molar) gas constant. At equilibrium (\(\Delta G = 0\)) the fraction of the concentrations of the two components in equation 3.129 is defined as equilibrium constant \(K_{eq}\). It follows:

\[
\Delta G^o = -RT \ln K_{eq}. \tag{3.130}
\]

With \(N_0\) as the total molar concentration of the protein \((c_A + c_B = N_0)\) and \(f_A\) and \(f_B\) as the mole fractions of \(A\) and \(B\) \((f_A + f_B = 1)\), respectively, \(K_{eq}\) can be obtained from the fitted fractions through

\[
K_{eq} = \frac{f_B N_0}{f_A N_0} = \frac{f_B}{f_A}. \tag{3.131}
\]

\(^{1}\)One mole of substance reacts completely under standard conditions.
Equation 3.130 in combination with equation 3.128 under standard conditions yields [Jelesarov and Bosshard 2004, Palmer 2000]

\[
\ln \frac{f_B}{f_A} = \frac{\Delta S^o}{R} - \frac{\Delta H^o}{R} \frac{1}{T}.
\] (3.132)

Plotting the natural logarithm of the fraction’s ratio versus the reciprocal temperature \( T \) in case of an equilibrium existing between the two spectral components accordingly results in a straight line, where the intercept equals the negative standard entropy change \( \Delta S \) divided by \( R \) and the slope equals the standard enthalpy change \( \Delta H \) divided by \( R \). Such plots are known as van ’t Hoff plots since differentiation of equation 3.132 yields the van ’t Hoff equation.

In the following, the expression \( \Delta H \) will be used instead of the standard form \( \Delta H^o \).

Error margins in the van ’t Hoff plots plots were obtained according to

\[
\Delta \ln K_{eq} = \left| \frac{\partial \ln K_{eq}}{\partial f_A} \right| \Delta f_A + \left| \frac{\partial \ln K_{eq}}{\partial f_B} \right| \Delta f_B
\] (3.133a)

\[
\Rightarrow \Delta \ln K_{eq} = \left[ \left( \frac{1}{f_A} \right) + \left( \frac{1}{f_B} \right) \right] 0.02, \quad (3.133b)
\]

where \( \Delta f_A = \Delta f_B = 0.02 \) was assumed.

\( \Delta H \) and \( \Delta S \) error margins (\( \Delta \Delta H \) and \( \Delta \Delta S \)) were obtained directly from the linear interpolations taking into account the errors for \( \Delta \ln K \).

Eventually, the error margins for the changes in the Gibbs energy \( \Delta G \) were derived from

\[
\Delta \Delta G = \left| \frac{\partial \Delta G}{\partial \Delta H} \right| \Delta \Delta H + \left| \frac{\partial \Delta G}{\partial \Delta S} \right| \Delta \Delta S
\] (3.134a)

\[
\Rightarrow \Delta \Delta G = \Delta \Delta H + T \Delta \Delta S. \quad (3.134b)
\]

Furthermore, from the reorientational correlation times of the spin label side chain’s motions introduced in section 3.1.3.5 the enthalpy of activation \( \Delta H^* \) can be calculated for an activation process induced by temperature changes. \( \Delta H^* \) provides insight into the energy barriers that have to be overcome by a particular spin label side chain investigated. According to [Steinhoff et al. 1989] \( \Delta H^* \) can be determined from

\[
\tau_c = \tau_{c,0} \cdot \exp \left( \frac{-\Delta H^*}{RT} \right),
\] (3.135)

where \( \tau_{c,0} \) and \( \Delta H^* \) are assumed to be temperature-independent in the temperature range applied here. Plotting the logarithms of values obtained for the reorientational correlation
times \textit{versus} the inverse of temperature (\textit{Arrhenius plot}) enables the determination of the activation enthalpies $\Delta H^*$ from the slopes of linear interpolations applied to the data:

$$\ln \tau_c = \ln \tau_{c,0} - \frac{\Delta H^*}{R} \frac{1}{T}.$$ \hspace{1cm} (3.136)

The corresponding error margins are directly obtained from the linear interpolations taking into account the estimated errors from the $\tau_c$ determination.
CHAPTER 4

MATERIAL & METHODS

4.1 Sample preparation

Protein samples used in this work have been prepared in collaboration with the group of Martin Engelhard from the MPI for Molecular Physiology in Dortmund. The protein expression of several NpSRII/NpHtrII-mutants has been performed in our laboratory. All chemicals used were of p.a. quality and from the following companies (exceptions are specified): Merck KGaA (Darmstadt, Germany), Riedel de Häen (Seelze, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich Group (Munich, Germany).

4.1.1 Bacterial strains and plasmids

The bacterial strain *E. coli* XL1 was used for cloning, *E. coli* BL21(DE3) or *E. coli* Rosetta (DE3) strains for gene expression.

- *E. coli* XL1
  - F’ Tn 10 proA+B+ lacP Δ(lacZ)M15/recA1 andA1 gyrA96
  - (NaI') Thi hsdR17 (rK− mK+) supE44 relA1 lac
  - [Bullock et al. 1987]
- *E. coli* BL21(DE3)
  - F− ompT [Ion] hsdSB (rB− mB+)
  - λ(DE3)
  - [Studier and Moffatt 1986]
- *E. coli* Rosetta (DE3)
  - F− ompT gal [dcm] [Ion] hsdSB (rB− mB−)
  - λ(DE3)
  - pRARE(CmR)
  - [Novagen, EMD Chemicals Inc., Darmstadt, Germany]

Receptor and transducer genes of different lengths were subcloned into pET27bmod expression vectors [Klostermeier et al. 1998] with C-terminal His-tags according to [Wegener et al. 2000]. The resulting plasmids pET27bmod-NpSRII-His [Hohenfeld et al. 1999], pET27bmod-t-HtrII-His [Wegener et al. 2001], pET27bmod-

*a Affinity-tags like polyhistidine (His)- or Streptavidine (Strep)-tags are peptides commonly used to simplify expression and purification (see also 4.1.3 Affinity chromatography).
nphtrII-1-230-His (C-terminal truncated transducers including residues 1-157 and 1-230, respectively) and pET27bmod-nphtrII-His (full length transducer) [MENNES et al. 2007] were used for two-step PCR mutagenesis according to the overlap extension method described previously [HO et al. 1989]. Positive clones were verified by DNA sequencing.

4.1.2 Protein expression

NpSRII-His, NpHtrII157-His and NpHtrII230-His as well as their respective mutants were expressed in E. coli BL21 (DE3) as described [SHIMONO et al. 1997], in case of NpHtrII-His and corresponding mutants E. coli Rosetta (DE3) was used according to [MENNES et al. 2007]. Transformed E. coli cells were grown either in LBb medium (5 g yeast extract, 10 g peptone, 10 g sodium chloride ad 1,000 ml, pH 7.2) or 2TY medium (10 g yeast extract, 16 g peptone, 5 g sodium chloride ad 1000 ml, pH 7.2) supplemented with 50 μg/ml kanamycin sulfatec. Starter cultures were shaken in 10 ml test-tubes and 200 ml flasks with baffles, main cultures in 2,000 ml flasks with baffles (volume ratio culture:flask = 1:2 - 1:3) at 310 K and 150 rpm in an Aerotron incubation shaker (Infors, Switzerland). Higher amounts of E. coli cells were grown in 10-50 l fermenters (Braun Biotech International GmbH, Melsungen, Germany). At an OD578nm of approximately 1.0 the overexpression of the protein was induced by addition of 0.5 mM (final concentration) IPTG. In case of NpSRII or corresponding mutants 10 μM (final concentration) all-trans retinal was added simultaneously. Cells were harvested 2-3 hours after induction via centrifugation (20 min, 5,000 rpm (≈4,200×g), 277 K) in a Sorvall Evolution Superspeed Centrifuge with a SLA3000 rotor and afterwards resuspended in a buffer containing 300 mM NaCl, 50 mM NaPi and 2 mM EDTA (pH 8.0).

4.1.3 Cell disruption and protein purification

The following buffers were used during the different preparation steps described below:

- A mem \(2\% (w/v)\) DDM, 300 mM NaCl, 50 mM NaPi, pH 8.0
- B mem \(0.05\% (w/v)\) DDM, 300 mM NaCl, 50 mM NaPi, pH 8.0
- C mem \(0.05\% (w/v)\) DDM, 25 mM imidazole, 300 mM NaCl, 50 mM NaPi, pH 8.0
- D mem \(0.05\% (w/v)\) DDM, 200 mM imidazole, 300 mM NaCl, 50 mM NaPi, pH 8.0
- E mem \(0.05\% (w/v)\) DDM, 10 mM Tris, pH 8.0
- F mem \(0.05\% (w/v)\) DDM, 10 mM Tris, pH 8.0
- F NpSRII mem \(0.05\% (w/v)\) DDM, 30 mM NaCl, 10 mM Tris, pH 8.0
- F NpHtrII mem \(0.05\% (w/v)\) DDM, 80 mM NaCl, 10 mM Tris, pH 8.0
- G mem \(0.1\% (w/v)\) DDM, 500 mM NaCl, 10 mM Tris, pH 8.0

b Known as lysogeny broth, Luria broth or Luria-Bertani broth.
c Aminoglycoside antibiotic for selectivity reasons, Sigma-Aldrich.
d \(1 \text{OD}_{578\text{nm}} = 2 \cdot 10^8\) cells per ml.
4.1 Sample preparation

The resuspended *E. coli* cells were disrupted by sonication for 1.5 min in pulsed mode\(^e\) with a Branson sonifier 250 accessorized with a Microtip (duty cycle 50\%). Meanwhile, the falcon tube containing the cells was also stored in an ice bath to prevent heating-up. This procedure was repeated twice. Higher amounts of *E. coli* cells were broken up by the use of a M-1105 Microfluidizer (Microfluidics Corporation, Newton, MA, USA). Afterwards, the membranes were sedimented (20,500 rpm (≈50,200×g), 277 K, 1 h) in a Sorvall Evolution Superspeed Centrifuge with a SS34 rotor, resuspended in buffer A\(_{mem}\) and stirred over night at 277 K to solubilize. *N. pharaonis* sensory rhodopsin II containing samples had to be stirred in the dark to avoid initiation of the photocycle. After another centrifugation step (20,500 rpm (≈50,200×g), 277 K, 1 h) the supernatant containing the solubilized proteins was loaded into a Ni\(^{2+}\)-NTA-agarose-column (type Superflow, Qiagen, Hilden, Germany), pre-equilibrated with buffer B\(_{mem}\) at ambient temperature. In this immobilized metal ion affinity chromatography (IMAC) the histidine-tagged recombinant protein is trapped in the solid phase of the column via coordinative covalent bindings of adjacent histidines\(^f\) to the Ni\(^{2+}\) metal ions, thereby forming a chelate complex. Molecules unspecifically bound to the nickel-matrix could then be removed by extensive washing with buffer C\(_{mem}\) containing 30 mM imidazole, a competitive binder to the matrix. Such moderate concentrations of imidazole are sufficient to wash out unwanted molecules looser bound to the matrix than the chelates. The purified samples were eluted from the column with buffer D\(_{mem}\) providing adequate amounts of imidazole to substitute the sample in the Ni-NTA-matrix.

To remove the imidazole in the Ni-eluate, subsequently, ion exchange chromatography\(^g\) with the positively charged resin DEAE was used. DEAE bound in the stationary phase of the column traps negatively charged molecules of a certain net charge from the mobile phase. Washing steps have to be performed by keeping the ionic strength of the mobile phase (same salt concentration in used buffer) at the same level. Elution of the sample from the column will be achieved through the use of a much higher ionic strength/salt concentration. Hence, the protein of interest will be screened from the solid phase since interaction between the protein and the ions in the mobile phase are favored with regard to those in the DEAE-matrix and the protein can elute.

In order to get the Ni-eluate trapped in the DEAE-matrix, it had to be diluted with buffer E\(_{mem}\) to a NaCl-concentration below 80 mM and 30 mM for NpHtrII and NpSRII containing samples, respectively. The diluted eluate was loaded on the pre-equilibrated (buffer E\(_{mem}\)) DEAE-column and washed extensively with F\(_{mem}^{NpSRII}\) and F\(_{mem}^{NpHtrII}\) for the respective protein samples to dispose the imidazole. Proteins were eluted from the matrix using buffer G\(_{mem}\) in all cases.

\(^e\)To minimize heat generation.

\(^f\)Imidazol side chains possess high binding affinity to metal ions.

\(^g\)Separation of ions/polar molecules based on coulombic interactions.
Protein concentrations were determined with an UV-VIS spectrophotometer (UV-2450, Shimadzu Corporation, Kyoto, Japan). In case of NpSRII samples, absorption of the retinal chromophore at 498 nm using a molar extinction coefficient of 40,000 l/mmol·cm [KLARE 2002] was measured. The concentration was then calculated through the Beer-Lambert law for liquids:

\[
E_\lambda = -\lg \left( \frac{I}{I_0} \right) = \epsilon_\lambda \cdot c \cdot l
\]  

or

\[
\epsilon_\lambda = \frac{E_\lambda}{c \cdot l}
\]  

where \(E_\lambda\) is the extinction at the wavelength \(\lambda\), \(I\) the intensity of the transmitted light, \(I_0\) the intensity of the incident light, \(\epsilon_\lambda\) the molar extinction coefficient at \(\lambda\), \(c\) the molar concentration and \(l\) the path length in the liquid sample. Protein purity could be estimated by comparison of the chromophore absorption with that of the existing aromatic amino acids at 280 nm. Purity above 95% was achieved in the case \(E_{280}/E_{498}\) equaled 1.3. In case of NpHtrII samples, the protein concentration was identified according to the method described by Ehresmann et al. [EHRESMANN et al. 1973]. In this method the concentration of proteins in solutions or cell-free extracts can be determined by measuring the absorbance at 228.5 nm and 234.5 nm. In the range from 0 to 125 μg/ml the relationship between absorbance and protein concentration is linear in the way that \(\Delta E = E_{228.5} - E_{234.5} = 1\) corresponds to a protein concentration of 317.5 μg/ml. It follows:

\[
c_{NpHtrII}(mg/ml) = \frac{\Delta E}{3.15}.
\]  

### 4.1.4 Spin labeling of cysteine mutants

For spin labeling according to [TODD et al. 1989], the NpSRII-mutants as well as those of NpHtrII of different C-terminal length, purification has been performed as described in 4.1.3 with some exceptions: i) A final concentration of 10 mM DTT\(^1\) was added to the Ni-NTA-eluate. The mixture was incubated for 1-3 h at 277 K. ii) The following DEAE chromatography was also used to remove the DTT from the protein sample before addition of the spin label, since remaining DTT would further on inhibit spin label binding through disulfide bridges and cut already existing ones. A permanent nitrogen atmosphere was used during the washing steps and the elution from the column to prevent oxidation of the cysteine side chain. Immediately after elution, the spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTS; Toronto Research Chemicals Inc. (TRC), Toronto, Canada) was added to the sample in a spin label to cysteine ratio of 10:1 according to Pfeiffer et al. [PFEIFFER et al. 1999]. In this study, an MTSSL stock solution of 100 mM in

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\(^{1}\)Prohibits oxidation of sulfhydryl groups (SH-) to disulfide bridges by use of oxygen.
acetonitril or DMSO was used. Addition of higher MTSSL concentrations was avoided due to the possible formation of unwanted biradicals in the protein sample. The MTSSL-protein solution was incubated for $12\ h$ at $277\ K$ in the dark. Excess spin label was removed by DEAE chromatography.

4.1.5 Extraction of polar lipids and bilayer composition

Extraction of polar lipids from *Halobacterium salinarum* and recently from *Na-tronomonas pharaonis* has been performed in the group of Martin Engelhard (Max-Planck-Institute, Institute for Molecular Physiology, Dortmund, Germany) according to Kates et al. [KATES et al. 1982]. The procedure is described in detail by Johann P. Klare [KLARE 2002] and leads to stable lipid stock solutions of $2\ \text{mg/ml}$.

4.1.6 Reconstitution into purple membrane lipids (PML)

For reconstitution of the NpSRII/NpHtrII complexes into polar lipids either from *H. salinarum* or *N. pharaonis*, the purified sample solutions in buffer $G_{mem}$ were used. The solubilized single constituents, NpSRII and NpHtrII, were mixed in a 1:1 molar ratio. In case solubilized protein complexes were required for experiments, the buffer was exchanged by the desired one and the sample was concentrated by use of centrifugal filter units (0.5 ml, 5/10/30 kDa NMWL; Millipore, MA, USA). The lipid stock solution was added to the NpSRII/NpHtrII mixtures in either 40-fold (w/w ratio lipids/complex $\approx 1:1$), 200-fold (w/w $\approx 1:5$) or 400-fold (w/w $\approx 1:10$) molar excess per 1:1 NpSRII/NpHtrII complex. After adjustment of the salt concentration to 1 M NaCl and addition of detergent-adsorbing Biobeads (SM2, 10 mg/mg of DDM; Boehringer Mannheim, Germany), the mixture was shaken ($16\ h$, $277\ K$) in the dark. Subsequently, Biobeads were removed by filtration and the membranes containing the NpSRII/NpHtrII complexes in their native 2:2 stoichiometry (see figure 4.1) were pelleted by centrifugation with a desktop centrifuge (12,800 rpm ($\approx 15,200\times g$, $277\ K$; Eppendorf, type 5415D). Sediments were resuspended in the desired buffers.

4.1.7 Denaturing gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for separation of proteins in the range from 1 to 100 $kDa$ was performed according to Schaegger and von Jagow [SCHAEGGER and VON JAGOW 1987]. The total percentage concentration of acrylamide was set to 10% or 16%, depending on the needed separation region of the proteins, at a crosslinkage of 3%. Prior to the application, protein samples were incubated in sample buffer for $30\ min$ at ambient temperature.

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3Nominal molecular weight limit.
4.2 EPR technique and performance

4.2.1 cw EPR

In order to investigate several issues describing the spin and the molecular system of interest, different experimental setups had to be used in this study. All of them are operating at microwave frequencies of about 9.5 GHz, in the so-called X-band. The basic assembly of the EPR spectrometers used for continuous wave (cw) measurements is presented in figure 4.2; amongst others further information on cw and pulse EPR can be obtained in [Charles P. Poole 1983, Schneider and Plato 1971, Hemminga and Berliner 2007]. The spectrometer can be divided into subunits describing the microwave part, the magnet and the detection system, respectively, where the resonator harboring the sample volume is located in an homogeneous magnetic field provided by an electromagnet. The microwave, produced in the case presented here by a reflex klystron, is directed to the resonator (cavity) through a waveguide system. It is worth mentioning that the klystron has to be matched to the waveguide impedance via an E/H-tuner to prevent reflections. One fraction of the microwave radiation is directly guided through the reference arm\(^1\) to the detecting unit (here: diode), acting as bias. This procedure ensures detection in the linear operation range of the diode, where the current through the diode is linear to the input voltage (i.e. to the square root of the microwave power). Amplitude and phase can be adjusted via the secondary attenuator and phase shifter in the reference arm. Another fraction of the microwave radiation passes the main attenuator and the circulator to reach the cavity\(^m\). At the resonance frequency of a matched cavity, no microwave will be reflected, thereby establishing the basis for measurement of the tiny power absorption due to EPR. The microwave is coupled into the cavity via the iris. A coupling screw in front of the iris

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\(^1\)Microwave radiation at selected frequency is maintained during measurement.

\(^m\)A cavity is a metal box of different shapes that stores microwave energy (called resonance).
4.2 EPR technique and performance

Figure 4.2: Schematic representation of the main units in EPR spectrometers.

EPR technique and performance can be used to adjust the size of the hole, so that the impedances of the resonator and the waveguide are matched and consequently no microwave reflects. Under these conditions the system is critically coupled \((R_0n^2 = r)\) in equation 4.3b). A resonator is characterized by the quality factor \(Q\) giving information about its ability to store microwave energy:

\[
Q = 2\pi \left( \frac{\text{energy stored}}{\text{energy dissipated per microwave period}} \right) \quad (4.3a)
\]

\[
= 2\pi \left( \frac{\nu_{mw} \cdot L}{R_0n^2 + r} \right), \quad (4.3b)
\]

where \(\nu_{mw}\) is the microwave frequency, \(L\) the inductivity of the resonator, \(R_0\) the impedance of the waveguide from the klystron to the cavity, \(n\) a parameter characterizing the coupling between waveguide and resonator, and \(r\) the impedance of the resonator. Energy losses in the cavity originate from heat production in the cavity walls through the electrical currents generated by the microwaves, transmission via coupling units and dielectric losses in the aqueous sample [Collie et al. 1947, Sueki et al. 1996].

A constitutional phenomenon of electromagnetic radiation is the coexistence of electric and magnetic field components perpendicular to each other. Thus, the standing wave in
the cavity provides the magnetic field $B_1$ necessary for EPR. Furthermore, the property of the two components of exhibiting a 180 degrees phase shift can especially be exploited for samples in aqueous environments with high dielectric losses. A sample location at high electric microwave fields decreases $Q$ and consequently the sensitivity of the setup, whereas the wanted EPR absorption increases with the magnetic field component. The conditions of a high magnetic and a low electric field component, for maximum absorption and highest sensitivity, beyond the cavity walls only apply for one position, i.e. the resonator’s center. In a rectangular cavity, capillary tubes such as Blaubrand intraMARK micropipettes, 50 μl, (Brand GmbH + Co KG, Wertheim, Germany) or flat cells containing the sample under investigation fit best the requirements, although quite high sample volumes (15 μl and higher) are needed. Loop gap resonators, however, can be used with much smaller sample volumes (1 μl and higher) since they possess a much higher filling factor. Though, this kind of resonators has a lower quality factor than cavities.

If the sample probe under conditions of critical coupling absorbs microwave energy, the energy losses in the cavity increase ($Q$ decreases) thereby changing the resonator’s impedance. The impedances of the resonator and the waveguide do not match anymore; a fraction of the microwave radiation proportional to the absorbed amount will be reflected and guided through the circulator to the detector. This gives the EPR absorption signal at the respective $B_0$-field.

Since the microwave power changes due to EPR absorption are only of minor amount, the signal is amplified by the widely used technique of field modulation and phase sensitive detection (see figure 4.3), using a so-called lock-in amplifier for the frequency selective measurement of periodic voltages. Here, the magnetic field strength has to be modulated sinusoidally at a certain modulation frequency (here: 50 kHz). The modulation coils needed are mounted laterally to the resonator, thus being localized in the static magnetic field $B_0$. Applying the modulation current to the coils leads to oscillations with the wanted frequency. In case of resonant absorption in the cavity, the reflected microwave radiation will be amplitude modulated with the same frequency. To amplitude-modulate the whole EPR absorption signal in the exact way, the modulation amplitude has to be optimized. In the best case, one B-field interval modulated exhibits a linearly changing absorption signal. Only then the sinusoidally modulated signal detected at the diode will yield an amplitude proportional to the slope in the absorption signal. Modulation amplitudes and/or frequencies chosen too high will affect the real signal shape. The lock-in amplifier applied only detects signals carrying the reference modulation frequency, thus suppressing noise from other sources and the background. After phase sensitive detection, the EPR signal present describes the low-noise amplified first derivative of the original absorption signal that, after A/D conversion, can be evaluated by a PC. It is worth mentioning that also the

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*Associated with the microwave electric field.

*Integral of the field amplitude $B_1$ over the sample divided by the integral of $B_1$ over the whole resonator.
4.2 EPR technique and performance

Figure 4.3: Effective magnetic field modulation to enhance sensitivity. Upper part: time-dependent fieldsweep of modulation, mid part: vertical magnetic field modulation interacts with the absorption signal to produce the horizontal EPR signal amplitude modulation (modulation amplitude enlarged to visualize better the effect), lower part: first derivative EPR signal after phase sensitive detection

The sampling time constant of the lock-in amplifier has to be chosen thoughtfully to maximize the sensitivity of the spectrometer in terms of filtering the signal of interest. One further aspect worth mentioning is the handling of a deviation of the microwave frequency from the resonance frequency of the cavity, e.g. due to temperature shifts. Such changes are detected and corrected by the klystron’s automatic frequency control (AFC). For this purpose, the reflector voltage of the klystron is also modulated sinusoidally with a variable amplitude and frequency (different from the field modulation). In case of a frequency deviation, the impedances from the waveguide and the resonator do not match anymore and microwave will be reflected. The voltage detected at the diode will be amplitude-modulated dependent on the frequency shift. After phase sensitive detection, this signals results in a first-derivative proportional error signal. Eventually, a correction voltage proportional to the error signal is inducted to the reflector voltage of the klystron.
4.2.2 Pulse EPR

To meet the requirements for the performance of pulse EPR, the technical setup has to be changed according to figure 4.4. Contrary to cw EPR, pulse EPR measurements depend on the excitation of spins exhibiting a certain distribution of resonance frequencies. Excitation bandwidths of at least a few MHz are needed. Due to the relation between field strength $B_1$, pulse duration $t_p$ and flip angle $\beta$ (see equation 4.5), very high microwave power $P_{mw}$ is required for non-selective pulses, when the pulse length should be kept at the same level. This high microwave power is achieved by additional amplification of the microwave source output. Mostly, a travelling wave tube amplifier (TWT), with typical output powers of 1 kW and high phase stability, is applied. A resonator’s bandwidth is defined as the frequency difference between the upper and lower frequencies where the power in the resonator is attenuated by a factor of two (so-called 3-dB points) compared to the center of the dip. Since critically coupled high-$Q$ resonators at X-band have a maximum bandwidth of about 2 MHz (sharp m.w. mode), they are not suitable and have to be overcoupled ($R_0n^2 > r$ in equation 4.3b) to decrease $Q$ and to increase the bandwidth. However, the shape of a microwave pulse is also dependent on $Q$ since new power levels of the incident microwave power in the cavity for both the power increase at the beginning and the decrease (cavity ringing) at the end of a pulse are reached exponentially. The higher the $Q$, the longer the response time constant [GALLAY and VAN DER KLING 1986]. Consequently, high $Q$ values result in long rise and fall times of the microwave field built up, and thus prolong the dead time $t_d$ that has to elapse after a pulse before a signal can be detected without disturbance. At the time of detection, the microwave pulse power decay (cavity ringing) should have reached a value significantly lower than the signal power. Consequently, the dimensions of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_4}
\caption{Schematic, simplified representation of a pulse EPR spectrometer (modified from [HEMINGA and BERLINER 2007]). Components required for the performance of ELDOR or ENDOR are displayed as dashed parts. PFU: pulse forming unit.}
\end{figure}
the parameters applied should be chosen with care in terms of obtaining maximum detection sensitivity as pointed out by the following equations:

\[ B_1 = \sqrt{\frac{2\mu_0 Q P_{mw}}{V_C v_{mw}}}, \]  

\[ \beta = F \frac{g\beta_e B_1}{h} t_p, \]  

where \( V_C \) is the resonator’s effective volume, and \( F = 1 \) with electron spin \( S = 1/2 \).

To further minimize the dead time \( t_d \), to avoid the disturbance of the signal by overlap of additional echos\(^\text{v}\), and thereby to minimize the signal losses during this time, a so-called phase-cycling [Fauth et al. 1986, Schweiger and Jeschke 2001] is applied in a set of pulse EPR experiments. However, at a very low \( Q \ t_d \) is no more determined by the resonator but by the amount of internal reflections in the transmission line between different units. Power reflections of some hundred watts already suffice to damage the kind of detectors used in these setups. Thus, a switch (S in figure 4.4) has to protect the detection part by closing the circuit during the application and the adequate decay of the high power pulses. Before the eventual detection of the signal, a downconversion of the signal to video frequencies is implemented by the mixer (M in figure 4.4). A mixer refers the signal coming from the sample to the phase of the microwave transmitted through the reference arm (directly from source). The fixed phase relation between the excitation field and the resulting signal present in pulse EPR thereby simplifies the separation of wanted and unwanted signals. Applying the mixer then leads to the measurement of the microwave frequency modulation, namely the difference frequency being in the range from zero to 100 MHz. In order to achieve a good signal-to-noise ratio, the signal coming from the sample needs to be pre-amplified before the mixer. The signal after the mixer also needs amplification to be adjusted to the operation range of the following analog-to-digital converter. The gain of this video amplifier has to be readjusted for each measurement.

The pulse forming unit (PFU in figure 4.4) is responsible for the creation of the pulse pattern needed for the experiment. Today’s spectrometers are equipped with four or even eight pulse channels. The adjustment of microwave phase and power is performed in the single channels before the pre-amplification takes place. In order to facilitate the fast change of interpulse delays necessary during the measurement, pin-diodes with response times between two and five nanoseconds are used. For the following pre-amplification the single channels are unified again so that the main attenuator and phase adjustment unit in the high power part is able to act on all of them.

In most cases the signal is detected via the integration of the resulting echo over a window of a certain, adjusted time, which is at least as long as the time resolution of the detection circuit. In general, echo investigation ranges from single point (mostly maximum of the

\(^{v}\text{Due to cross interactions between the single pulses.}\)
echo) to whole echo integration. The echo intensity is then recorded as a function of one ore two variables depending on the kind of experiment performed.

4.2.3 Pulse ENDOR

The main features needed in a conventional EPR spectrometer to perform pulse ENDOR measurements are indicated in figure 4.4 by dashed lines. Beyond the above mentioned literature, further information can be obtained in [KURRECK et al. 1988].

Since in pulse ENDOR radio frequency radiation is used to excite nuclear transitions during the measurement of an electron spin echo, a r.f. source, a pulse forming unit (PFU) for the needed r.f. pulses, a r.f. amplifier and r.f. coils at the sample have to be used. In X-band, the operation range of source and amplifier should at least cover 1 to 30 MHz, the assembly should be able to provide r.f. pulse powers of a few hundred watts at typical pulse lengths of 1 to 200 μs and allow repetition times between 1 and 10 ms. The most delicate part in this context is the application of the r.f. coils to the sample location. In general, two possibilities are available: i) the m.w. resonator walls can be made transparent for the r.f. radiation produced by the coils outside the cavity, but has to remain imper- vious for the m.w. radiation; ii) the r.f. coils have to be positioned inside the resonator, preferably close to the sample volume. Both alternatives feature advantages and disadvantages regarding thermal effects or optimal power use. However, it has been shown that at higher frequencies (95 GHz and higher) r.f. coils positioned outside a TE₀₁₁ cylindrical cavity [BURGHAUS et al. 1992] are preferable, whereas at lower frequencies (e.g. in our case in X-band) the most successful solution is given by r.f. coils placed inside the resonator. The latter possibility can be achieved by the use of a Helmholtz coil with rectangular shape [BIETSCH and VON SCHÜTZ 1993] in a dielectric resonator. Moreover, the matching between the r.f. transmission line and the coils is of notably importance. In a typical ENDOR setup, this task is performed by a matching network⁴ consisting of capacitances and in some cases also of resistances.

4.2.4 Amendatory equipment and experimental procedure

Due to the diverse nature of the experimental X-band EPR methods applied, respective setup compositions have to vary accordingly. The main setup distinctions and the particular procedures are described below. Sample volumes, capillary tube properties and probe positionings were chosen in a way to achieve optimal measurement sensitivity and to minimize dielectric losses. For all cw EPR experiments except for those to determine the accessibilities to paramagnetic quenchers, the B-field scan covering the whole EPR spec-

⁴Divided into broadband matching networks and matching networks to enhance the r.f. magnetic field component $B_2$. 
trum was in the range from 100 to 130 Gauss. To enhance the signal-to-noise ratio, cw EPR spectra were accumulated up to 15 times and added up directly by the software used.

**Room temperature** (296 ± 2 K) cw EPR measurements were performed using a commercially available MiniScope benchtop EPR spectrometer (MS200; Magmetech GmbH, Berlin, Germany) with a rectangular TE$_{102}$ resonator. Due to heat production in the resonator during operation, the sample environment was fluxed with gaseous nitrogen keeping the temperature stable. The microwave power was set to 10 mW and the B-field modulation amplitude to 0.15 mT. EPR glass capillaries (0.9 mm inner diameter) were filled with sample volumes of 15 μl at final concentrations of 100 to 300 μM (ideal case).

For **temperature dependent cw EPR** measurements, a homebuilt EPR spectrometer equipped with a dielectric resonator (Bruker Biospin; see above) was used. To adjust and stabilize the sample temperature in the range between 263 and 343 K, a liquid flow cryostat was connected to a reservoir unit able to heat and cool the liquid inside consisting of a 30% ethylene glycol / 70% water mixture. The microwave power was set to 1 mW and the B-field modulation amplitude to 0.15 mT. In case several samples were measured during one temperature cycle, the samples not located in the resonator were stored in the water/ethylene glycol bath to prevent rapid cooling and heating of the proteins.

**Accessibilities for the paramagnetic quenchers** oxygen and NiEDDA (Ni(II)ethylenediamine diacetate, uncharged; in former experiments the charged chromium oxalate (CrOx) was used) were obtained using a homebuilt cw EPR spectrometer equipped with a loop gap$^a$ resonator. According to the method of cw power saturation, the microwave power was applied to the sample in the range from 0.1 to 65 mW. A motor controlled by the measuring software was used to change the main attenuator during the measurement. Reusable, gas permeable TPX (Polymethylpenten) capillaries (RototecSpintec GmbH, Biebesheim, Germany) were mounted containing 5 μl of sample. For reference measurements the sample was deoxygenated via the presence of a nitrogen gas flux around the sample. In order to obtain accessibilities for oxygen, the nitrogen was replaced by air (21% O$_2$). For water-accessibilities, a NiEDDA solution was added to the sample to a final concentration of 20 mM and 3 mM, respectively, and the nitrogen gas flux was restored. In each case the sample was fluxed with the respective gas 20 min before the experiment was started to prevent the overlap of different exchange mechanisms. Contrary to the other cw EPR measurements, the B-field scan in accessibility experiments only covers the central resonance line due to the investigation of its amplitude changes.

**Cw EPR** spectra for **inter-spin distance determination** in the range from ≈ 0.8 to 2.0 nm were obtained on a homebuilt cw EPR spectrometer accessorized with a rectangular cavity (AEG H$_{103}$; AEG, Berlin, Germany) and recently with a Super High Sensitivity

$^a$Acting as cryoprotectant.

$^a$Has lower intrinsic Q than cavities but a high filling factor allowing measurement performance with very small sample volumes.
Probehead (Bruker Biospin GmbH, Rheinstetten, Germany). A B-NM 12 B-field meter (Bruker Biospin, see above) was accessorized for exact measurement of the external magnetic field (B₀). For sample cooling and temperature stabilization to 160 K, a continuous flow cryostat (ESR900; Oxford Instruments, Oxfordshire, UK; see figure 4.5: more detailed information can be obtained at www.oxinst.com) sucking gaseous\(^*\) \( N₂ \) was used in combination with an intelligent temperature controller (ITC 4; Oxford Instruments). The needle valve and hence the nitrogen flow of the transfer line transporting the nitrogen from the reservoir to the cryostat was completely open during the cooling procedure but was closed slightly for temperature stabilization and measurement. To control the sample temperature, a thermocouple element was connected to the ITC. The microwave power was set to 0.2 \( mW \) and the B-field modulation amplitude to 0.25 \( mT \). EPR quartz capillaries (3 \( mm \) inner diameter) were filled with sample volumes of 40 \( \mu l \). Freezing the samples prior to their insertion in the resonator was performed to avoid a possible burst due to sample volume expansion inside the capillary in the dewar.

\[^*\]Liquid \( N₂ \) had to be used when the final measurement temperature was too low to be reached with the gaseous \( N₂ \).

\[\text{Figure 4.5: Schematic representation of an ESR900 continuous flow cryostat.}\]
For pulse EPR experiments presented in this study, a Bruker Elexsys 580 spectrometer equipped with a Flexline split-ring resonator ER 4118X-MS3 (Bruker Biospin GmbH, Rheinstetten/Karlsruhe, Germany) was used. To achieve broad excitation bandwidths, the resonator was overcoupled to \( Q \approx 100 \). A continuous flow cryostat (CF-935; Oxford Instruments, Oxford, UK) was used for cooling to 50 \( K \) with liquid helium. Contrary to the liquid flow cryostat described in figure 4.5, this cryostat immerses the entire sample and cavity in the cryogen flow, providing experimental flexibility by compatibility with many different cavities. Temperature setting and stabilization was again controlled by an intelligent temperature controller (ITC 503S; Oxford Instruments). Quartz capillaries (inner diameter 3.5 \( mm \)) have been loaded with 40 \( \mu l \) of sample containing 15 to 20\% of deuterated glycerol. Deuterated glycerol was used due to its effect on the spin-lattice relaxation and the connected enhancement of the spin-spin/transverse relaxation time \( T_2 \) described in section 3.1.3.2. Freezing the samples prior to their insertion in the resonator was again used to avoid a possible burst inside the cavity.

Previous to interspin distance determination experiments, two-pulse electron spin echo envelope modulation (ESEEM) spectra were recorded for obtaining the relaxation in terms of the phase memory time \( T_m^u \). Here, a simple \( \frac{\pi}{2} - \tau - \pi - \tau - \text{echo} \) pulse sequence is used to measure the exponential decay of the echo height due to dephasing. For ESEEM measurements a single point (the echo maximum) or an integral over the echo is sampled. The echo decay is measured against the stepwise increase of the interpulse-delay time \( \tau \), starting at \( \tau = 104 \, ns \) and increasing by 4 \( ns \). Pulse lengths of 16 \( ns \) (\( \frac{\pi}{2} \)) and 32 \( ns \) (\( \pi \)) were used (+x-channel).

Furthermore, to determine the \( B_0 \)-field value corresponding to the maximum of the cw EPR absorption spectrum, an echo-detected fieldsweep was performed using the \( \frac{\pi}{2} - \tau - \pi - \tau - \text{echo} \) pulse sequence (in the + \( x \)-channel) with fixed inter-pulse delay times of \( \tau = 200 \, ns \). In this case, an integration over the complete echo was applied during detection while the magnetic field is swept through the whole spectrum. By integrating the area under the echo, a filtering can be achieved to cancel out so-called power broadening effects\(^v\)

Finally, four-pulse DEER experiments were performed using the PulseSPEL program written by G. Jeschke, described amongst others in [Dressel et al. 2004], with the pulse sequence \( \frac{\pi}{2} (\nu_{\text{obs}}) - \tau_1 - \pi (\nu_{\text{obs}}) - t' - \pi (\nu_{\text{pump}}) - (\tau_1 + \tau_2 - t') - \pi (\nu_{\text{obs}}) - \tau_2 - \text{echo} \). Quadrature detection\(^w\) was used. For the DEER pulses in the observer frequency \( \nu_{\text{obs}} \) the \( +x \), \( -x \)-channels were used. A two-step phase-cycling (\( +<x>/-<x> \)) was performed on the first pulse; the two signals were added. All measurements were performed using observer pulse lengths of 16 \( ns \) for \( \frac{\pi}{2} \) and 32 \( ns \) for the \( \pi \)-pulses. The \( \pi \)-pulse at the pump frequency

\(^v\)Comprises all processes causing echo dephasing, also transverse relaxation time \( T_2 \).

\(^w\)To be distinguished from power broadening in cw EPR.

\(^w\)Both the real and imaginary part of the spectrum are observed.
\( \nu_{pump} \) had a length of 12 ns to enhance sensitivity [Jeschke et al. 2004a]. The power of the pump pulse was adjusted for maximum flip angle using an inversion recovery sequence \( \pi_{\text{pump}} - T - \frac{\pi}{2} - \tau - \pi_{\text{obs}} - \tau - \text{echo} \) with coinciding pump and observer frequency at the position in the microwave mode where the pump pulse is applied. Time \( t' \) was varied, whereas \( \tau_1 \) and \( \tau_2 \) were kept constant. \( \nu_{\text{pump}} \) was set to the center of the resonator mode and to coincide with the global maximum of the nitroxide spectrum (to maximize modulation depth), whereas \( \nu_{\text{obs}} \) was set 65 MHz higher to the local maximum at the low-field part of the spectrum (see figure 4.6). To suppress nuclear modulation, traces were added up for eight equidistant values for \( \tau_1 \) between 200 and 256 ns in case of protons and between 400 and 792 ns in case of deuterons. \( \tau_2 \) was chosen separately for each measurement depending on transverse relaxation, signal strength and the longest distances that had to be measured. Data were analyzed for dipolar evolution times \( t = t' - \tau_1 \geq 0 \). Accumulation of the DEER traces was performed up to 24 hours.

![Figure 4.6: Observer and pump pulse settings in the four-pulse DEER experiment. Echo-detected field swept EPR spectrum of a nitroxide (left) and a simulated idealized microwave mode assuming very low Q (right).](image)

**Pulse ENDOR** measurements were performed using a Bruker Elexsys E580 spectrometer equipped with a DICE (Digitally Computed Excitation offering FM, AM, FSK (Frequency Shift Keying) or field modulation and utilizing the Direct Digital Synthesis (DDS) concept for the E560-D-P pulsed ENDOR accessory) unit including a radiofrequency amplifier (Amplifier Research 250A250A), and a dielectric ring ENDOR resonator (EN4118X-MD-4-W1; Bruker, see above). The resonator was immersed in a nitrogen continuous flow cryostat (CF-935; Oxford Instruments, see above) being controlled by an intelligent temperature controller (ITC 503S; Oxford Instruments, see above). All measurements were performed at 80 K. 40 \( \mu l \) of sample containing 15% of either glycerol or deuterated glycerol to enhance the relaxation time were filled in EPR quartz capillaries (inner diameter 4 mm). Also in these measurements, samples have been frozen prior to their insertion in the resonator. ENDOR spectra were recorded at the maximum intensity of the two-pulse field swept electron spin echo EPR spectrum. For Davies-type ENDOR, the microwave pulse sequence \( \pi - t - \frac{\pi}{2} - \tau - \pi - \tau - \text{echo} \) was used with 64 ns \( \frac{\pi}{2} \) and 128 ns \( \pi \) pulses highlighting the
ENDOR nature of using selective pulses. Separation times $t$ and $\tau$ were set to 13 $\mu$s and 500 $ns$, respectively. The radio frequency pulse applied between the first and second microwave pulse, was set to a length of 10 $\mu$s.

4.3 Data handling

One of the most delicate parts of EPR spectroscopy is data handling. Since each kind of experiment was designed to address certain issues, a set of analysis methods is needed. The following sections will briefly explain the methods used to analyze the EPR spectra obtained in this study.

4.3.1 Fast and slow motion cw EPR spectra

To compare room temperature cw EPR spectra with each other, two types of normalization are common. The easiest way is to normalize the spectra to the value of maximum amplitude of the EPR first derivative spectrum, which in the kind of spectra presented here always leads to a normalization to the maximum of the central resonance line. A more sophisticated method is to normalize the spectra to the area of the EPR absorption spectrum. This area is directly proportional to the spin concentration in the sample and can be used to calculate the sample’s concentration when compared to the area integral of a reference spin probe (here 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL)) of known concentration $c_{\text{ref}}$:

$$c_{\text{samp}} = \frac{N_{\text{samp}} \cdot c_{\text{ref}}}{N_{\text{ref}}}, \quad (4.6)$$

where $N_{\text{samp}}$ and $N_{\text{ref}}$ are the evaluated integral areas of the absorption spectra for the sample and the reference, respectively. If the sample concentration has already been determined by other techniques (here $c_{\text{sampII}}$), the obtained sample concentration $c_{\text{samp}}$ can be used to calculate the spin labeling efficiency (SLE) when the reference used is known to exhibit a SLE of 100%:

$$\text{SLE} = \frac{c_{\text{samp}}}{c_{\text{sampII}}}, \quad (4.7)$$

It is worth mentioning that the determination of the sample concentration with relation 4.6 may result in large errors since the SLE is known to deviate from 100%.

Due to the more scientific character of the normalization to the area of the EPR absorption spectra, all spectra presented in this study (except the RT cw X-band EPR spectra presented for solubilized samples in section 5.1.3) were treated in this manner. Moreover, figure 4.7 obviously demonstrates the easier visual comparison of spectra of different shape by the use of area-normalization.
Figure 4.7: Comparison of amplitude- (left) and area- (right) normalization each showing one spectrum with (dotted line) and without (solid line) dipolar broadening.

In the case the measuring program itself does not add up the single spectra during accumulation, the program uniform written by C. Beier in the group of H.-J. Steinhoff was used. Besides the addition of spectra belonging to a set of measurements, possible magnetic field shifts (e.g. due to hysteresis) between scans being recorded while the magnetic field was scanned from low to high values (up-scans) and those being recorded while the magnetic field was scanned from high to low values (down-scans) could be eliminated using uniform.

Temperature dependent cw EPR spectra were analyzed in terms of an equilibrium between the different components present in spectra (except those measured at 160 K) investigated so far. In this regard, the program freedfit (by R. Fiege and H.-J. Steinhoff according to [Freed 1976]) was used. A brief theoretical introduction is already given in section 3.1.4.1. Fitting of simulated EPR spectra to the experimental ones detected between 263 and 343 K was performed according to a simple Brownian model of isotropic reorientational diffusion of the nitroxide. The spectra were fitted with two distinct spectral components. Component 1 was characterized by longer correlation times, and due to the inherent more complex influence of reorientational potentials in determining the real spectral shape, the simulation must be considered strongly to be approximated. The shape of component 2 on the other hand, was typical for an almost isotropic reorientational motion of the nitroxide, thus the simulated spectral features were better representing the real spectral shape. The $g$ and $A$ tensor values used as input parameters were determined prior to these simulations by spectral analysis of cw EPR spectra measured at 160 K (see section 4.3.3).

The reorientational correlation time $\tau_c$ was calculated from the obtained rotational diffusion constant ($R$) as $\tau_c = (6 \cdot R)^{-1}$ (see equations 3.58 and 3.79) for the two components. The rotational diffusion constants and the ratio of the two spectral components were allowed to vary according to the temperature. The logarithm of the ratio of the two components was plotted versus the inverse of temperature and analyzed in terms of a simple thermodynamic equilibrium between two states. The equilibrium constant $K$ was derived from the van’t Hoff plots. Prior to the fittings, all spectra were normalized to the area of the EPR ab-
sorption spectrum using the program *unispec* written in the group of H.-J. Steinhoff by C. Beier. *Unispec* is a program that amongst others calculates the spectrum’s second integral and the area-normalized spectrum.

Central resonance lines from accessibility measurements have been analyzed using the program *powerfit* written by Martin Kühn [Kühn 2003]. With this software, pseudo Voigt lines\(^8\) fitted to the central resonance lines of the cw EPR spectra give the resonance line’s peak-to-peak amplitude \(Y'_{pp}\) and width \(\Delta H_{pp}\). In the implemented program *satfit*, the particular peak-to-peak amplitudes are plotted against the square root of the microwave power calculated from the corresponding settings of the microwave main attenuator. Through fitting of the relation

\[
Y'_{pp} = A \frac{\sqrt{P}}{(1 + 2^{1/2} P_{1/2})^{\epsilon}},
\]

(with \(A\), \(P_{1/2}\) and \(\epsilon\) as adjustable parameters) to the corresponding dataset, the value for \(P_{1/2}\) is determined, giving the incident microwave power where the first derivative amplitude \(Y'_{pp}\) is reduced to half of its unsaturated value (see figure 3.9). With the equation already described (see equation 3.65), the accessibility parameter \(\Pi_{pq}\) of a certain paramagnetic quencher \(pq\) can be obtained for comparison with literature [Farahbakhsh et al. 1992, Oh et al. 2000]:

\[
\Pi_{pq} = \left( \frac{P_{pq}^{\frac{N_2}{1/2}} \Delta H_{pp}^{DPPH}}{P_{pq}^{\frac{1/2}{1/2}} \Delta H_{pp}^{N_2}} \right) \cdot \frac{\Delta H_{pp}^{DPPH}}{P_{1/2}^{DPPH}}.
\]

Again, the index \(N_2\) stands for the reference measurements performed in the absence of paramagnetic quenchers and in the presence of nitrogen, and the index DPPH stands for the standard Diphenyldipicrylhydrazide used. \(\Delta H_{pp}\) describes the peak-to-peak linewidth of the central resonance line under conditions of non-saturating microwave power. Solid DPPH is commonly used as standard substance to normalize accessibility values for international comparisons. Note that in the study presented, no normalization of the \(\Pi_{pq}\) values was performed since DPPH has shown to be no good choice in terms of this standardization [Kühn 2003]. Hence, to evade the delicate DPPH measurements and to give a more reliable value, the exchange frequency \(W_{ex}\) between the nitroxide group and the respective paramagnetic quencher has been calculated directly (see section 4.3.2).

4.3.2 Calculation of exchange rates \(W_{ex}\)

As already mentioned, in power saturation measurements to determine accessibilities for paramagnetic quenchers, DPPH is not a good choice to normalize for variations in res-

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\(^8\) Convolution of a Lorentz- and a Gauss-profile.

\(^9\) For definition of the parameters see section 3.1.3.
onator efficiency to facilitate comparison of $\Pi_{pq}$ values obtained with different resonators (e.g. inter-laboratory comparisons). First, DPPH is a powder exhibiting an immense EPR signal and consequently has to be diluted with KCl or MgO. Since membrane proteins in the detergent-solubilized as well as in the lipid-reconstituted form are resuspended in an aqueous solution, the dielectric properties of the sample and the reference are not consistent. Second, powdery DPPH samples are not measured in the gas-permeable TPX capillaries used for the accessibility experiments, but in quartz tube capillaries of similar diameter leading to different $B_1$-field distributions at the sample location. Eventually, an equal filling height of the aqueous samples and the reference is hard to manage due to their different states of aggregation. In the following paragraphs a method to determine Heisenberg exchange rates from power saturation measurements is described according to Altenbach et al. [Altenbach et al. 2005].

Heisenberg exchange between a nitroxide and a paramagnetic quencher molecule (here: NiEDDA (in former experiments the charged chromium oxalate (CrOx) was used) and molecular oxygen) requires a direct contact interaction in an encounter complex. The exchange rate $W_{ex}$ is consequently a measure of the exposure of the spin label to the medium containing the respective paramagnetic quencher and is thus directly proportional to the accessibility parameter $\Pi_{pq}$ already introduced:

$$\Pi_{pq} = \alpha W_{ex}, \quad (4.10)$$

where $\alpha$ is the proportionality constant to be specified. Since $\alpha$ is independent of the paramagnetic reagent itself, in the following only measurements in the presence of the water soluble NiEDDA are discussed. It is noteworthy that the paramagnetic species used for the determination of water accessibilities has to be electrically neutral (to avoid unwanted interactions) and of the size of the nitroxide for sterical reasons. Additionally, the exchange rate between the nitroxide and the paramagnetic reagent has to be solely diffusion controlled as applies in the strong exchange limit. Thus, to determine $\alpha$ using NiEDDA, only spin label positions at water exposed sites of the complex are suitable for this investigation. Finally, the constraint $T_{1PQ} < \tau_E$, with $T_{1PQ}$ describing the longitudinal relaxation time of the paramagnetic quencher and $\tau_E$ the encounter complex lifetime, ensures the nitroxide/paramagnetic quencher interaction to be solely specified by Heisenberg exchange with minor contributions of distance-dependent magnetic dipolar interactions.

The collision frequency with a reagent experienced by the nitroxide is furthermore given by the relation

$$W_{ex} = k_{ex} C_{PQ}, \quad (4.11)$$
with $k_{ex}$ describing the exchange rate constant and $C_{PQ}$ the reagent’s concentration. In case of diffusion controlled Heisenberg exchange, the exchange rate constant is defined by

$$k_{ex} = P_{max} f k_D = P_{max} f 4\pi \frac{N_A}{1000} (D_N + D_{PQ})r_c,$$

(4.12)

with $P_{max}$ as maximum exchange efficiency, $f$ as “steric factor” and $k_D$ as the diffusion controlled rate constant. In the strong exchange limit and with $T_1PQ < T_E$ it follows that $P_{max} = 1$. $k_D$ is furthermore described by $D_N$ and $D_{PQ}$, the diffusion constants for the nitroxide (N) and the paramagnetic quencher (PQ), respectively, and the collision radius $r_c = r_N + r_{PQ}$ with $r_N$ and $r_{PQ}$ as effective radii of the particular species.

Although Heisenberg exchange under the assumptions mentioned equally changes longitudinal ($T_1$) and transverse ($T_2$) relaxation time of the nitroxide (see equation 4.13), only effects on $T_2$, visible in Lorentzian line broadening, are considered here.

$$W_{ex} = \Delta \left( \frac{1}{T_1} \right) = \Delta \left( \frac{1}{T_2} \right)$$

(4.13)

The cw EPR spectrum of the nitroxide subject to the interaction with the paramagnetic quencher can be constructed by convolution of the cw EPR spectrum in the absence of exchange with a Lorentzian line (see figure 4.8). It follows:

![Figure 4.8: Convolution of the spectrum in absence of NiEDDA (black, left) with a Lorentzian line of certain FWHM value (olive, center) leads to the broadened spectrum showing exchange interaction of the nitroxide with the paramagnetic quencher (light blue, right).](image)

$$W_{ex} = \Delta \left( \frac{1}{T_2} \right) = \frac{1}{2} \gamma \Delta H_{1/2} = 4.4 \times 10^6 (g) \Delta H_{1/2},$$

(4.14)

with $\gamma$ as the gyromagnetic ratio and $g$ as the g-factor of the nitroxide. Here, $\Delta H_{1/2}$, the broadening due to the paramagnetic quencher given, is in Gauss (G) and $W_{ex}$ accordingly in Hertz (Hz). Using equations 4.10, 4.11, 4.14 and a comparison of line broadening data with that of cw power saturation for different solvent exposed sites in solubilized colicin samples (further information on colicin can be obtained from L. Pulagam [PULAGAM 2007]) enables the calculation of $\alpha$. Note that the $\Pi_{pq}$ values obtained through accessibility measurements have not been normalized for variations in resonator efficiency and consequently the obtained $\alpha$ values are cavity dependent (only for measurements performed on the spec-
trometer specified in section 4.2.4). It is moreover noteworthy that samples to be used for this approach have to be characterized by a single spectral component since distinction of the effects of several spectral components can hardly be done in accessibility measurements. To obtain the $k_{ex}$ values from the different samples, cw X-band EPR measurements have been performed in terms of different final NiEDDA concentrations in the sample volumes. Fitting of simulated cw EPR spectra to those in the presence of NiEDDA yielded the $\Delta H_{1/2}$ values neccessary for the calculation of the $k_{ex}$ values that are described by the slopes of the linear regressions as displayed in figure 4.9. The program used was on the basis of the well-established EPR spectrum simulation program easyspin, a Matlab toolbox written and maintained by S. Stoll (University of California, Davis, USA), combined with an additional algorithm to convolute the Lorentzian with the EPR spectrum written by H. Reyher (University of Osnabrueck, Germany).

With the values determined for the exchange rate constant $k_{ex}$, accessibility values obtained earlier and the NiEDDA concentrations used, $\alpha$ could be calculated for each sample as follows:

$$\alpha = \frac{k_{ex} \cdot C_{NiEDDA}}{\Pi_{NiEDDA}}. \quad (4.15)$$

**Figure 4.9:** Plots of exchange frequency $W_{ex}$ versus NiEDDA concentration $C_{NiEDDA}$ for the water exposed colicin residues 33R1, 62R1, 115R1 and 166R1. $W_{ex}$ values were calculated with the far right term of equation 4.14 from cw EPR spectra performed. According to equation 4.11 $k_{ex}$ can be obtained from the slopes of the linear regressions of the particular data set (light blue lines).
Data handling

Error margins have been obtained according to:

\[
\Delta \alpha = \left| \frac{\partial \alpha}{\partial \Pi} \Delta \Pi \right| + \left| \frac{\partial \alpha}{\partial k_{ex}} \Delta k_{ex} \right| + \left| \frac{\partial \alpha}{\partial C_{NiEDDA}} \Delta C_{NiEDDA} \right| \tag{4.16a}
\]

\[
\Rightarrow \Delta \alpha = \left( \frac{\Delta \Pi}{\Pi} + \frac{\Delta k_{ex}}{k_{ex}} + \frac{\Delta C_{NiEDDA}}{C_{NiEDDA}} \right) \alpha, \tag{4.16b}
\]

where \( \Delta \Pi \) as well as \( \Delta C_{NiEDDA} \) were assumed to be \( \pm 10\% \) and \( \Delta k_{ex} \) was obtained from the errors of the linear regressions including minor errors for the determination of the Lorentzian FWHM values by the fitting procedure. Eventually, the mean \( \alpha \) was found to be

\[
\alpha = 1.87 \pm 0.43 \frac{1}{MHz}. \tag{4.17}
\]

However, inhomogeneities in the B-field have still to be considered.

4.3.3 cw EPR powder spectra

Simulated dipolar broadened EPR powder spectra were fitted to experimental data obtained in cw EPR distance measurements performed at 160 K to determine the inter-spin distances. The software dipfit used for this purpose written by H.-J. Steinhoff and coworkers is described more detailed in [Steinhoff et al. 1997]. A brief introduction to the theory is given in section 3.1.4.2. To account for a range of distances expected to arise from different spin label side chain orientations, a Gaussian distribution of inter-spin distances with a distribution width of 0.2 nm was assumed. During the fitting procedure, the g tensor values, those for \( A_{xx} \) and \( A_{yy} \) of the hyperfine tensor as well as the Lorentzian and Gaussian line width parameters were fixed to the values found for the corresponding reference spectrum without dipolar broadening representing the singly labeled species. The g tensor parameters used were determined by fitting of low temperature spectra detected at 95 GHz [Brutlach et al. 2006]. \( A_{xx} \) and \( A_{yy} \) were fixed to 0.58 and 0.42 mT, respectively, whereas \( A_{zz} \) was varied to account for differences in the polarity in the immediate spin label environment. The spectra were convoluted with a field-independent Voigt line shape function composed of a superposition of 29% Lorentzian and 71% Gaussian of 0.3 and 0.37 mT width, respectively. The fitting parameters for the determination of the dipolar broadening are thus the average inter-spin distance, \( A_{zz} \) of the hyperfine tensor and the fraction of the singly spin labeled component. The latter one can also be calculated prior to the simulations via the spin label efficiency (see section 4.3.1). In case of doubly labeled mutants, statistical calculations about the individual, singly labeled spin label sites have to be taken into account and weightened accordingly. The spin label efficiency in this study was for all lipid-reconstituted mutants investigated in the range from 60 - 80%.

To visualize dipolar broadenings present in the powder spectra, subsequent to the fittings all spectra were normalized to the area of the absorption spectra using unispec.
4.3.4 Pulse EPR data

Inter-spin distance distributions and average numbers of spins in the sample were obtained from the dipolar time evolution data from pulse EPR measurements by the program \textit{DEERAnalysis2006} written by G. Jeschke [Jeschke et al. 2006]. The program handles dead-time free pulse ELDOR data and is based on algorithms described previously [Jeschke et al. 2002, Jeschke et al. 2004b, Chiang et al. 2005, Jeschke and Polyhach 2007]. In properly adjusted four-pulse DEER experiments, the signal should be entirely in the real part of the data set. However, since for very weak signals often obtained for membrane protein samples the exact adjustment of the phase during setup is quite difficult, part of the signal will be in the imaginary data set. Small phase drifts during spectra accumulation are also likely to occur. Therefore, a phase correction had to be applied before the fitting. Here, the program by default determines the corresponding phase correction directly by minimizing the root mean square deviation of the imaginary part for the last three quarters of data (see figure 4.10a). Since in most cases only distances within the complex under investigation are of interest, the spectral contribution of inter-molecular distances has to be suppressed. This is done by the background correction (see figure 4.10a), a separation of the signal \( V(t) = \{1 - [1 - \Delta D(t)]\} \) \( B(t) \) into a dipolar evolution function \( D(t) \) (see figure 4.10b) for the nanoobject itself and the background decay \( B(t) \) due to neighboring spin labels. In this study a homogeneous distribution of the objects with \( d = 3 \) dimensions has been shown to fit well. It follows:

\[
B(t) = \exp \left( -kt^2 \right),
\]

\[\Rightarrow B(t) = \exp (-kt).\]  

(4.18a)

(4.18b)

Due to possible pump pulse interference with the last observer pulse or noise, in some cases data points have to be excluded at the end of the spectra during analysis. Though the program does not cut off data points automatically, a suggestion for cutoff is displayed in the dipolar evolution plot showing the data after background correction. To do so, the noise is calculated by the program and compared to an acceptable noise level that has to be preserved. Finally, realistic distance distributions \( P(r) \) (see figure 4.10d) are determined using \textit{Tikhonov regularization} incorporating the constraint \( P(r) > 0 \), where the compromise between noise suppression (smoothness) and resolution of the distance distribution is quantified by the regularization parameter \( \alpha \). It is worth mentioning that this is a delicate task. Though the characteristics of the whole distance distribution (e.g. mean distance \( \langle r \rangle \) and standard deviation \( \sigma_r \)) are stable, the positions and peaks of the single distribution peaks do strongly depend on the noise. Minimizing the \textit{target function} given in equation 4.19 provides an adequate way to solve this issue. In

\[
G_{\alpha}(P) = \|S(t) - D(t)\|^2 + \alpha \left\| \frac{d^2}{dr^2} P(r) \right\|^2
\]

(4.19)
4.3 Data handling

Figure 4.10: Plots from DEERAnalysis2006. a) Real (black trace) and imaginary (magenta trace) part of data set after phase correction, setting of zero time (green vertical line), start of the range for the background fitting (light blue vertical line) and the fitted background function (red trace). b) Renormalized dipolar evolution function after background correction (black trace) and fit of the dipolar evolution function (red trace). Δ: modulation depth. c) L curve for α = 0.001, 0.01, 0.1, 1, 10, 100, 1,000, 10,000, 100,000 with the suggested best compromise (red dot, corner, α = 100). d) Distance distribution computed by Tikhonov regularization (black trace) between the lower (blue vertical line) and upper (magenta vertical line) end of the fit range (here 1.5-8 nm).

$S(t)$ describes a simulated time-domain signal from the given distance distribution $P(r)$ and $\|\cdot\|$ the Euclidean norm. A large value for $\alpha$ corresponds to a strong suppression of artifacts as well as to a strong broadening of the peaks in the distance distribution. Determination of an optimum $\alpha$ is done using the so-called L-curve criterion (described in detail in [Jeschke et al. 2006]) by plotting the logarithm of the smoothness $\eta$ (second term on the right hand side of equation 4.19 without $\alpha$) against the logarithm of the mean square deviation $\rho$ between the simulated and experimental dipolar evolution function (first term on the right hand side of equation 4.19) to find out the best compromise between them (see figure 4.10b). Since this approach describes a purely mathematical criterion that does not take into account any other knowledge, the final solution for each distance distribution obtained in this study was constructed by finding the most reasonable compromise between $\alpha$ and the expected results.

The average number of spins $\langle n \rangle$ is determined via the total modulation depths

$$\Delta = 1 - \exp [\lambda(\langle n \rangle - 1)], \quad (4.20)$$

where $\lambda$ is the modulation depth factor, which is influenced by the shape of the pump pulse and the position of the pump frequency with respect to the mode’s center. In order to get
realistic results, the program was tested with synthesized nitroxide biradicals having 100% labeling efficiency. More detailed information can be obtained from [Jeschke et al. 2006, Jeschke 2006] and therein cited references.

4.3.5 Pulse ENDOR data

Spectra recorded in Davies-type pulse ENDOR measurements were analyzed qualitatively without the use of any fit programs. It is noteworthy that since no modulation was used in these experiments, the ENDOR spectra are in absorption. Due to very small signal amplitudes in some cases and resulting measuring times of up to 24 hours, wrong phase settings occurring during experimental setup and permanent minor phase drifts adding up during spectra accumulation had to be compensated. The measuring program Xepr provided by Bruker Biospin together with the spectrometer used was able to apply phase corrections to the real and imaginary part of the saved experimental data set. Baseline problems are apparent to a greater or lesser extent for each particular experiment, but have shown not to be dependent exclusively on the sample concentration. Hence, a normalization of the spectra to the same area or the maximum amplitude is not reasonable. However, an approximated normalization of the high-field ends (between 18 and 24 MHz) of the spectra to the same slope was assumed to be an adequate method. Subsequently, to increase the signal-to-noise ratio and to aid interpretation, the ENDOR spectra were symmetrized with respect to the proton nuclear Zeeman frequency at 14.65 MHz (see figure 4.11). The spectra are displayed on an hyperfine coupling axis centered at the proton nuclear Zeeman frequency. To visualize the differences between the respective spectra, the part of the symmetrized spectra not describing any ENDOR signal was cut according to the red, dotted horizontal line in the right part of figure 4.11.

Figure 4.11: Symmetrization of Davies-type pulse ENDOR spectra. A copy of the original spectrum (left) is mirrored with respect to the proton nuclear Zeeman frequency $\nu_H$ (center, red). The sum of the two spectra gives the symmetrized result (right).
CHAPTER 5

RESULTS

For structural and functional elucidation of the spin labeled HAMP domain, the NpSRII/NpHtrII complex was investigated in terms of spin label side chain dynamics, accessibilities for paramagnetic quenchers, polarity, the spin label’s proton-microenvironment, and inter-spin distances. The results are illustrated in the following sections, particularly with respect to the relevance of the environmental salt concentration for the properties of the HAMP domain. For clarity reasons, the following paragraphs are divided in subsections according to the EPR information obtained.

In the following, the HAMP residues chosen for the investigations will be depicted in a simple schematic representation of the HAMP domain showing the protein structure according to secondary structure analysis (AS1 (α-helical)-connector (undefined structure)-AS2 (α-helical), see figure 2.6). This schematic HAMP model will be presented as a simple prolongation of helices TM2 and TM2’ of the transmembrane part of the trasducer in complex with NpSRII from the available X-ray structure (see e.g. figure 5.1).

5.1 Side chain dynamics subject to environmental inputs

The overall dynamics of a spin label side chain attached to a selected position in the protein under investigation can be obtained in terms of the side chain mobility described by $\Delta H_{pp}^{-1}$ and line shape analysis. These properties have been proven to be a fundamental source of information, particularly when environmental conditions (e.g. salt concentration) are thought to induce major rearrangements in the protein sidechains. In the following paragraphs a detailed inspection of the EPR spectral features present under different environmental conditions is done in order to derive their molecular origin. To do so, several positions in the HAMP domain of the truncated transducer (NpHtrII$_{157}$) in complex with NpSRII reconstituted in PML were chosen for site-directed spin labeling EPR. Where no representation of the residues’ location is given, the reader is referred to figure 5.1 for a general overview. For comparison, three positions (S154, K157 and L159) in helix F of the receptor NpSRII in complex with NpHtrII$_{157}$ reconstituted in PML, position S226 in
bacteriorhodopsin in purple membranes, position L105 in Colicin A as well as 5-doxylstearic acid enriched purple membranes were investigated.

Figure 5.1: Model representation of the HAMP domain fused to the ribbon diagram of the crystal structure of the transmembrane part (NpSRII: blue, NpHtrII: gray blue) showing the approximate locations of the transducer residues L75, A79, L93, M100, L105, E116, and Y121. The schematic HAMP motif was constructed according to secondary structure predictions (AS1-connector-AS2), where the two amphipathic sequences due to their expected \( \alpha \)-helical structure are depicted by gray barrels, which are overlayed by slightly transparent \( \alpha \)-helices. The brownish area indicates the lipid bilayer. \( C_\beta \) atoms of the residues chosen are displayed by the light yellow balls designated by the 1-letter amino acid code followed by the amino acid’s number in the sequence.

5.1.1 Effects of high salt concentrations and high lipid contents

At low salt concentrations as 150 mM KCl, the spectra obtained for the HAMP residues are characterized by the presence of two distinct components. In figure 5.2A, the cw X-band EPR spectra detected for four residues representative for the different HAMP subdomains at ambient temperature are presented. Component 1 represents a fraction of the nitroxide population where the spin label is engaged in secondary and tertiary interactions reducing its reorientational freedom. The resulting EPR spectrum is characterized by broad lines and large apparent hyperfine splittings. Contrarily, component 2 represents a nitroxide subpopulation exhibiting narrower lines, indicating high spin label mobilities, which can be correlated to lack of secondary/tertiary constraints in the spin label microenvironment and/or backbone fluctuations. As illustrated in section 3.1.3.5, the term mobility is used
5.1 Side chain dynamics subject to environmental inputs

Figure 5.2: Spin normalized room temperature X-band EPR spectra of spin-labeled NpHtrII variants in complex with NpSRII reconstituted in PML under different environmental conditions. The respective HAMP subdomain harboring the residue presented is given in parentheses with the position’s denomination (top). Spectra of samples recorded in the presence of 400-fold molar excess of lipids per 1:1 complex are shown with dashed lines. For the sake of comparison, some of the spectral amplitudes were multiplied by scaling factors depicted at the high-field ends of the respective spectra. The samples were measured in Tris-HCl buffers (pH 8) containing: A) 150 mM KCl, the two spectral components (1 and 2) are highlighted by the black arrows; B) 2 M KCl and 20% (v/v) glycerol as cryoprotectant (experimental conditions used for high salt concentration in low temperature experiments); C) 3.5 M KCl; D) 150 mM KCl and 40% (w/v) sucrose.

in a more general sense defining the motional freedom of the MTSSL side chain. Fitting of simulated spectra to the experimental ones (according to the method described in section 4.3) allowed to obtain an effective isotropic reorientational correlation ($\tau_c$) time of approximately 10 ns for component 1 and of approximately 1 ns for component 2. The latter component exceeds the mobility observed for positions located in very dynamic loop regions [Mchaourab et al. 1996, Isas et al. 2002], thus, we suggest that protein backbone fluctuations are involved in determining the narrow spectral lineshapes observed.

It is furthermore quite evident that the ratio between the two components is dependent on the location of the spin label in the three different HAMP subdomains (AS1, connector, AS2). The fraction of the component characterized by narrow lines is minimal at the beginning of the AS1, where the HAMP domain is in close proximity with the transmembrane region. Analysis of the subpopulations’ fractions requires the determination of the spectral areas, which can be obtained by fitting of the spectra, but not from comparison of peak intensities.

To mimic the physiological conditions, the salt concentration was increased to 2 M KCl and 3.5 M KCl (see figure 5.2B and C, respectively). This led to a relevant suppression of component 2 and a concomitant increase of component 1 for all positions investigated (see

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*a* The additional presence of 20% (v/v) glycerol (viscosity $\eta_{20\%\text{glycerol}} = 1.7 \text{ cp}$) in the sample has almost no effect on the spectral features (data not shown).
e.g. position 121). With respect to the signal amplitudes, the effects induced by high salt concentrations are more evident for residues, where the second component is characterized by very sharp lines as e.g. in the case of L105R1. However, complete elimination of component 2 was never obtained, with exception of position 89, close to the transmembrane region of the complex.

Due to the fact that the extent of protein crowding in the native membrane is not fully understood, and that possible aggregation mechanisms might influence the EPR spectral features, the NpSRII/NpHtrII complex was additionally reconstituted using a 400-fold molar excess of lipids instead of the normally used 40-fold molar excess of lipids per 1:1 complex. The resulting spectra illustrated in figure 5.2A to C clearly indicate that dilution of the proteins in the membrane did not modify the spectral changes, thus suggesting that the properties observed are not due to protein aggregation.

To investigate a possible additional influence of the viscosity of the medium on the spectral changes detected, the spectra of NpSRII/NpHtrII$_{157}$-S89R1 and NpSRII/NpHtrII$_{157}$-L105R1 were also recorded in the presence of 40% (w/v) sucrose. The viscosity of $\eta_{40\%\ sucrose}$ is 6.2 cp, whereas the viscosity of water at 293 K is 1.002 cp, and for 2 M and 3.5 M KCl are 0.99 cp and 1.02 cp, respectively (all viscosity values are taken from [Lide 2008]). Although the change in viscosity induced by sucrose is much larger than that induced by high salt concentrations, the salt-induced spectral effects are obviously much more pronounced. Hence, solely viscosity-based spectral changes can be excluded.

An issue worth to address is the influence of the transducer truncation on the salt-induced effects and in general on the overall HAMP structure. Therefore, cysteines were also engineered and spin labeled in the NpHtrII$_{1-230}$-C173S construct, comprising both HAMP domains, and in the full length transducer NpHtrII-C173S, where the single native cysteine in the transducer protein sequence was in each case exchanged by a serine to avoid the binding of the MTSSL to this residue. Comparison of the room temperature spectra for a selected position in the AS1, i.e. NpSRII/NpHtrII$_x$-A94R1, measured under low and high salt conditions, is presented in figure 5.3.

The three spectra recorded for 150 mM KCl (black lines) show both spectral components. Only minor differences are visible particularly with respect to the narrow lines of component 2. It is noteworthy that minor changes in the spectral component 2 were also found in different batches of the same mutant (data not shown), indicating the sensitive nature of the second component.

To compare the three spectra at high salt conditions, a magnified view of the low-field spectral region is presented in figure 5.3, right. Obviously, the presence of the high salt concentration led to the almost complete suppression of the second component in all three cases, and to the appearance of similar spectral features correlated to component 1. This indicates that the salt effect described is a specific property of the HAMP domain, and the
5.1 Side chain dynamics subject to environmental inputs

Figure 5.3: Comparison of spin normalized room temperature cw X-band EPR spectra of NpHtrII-A94R1 in complex with NpSRII reconstituted in PML. Left: NpHtrII variants truncated at positions 157 (NpHtrII<sub>157</sub>) and 230 (NpHtrII<sub>1−230</sub>-C173S) as well as the full length transducer variant (NpHtrII-C173S) were measured at 150 mM KCl (black lines) and 3.5 M KCl (olive lines). The two spectral components (1 and 2) are highlighted by arrows. Right: Magnified view on the low-field peak region of the spectra detected for the variants NpHtrII<sub>157</sub> (gray), NpHtrII<sub>1−230</sub>-C173S (black) and NpHtrII-C173S (olive) under high salt conditions. Due to the almost complete suppression of component 2 at high salt concentration, only component 1 is visible. The asterisks denote the complex spectral shape of component 1, where two contributions arising from different rotameric states of the spin label side chain are visible.

Influence of the cytoplasmic domain is negligible. Consequently, the truncated version of the transducer is suitable for the following investigation.

An intrinsic feature of component 1 is the complex spectral shape. It comprises two contributions from different rotameric states of the nitroxide side chain that are highlighted by the asterisks in the right part of figure 5.3. The first contribution is expressed by the broad spectral feature (see left asterisk in the right part of figure 5.3) easily visible in all spectra presented, whereas the second contribution is much harder to recognize since it is expressed in the spectrum very close to component 2 (see right asterisk in the right part of figure 5.3). The origin of the rotamers with different mobility simultaneously present in the X-band EPR spectra has been studied extensively by J. H. Freed and coworkers in terms of computational calculations and experiments on MTSSL spin probes attached to “ideal” α helices [TOMBOLATO et al. 2006a, TOMBOLATO et al. 2006b]. By means of the restrictions deriving from the MTSSL chain geometry (characterized by the five dihedral angles depicted in figure 2.12) and local constraints, they could identify a limited number of allowed spin label conformers that undergo torsional fluctuations (damped oscillations) and conformational jumps. After comparison of the time scales and amplitudes of the different motions present, the major role played by rotations about the outermost bonds of the nitroxide side chain, i.e. the dihedral angles $\chi_4$ and $\chi_5$ (see figure 2.12), could be obtained [TOMBOLATO et al. 2006a, TOMBOLATO et al. 2006b]. The relevance of certain
MTSSL R1 side chain rotamers on the 9.5 GHz EPR time scale has been supported recently by Hubbell and coworkers [Guo et al. 2007, Guo et al. 2008]. Thus, jumps and librations about the energy landscape’s minima of these two dihedral angles are considered here to be responsible for the complex features of the spectral component 1.

To reveal the importance of the salt’s anions nature on the observed spectral changes, experiments using the NpSRII/NpHtrII157-S91R1 variant were carried out with diverse anions (Cl\(^-\), SCN\(^-\) and SO\(_4\)\(^{2-}\)). Same effects were observed also for NpSRII/NpHtrII157-A88R1, -S89R1, -L90R1 and -T92R1 (data not shown). The resulting spectra given in figure 5.4 are illustrated both with respect to their molar concentrations and their ionic strengths I.

![Figure 5.4](image_url)

**Figure 5.4:** Effects of different salts on the shape of the room temperature cw X-band EPR spectra obtained for NpSRII/NpHtrII157-S91R1. For better comparison, the magnified low-field lines of the spin normalized spectra obtained are presented, and the dotted horizontal lines representing the spectral baselines are given to better guide the eyes. According to the salt species (top) used, the spectra are arranged in three sets. Measurements were performed for different molar concentrations (left of each spectrum) in each set. The ionic strengths are given in dependence of the molar concentration [M] at the top.

The consistent suppression of the more dynamic component’s fraction with higher salt concentrations is observable for all anions tested, whereas the extent of the effect for each salt is different and does not depend on the ionic strength, rather on the molar concentration. In fact, the suppression of the second component in case of the used NaCl is more pronounced compared to that in the presence of KSCN, although the two species possess the same ionic strength (I = 1 [M]). On the other hand, in terms of molar concentrations, Na\(_2\)SO\(_4\) is more effective to induce spectral changes, and in terms of ionic strength the two salts affect the HAMP structure in a similar way. These findings point to the fact that the salt-induced changes are not solely due to ionic strength effects.
The dependence of the changes monitored can be correlated with the "Hofmeister series" of neutral salts after Franz Hofmeister, who first described the mentioned phenomenon in 1888 [Hofmeister 1888]. Generally, the Hofmeister effect is based on salt-specific changes of the properties at the protein/water-interface. Two opposite mechanisms exist: i) so-called kosmotropic ions cause the salting-out of nonpolar groups what is known to stabilize protein structures, the rank order of effectiveness of anions in salting-out is $\text{SCN}^\text{-} < \text{Br}^- < \text{Cl}^- < \text{F}^- < \text{SO}_4^{2-}$ and the rank of order of cations $\text{Li}^+ < \text{Na}^+ < \text{K}^+ < \text{Mg}^{2+} < \text{Ca}^{2+}$ [Baldwin 1996, Melander and Horvath 1977, Nandi and Robinson 1972b]; ii) so-called chaotropic ions cause the salting-in of the peptide groups thereby destabilizing the proteins in action, the rank order of effectiveness of anions in salting-in is $\text{SO}_4^{2-} < \text{F}^- < \text{Br}^- < \text{I}^- < \text{SCN}^\text{-}$ and the rank order of cations $\text{Li}^+ < \text{Na}^+ < \text{K}^+ < \text{Ca}^{2+}$ [Nandi and Robinson 1972a]. The null point in the Hofmeister series, separating protein denaturants from stabilizers, can accordingly not be defined sharply since always a balance between the two mechanisms has to be regarded [Vogel et al. 2001]. It is worth mentioning that divalent ions are more effective in salting-out than monovalent ones, that the effectiveness of the influence of the anions is supposed to be stronger compared to the cations, and that almost all Hofmeister ions salt out nonpolar molecules from aqueous solutions [Baldwin 1996]. Although the Hofmeister effect is considered to be exerted indirectly, namely water-mediated, the question of how this process takes place in detail is still not unraveled. However, the most kosmotropic anion $\text{SO}_4^{2-}$ induced the strongest suppression of the fraction of component 2. The effects observed could be supported by measurements on further transducer mutants as NpSRH/NpHtrII157-A88R1, -S89R1, -L90R1 and -T92R1 (data not shown). Altogether, the data obtained allow to suggest a conformational change in the HAMP domain induced by higher salt concentration.

A more detailed picture of the possible conformational change in the HAMP domain can be obtained by a nitroxide scanning, where the residues in the protein sequence are successively mutated, spin labeled and investigated via EPR spectroscopy. This has been done for all residues in the amphipathic sequence 1 and selected ones in the predicted connector region and in the amphipathic sequence 2. The resulting spectra obtained in the presence of 3.5 $M$ KCl (and in some cases also for 4 $M$ NaCl) are compared to the spectra obtained at 150 $mM$ KCl [Doebber 2005, Bordignon et al. 2005] in figure 5.5.

Under low salt conditions, the two spectral components are visible in each case but with different ratios. The first residue depicted, i.e. V78R1, describes a position located in the transmembrane part of the transducer helix TM2, where the motional freedom of the attached side chain is expected to be restricted due to secondary and tertiary interactions. Broad spectral features are indeed recorded, where no indications for the second spectral component are present. On the other hand, all positions investigated in the predicted $\alpha$-helical AS1 exhibit distinct spectral components 1 and 2. Interestingly, the spectral shapes
Chapter 5 Results

Figure 5.5: Spin normalized cw X-band EPR spectra obtained under low (left, previously published data) and high salt (right; black: 3.5 M KCl, blue: 4 M NaCl) conditions for selected NpSRII/NpHtrII$^{157}$ mutants reconstituted in PML. A schematic representation on the right showing the AS1, the connector and the AS2 (not scaled) is adapted to the location of the residues investigated. For better comparison, some spectral amplitudes were multiplied by a scaling factor.
5.1 Side chain dynamics subject to environmental inputs

In this subdomain of the HAMP differ despite the consistent secondary structure predicted. From the EPR analysis, the spectra can be divided into two groups: the first one comprising residues A88R1 to K96R1, possessing both a pronounced first and second component, whereas the second group of residues from A97R1 to M100R1 exhibits a smaller fraction of component 1 and narrower spectral features for component 2. Selected positions in the connector region (G101R1 to D115R1) were found to have similar shapes and compositions of the two components as found in the latter part of the AS1. Indeed, this is an interesting finding since the connector is predicted to have no defined secondary structural elements. The few residues examined in the predicted \( \alpha \)-helical AS2 (residues G118R1 to A122R1) and in the following cytoplasmic region (A148R1) also exhibit very narrow lines (higher fraction of component 2), comparable to those obtained in the connector region.

Increasing the KCl concentration to 3.5 \( M \), results in the expected suppression of component 2 except for those positions that are already characterized by a spectral shape indicating a compact structure at low salt concentration (e.g., V78R1). The spectral features distinguishing the two AS1 subregions are not anymore present under high salt conditions. The high-salt spectrum detected for NpSRII/NpHtrII\(_{157}\)-R99R1 is the only variant exhibiting a sharper second component. Since the second component in most of the spectra observed for the connector and the AS2 residues is particularly for L105R1, D115R1, A122R1 and A148R1 (see figure 5.5) still visible, they differ slightly from those mentioned before. Notably, a complex spectral shape was obtained for all positions under high salt conditions, which can be described by the presence of two spin label rotamers within a well defined secondary structure.

High-salt measurements performed with selected transducer residues in the HAMP domain (A88R1, A94R1, L105R1 and A122R1) in the presence of 4 \( M \) NaCl showed no difference with respect to KCl (see figure 5.5, blue lines). With the exception of very minor differences, the EPR spectra obtained for both high-salt experiments are completely in line, pointing to the exchangeable nature of the salt used as long as the anion is maintained. Particularly in the case of the NpSRII/NpHtrII\(_{157}\) complex investigated in this study, the two salts KCl and NaCl are of major importance due to their occurrence in the native environment of the membrane protein. According to [CHRISTIAN and WALTHO], the ionic composition of the intracellular milieu differs greatly from that of the outside medium. Estimated ionic concentrations in the medium are 4.0 \( M \) for \( Na^+ \) and 0.032 \( M \) for \( K^+ \), whereas the intracellular milieu contains 1.37 \( M \) \( Na^+ \) and 4.57 \( M \) \( K^+ \). These conditions allow an in vivo balance of the osmotic pressure. In case of \( H. \) salinarum and \( N. \) pharaonis, potassium has shown to be a compatible solute. Since the residues investigated in this study are located in the HAMP domain that is protruding into the cytoplasm of the cell, the use of KCl is preferable although NaCl is shown to induce the same effect.
In order to evaluate the motional freedom of the positions investigated, the mobility parameter $\Delta H_{pp}^{-1}$ (the inverse of the central resonance line width) was calculated in the presence of low and high salt concentration (see figure 5.6).

![Figure 5.6: Comparison of spin label mobility data at low and high salt concentrations. Mobility parameters $\Delta H_{pp}^{-1}$ (150 mM KCl: black circles; 3.5 M KCl: olive squares) extracted from the RT cw X-band EPR spectra depicted in figure 5.5 are presented versus the residue number. Errors are estimated to ±10% due to uncertainties in line width determination. Values obtained for directly neighboring residues are connected via dashed lines. The connector region between the two amphipathic sequences is shaded in gray due to its predicted lack of fixed structure. Mobility values for positions 79 to 87 were determined by A. A. Wegener (formerly Max-Planck Institute for Molecular Physiology, Dortmund, Germany).](image)

The mobility profile observed at low salt concentration (see black circles in figure 5.6) were obtained during my diploma thesis [Doebber 2005, Bordignon et al. 2005]. They are briefly explained here in order to provide a basis for the recently obtained data. Low mobilities are observed for positions located in the transmembrane part of the transducer helix TM2 (residues V78 to L82) and the N-terminal end of the AS1 (residues G83 to A87), typical for buried residues and very strong interactions of the nitroxide side chain with secondary and tertiary structural elements in its microenvironment. The following part of the AS1 (residues A88 to A94), where the spectra possess both a pronounced component 1 and 2, a periodic pattern in the $\Delta H_{pp}^{-1}$ values is visible, indicative for a periodic structure. The presence of this $\alpha$-helical structure was also demonstrated by high-field EPR analysis [Brutlach et al. 2006]. The absence of such a periodic motif in the remaining part of the AS1 (residues A95 to M100), and the presence of very high mobility values suggested a very dynamic end of the AS1 under low salt conditions. It is noteworthy that due to the determination of the $\Delta H_{pp}^{-1}$ values from the line width of the central resonance line, the resulting value is biased to the second component since the corresponding narrow line dominates the shape of this resonance line. No information on the first spectral component.
5.1 Side chain dynamics subject to environmental inputs

can then be inferred from the mobility data presented. The presence of a pronounced component 2 as in case of residues A95 to M100 in the AS1, G101 to D115 in the connector region and G118 to A122 in the AS2 correspondingly results in very high values for $\Delta H_{pp}^{-1}$ (see figure 5.6), in line with a very dynamic helix, or a partially unfolded structure.

Increasing the salt concentration to 3.5 M KCl results in minor changes of mobility for residues V78 and A88 to L91. With the exception of two positions (R99 and E116), the presence of 3.5 M KCl (see figure 5.6, olive squares) leads to an overall decrease in mobility. The described prevalence of component 1 visible in all spectra detected, and the greater similarities between them is thus reflected in the mobility parameter profile. Strikingly, huge differences between the low and high-salt mobility parameter values (up to about 3.5 ($mT$)$^{-1}$) can be observed for residues located at the C-terminal end of the AS1 (A95 to M100, except for position R99), indicating the relevant suppression of the mobile component 2 in favor of the restricted spectral component 1. The mobility values obtained at high salt concentration reflect the properties of the major spectral component 1 and are typical for tertiary-interaction and helix-surface sites (see figure 3.8). Eventually, periodical patterns, appearing in the mobility plot, suggest the presence of a periodoc structure for the C-terminal end of the AS1.

Altogether, these findings point to the presence of a dynamic HAMP domain (henceforth denoted as $d$HAMP, correlated with the spectral component 2) prevalent at low salt concentrations, and a more compact HAMP structure (henceforth denoted as $c$HAMP, correlated with the spectral component 1) prevalent at high salt concentrations.

In order to find out if the salt-induced effects can also be observed for residues not located in the HAMP domain, and to address the effects of high salt concentrations on the purple membrane lipid bilayer, measurements were also performed on three residues in the receptor molecule NpSRII and on spin labeled lipid bilayers.

Spin labeled positions in the NpSRII E-F-loop (S154R1), located at the interface with the transducer helix TM2, and on NpSRII helix F (K157R1, L159R1), located close to TM2 and helix G, respectively (see figure 5.7, left), were investigated in the presence of both low and high salt concentrations. The resulting spin normalized EPR spectra are presented in the right part of figure 5.7.

At low salt concentration, the spectral shapes of the NpSRII variants examined already possess quite low degrees of motional freedom compared to that observed for residues in the transducer HAMP domain. The presence of complex spectral features can be recognized in all spectra shown. NpSRII-L159R1/NpHtrII_{157} exhibits a spectrum indicative for restrictions in the spin label motions due to secondary/tertiary interactions. Position 154, located in a loop region, exhibits spectral features indicative for a more mobile spin label side chain.
Increasing the salt concentration to 3.5 M KCl leads to a considerable spectral change for position 154 and minor changes for position 159 (see olive lines in figure 5.7). Even though the spectral change has the same nature compared to that monitored for the transducer residues, namely a decrease in the overall mobility, the extent of the changes is much less pronounced, and the nature of the mobile component suppressed is different from that observed in the HAMP residues. The salt-induced effect in the NpSRII residues is considered to be indirect, and therefore indicating that only the signal transduction domain undergoes a specific transition between two different conformational states.

To address furthermore the stability of the purple-membrane lipid bilayer at different salt concentrations, and to define the significance of the structural rearrangement of the HAMP domain with respect to possible changes in the lipid environment, spin labeled lipid bilayers were investigated under low and high salt conditions. 5-doxylstearic acids (5DSA, see figure 5.8, top) containing a nitroxide attached to the fifth carbon atom in the fatty acid chain were inserted into the native purple membrane layer in a 1:100 stoichiometric ratio.
5.1 Side chain dynamics subject to environmental inputs

Figure 5.8: Spin normalized cw X-band EPR spectra of 5-doxyllstearic acid (5DSA) enriched purple membrane lipid bilayers in the absence (upper panel) and presence (lower panel) of the reconstituted NpSR/II/NpHtrII<sub>157</sub> complex. At the top a schematic representation of the 5DSA structure is depicted with the nitroxide group carrying the unpaired electron. Measurements were performed at different salt concentrations (150 mM KCl, black lines; 3.5 M KCl, olive lines) and different temperatures (298 K, left panel; 343 K, right panel).

At room temperature and in the presence of 150 mM KCl, the EPR spectrum of the 5DSA in PML (see figure 5.8 black upper left spectrum) exhibits the shape of a strongly immobilized spin label. The nitroxide ring in this case is directly attached to the aliphatic chain, thus no different rotameric conformers induce complex lineshapes, as on the contrary observed for the MTTSL bound to protein sites. The salt-induced effects are then expected to be easily visualized. Increasing the salt concentration to 3.5 M KCl (see figure 5.8), results in a slight decrease in mobility of the spin labeled lipids, pointing to a more compact lipid bilayer in the presence of high salt concentrations. Increasing the temperature from 298 K to 343 K (see figure 5.8 upper right part), leads to an increase in mobility of the lipid chains possibly due to the temperature-driven changes in the viscosity of the medium and additional changes in the lipid phase. Increasing the salt concentration at high temperatures induced as well a slight decrease in mobility of the 5DSA radical. Differences between the high-salt spectra recorded at 289 K and 343 K are comparable to those between the analogs measured for low salt concentrations. Reconstitution of the NpSR/II/NpHtrII<sub>157</sub> complex into the spin labeled lipid bilayer (see figure 5.8 lower panel) leads to a strong restriction in motion of the 5DSA radical. This points to a more compact composition of the lipid bilayer in the presence of the NpSR/II/NpHtrII<sub>157</sub> complex. However, most notably the salt-induced as well as temperature-driven changes in the protein-containing spin labeled
PML yields much less pronounced effects compared to the protein-free PML, highlighting the stabilizing nature of the protein component on the membrane bilayer.

Altogether, the data obtained for 5DSA-enriched PML furthermore suggest that the spectral changes observed for the spin labeled positions in the HAMP domain of the complex embedded in PML cannot exclusively be caused by a major rearrangement of the membrane bilayer.

5.1.2 Effects of acidic pH and different environmental agents

As cells had to adapt to a variety of external conditions in order to survive during evolution, the issue of protein stability and protein solubility is quite delicate. Cell exposure to environmental stress may accordingly induce effects on protein stability ranging from denaturation to stabilization, or even folding of unfolded proteins. Both mechanisms are exploited by cells in the nature. Accordingly, testing the influence of different environmental inputs on structural changes is an interesting point, particularly with respect to the two components described here. The following paragraphs shall gain insight into the nature of the predicted dHAMP and cHAMP conformations.

First, according to the studies carried out by Kawagishi and coworkers [Umemura et al. 2002] on the E. coli Tsr and Tar, two chemoreceptors mediating opposite responses to the same changes in the cytoplasmic pH, the electrostatic properties of a short region within the linker domain is critical for signaling during pH sensing. Despite the high discrepancies between the extracellular pH ranges of the investigated chemoreceptors from E. coli (pH 5 - 9) and the NpSRII/NpHtrII complex from N. pharaonis (pH 8-11, e.g. [Falb et al. 2005]) and due to the high homologies between the cytoplasmic domains in chemoreceptors and the transducer NpHtrII, the effect of acidic pH on the two spectral components present has been examined. The EPR spectra recorded for selected transducer mutants representative for the different HAMP subregions (A80R1:TM2, L90R1:AS1, R99R1:C-terminal end of AS1, D115R1:connector and A122R1: N-terminal end of AS2) in complex with NpSRII reconstituted in PML are presented in figure 5.9. Second, measurements in the presence of PEG (polyethylene glycol) were carried out in order to understand which of the two conformations is the most stable one. To avoid protein aggregation induced by PEG (see amongst others [Bolen 2004]), we decided to use a 200-fold molar excess of lipids per 1:1 protein complex instead of the usual 40-fold molar excess.

Upon the use of acidic pH, all residues are engaged in a very compact structure as is clearly observable by the suppression of the second component’s fraction. In case of the transmembrane residue A80R1, changes in the motion of the already restricted nitroxide side chain are hardly visible. Although the pH-induced spectral changes in the spectra of the remaining variants are differently pronounced, the direction of the effect is coincident
5.1 Side chain dynamics subject to environmental inputs

Figure 5.9: Effect of different environmental constraints on the two spectral components. The low field parts of spin normalized cw X-band EPR spectra of selected transducer residues residues (A80R1, L90R1, R99R1, D115R1, A122R1) are depicted. For better comparison, the spectral amplitudes of the spectra detected for NpSRII/NpHtrII<sub>157</sub>-R99R1 were multiplied with a scaling factor (1/2). Measurements performed in the presence of pH 3.4 (150 mM KCl, 10 mM citrate-HCl; narrow dotted lines), 30% (w/v) PEG 1000 (150 mM KCl, 10 mM Tris-HCl, pH 8; broad dotted line) and 30% (w/v) PEG 3350 (150 mM KCl, 10 mM Tris-HCl, pH 8; dashed line) are compared to the 3.5 M KCl (10 mM Tris-HCl, pH 8; solid line) spectra. For the transducer mutant A80R1 also the spectrum detected in the presence of 150 mM KCl (10 mM Tris-HCl, pH 8; broad gray line) is depicted. The NpSRII/NpHtrII<sub>157</sub> complex was reconstituted in PML in a 1:200-fold molar excess of lipids per 1:1 protein complex to prevent aggregation.

with that observable upon the use of increasing salt concentrations. All spectra are biased towards component 1 at low pH. If this effect can be assigned to the importance of pH changes for the activity of the protein complex, or simply to the fact that acidic pH values increase the hydrophobicity of the hydrophobic residues, which are except in the charged connector region quite numerous, cannot be distinguished from these experiments. Nevertheless, acidic pH has shown to resemble the compact structure present at high salt concentrations.

Furthermore, osmolytes such as PEG are known to make proteins less soluble, and are thus acting as effective precipitants for protein crystallization. In case of proteins exhibiting a conformational equilibrium between two states, such osmolytes may also drive the protein to the less hydrated, more folded state [Kim et al. 2008]. In order to test if PEG has a stabilizing function on one of the components present in the NpSRII/NpHtrII<sub>157</sub> spectra, measurements were carried out in the presence of 30% (w/v) PEG with the same variants
used for pH-dependent experiments (see figure 5.9). Since the effect of the osmolyte is often strongly dependent on the size of the species used, PEGs of different molecular weights have been used in this study: the commonly used PEG3350 as well as one smaller (PEG1000) and one larger (PEG8000) entity. The measurements were performed in the presence of low salt concentrations. Because in case of the used PEG8000 we could not unambiguously exclude partial protein precipitations, which are to be expected when the osmolyte affects the protein properties in an extreme way, the data obtained is excluded from the considerations made here. Although the viscosity based experiments performed in the presence of 40\% (w/v) sucrose mentioned above did show not to have major influence on the spectral shapes, it is worth mentioning that the viscosity of 30\% (w/v) PEG1000 in water ($\eta_{30\% \text{PEG1000}} \approx 6.4$ cp) is comparable to that of 40\% (w/v) sucrose ($\eta_{40\% \text{sucrose}} = 6.2$ cp). The spectra obtained for PEG1000 and PEG3350, where definitely no aggregation/precipitation effects were existent, are presented in figure 5.9. The presence of the stabilizing osmolyte leads to a restriction in the side chain motion in all cases except for residue A80R1 due to the already minor degree of motional freedom present under low salt conditions. Moreover, PEG-size-dependent differences are clearly observable since the suppression of component 2 using the smaller PEG1000 is of minor extent compared to that induced by PEG3350. However, the addition of 30\% (w/v) PEG to the 150 mM KCl containing samples is not as efficient in suppressing component 2 as the high salt concentration. The measurements performed in the presence of PEG can however suggest the stable nature of the conformation related to the compact component 1 and raises the question about a possible equilibrium between the two conformations reflected by the two components present in the spectra.

In order to continue investigation of the origin of component 1 and 2, and to understand their possible mutual relation in terms of a “two-state” equilibrium, titration experiments with NiEDDA, ascorbic acid, and urea were carried out.

As described in sections 3.1.3.6 and 4.3.2, the water soluble NiEDDA enables to distinguish if a spin label side chain is located in the water phase since encounters between the nitroxide and the fast-relaxing NiEDDA result in a broadening of the EPR line. Because the exchange interaction between the NiEDDA and the spin label solely decreases the nitroxide’s relaxation times, the line broadening is reversible. In the case of an equilibrium existing between a water-exposed component and one that is not accessible for water (buried, or deep in the lipid), NiEDDA-induced effects on the spectral shapes are only expected for the water-exposed species. Equal to NiEDDA, the reducing agent ascorbate is solely soluble in the water phase, thereby only reducing the nitroxide of the water-exposed component. However, the decisive difference between the two types of experiments is the irreversible reducing nature of ascorbate. If a “two-state” equilibrium exists between a non-water-exposed and a water-exposed spin label species, both spectral components are
expected to be decreased in the same way due to the interexchangeable nature of the two components.

The results of the measurements carried out for NpSRII/NpHtrII\textsubscript{157}-A97R1, a position located in the amphipathic sequence 1, reconstituted in PML in the presence of different NiEDDA concentrations are illustrated in figure 5.10A. Under low salt conditions, a gradual broadening of the dynamic component 2 also visible in a decrease of the component's spectral amplitude is observable with higher NiEDDA concentrations. At a final concentration of 52 mM NiEDDA, the component 2 is completely suppressed. Analogous to these facts, a complete elimination of component 2 can be observed upon the addition of 52 mM NiEDDA in the presence of high salt concentrations (see figure 5.10A, right). Though a broadening of component 1 (cHAMP) is not evident in both cases (low and high salt) it cannot be excluded. This is due to the quite difficult observation of line-broadening mechanisms in spectral components already characterized by powder-like spectral features. However, the cHAMP conformation is considered to be less accessible to the water phase than the dHAMP conformation.

The effects of the addition of a final concentration of 5 mM ascorbate to the NpSRII/NpHtrII\textsubscript{157}-A97R1 protein sample are presented in figure 5.10B. Despite the obviously different ratios between the two spectral components under low (see figure 5.10B, left panel) and high (see figure 5.10B, right panel) salt conditions, the overall spectral intensity is decreased with increased ascorbate incubation times in both cases. The superposition of the spectra obtained in the absence of ascorbate (0' incubation time) and after prolonged incubation under both low (see figure 5.10B, upper center, gray bold line) and high (see figure 5.10B, lower center, gray bold line) salt conditions unambiguously shows the maintenance of the two component's ratio. Since from the presented NiEDDA data the cHAMP conformation was found to be less water accessible, these results prove the existence of a thermodynamic equilibrium between the cHAMP and the dHAMP.

The fact that the very high mobility values obtained for the spectral component 2 can neither be ascribed to properly folded protein structures, nor to fully denatured/unfolded protein portions, urea titration experiments were performed with NpSRII/NpHtrII\textsubscript{157}-A122R1 (see figure 5.11) to define the nature of the dHAMP conformation. Urea is a commonly used denaturant, which is thought to unfold proteins by altering electrostatic interactions either by solvating the charged residues of a protein, or by forming hydrogen bonds with the protein backbone [O´Brien et al. 2007].

As apparent from figure 5.11A and B, the addition of increasing urea concentrations to the NpSRII/NpHtrII\textsubscript{157}-A122R1 (N-terminal end of AS2) sample results in a rising spectral intensity of component 2 at both low and high salt concentrations. Strikingly, at low salt concentrations the effects of urea on the spectral shapes are small (see figure 5.11A, upper part). The spectra obtained in the presence of a final urea concentration of 1.5 M and 6 M are coincident, indicating the completion of the unfolding process already at 1.5 M
Figure 5.10: Effects of NiEDDA and ascorbic acid on the two spectral components under low (150 mM KCl, left panel) and high (3.5 M KCl, right panel) salt conditions. A) Spin normalized cw X-band EPR spectra of NpSR II/NpHtr II A97R1 performed with increasing NiEDDA concentrations designated on the left. B) Time evolution of ascorbate induced changes on the spin normalized spectra of the investigated mutant. The spectra are arranged according to the residence time (depicted on the left of each spectrum) of the ascorbate (final concentration 5 mM) added. The insets show the spectra in the absence of ascorbate (0’, black lines) compared to that after prolonged incubation (60’ (150 mM KCl, upper part, gray bold line) and 34’ (3.5 M KCl, lower part, gray bold line)) normalized to the same amplitude. The measurements presented were performed by E. Bordignon (former member of the work group of H.-J. Steinhoff).

urea. This clearly suggests that the HAMP domain is already partially destabilized at low salt concentration, and that the spin label associated with component 2 is characterized by motions similar to those of a partially unfolded transducer. On the contrary, in the presence of high salt concentration the urea effect on the spectral shape is more pronounced (see figure 5.10A, lower part). A stepwise unfolding of the protein structure is visible by the increasing spectral intensities with higher urea concentrations. This process terminates at a final urea concentration of 4 M, where the corresponding spectrum resembles that one recorded in the presence of 6 M urea. This indicates the stabilized character of the protein.
5.1 Side chain dynamics subject to environmental inputs

Figure 5.11: Urea-induced effects on the two spectral components in case of NpSRII/NpHtrII_{157}-A122R1. A) For comparison, the low field lines of the spin normalized cw X-band EPR spectra detected for different urea concentrations and measured in the presence of 150 mM (upper part) and 2.9 M (lower part) KCl are presented. The spectra are depicted according to the urea concentration used (see legends). For the sake of clarity, the entire spectra obtained for urea-free (B, 0 M, black lines) and 6 M urea (B, gray bold lines) containing samples are compared in the low (B, upper part) and high (B, lower part) salt conditions. D) Difference spectrum obtained by subtracting the high-salt from the low-salt spectrum in B. The spectra presented in B and C are normalized to the same integral. The spectral amplitudes are multiplied by a scaling factor depicted at the high-field ends of the spectral parts in some cases.

at high salt concentrations. Though the changes observed for the spectra in the presence of high salt concentrations are of greater extent than those for the low salt analogs, the unfolding in the first case does not reach the same level as the latter one. However, as apparently visible in the spectra presented, even at low salt concentrations the commonly used urea concentration of 6 M to fully denature/unfold proteins does not suffice to fully unfold the NpSRII/NpHtrII_{157} complex investigated here. It is therefore noteworthy that the samples after the addition of 6 M urea were still orange, as they are under physiological conditions. This indicates intact membranes with reconstituted NpSRII and thus properly bound transducers. Consequently, the membrane anchor is supposed to be very stable against external inputs, preventing the complete denaturation of the NpSRII/NpHtrII complex, and that this effect is enhanced at high salt concentrations. Eventually, for comparison between the urea unfolded component and the pure spectral component 2 ($d$HAMP), the latter one was
obtained by subtracting the urea-free spectrum detected at high salt concentration from the one at low salt concentration (see figure 5.11C). The extremely dynamic nature of the \( dHAMP \) conformation is thereby revealed. Altogether, the results associate the \( dHAMP \) dynamics to backbone fluctuations similar to those present in partially unfolded proteins.

### 5.1.3 Micelle vs. lipid bilayer

As shown in the previous section, lipid-protein interactions play a crucial role in the stability of the membrane. Vice versa, with respect to protein structure and physiological activity, the effects of the lipid bilayer on the integral membrane protein are of major importance. Understanding effects of the solubilization of the complex in detergents in terms of structure and function is significant since membrane protein purification techniques as described in section 4.1 require the protein extraction from the membrane of the bacterial host into detergent micelles. Moreover, most of the information available for membrane proteins and membrane protein complexes stems from solubilized samples due to the exclusive application of techniques such as absorption spectroscopy, circular dichroism, and high-resolution NMR to dissolved samples. In order to illuminate possible structural changes in the sensitively acting signal transduction domain proximal to the membrane/micelle upon solubilization, \( \text{NpHtrII}_{157} \) spin labeled variants in the HAMP domain in complex with \( \text{NpSRII} \) solubilized into DDM (N-Dodecyl-\( \beta \)-D-maltoside) have been investigated via EPR. In contrast to the 2:2 stoichiometry observed for the lipid-reconstituted protein complexes, this native stoichiometric ratio was shown to be affected in the DDM-solubilized form, where only 1:1 complexes were found [Wegener et al. 2001, Sudo et al. 2001].

The RT cw X-band EPR spectra obtained for \( \text{NpHtrII}_{157} \) mutants in the TM2 (V78R to T82R), the predicted AS1 (G83R to M100R), the connector (G101R, L105R, D106R, D115R and E116R), the predicted AS2 (A122R) and the following cytoplasmic portion (A148R) in complex with \( \text{NpSRII} \) in the DDM-solubilized form (red lines) are compared to those in the PML-reconstituted form (black lines) in figure 5.12.

Interestingly, the overall spectral features are retained in the detergent-solubilized sample, where also two conformations are visible. However, as evident from the comparison of the room-temperature cw X-band EPR spectra recorded in the detergent-solubilized and the lipid-reconstituted \( \text{NpSRII/NpHtrII}_{157} \) complexes (see figure 5.12), the compact component visible in the spectra of the solubilized samples represents a conformation different from the \( cHAMP \). Component 2, on the other hand, represents a conformation similar to the \( dHAMP \). The spectral changes occurring upon solubilization can generally be separated in two groups. The transmembrane residues and those located at the N-terminal end of the AS1 up to position 94 as well as residues M100 and G101 at the AS1/connector-transition show increased spectral amplitudes of component 2. Contrary, the following residues investigated at the C-terminal end of the AS1, the connector (except M100 and G101), the AS2 and
5.1 Side chain dynamics subject to environmental inputs

Figure 5.12: Comparison between cw X-band EPR spectra obtained for NpHtrII<sub>157</sub> mutants in complex with NpSRII in the PML-reconstituted (black lines) and detergent-solubilized (red lines) form. Contrary to other cw X-band spectra presented in this study, for better comparison the spectra are normalized to the same positive amplitude. The spectra are designated according to the residue mutated. As e.g. depicted in figure 5.1, the residues are located in the TM2 (V78 to T82), in the AS1 (G83 to M100), in the connector region (G101, L105, D106, D115 and E116), in the AS2 (A122) and in the following cytoplasmic domain (A148).
the following cytoplasmic portion exhibit suppressed amplitudes of component 2. In order to specify the changes observed, the mobility parameters $\Delta H^{-1}_{pp}$ were calculated from the spectra presented and compared to that of the reconstituted NpSRII/NpHtrII$^{157}$ complexes already described (see figure 5.13).

![Figure 5.13: Comparison of spin label mobility data for detergent-solubilized (red squares) and lipid-reconstituted (gray circles) for the investigated NpHtrII$^{157}$ variants in complex with NpSRII. Mobility parameters $\Delta H^{-1}_{pp}$ extracted from the RT cw X-band EPR spectra depicted in figure 5.12 are presented versus the residue number. Errors are estimated to ±10% due to uncertainties in line width determination. Values obtained for directly neighboring residues are connected via dashed lines. The connector region between the two amphipathic sequences is shaded in gray due to its predicted lack of fixed structure. Mobility values for positions 79 to 87 in the reconstituted complexes were determined by A. A. Wegener (formerly Max-Planck Institute for Molecular Physiology, Dortmund, Germany.)](image)

The described increasing spectral amplitudes in component 2 are clearly retrievable in the mobility parameter versus residue plot since the $\Delta H^{-1}_{pp}$ values are always biased by the dynamic component as noted before. Starting with the changes occurring in the transmembrane part of the transducer, a drastic side chain mobilization of about 1.5 ($mT)^{-1}$ is observable for residue V78R1. Since this side chain is not considered to be in direct contact with the membrane in the lipid-reconstituted form, but to be pointing towards the transducer/transducer-interface, and thereby restricted in its freedom to move by the presence of the second NpHtrII molecule, the changes observed can unambiguously be assigned to the exchange of the second transducer by the detergent molecules. Analyzing from this point of view the following residues in the transmembrane helix and the N-terminal end of the AS1 up to position 94, the absence of the second transducer molecule is clearly visible in the increased mobility values. The periodic pattern recognizable in the mobility values of the solubilized samples at least up to residue A87 indicates the presence of $\alpha$-helical structure. Here, minor differences between the obtained $\Delta H^{-1}_{pp}$ values for the reconstituted and solu-
5.2 Temperature-driven dynamics

In the previous sections the existence of the “two-state” equilibrium between the compact conformation (cHAMP) and the dynamic conformation (dHAMP) was revealed. We have also seen that this equilibrium can be shifted towards the compact conformation by increasing the salt concentration. In order to specify the changes observed in terms of thermodynamic parameters, temperature-dependent measurements were performed.

Figure 5.14 clarifies why the inspection of the behavior of the two spectral components can excellently be performed in the frame of temperature-dependent measurements. Increasing the temperature up to 343 K at low salt concentrations (see figure 5.14 left panel) leads in the case of the PML-reconstituted NpSRII/NpHtrII\textsubscript{157}-A94R1 to the nearly complete sup-
Figure 5.14: Temperature effects on the spectral features of the NpHtrII\textsubscript{157}-A94R1 variant in complex with NpSRII reconstituted in PML. Measurements were performed under low (150 mM KCl, left panel) and high (3.5 M KCl, right panel) conditions at temperatures ranging from 283 K to 343 K as depicted on the left. The spin normalized spectra recorded (solid lines) are superimposed by the fittings of the experimental spectra (dotted lines) to obtain the isotropic reorientational correlation times and ratios of the spectral components 1 (cHAMP) and 2 (dHAMP), which are highlighted by the gray arrows.

pression of component 1, and dramatic increase in the spectral amplitudes of component 2. That the extreme case observed at 343 K is the result of a process, where the cHAMP conformation gradually vanishes and the dHAMP conformation gradually raises with increasing temperatures, is unambiguously traceable in figure 5.14. The same effect can be observed under high salt conditions, though the effect is much less pronounced compared to the low salt case. This finding supports the assumption of a more stabilized structure present under high salt concentrations, which maintains its stability even at high temperatures. However, the changes obtained in the presence of 150 mM and 3.5 M KCl underline the interexchange between the two spectral components. Lowering the temperature is shown to act as the increase of salt concentration on the spectral features, both conditions shifting
5.2 Temperature-driven dynamics

the equilibrium towards the cHAMP conformation. According to section 3.2, the thermodynamic properties of the process observed can be derived from the changes of the two component’s fractions with the temperature. The fractions of the two components in each spectrum as well as the respective reorientational correlation times $\tau_c$ can be obtained from the fitting of simulated spectra to the experimental ones as described in section 4.3.1. The fittings performed with the program Freedfit, where a simple Brownian model according to isotropic reorientational diffusion of the nitroxide was used (see section 4.3), are depicted in figure 5.14. In this regard it is worth noting that the main reason for these fittings was to determine the areas/fractions of the two spectral components rather than to determine the “real” reorientational correlation times. However, only minor discrepancies can be observed between the experiments (see figure 5.14 solid lines) and the simulations (see figure 5.14 dotted lines). All relevant values obtained for the reorientational correlation time and the fractions of the two spectral components are registered in the supplementary data part in the appendix (see tables A.1 and A.2).

Since proteins are known to denature when they are far-off their physiological conditions and tend to aggregate, or unfold, the plausibility of the very dynamic component 2 at high temperatures has to be checked. Therefore, the reorientational correlation time obtained for component 2 (e.g. $\tau_c = 0.21$ ns for NpSRII/NpHtrII157-A122R1, 150 mM KCl, 343 K) was compared to those obtained from the spectra of a short 9-mer peptide soluble in water labeled with a proxyl nitroxide label at the 5th cystein in the amino acid sequence, of a tempol nitroxide dissolved in water, and of the low salt urea (6 M) denaturated A122R1 transducer variant (see figure 5.15). Fitting of simulated spectra to the experimental ones results in reorientational correlation times of $\tau_c = 0.08$ ns for the proxyl label attached to the 9-meric peptide, $\tau_c = 0.06$ ns for the tempol solution, and $\tau_c = 0.24$ ns for the urea treated sample. Despite the very high motional freedom of the spin label side chains obtained for the transducer’s spectral component 2 at high temperatures, the corresponding $\tau_c$ values are still in the range, where motions of intact, functional entities can be observed. Interestingly, the reorientational correlation time observed for component 2 in the urea denaturated case of residue A122R1 exhibits a slightly higher value than that obtained for the high-temperature analog, pointing to the very high dynamics present at high temperatures.

One further aspect worth mentioning in terms of the questionable functionality of the very dynamic second component, is the complete reversibility of the effects observed. That means, lowering the temperature back to ambient temperature (298 K) after the NpSRII/NpHtrII157 has been heated up, yields, with the exceptions of minor fractions of released free spin label in some cases, the same spectra as before. This also suggests the equilibrium’s existence and that component 2 is not merely a misfolded fraction of the transducer in the sample.
Figure 5.15: Exemplification of fitted isotropic reorientational correlation times. Measurements were performed for a 9meric peptide with a proxyl nitroxide label (schematic representation top left, kindly gifted from Dr. Ruper Abele, Johann Wolfgang-Goethe Universität Institut für Biochemie, Biozentrum, Frankfurt) attached to the 5th cysteine in the amino acid sequence and for tempol (schematic representation top right), both dissolved in water. Spin normalized cw X-band EPR spectra obtained (solid lines) are superimposed by the fittings of the experimental spectra (dotted lines) to obtain the isotropic reorientational correlation times \( \tau_c \) (depicted above the respective spectra). For comparison, the experimentally obtained spectrum of NpSRII/NpHtrII\textsubscript{157}-A122R1 (solid gray line, \( \tau_c = 0.21 \) ns for spectral component 2) under low salt conditions at 343 K, as well as the low salt urea (6 M) denaturated A122R1 variant (298 K, dashed gray line, \( \tau_c = 0.24 \) ns for spectral component 2) is added in the tempol overview.

In the case denaturing processes occur during the temperature-dependent measurements they will be identified in Arrhenius and van’t Hoff plots since such side effects would lead to a non-linear behavior of the logarithms of the correlation times and fractions observed.

To determine the thermodynamic parameters of the processes occurring in the HAMP domain, the temperature dependence of the spectral shape of several NpHtrII\textsubscript{157} variants in complex with NpSRII were analyzed. Representative for each subdomain, residues S91, A94, S98 and R99 in the AS1, D106 in the connector and Y121 in the N-terminal end of the AS2 were investigated. Although the amount of lipids used in this study has already been shown not to affect the spectral properties, temperature-dependent measurements for some mutants were carried out in both lipid-reconstituted forms containing 40-fold molar excess of lipids and 400-fold molar excess of lipids per 1:1 NpSRII/NpHtrII\textsubscript{157} complex. Exemplificative for the measurements performed, the low-field parts of the spin normalized spectra detected at different temperatures for residues A94, S98, D106 and Y121 are presented in figure 5.16 under both low and high salt conditions.
5.2 Temperature-driven dynamics

Figure 5.16: Temperature-dependent spectral changes of NpHtrII<sub>157</sub> variants in complex with NpSRII reconstituted in PML at low (150 mM KCl, left panel) and high (3.5 M KCl, right panel) salt concentrations. Spectra recorded for positions 94 and 98 (AS1), 106 (connector) and 122 (AS2) are presented. For comparison, the low-field lines of the spin normalized spectra are depicted, and in some cases the spectral amplitudes are multiplied by a scaling factor. The insets are shown to highlight the changes in component 1. The spectra are colored according to the legend. Oriented arrows indicate the direction of the changes with lower temperatures in the two spectral components.
In all cases presented, the gradual increase of component 1, and the opposite effect for component 2 is clearly observable with decreasing temperature. Inspection of the measurements carried out under low salt conditions (see figure 5.16 left panel) yields the much more pronounced increase in the dHAMP conformation upon temperature increase for the two residues D106 and Y121 compared to the two residues chosen in the AS1 (A94 and S98). However, higher amplitudes are correlated with smaller linewidths, so faster reorientational correlation times, as presented in tables A.1 and A.2 in the appendix. Interestingly, the narrow lines in the NpSRII/NpHtrII_{157}-D106R1 and NpSRII/NpHtrII_{157}-Y121R1 spectra enables the clear recognition of the two rotameric contributions of component 1 (see insets in figure 5.16), and their temperature-dependent changes as well. The apparent shift from the more to the less restricted spin label side chain rotamer with higher temperatures shows that also these motions follow the observed trend of the temperature-driven side chain dynamics. Less pronounced changes are observable in the high-salt spectra (see figure 5.16 right panel). However, the main changes upon the temperature increase are analogous to that described under low salt conditions.

From the fitting of simulated spectra to the experimental ones the spectral component’s reorientational correlation times as well as their respective fractions of the spectral integrals were obtained (see tables A.1 and A.2 in the appendix). As pointed out in section 4.3.1, the different rotameric contributions of component 1 were fitted/simulated by solely one component. The resulting simulation has accordingly to be treated carefully due to this approximation. The reorientational correlation times $\tau_c$ obtained for the NpHtrII_{157} mutants investigated in complex with NpSRII and reconstituted either with 40-fold or 400-fold molar excess of purple membrane lipids per 1:1 complex are presented in Arrhenius plots in figure 5.17A. The reorientational correlation times $\tau_c$ of each component are clearly shifted towards decreased $\tau_c$ values with higher temperatures. The linear correlation between the logarithms of $\tau_c$ and the inverse of temperature illustrated by a linear interpolation for each set of correlation times obtained show only minor deviations. As discussed in detail in [Steinhoff et al. 1989] and [Steinhoff, 1990] such a linear behavior is expected for the temperature-dependence of an activated process. A parameter that has to be accounted for when reorientational correlation times of spin label side chains are examined in terms of temperature-dependent measurements is the solvent viscosity changing with temperature. As has been investigated and illustrated in [Steinhoff, 1990], these changes are not negligible in the temperature range applied here. The effects of the solvent viscosity on the fluctuations of the investigated side chain is moreover strongly dependent on the extent to which the residue observed is exposed to the solvent. In the case the motion of the side chain mainly takes place in the protein-interior, the effect of the solvent viscosity is attenuated and shielded compared to motions performed on protein surfaces [Steinhoff, 1990]. Thus, the nitroxide microenvironment has to be considered as beyond the mentioned viscosity-induced
5.2 Temperature-driven dynamics

Figure 5.17: Arrhenius and van’t Hoff plots for investigated NpHtrII_{127} variants (S91R1, A94R1, S98R1, R99R1: AS1; D106R1: connector; A122R1: AS2) in complex with NpSRII reconstituted in PML. Measurements were performed under the following conditions: 150 mM KCl, low lipid content: black squares; 150 mM KCl, high lipid content: gray circles; 3.5 M KCl, low lipid content: olive squares; 3.5 M KCl, high lipid content: light green circles. The asterisks denote the full length transducer. A) Plot of the logarithm of the reorientational correlation times $\tau_c$ (component 1: upper parts of graphs; component 2: lower parts of graphs) versus the inverse of temperature. Errors are obtained from an estimated ±10% error for the fitting parameter R (see section 4.3.1). B) Natural logarithm of the concentration ratio of the two HAMP conformations represented by the two spectral components, $\ln \frac{f_2}{f_1}$, versus the inverse of temperature. Errors are estimated to be in the 2% range for all fractions obtained from the fittings. The horizontal light yellow areas indicate conditions comparable to the physiological ones (3.5 M KCl and 313 to 328 K). The linear interpolations of the data are colored according to the given code.
effects, generally, the interactions of the side chain with secondary/tertiary structural elements of the protein influence the values of $\tau_c$.

In consideration of the aspects mentioned, particularly the differences observable between the two spectral components in the Arrhenius plots presented in figure 5.17A are plausible. Minor deviations visible for the plots of the correlation times using samples with 400-fold molar excess of lipids per 1:1 complex (see gray and light green circles and lines in figure 5.17A) are found to be negligible for all NpHtrII<sub>157</sub> variants investigated.

The enthalpies of activation $\Delta H^*$ calculated for NpHtrII<sub>157</sub> variants investigated under low and high salt conditions from the slopes of the linear interpolations in figure 5.17A are given in table 5.1. Different $\Delta H^*$ values for the NpHtrII<sub>157</sub> variants investigated under different salt conditions indicate the dependence of the energy barrier properties of the spin label side chain motions on the specific locations of the labels. However, with the exceptions of residues S91 and Y121, all activation enthalpies presented do not differ strongly, pointing to comparable activation energies that have to be overcome in both environments containing low and high salt concentrations, and are found to be in agreement with previously published data [Steinhoff et al. 1989]. The differences observed in the $\Delta H^*$ values calculated for residue S91 indicate the position’s location in a very compact protein region, where the

<table>
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<th>NpHtrII&lt;sub&gt;157&lt;/sub&gt; variant</th>
<th>KCl content in M</th>
<th>$\Delta H^*$ in kJ mol&lt;sup&gt;-1&lt;/sup&gt; cHAMP</th>
<th>$\Delta H^*$ in kJ mol&lt;sup&gt;-1&lt;/sup&gt; dHAMP</th>
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<td>16 ± 4</td>
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Table 5.1: Enthalpies of activation $\Delta H^*$ calculated from the slopes of the linear interpolations in the logarithm of $\tau_c$ versus the inverse of temperature plots (see figure 5.17A). The full-length transducer variant A94R1 is denoted by an asterisk.
side chain has to overcome a higher energy barrier to move. The fitting results obtained for component 1 in case of residue Y121 measured in the presence of 150 mM KCl were found not to represent the experimental data in a satisfying way, thus, the corresponding enthalpy of activation is not illustrated in table 5.1. The enthalpies of activation in case of higher lipid contents (400-fold molar excess of lipids per 1:1 complex) used are fully in line with the data presented (data not shown), supporting the assumption that the lower lipid content usually taken does not affect structure and function of the NpSRII/NpHtrII\textsubscript{157} complex.

Beyond the effects visible in the changes of the reorientational correlation times of the two spectral components, temperature is found to influence their spectral fractions as well. The variation of the components’ ratio with temperature in the presence of 150 mM and 3.5 M KCl, respectively, is shown in van’t Hoff plots (see figure 5.17B). Here, the natural logarithm of the ratio of the two spectral components (\(\frac{\text{fraction}_{\text{comp}2}}{\text{fraction}_{\text{comp}1}}\)) is plotted versus the inverse of temperature in order to trace the shifts of the equilibrium towards the more dynamic component 2 with higher temperatures. Differences between the data collected for measurements performed under low and high salt conditions (see figure 5.17B black and olive squares, respectively) in the plots for the transducer variants A94R1 (AS1), S98R1 (AS1), R99R1 (AS1), D106R1 (connector) and Y121R1 (AS2) are visible. Changes upon the use of higher amounts of lipids (see gray (150 mM KCl) and light green (3.5 M KCl) circles and lines in figure 5.17B) are of relevant degree only in the high-salt case of residues R99 and Y121. Data obtained for the full-length transducer variant A94R1 (in figure 5.17 denoted by the asterisks and colored analogous to the NpHtrII\textsubscript{157} version) are within the experimental errors in line with the data for the analogous position in the truncated NpHtrII\textsubscript{157}. An interesting point concerning the spectral composition of the two components is highlighted by the horizontal yellow bars in figure 5.17B. Each bar indicates the region in the single van’t Hoff plots exhibiting the same spectral composition as obtainable under physiological conditions (high salt concentration and temperatures between 313 and 328 K). Low salt concentrations in combination with low temperatures can mimic the physiological relevant equilibrium.

Calculation of the changes in the thermodynamic parameter enthalpy (\(\Delta H\)), entropy (\(\Delta S\)) and Gibbs energy (\(\Delta G\)) as well the equilibrium constant \(K_{eq}\), characterizing the equilibrium existing between the cHAMP and the dHAMP conformation, yields the values given in table 5.2. As evident from the errors for each of the parameters calculated, minor changes in the spectral composition in terms of the two component’s ratio may produce major deviations in the thermodynamic parameters. Detailed predictions about the processes taking place for each residue investigated are accordingly hard to manage, only general considerations are possible. Inspecting first of all only the entropic and enthalpic changes given in table 5.2, no plausible correlation seems to be apparent. In most of the cases, higher
<table>
<thead>
<tr>
<th>NpHtrII&lt;sub&gt;157&lt;/sub&gt; variant</th>
<th>KCl cont. in M</th>
<th>$\Delta H$ in $k}\text{J/mol}$</th>
<th>$\Delta S$ in $k}\text{J/mol K}^{-1}$</th>
<th>$\Delta G_{298~K}$ in $k}\text{J/mol}$</th>
<th>$K_{eq<del>298</del>K}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S91R1</td>
<td>0.15</td>
<td>7 $\pm$ 5</td>
<td>12 $\pm$ 15</td>
<td>3 $\pm$ 4</td>
<td>0.3 $\pm$ 0.4</td>
</tr>
<tr>
<td>A94R1</td>
<td>0.15</td>
<td>17 $\pm$ 3</td>
<td>40 $\pm$ 10</td>
<td>5 $\pm$ 3</td>
<td>0.2 $\pm$ 0.2</td>
</tr>
<tr>
<td>A94R1*</td>
<td>3.50</td>
<td>47 $\pm$ 11</td>
<td>124 $\pm$ 33</td>
<td>10 $\pm$ 10</td>
<td>0 (0.02) $\pm$ 0.1</td>
</tr>
<tr>
<td>A94R1*</td>
<td>0.15</td>
<td>24 $\pm$ 3</td>
<td>64 $\pm$ 9</td>
<td>5 $\pm$ 3</td>
<td>0.2 $\pm$ 0.1</td>
</tr>
<tr>
<td>A94R1*</td>
<td>3.50</td>
<td>41 $\pm$ 6</td>
<td>109 $\pm$ 17</td>
<td>8 $\pm$ 5</td>
<td>0 (0.04) $\pm$ 0.1 (0.08)</td>
</tr>
<tr>
<td>S98R1</td>
<td>0.15</td>
<td>11 $\pm$ 2</td>
<td>31 $\pm$ 5</td>
<td>2 $\pm$ 1</td>
<td>0.5 $\pm$ 0.3</td>
</tr>
<tr>
<td>S98R1</td>
<td>3.50</td>
<td>20 $\pm$ 4</td>
<td>38 $\pm$ 13</td>
<td>9 $\pm$ 4</td>
<td>0 (0.02) $\pm$ 0 (0.04)</td>
</tr>
<tr>
<td>R99R1</td>
<td>0.15</td>
<td>6 $\pm$ 2</td>
<td>15 $\pm$ 5</td>
<td>1 $\pm$ 1</td>
<td>0.6 $\pm$ 0.3</td>
</tr>
<tr>
<td>R99R1</td>
<td>3.50</td>
<td>26 $\pm$ 9</td>
<td>64 $\pm$ 27</td>
<td>7 $\pm$ 7</td>
<td>0.1 (0.06) $\pm$ 0 (0.02)</td>
</tr>
<tr>
<td>D106R1</td>
<td>0.15</td>
<td>30 $\pm$ 2</td>
<td>96 $\pm$ 7</td>
<td>2 $\pm$ 2</td>
<td>0.4 $\pm$ 0.3</td>
</tr>
<tr>
<td>D106R1</td>
<td>3.50</td>
<td>21 $\pm$ 6</td>
<td>44 $\pm$ 19</td>
<td>7 $\pm$ 6</td>
<td>0.1 (0.05) $\pm$ 0.1</td>
</tr>
<tr>
<td>Y121R1</td>
<td>3.50</td>
<td>61 $\pm$ 8</td>
<td>170 $\pm$ 25</td>
<td>10 $\pm$ 7</td>
<td>0 (0.02) $\pm$ 0.1 (0.05)</td>
</tr>
</tbody>
</table>

Table 5.2: Changes in the thermodynamic parameters enthalpy ($\Delta H$), entropy ($\Delta S$) and Gibbs energy ($\Delta G_{298~K}$ at 298 K) as well as the equilibrium constant $K_{eq~298~K}$ at 298 K calculated from the slopes depicted in the plots of the natural logarithms of the component ratios versus the inverse of temperature (see figure 5.17B) for the transducer residues investigated. The A94R1* demonstrates the results for residue A94R1 investigated in the full-length NpHtrII.

Values were calculated for residues investigated at high salt concentrations, indicating the greater "extent" of changes occurring under high salt conditions since the enthalpy changes can be considered as the energy released in the reaction and the entropy changes as ability of the system to work. High values were obtained for $\Delta H$ and $\Delta S$ in case of residue A94 in both the truncated (NpHtrII<sub>157</sub>) and the full-length (NpHtrII) transducer in the presence of 3.5 M KCl. Comparable and even higher values were calculated for Y121 in the presence of 3.5 M KCl. Comparably low values for the high-salt measurements can on the other hand be found in case of residues S98 and R99 located at the C-terminal end of the AS1 and D106 in the connector. A peculiarity in the values presented is the fact that the data obtained under low salt conditions for transducer variant D106R1 describe higher changes compared to the high-salt analogs. Obviously, the values of the enthalpic and entropic changes determined show site-specific stabilization or destabilization of the HAMP domain structure by nitroxide incorporation. However, despite these differences in the $\Delta H$ and $\Delta S$ values, the changes in the decisive factor, the Gibbs energy, characterizing the reaction between the eHAMP and the dHAMP conformation, were found to be the same within the errors for all measurements performed in the presence of low and high salt concentrations, respectively (see table 5.2 $\Delta G$). The resulting agreement of the equilibrium constants $K_{eq}$ determined for positions in
the different HAMP subdomains AS1, connector and AS2 strongly supports the hypothesis of a salt-dependent “two-state” equilibrium existing between the two spectral components and the corresponding conformations, cHAMP and dHAMP. It is worth mentioning that the data obtained from measurements performed with high lipid content (400-fold molar excess per 1:1 complex) is in agreement with the presented data (not shown).

For comparison of the values obtained for the different thermodynamic parameters, spin labeling EPR temperature-dependent measurements have been performed for residue L159 located in helix F of NpSRII (see figure 5.7), residue BR-S226R1 located at the C-terminal end (loop) of helix G in bacteriorhodopsin (see amongst others [WEGENER et al. 2000]), and ColA-A105R1 located at the C-terminal end of helix 5 in colicin A (see amongst others [PULAGAM 2007]). NpSRII-L159R1 was complexed with NpHtrII\(\text{157}\) and as well as BR in purple membranes, whereas the colicin A sample was water soluble. In case of the NpSRII and the BR mutant, the EPR spectra show a rigid-limit behavior (data not shown), whereby the resulting reorientational correlation times obtained from the fittings of simulated to experimental spectra are not significant for the analysis carried out here. However, it is noteworthy that the changes in the EPR spectra in the temperature-dependent measurements are considered not to show a shift between two protein conformations but rather a shift in the fractions of two spin label rotamers (data not shown), comparable to those observed for the compact component for the transducer variants. Such an absence of a second protein conformation in NpSRII and BR supports the HAMP-specific character of the “two-state” equilibrium found for NpHtrII.

The \(\tau_c\) values obtained for the colicin A mutant are presented in table A.2 in the supplementary data part in the appendix, the corresponding Arrhenius and van’t Hoff plots are shown in figure 5.18A. A linear behavior of the changes in the logarithms of \(\tau_c\) with the temperature is clearly visible as well as the different nature of the side chains’ motions in the two protein subpopulations.

The enthalpies of activation calculated for the process observed are presented in table 5.3. Strikingly, they are comparable to those activation enthalpies obtained for the NpHtrII\(\text{157}\) mutants (see table 5.1).

<table>
<thead>
<tr>
<th>variant</th>
<th>(\Delta H^*) comp 1 in (kJ/mol)</th>
<th>(\Delta H^*) comp 2 in (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ColA-A105R1</td>
<td>6 ± 2</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

Table 5.3: Enthalpies of activation \(\Delta H^*\) calculated from the slopes of the linear interpolations in the logarithm of \(\tau_c\) versus the inverse of temperature plots (see figure 5.18A).

Moreover, the thermodynamic parameters determined are given in table 5.4. As pointed out earlier, the interpretation of the enthalpic and entropic changes is difficult, particularly when solely one residue is investigated in the protein sequence. However, comparison of
Figure 5.18: Arrhenius and van’t Hoff plots for the investigated water soluble ColA-A105R1 mutant located at the C-terminal end of helix 5 in colicin A. Measurements were performed with 10 mM potassium phosphate buffer (pH 6.8) according to Pulagam [2007]. A) Plot of the logarithm of the reorientational correlation times $\tau_c$ (component 1: upper part of graph; component 2: lower part of graph) versus the inverse of temperature. Errors are obtained from an estimated ±10% error for the fitting parameter R (see section 4.3.1). B) Natural logarithm of the concentration ratio of the two HAMP conformations represented by the two spectral components, $\ln(\frac{f_2}{f_1})$, versus the inverse of temperature. Errors are estimated to be in the 2% range for all fractions obtained from the fittings. The linear interpolations of the data are colored according to the given code.

The Gibbs energy change calculated for the colicin A variant with those determined for the NpHtrII157 mutants examined under low salt conditions (see table 5.2) shows striking similarities. In this regard it is worth mentioning that colicin A, a water-soluble toxin forming a voltage-gated channel in the cytoplasmic membrane of target bacteria, was found to exhibit an equilibrium between a molten-globule-like structure (at $\approx$ pH 2) in order to penetrate the membrane and a folded state [Pulagam 2007]. Thus, colicin A uses a very dynamic functional conformation that could have been identified with temperature-dependent measurements as well. The analogy observed between colicin A and the HAMP domain might suggest similar equilibria between a dynamic and a more compact state.

<table>
<thead>
<tr>
<th>variant</th>
<th>$\Delta H$ in kJ mol$^{-1}$</th>
<th>$\Delta S$ in kJ mol$^{-1}$K</th>
<th>$\Delta G_{298}$ K in kJ mol$^{-1}$</th>
<th>$K_{eq}$ 298 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>ColA-A105R1</td>
<td>61 ± 3</td>
<td>193 ± 10</td>
<td>4 ± 3</td>
<td>0.2 ± 0.2</td>
</tr>
</tbody>
</table>

Table 5.4: Changes in the thermodynamic parameters enthalpy ($\Delta H$), entropy ($\Delta S$) and Gibbs energy ($\Delta G_{298}$ K at 298 K) as well as the equilibrium constant $K_{eq}$ 298 K at 298 K calculated from the slopes depicted in the plot of the natural logarithms of the component ratios versus the inverse of temperature (see figure 5.18B).
5.3 Side chain accessibilities for quencher reagents

5.3.1 Effects of high salt concentrations and high lipid content

In order to specify the structural and functional data obtained from the investigation of the side chain dynamics subject to different environmental conditions, accessibility measurements were carried out in the presence of low and high salt concentrations. Due to its relevance in the literature in terms of providing a predicted prototype structure for HAMP domains (see section 2.1 and figure 2.8), direct comparison of the data obtained with that from the hyperthermophile \( A. fulgidus \) [HULKO et al. 2006] is done.

Accessibility measurements were carried out to distinguish between residues exposed to the bulk water (buffer), oriented towards the lipid phase, or buried in the protein interior. High collision frequencies \( W_{ex} \) (in \( \text{MHz} \)) between the water-soluble reagent NiEDDA (or the analogous chromium oxalate, CrOx) and the nitroxide characterize water-exposed residues, whereas, due to its preferential penetration of the lipid bilayer, frequent encounters with molecular oxygen point to a side chain location in the membrane. Low accessibilities for both paramagnetic quencher molecules are typical for positions oriented into the protein interior.

As described in section 5.1.2, using higher NiEDDA concentrations leads to more pronounced line-broadenings in the EPR spectra of water-exposed spin labels. Figure 5.19 shows that this effect has to be accounted for particularly with respect to the two spectral components present. The spectra of the two HAMP mutants exemplified are characterized

![Figure 5.19: Effect of 3 and 20 \( \text{mM} \) NiEDDA on the central resonance lines of cw X-band EPR spectra recorded for NpHtrII\textsubscript{157} variants in complex with NpSRII reconstituted in PML. The mutants illustrated present different subdomains of the HAMP: NpSRII/NpHtrII\textsubscript{157}-R99R1 (left), AS1; NpSRII/NpHtrII\textsubscript{157}-L105R1 (right), connector. Addition of different NiEDDA concentrations (3 \( \text{mM} \) (dotted lines), 20 \( \text{mM} \) (dashed lines)) to a NiEDDA-free sample (0 \( \text{mM} \) NiEDDA (solid lines)) leads to individual line broadenings for the two spectral components highlighted by arrows (cHAMP: 1, dHAMP: 2) for different residues investigated. For comparison, the spectra detected in the absence of NiEDDA are normalized to unity.](image-url)
by different ratios of the two spectral components. In case of residue R99R1 both components are pronounced, whereas component 2 is more pronounced in case of residue L105R1. Addition of 20 mM NiEDDA results in relevant broadenings (thus decrease in the amplitudes) of the lines representing component 2 for both mutants, indicating that residues in the \(d\)HAMP are water exposed. The associated decrease in the EPR signal amplitude is more evident for the connector position 105, showing higher encounter rates with NiEDDA compared to the R99R1 variant. Addition of 3 mM NiEDDA on one hand supports the higher water exposure of component 2 for residue L105R1 compared to R99R1, but on the other hand also reveals the nonlinear behavior of the line-broadening mechanism with the NiEDDA concentration. The suppression of the \(d\)HAMP signal amplitude for 3 mM NiEDDA is almost of comparable extent to that observed for 20 mM in case of residue L105R1. Moreover, since also the signal amplitude of the \(c\)HAMP component decreases visibly in the presence of both 3 and 20 mM NiEDDA in the spectra recorded for residue L105R1, this residue seems also to be water exposed in the \(c\)HAMP conformation in spite of its compact nature. Contrarily, no line broadening effects are evident for component 1 in case of the R99R1-spectra, thus pointing to its lower water accessibility. Altogether, the spectra of the two residues exemplified showed the peculiar behavior of the spectral shape upon the addition of paramagnetic quencher molecules. The two components are considered to behave differently with increasing reagent concentrations, and with higher NiEDDA concentrations the decisive spectral feature in the determination of spin label side chain accessibilities is indeed the more compact \(c\)HAMP, due to the suppression of the more mobile water-exposed \(d\)HAMP.

Accessibilies determined for NpHtrII\(_{157}\) mutants in complex with NpSRII reconstituted in PML measured under low salt conditions (150 mM KCl), are presented in figure 5.20B. The data shown was obtained during my diploma thesis and has been described in [DOEBBER 2005] and [BORDIGNON et al. 2005]. A brief description is given here in order to gain insight into the general structural features of the HAMP domain and neighboring regions. Furthermore, due to the fact that former accessibilities of the R1 side chain to paramagnetic quencher reagents were presented \textit{via} the accessibility parameter \(\Pi\) described in detail in sections 3.1.3.6 and 4.3.1, conversion to the currently used exchange frequency \(W_{ex}\) (Heisenberg exchange rate, used later on) has to be done. In order to provide a reliable interconversion between the two factors, the control on the B-field axis would be required. Since this is not the case for the past measurements, only strongly approximated interconversions can be done. The \(\Pi\) \textit{versus} residue number plot determined for residues of a cytoplasmic loop region in bacteriorhodopsin [PFEIFFER et al. 1999] (from position 154 at the C-terminal end of helix E to position 171 at the N-terminal end of helix F) was used to at least enable such an approximative comparison. Contrary to the NpSRII/NpHtrII complex, bacteriorhodopsin was found to be a good candidate for this issue since the room temperature cw X-band EPR spectra have been shown to be insensitive to aging of the protein, or
5.3 Side chain accessibilities for quencher reagents

Figure 5.20: Accessibilities of the R1 side chains of spin labeled NpHtrII \(_{157}\) mutants in complex with NpSRII reconstituted in PML. A) Interconversion of the formerly used accessibility parameter \(\Pi\) and the exchange frequency \(W_ex\) on the basis of an accessibility parameter \(\Pi\) versus residue number plot obtained for a cytoplasmic loop region in bacteriorhodopsin [Pfeiffer et al. 1999]. \(\Pi\) values calculated from CrOx (50 mM) measurements (filled diamonds, solid lines) are compared to \(W_ex\) values obtained from NiEDDA (3 mM) experiments (left scale), whereas \(\Pi\) values determined for oxygen (21% \(O_2\), air) accessibilities (open diamonds, dashed lines) are compared to the analogous exchange frequencies (right scales). The scales have to be considered as strongly approximated (see text). B) Accessibility parameter (\(\Pi\)) values calculated for different HAMP residues investigated under low salt conditions. Values obtained from experiments in the presence of oxygen (21% \(O_2\), air) and CrOx (50 mM) are depicted in red and blue, respectively. A magnified view of the values obtained for residues V78R1 to A87 (measured and calculated by A. A. Wegener (former group member)) is given in the inset. Errors are estimated to \(\pm 10\%\) mainly due to changes in the reagent’s concentration and uncertainties in the determination of the power during the experiments.

Environmental changes. Two residues P165R1 and E166R1 located at the N-terminal end of helix F in the aqueous phase were investigated in terms of power saturation measurements using both NiEDDA and CrOx, as well as oxygen. Exchange frequencies were calculated according to section 4.3.2 and the corresponding scales fitted to figure 5.20A. For comparison between values obtained with NiEDDA or CrOx one has to take into account that...
since NiEDDA has shown to be about twice (1.8) as effective as CrOx concerning the line broadening, the two reagents affect the spectral components differently (see figure 5.19) and thus may lead to different accessibility values depending on their relative concentrations.

The accessibility profile presented in figure 5.20B shows very low values for both $\Pi_{CrOx}$ and $\Pi_{oxygen}$ up to residue 86, pointing to a very dense packing of the NpSRII/NpHtrII$_{157}$ complex in this region. A periodicity of the accessibility values for oxygen measurements of about 3.5 (see inset in figure 5.20B) clearly indicates $\alpha$-helical content of this region as predicted by secondary structure analysis. Up to residue A94 the CrOx accessibilities first increase gradually indicating the protrusion of the nitroxide side chains into the bulk water, and then approach values typical for water-exposed residues from position 95 on (C-terminal end of the AS1). Strikingly, the $\Pi_{CrOx}$ values for residues L93, A94, A97 and S98 show dramatically decreased values compared to those of the neighboring residues indicating strong secondary/tertiary interactions of the nitroxide side chains inhibiting the CrOx diffusion toward the respective side chain. The accessibility parameter values obtained in the presence of oxygen analogously increase from position 86 to 91 supporting the increasing proximity of the side chains investigated to the bulk water. From residue T92 to the end of the predicted AS1, the almost constant low level of the $\Pi_{oxygen}$ values is typical for water-exposed side chains. Although the $\Pi_{CrOx}$ values obtained are slightly lower than expected, the accessibility profile presented is mainly in accordance with the side chain mobilities of the respective residues investigated in the presence of 150 mM KCl. Though a slightly disturbed periodical pattern can be observed at the C-terminal end of the AS1, it cannot be assigned fully to a region of $\alpha$-helical content due to the disagreement with the mobility data. It is noteworthy that a distinct periodicity describing an $\alpha$ helix might well be disturbed by secondary/tertiary interactions of the residues’ side chains.

Results of oxygen and NiEDDA accessibility measurements performed in the presence of 150 mM and 3.5 M KCl for both low (40-fold molar excess of lipids per 1:1 NpSRII/NpHtrII$_{157}$ complex) and high (400-fold molar excess per 1:1 complex) lipid contents are presented in terms of $W_{ex}$ in figure 5.21. The oxygen accessibilities obtained at low salt concentrations (see figure 5.21A, black squares) are within the experimental errors in line with the data presented in figure 5.20B. Reference measurements with 5-doxylstearic acids (5DSA, see figure 5.8, top) and 16-doxylstearic acids containing a nitroxide ring compound attached to the 5th and 16th carbon atom in the fatty acid chain, respectively, were inserted in PML ($\approx$ C$_{20}$) in a 1:100 stoichiometric ratio and investigated concerning their oxygen and NiEDDA accessibilities. The exchange frequency of oxygen with the nitroxide near the membrane/water interface (5DSA) was found to be about 5 MHz, whereas the corresponding value for the nitroxide deeper in the bilayer (16DSA, near the center of the membrane) was 8.5 MHz. Comparison of these values with the recently obtained oxygen accessibilities for the low salt measurements clearly show that they are not located in the lipid phase but rather water exposed. Some of the exchange frequencies derived from high
Figure 5.21: Accessibilities of the nitroxide side chains of spin labeled NpHtrII₁₅₇ variants in complex with NpSRII reconstituted in PML. A) Accessibilities for oxygen (21%, air) depicted in terms of the Heisenberg exchange rates $W_{ex}$ between the R1 side chain and the paramagnetic species versus residue number. Measurements were performed at low (150 mM KCl, black/gray) and high (3.5 M KCl, olive/light green) salt concentrations for 40-fold (squares) and 400-fold (circles) molar excess of lipids per 1:1 NpSRII/NpHtrII₁₅₇ complex. B) Heisenberg exchange rates from NiEDDA measurements (20 mM, closed squares; 3 mM normalized to 20 mM, open squares) versus residue number. Errors are estimated to ±20% mainly due to uncertainties in the determination of the quencher’s concentration and of the $\alpha$ values (see section 4.3.2). For comparison, the lower part shows a magnified view of the region of low NiEDDA accessibilities indicated by the dashed lines and the arrows. Values obtained for directly neighboring residues are connected by lines and the connector region is highlighted by gray boxes in all graphs.
salt (3.5 M KCl) measurements, where about 90% of the spin label population can be ascribed to the cHAMP conformation, are quite low for water-exposed sites in terms of the Heisenberg exchange frequency. Particularly the oxygen accessibilities for residues A97 and M100 possess very low values ($W_{ex} \approx 0.25 MHz$). However, comparison of the trends of the low- and high-salt data obtained yields minor difference between them. The most notably difference is the indication of a periodicity in the $W_{ex}$ values for the high-salt conformation at the C-terminal end of the AS1. This supports the assumption of more defined structural elements/well defined tertiary interactions present at high salt conditions. Measurements performed in the presence of higher lipid contents (400-fold instead of 40-fold molar excess of lipids per 1:1 complex; see figure 5.21A, gray and light green circles) did not show relevant differences neither at low, nor at high salt concentrations.

Heisenberg exchange frequencies for water accessibilities under low salt conditions were obtained using 3 and 20 mM NiEDDA as already mentioned. The inconsistency of the $W_{ex}$ values in the presence of 3 (normalized to 20 mM) and 20 mM NiEDDA apparent from figure 5.21B (upper part) clearly shows the nonlinear relation between NiEDDA concentration and accessibility, and thus immediately suggests the presence of the two spectral components representing two conformations. The very high water accessibilities observed for 3 mM-NiEDDA measurements can exclusively be assigned to the dynamic component 2 ($d$HAMP) being fully exposed to the water phase for each residue investigated. For better comparison, a magnified view of the low NiEDDA accessibility values is depicted in the lower part of figure 5.21B. Interestingly, similar values can be observed for both low and high salt measurements using a final NiEDDA concentration of 20 mM. This demonstrates that the spectral component 1 at low salt concentration and the prevalent spectral component at high salt concentration both represent the cHAMP conformation. Aside from this general consistency, the values of the NiEDDA (20 mM) accessibilities in the presence of 150 mM KCl are slightly higher than the high-salt analogs in most of the cases, pointing to the incomplete “suppression” of the $d$HAMP component. However, in comparison with the reference data obtained from NiEDDA (20 mM) measurements with 5DSA and 16DSA resulting in $W_{ex} \approx 2.5$ and 0.3 MHz, respectively, exchange frequencies obtained from high salt measurements and most of those from low salt experiments possess quite low values. The data suggests these residues to be located in the protein interior. Higher NiEDDA accessibilities and consequently water-exposed locations can only be found for residue L105 in the connector in the presence of low and high salt concentrations, for residue Y121 under low and A122 under high salt conditions. Moreover, a periodical motif at the C-terminal end of the AS1 is indicated by the few residues investigated, supporting the assumption of $\alpha$-helical content of this region at least for the high-salt conformation. Differences upon the use of higher amounts of lipids (400-fold instead of 40-fold molar excess of lipids per 1:1 complex; see figure 5.21B, gray and light green circles) cannot be detected, excluding lipid-induced effects on the accessibilities due to inter-complex interactions.
To unravel the molecular details of the compact cHAMP conformation, the water accessibilities for the different NpHtrII\textsubscript{157} variants described in figure 5.21B under high salt conditions (3.5 M KCl) were compared to the solvent accessibilities of the corresponding positions in the Af1503 HAMP domain from \textit{A. fulgidus} obtained \textit{in silico}. In order to gain insight into the approximate locations of the residues investigated in the \textit{N. pharaonis} HAMP and the analogous positions in the Af1503, a schematic overview is given in figure 5.22. Additional comparison of the residues chosen for this investigation can be obtained from the sequence alignment of the two HAMP domains superimposed to the predicted secondary structure, which was already introduced in figure 2.8.

The solvent accessibilities of the Af1503 residues corresponding to the side chains investigated in the \textit{N. pharaonis} HAMP are presented in terms of the solvent-accessible surface (SAS) in figure 5.23. The values were extracted from [Swain and Falke 2007], where a standard rolling water molecule method was used to obtain the data. The SAS of a biomolecule first described by Lee and Richards [Lee and Richards 1971] is the surface area, usually given in square angstroms (Å\textsuperscript{2}), that is accessible to a solvent (here water).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.22.png}
\caption{Models for the HAMP domains from \textit{N. pharaonis} HtrII (left, see figure 5.1) and \textit{A. fulgidus} Af1503 (right, see figure 2.8). \textit{C}_\beta atoms of some residues investigated are represented by colored balls in the HAMP domain from \textit{N. pharaonis}, in case of glycines \textit{C}_\alpha atoms are shown instead. Analogous sites are highlighted in the isolated HAMP domain from \textit{A. fulgidus} derived from NMR studies. For the sake of clarity the top (top right) and the side (bottom right) view is shown.}
\end{figure}
Figure 5.23: Comparison of Heisenberg exchange rates $W_{ex}$ (olive squares) determined for NpHtrII variants in complex with NpSRII reconstituted in PML with the solvent accessibilities calculated for corresponding positions in the archaeal HAMP NMR structure from A. fulgidus (open circles, values taken from [Swain and Falke 2007]). The NpHtrII $W_{ex}$ values were determined from measurements performed in the presence of 3.5 M KCl and 20 mM NiEDDA (see figure 5.21B, olive squares). Values for directly neighboring residues are connected via solid/dotted lines. The connector region between the two amphipathic sequences is highlighted by the gray box. The residue numbers given are those from the N. pharaonis HAMP.

For an atom it is defined as the surface area of a sphere of radius $R$ where on each point the center of a solvent molecule can be placed in contact with the van der Waals sphere around the atom without penetrating any other atom in the biomolecule. Accordingly, the radius $R$ is given by the sum of the atom’s van der Waals radius and the radius of the solvent chosen [Bustamante et al. 2000]. A typical value for the latter one approximating a water molecule is 1.4 Å. Using the rolling-water-sphere algorithm [Shrake and Rupley 1973] to determine the SAS is a common method, where a water molecule is rolled over the van der Waals radii of the atoms of the biomolecule under inspection and the surface area touched by the water molecule, i.e. the solvent accessible surface, is calculated.

Comparison of the trends of the NiEDDA (20 mM) $W_{ex}$ values and the solvent accessibilities in figure 5.23 shows a striking consistency in the AS1 (here A95 to M100) as well as in the AS2 (here G118 to A122), although the deviations visible for positions 98 and 99 are not in the range of the experimental errors. Due to the few residues investigated in the connector, an analogous comparison could not be done. However, although the values of the two residues investigated in the connector (L105 and D115) do not match fully the solvent accessibilities they can be considered comparable since the connector region is assumed to lack defined secondary structural elements and accordingly defined spatial arrangement with respect to the AS1 and AS2. Altogether, the cHAMP conformation is suggested to be in line with the four helical bundle structure obtained by NMR for the HAMP domain of A. fulgidus.
5.3 Side chain accessibilities for quencher reagents

5.3.2 Micelle vs. lipid bilayer

To further characterize the changes observed in the mobility pattern in the DDM-solubilized NpSRII/NpHtrII complex (see section 5.1.3), the collision frequencies between the paramagnetic quenchers CrOx and oxygen and the nitroxide were also determined for the solubilized samples. Analogous to the formerly performed accessibility measurements on the reconstituted variants, the accessibility parameter $\Pi$ is used here to present the data. An approximate conversion of the $\Pi$ values to the currently used Heisenberg exchange frequency $W_{ex}$ is given in figure 5.20A.

![Graph showing accessibility of R1 side chains for detergent-solubilized NpHtrII variants in complex with NpSRII. The accessibility parameter values ($\Pi$) obtained from measurements in the presence of 50 mM of the water-soluble CrOx are depicted via blue circles, the analogous values obtained from oxygen (21% O$_2$, air) measurements via red circles. Errors are estimated to ±10% mainly due to changes in the reagent’s concentration and uncertainties in the determination of the power during the experiments. For comparison the values for corresponding residues investigated in the lipid-reconstituted NpSRII/NpHtrII complex are added (gray circles, errors not shown).](image)

$\Pi_{CrOx}$ values obtained from accessibility measurements carried out for NpHtrII residues A95 to G101 at the C-terminal end of the AS1 clearly show the less water accessibility of these residues in the solubilized form compared to the reconstituted one (see figure 5.24 upper part). The information obtained from these accessibility measurements indicates the likely appearance of additional tertiary interactions between the transducer residues investigated and neighboring side chains after solubilization. However, it is noteworthy that also in the solubilized complex high quencher concentrations (CrOx, NiEDDA) may lead to the apparent suppression of the spectral component 2, yielding the obtained additional tertiary interactions.
5.4 Environmental polarity

5.4.1 Effects of high salt concentrations and high lipid content

Changes observed in the mobility and accessibility data in the presence of high salt concentrations indicate a higher compactness of the NpHtrII structure under high salt conditions. It is obvious that a decrease in the amount of water molecules surrounding the nitroxide side chain is linearly correlated with decreasing polarities of the considered environment when further effects due to e.g. very close charged protein regions are excluded. The \( A_{zz} \) parameter obtained from the low temperature (160 K) spectra of the NpHtrII residues investigated describes the environmental polarity and provides thus a further mean to corroborate the findings obtained in the former sections. \( A_{zz} \) values for HAMP residues V78 to G101, L105, D115 and A122 in the presence of 150 mM KCl (see figure 5.25) were obtained during my diploma thesis [Doebber 2005, Bordignon et al. 2005]. They are explained briefly here, the corresponding experimentally obtained cw X-band EPR spectra are illustrated in figure A.2 in the supplementary data part (appendix).

Quite high oscillations in the \( A_{zz} \) values of residues V78 to L82 located in the lipid bilayer are evident, indicating residue specific interactions between the nitroxide side chain and polar regions of the protein, the alkyl chains of the lipids, or water molecules that penetrated the membrane. Following the \( A_{zz} \) profile up to residue A94 reveals a slight increase in the environmental polarity (\( A_{zz} \approx 3.5 \text{ mT} \)) typical for residues that are in
Environmental polarity

5.4 Environmental polarity

Contact with the water phase, but still hindering the water molecules to completely access the sites under investigation. This indicates the stepwise extension of the residues from the lipid phase to the water phase, and that interactions with the secondary/tertiary structure are present in this region of the transducer. Slightly increased polarities ($A_{zz} \approx 3.55 \text{ mT}$) are observable for residues inspected at the C-terminal end of the AS1, where from the mobility and accessibility data already a very dynamic structure was suggested. The values obtained here are in line with a gradual protrusion of the spin label side chains to the bulk water. However, according to [Plato et al. 2002] the $A_{zz}$ values expected for nonpolar environments account for 3.36 mT, and polar environments are defined by values of 3.64 mT, showing that the $A_{zz}$ presented here are smaller than expected for water-exposed side chains. Very high polarity values unambiguously assignable to complete water exposure can only be found for positions 105 in the connector and 122 at the N-terminal end of the AS2, whereas residue D115 located at the connector/AS2 interface again exhibits a slightly lower value ($\approx 3.6 \text{ mT}$). As revealed in the temperature-dependent measurements, the equilibrium between the two conformations can be shifted to the cHAMP conformation either by high salt concentrations, or by lowered temperatures (see section 5.2). Thus, the polarity data is rather representative for the spectral component 1 than for component 2. Nonetheless, the decrease in the water accessibilities observed around position 97 in the AS1, which was assigned to the side chain’s interactions with secondary/tertiary structural elements in the room temperature structure, is retrieved in the $A_{zz}$ pattern presented.

In order to test the relevance of the salt-induced effect at low temperatures, recent experiments were performed with selected NpHtrII157 variants in the presence of both 150 mM and 2 M KCl (see black and olive squares in figure 5.26). Increasing the salt concentration to 2 M KCl leads to deviations from the low salt data in the $A_{zz}$ values, which are negligible within the experimental errors. That, despite the major discrepancies between the low and high-salt data observed in the mobility and accessibility analysis, a consistency of the corresponding $A_{zz}$ values could be observed, confirms the assumption of the equilibrium-shift to the compact cHAMP conformation at low temperatures. Moreover, $A_{zz}$ values derived from measurements performed in the presence of 40% (w/v) sucrose (see open triangles in figure 5.26) to rule out effects induced by changes in the solvent viscosity, were found to be similar to the values obtained for sucrose-free samples. Increasing the lipid content from 40-fold to 400-fold molar excess of lipids per NpSRII/NpHtrII157 complex (see circles in figure 5.26) shows neither under low salt, nor under high salt conditions remarkable changes in the environmental polarity. Lipid-induced effects in terms of inter-complex interactions can therefore be excluded.
Figure 5.26: Polarity parameters ($A_{zz}$) determined from the fitting of simulated cw X-band EPR spectra to the experimental ones performed at 160 K for NpHtrII<sub>157</sub> mutants in complex with NpSRII reconstituted in PML. Values obtained from measurements carried out in the presence of low (150 mM KCl, black squares) and high (2 M KCl, olive squares) salt concentrations are compared. Moreover, $A_{zz}$ values obtained from experiments performed with 40% (w/v) sucrose (150 mM KCl, open triangles) and with high lipid contents (400-fold instead of 40-fold molar excess of lipids per NpSRII/NpHtrII<sub>157</sub> complex) for both 150 mM (gray circles) and 2 M KCl (light green circles) are introduced. Errors are estimated to ±0.015 mT due to uncertainties in the determination of the local maxima needed for the $A_{zz}$-value calculation. In all high salt (2 M KCl) experiments performed 20% (v/v) of the cryoprotectant glycerol was added to the samples. The connector region between the two amphipathic sequences is highlighted by the gray box.

5.4.2 Micelle vs. lipid bilayer

As for the reconstituted samples, the $A_{zz}$ parameter provides useful means to clarify the predictions resulting from the mobility and accessibility analysis done for the transducer mutants investigated in the detergent-solubilized NpSRII/NpHtrII<sub>157</sub> complex. Figure 5.27 shows the comparison of the polarity data for the reconstituted complex described above, and the $A_{zz}$ values obtained for the solubilized samples. Lower polarity values defined by decreased $A_{zz}$ values are found for the solubilized samples compared to the reconstituted ones. Most notably, residues L90 and S91 exhibit $A_{zz}$ values typical for nonpolar environments ($\approx 3.36 \text{ mT}$ [Plato et al. 2002]), pointing to the presence of very strong secondary/tertiary interactions of the two side chains with neighboring residues, or other parts of the protein complex, or the micelle. On the one hand, it is worth reminding that in the solubilized form the complex exhibits a 1:1 stoichiometry, i.e. one transducer and one receptor, thereby excluding the possible strong interaction of the side chains with the second transducer. On the other hand, the micelles might also cover residues that were in contact with the second transducer before. As no distinction between different secondary/tertiary interactions is possible from the polarity data alone, decreased $A_{zz}$ values might be due to both side chains interacting with the receptor, and positions that are covered by the micelle.
5.5 Structural properties of NpSRII/NpHtrII in membranes

Figure 5.27: Polarity parameters (A_{zz}) determined from the fitting of simulated cw X-band EPR spectra to the experimental ones performed at 160 K for NpHtrII_{157} mutants in complex with NpSRII in the detergent-solubilized form (black squares). Errors are estimated to ±0.015 mT due to uncertainties in the determination of the local maxima needed for the A_{zz}-value calculation. For comparison, the polarity values obtained for the corresponding positions in the lipid-reconstituted form (150 mM KCl, see open squares in figure 5.25) are introduced (gray circles, not errors shown).

The polarity analysis performed clearly shows structural rearrangements taking place in the membrane-adjacent region of the transducer HAMP domain upon solubilization of the NpSRII/NpHtrII_{157} complex, in line with the mobility and accessibility data. Further information on the relative orientation of the transducer with respect to the receptor will be given in the following based on inter-spin distance determination in solubilized complexes.

5.5 Structural properties of NpSRII/NpHtrII in *N. pharaonis* and *H. salinarum* membranes

As pointed out in section 2.2, due to expression problems of the native lipid bilayer from *Natronomonas pharaonis*, generally reconstitution in polar lipids has been performed using the membrane extracted from *Halobacterium salinarum*. Besides their different lipid composition, the *N. pharaonis* bilayer exhibits slightly longer alkyl chains (C_{25}) compared to those from *H. salinarium* (C_{20}). Structural rearrangements occurring at the water/membrane interface of the NpSRII/NpHtrII complex on the basis of these distinctions were investigated via pulse ENDOR measurements.

The main constituent in the lipid bilayers from both bacteria is the polar lipid PGP(-Me) possessing two ^{31}\text{P} (100\% natural abundance) nuclei in the head group (see section 2.2). Its nuclear spin I = \frac{1}{2} enables the application of ENDOR experiments to determine distances
up to about 1 \( \text{nm} \) between the phosphorus and the unpaired electron of the attached nitroxide spin label. The distances between the lipid headgroups and several spin labeled NpSRII mutants in complex with the transducer NpHtrII reconstituted in the respective lipid bilayer in different salt conditions were investigated.

For all pulse ENDOR measurements performed the \(^{14}\text{N} \) (nuclear spin \( I = 1 \)) spin label was exchanged by the \(^{15}\text{N} \) (nuclear spin \( I = \frac{1}{2} \)) analogon in order to reduce the number of lines in the EPR absorption spectrum. Moreover, the \(^{15}\text{N} \) spin label was fully deuterated to decrease\(^b\) the line width in the EPR spectrum.

The receptor mutant NpSRII-L20R1 in complex with NpHtrII\(_{157}\) possesses a side chain not interacting with the transducer, or other receptor helices (see figure 5.28), and extending into the lipid phase. It is expected to exhibit an average distance of 0.5 \( \text{nm} \) between the unpaired electron of the nitroxide and the phosphorus, thus representing an ideal variant for the distance determination with pulse ENDOR.

The resulting (non-symmetrized) X-band pulse ENDOR spectrum is depicted in figure 5.29. The most prominent lines in this spectrum are arranged at and around the proton nuclear Zeeman frequency (\( \nu_H \approx 14.7 \text{ MHz} \) \[\text{Kay et al. 2006}\]) describing couplings of the electron spin with the surrounding protons. Couplings with the spin label deuterons are also easily recognizable around the \(^2\text{H} \) ENDOR frequency of about 2.5 \( \text{MHz} \) \([\text{approximated from [Kurreck et al. 1984]})\). However, these are not the couplings that were to be investigated here. The expected \(^{31}\text{P} \) couplings at the \(^{31}\text{P} \) nuclear Zeeman frequency of \( \approx 6 \text{ MHz} \) \[\text{Zänker et al. 2005}\] (highlighted by the reddish open circle in figure 5.29) providing the desired information, could not be resolved at all. Since this sample had a very high spin

\(^b\)\(^{1}\text{H} \) dipolar relaxation of the electron spin with the nearby proton nuclear spins is much faster than \(^2\text{H} \) dipolar relaxation, thereby “enhancing” the spin-lattice and accordingly the spin-spin relaxation time.
5.6 Non-exchangeable environmental protons

A more general information compared to a distance analysis obtainable from the couplings resolved in a pulse ENDOR spectrum is given by the possibility to distinguish between exchangeable and non-exchangeable protons in the spin label microenvironment. Since cw EPR accessibility measurements (see section 5.3) indirectly detect the “amount” of exchangeable protons from the bulk water, and high-field EPR experiments (not used in this study) allow the determination of the number of hydrogen bonds to the nitroxide, the data obtainable from pulse ENDOR spectroscopy represents a complementary method. As an evident consequence, the derivation of structural features and/or rearrangements in the protein/water interfaces from the pulse ENDOR spectra is possible. To what extent variations in the salt concentration of the buffer have influence on these properties is also to be presented in this section.

The residues investigated here are depicted in figure 5.28 (two receptor mutants: L20R1 and W24R1) and 5.1 (seven transducer variants: L75R1, A79R1, L93R1, M100R1, L105R1,
E116R1 and Y121R1). Due to their different locations in the NpSRII/NpHtrII\textsubscript{157} complex, distinctions between the resolved couplings in the respective ENDOR spectra are expected.

At first, measurements were performed with the PML-reconstituted complex under low salt conditions, i.e. 150 mM KCl, in a protonated buffer (H\textsubscript{2}O) containing 10 mM Tris HCl (pH 8). In order to interpret the couplings resolved in the spectra depicted in figure 5.30, possible proton sources have to be identified. Depending on the position of the attached spin label, potential sources are the bulk water, the protein backbone, the lipid bilayer, neighboring amino acid side chains as well as tertiary structure elements. As the MTS spin label described in section 2.3 has been exchanged by a fully deuterated analogon for ENDOR experiments, no couplings with the intrinsic protons of the spin label are expected. The hyperfine couplings visible in the pulse ENDOR spectra will be analyzed in a qualitative way, whereby misinterpretation cannot be excluded.

The ENDOR spectra given in figure 5.30 are centered around the \(^1\)H (proton) nuclear Zeeman frequency \(\nu_H \approx 14.7 \text{ MHz}\). Considering only weak couplings to be present here, each set of equivalent protons gives rise to a pair of lines centered at \(\nu_H\). Due to second-order
5.6 Non-exchangeable environmental protons

effects, the doublet distance may deviate slightly from $A$ (see section 3.1.5.2). The most prominent features in all spectra shown are the matrix line at $\nu_H$ and the doublet possessing a hyperfine coupling of $\approx 0.5 \text{ MHz}$ around $\nu_H$. These couplings cannot unambiguously be assigned to a certain species of protons. In fact, a “matrix region” can be defined with $\nu_H - 1.25 \text{ MHz} \leq \nu \leq \nu_H + 1.25 \text{ MHz}$, where $\nu$ describes the radio frequency applied in MHz [Weber et al. 2001]. The couplings appearing in this region define a group of signals with contributions from weakly coupled protons from the spin label environment, e.g. from the bulk water, amino acid side chains, the protein backbone and others. A signal exhibiting hyperfine coupling components of about $4.4 \text{ MHz}$ with lines at 12.4 and 16.8 MHz (approximated values) is definitely not part of the matrix region and can correspondingly be assigned to a certain proton species. In the literature similar hyperfine couplings have been obtained describing the interaction of an electron spin with a proton bound to a neighboring C-atom [Kay et al. 1999]. In terms of the distance between the two species, the resolved coupling is comparable to a hydrogen bound to the nitroxide’s oxygen, or similar couplings.

The spectra of the two receptor mutants NpSRII-L20R1/NpHtrII_{157} and NpSRII-W24R1/NpHtrII_{157} show an extremely pronounced matrix region, indicating the interaction of the electron spin with a large amount of protons in the spin label microenvironment. Since these two residues are expected to be located in the transmembrane part of the complex pointing into the lipids (see figure 2.10), the matrix region is thought to mainly comprise couplings with the lipid protons. The matrix region of the remaining spin labeled mutants shows much weaker couplings with the environmental protons.

L75 and A79, TM2 helix residues located in the membrane and near the membrane/water interface, expected to point towards helix TM1 of the second transducer and into the lipids, respectively, exhibit quite low/intermediate amplitudes of the couplings in the matrix region. The very low extent of weakly coupled protons of the L75R1 variant can be explained by the side chain’s extension into the transducer/transducer interface, where, compared to the lipid phase, or the bulk water, protons are only available in minor amounts. The weak couplings resolved in the spectrum obtained for residue A79R1 are on the contrary suggested to mainly originate from interactions with water molecules penetrating the membrane and the lipid tails.

The trend of the increasing amount of proton couplings in the matrix region of the NpHtrII_{157} HAMP mutants L93R1, L105R1, E116R1 and Y121R1 fully agrees with the assumption of the protrusion of the HAMP domain into the water phase. On the other hand, in the case of a linear extension of the transducer helix TM2 into the bulk water, one might have expected differences in the amount of weakly coupled environmental protons at least for the connector positions (L105 and E116), which are expected to be completely surrounded by water molecules, compared to position L93. However, the NpSRII/NpHtrII_{157}-L93R1 environment might comprise protons from both tertiary interactions with the second transducer and from the solvent.
Contrary to the just mentioned reasonable extension into the solvent, M100R1 located at the cytoplasmic end of the amphipathic sequence 1 shows a very low matrix region comparable to a quite buried side chain as e.g. L75 but not typical for a residue that is expected to be fully exposed to the water.

The well resolved hyperfine coupling ($\approx 4.4 \text{ MHz}$) found could be assigned to hydrogen bonds, thus it is possible that this position is characterized by a high degree of anisotropy. Since we are working with dynamic functional units, the couplings resolved in the spectra are broadened/weakened. The dynamicity might also cause differences in the number of hydrogens bound to the spin label sites in the distributed protein complexes, thereby allowing numbers not describing an integer.

In this qualitative analysis the 4.4 MHz couplings resolved in the pulse ENDOR spectra are divided into three groups showing different amounts of hydrogen bonds. According to Jeffrey [Jeffrey 1997], the strength of a hydrogen bond can be classified into strong, intermediate and weak. The presence of hydrofluoric acid, which is not supposed to be present in our samples was shown to cause strong H-bonds, whereas weak ones are generated mainly in protein-protein interactions. An intermediate strength was found for H-bonds involving water molecules, the case mainly considered in the following.

The spectra of the transducer mutant L75R1, and the receptor mutant L20R1, hardly exhibit hyperfine couplings at 12.4 and 16.8 MHz. Thus, they are considered not to have access to high amounts of water molecules for the hydrogen bonds. In the case of position L75, its direction towards the transducer/transducer interface is suggested to explain the lack of couplings, whereas for position L20, pointing into the lipid phase, the depth of the lipid bilayer excluding water molecules in higher amounts is assumed to be the decisive point.

The spectra of the NpSRII mutant W24R1, and of the NpHtrII$_{157}$ variants A79R1, L93R1, L105R1, E116R1 and Y121R1 show intermediate amplitudes of the observed 4.4 MHz hyperfine coupling. A reason for the similar affinity to bind hydrogens of positions that are expected to be more or less completely exposed to the water (L93, L105, E116, Y121) and positions that are located in the lipid bilayer and oriented towards the lipids (W24, A79), might be that the proximity of W24 and A79 to the membrane/water interface is sufficient in terms of water penetration to achieve the same effect. Additional bonds of these two residues with hydrogens from the alkyl chains of the lipids are also not excluded.

As described above, residue M100R1 possesses the lowest amount of proton couplings in the matrix region, whereby its exposure to the solvent is excluded. That is why the most pronounced hyperfine coupling at 4.4 MHz obtainable from the NpSRII/NpHtrII$_{157}$-M100R1 spectrum cannot be explained by hydrogen bonds with water molecules. In fact, this spectral feature can only be ascribed to the presence of a tightly packed protein conformation involving enormous protein-protein interactions and thereby formed H-bonds.
However, it is worth mentioning that the extent of couplings resolved in the matrix region may influence the shape of stronger couplings due to the superposition of the different lines.

Exchanging the protonated (H$_2$O) buffer by a deuterated (D$_2$O) one results in the gray spectra (solid lines) given in figure 5.31. Since after the H/D-exchange only non-exchangeable protons are left for interactions between the MTS and the proton nuclear spins, in fact a consistent decrease in the resolved couplings is observable for all transducer

![Figure 5.31: X-band pulse ENDOR spectra of receptor and transducer variants in the NpSRII/NpHtrI$_{157}$ complex reconstituted in PML. Measurements were performed in the presence of 150 mM KCl (solid lines) and 2 M KCl (dashed lines), respectively, using both a protonated (H$_2$O; black) and a deuterated (D$_2$O; gray) buffer. The spectra were normalized to the same slope of the high-field ends of the spectra, and symmetrized with respect to the $^1$H nuclear Zeeman frequency ($\nu_H \approx 14.7$ MHz). To visualize the differences between the respective spectra, the part of the symmetrized spectra not describing any ENDOR signal was cut according to figure 4.11. The x-axis has been adapted to represent the important spectral features in the best way; the spectra of largest amplitude from each set are scaled to unity.](image-url)
mutants investigated except for residue M100R1. On the contrary, the spectra detected for NpSR II-L20R1/NpHtrII and NpSR II-W24R1/NpHtrII hardly show changes upon solvent deuteration, in line with the assignment done before.

A slight decrease in the 4.4 MHz hyperfine couplings of the L20R1-spectrum supports the assumed water-dependent character of this interaction. Here, the spectrum measured in the presence of the D2O buffer shows that also minor amounts of water molecules can be present deeper in the lipid bilayer, and can be exchanged completely. The nearly constant level of the couplings in the matrix region indicates once more the weak couplings with protons from the lipid tails and not from water molecules. An analogous interpretation of the D2O spectrum performed for position W24 in the receptor is reasonable since the stronger couplings resolved at 12.4 and 16.8 MHz, which are considered to be water-dependent, vanish entirely upon the H/D-exchange, whereas no changes are observable for the weak proton couplings.

In the case of the two transducer variants located in the lipid bilayer, L75R1 and A79R1, the changes for position L75 can be ascribed to the exchange of H2O by D2O in the membrane as described above. Though the L75 side chain is expected not to point directly into the lipids, the interactions between the buried nitroxide and the protons from the water around the transducer/transducer interface may be resolved in the matrix region, where a decrease is clearly recognizable. The spectrum for position A79 pointing into the lipids shows, besides the expected decrease in the matrix region, only a minor decrease in the 4.4 MHz hyperfine couplings, in line with the interpretation previously given of a dual origin of these interactions. Remaining couplings visible in the presence of D2O can accordingly be ascribed to the interactions with the alkyl chains, or the protein surface.

Changes observable in the spectra of NpSR II/NpHtr II-A93R1 and NpSR II/NpHtr II-Y121R1 upon H/D-exchange are in line with their location at a helix surface that is surrounded by the bulk water. Both the weak couplings in the matrix region as well as the stronger interactions with hyperfine couplings of 4.4 MHz are decreased in the deuterated samples. The remaining couplings are assumed to reflect interactions with the protein backbone and neighboring amino acid side chains. However, hyperfine couplings with tertiary structure elements cannot be excluded.

Spectral examination of the deuterated M100R1 sample supports the suggested tight packing of the protein at this position since no changes are visible at all. As this assumption is in agreement with the compact HAMP conformation (cHAMP) and contradictory to the already presented experimental data obtained for the dHAMP conformation, the equilibrium-shift towards the compact conformation at low temperatures (here 80 K) is supported by this data. Moreover, the behavior of the L105R1-spectrum is quite interesting as in this case a dramatic decrease in the matrix region is combined with a complete preservation of the 4.4 MHz couplings. This further suggests this region to be involved in tertiary interactions.
Eventually, measurements have been performed for selected residues expected to be accessible to the water phase in the presence of 2 $M$ KCl in protonated and deuterated solvents. The resulting spectra for positions A93 (only protonated buffer), M100, L105, E116 and Y121 are depicted in figure 5.31.

The absence of major spectral changes in the presence of higher salt concentrations for the investigated side chains A93, M100, L105 and Y121, also supports the finding that the EPR data recorded at low temperatures is rather representative for spectral component 1 than for component 2. In contrast to this finding, the pulse ENDOR spectrum for the NpSRII/NpHtrII$_{157}$-E116R1 mutant shows changes at 2 $M$ KCl at least for interactions with weakly coupled protons. The additional use of a deuterated solvent clearly decreases all resolved couplings. Thus, this residue, located at the connector/AS2 transition, shows decreased weak couplings but nearly unchanged stronger couplings (4.4 MHz) under high salt conditions.

It is finally worth mentioning that several washing steps were needed to fully exchange the protonated by the deuterated solvent (and vice versa). Since the sample concentration has shown to be affected by too many washing steps a compromise between the two features had to be found. Hence, the presence of remaining water molecules hard to eliminate (e.g. in clefts) cannot be excluded. Exemplificative, the effect of washing steps performed beyond the usual procedure is illustrated in figure 5.32a. Although differences are visible in the two spectra presented, they are not of dramatic extent. That the effects shown upon the H/D-exchange in the sample buffer are moreover fully reversible is apparently recognizable from figure 5.32b.

![Figure 5.32: X-band pulse ENDOR spectra of two transducer mutants. The two sets of spectra are normalized as described in figure 5.31. a) Effect of additional washing steps (gray line) on the spectral shape of NpSRII/NpHtrII$_{157}$-Y121R1 compared to the used washing procedure (black line). b) Reversibility of the spectrum for the NpSRII/NpHtrII$_{157}$-L105R1 mutant. The original spectrum (150 mM KCl, protonated buffer; black line) is compared to the spectrum obtained after deuteration and reprotonation of the sample (gray line).](image-url)
5.7 Inter-spin distance analysis

Analysis of spin-spin interactions in a dimeric protein complex is a valuable method to investigate the structure of the dimer as well as its conformational changes. In this study cw and pulse EPR methods were used to gain information on the general topology of the membrane-adjacent region of the HAMP domain in the NpSRII/NpHtrII complex. First, inter-transducer distances between singly labeled NpHtrII variants in complex with NpSRII reconstituted in PML were obtained to gain insight in the HAMP-HAMP interface interaction. Second, possible rearrangements of the transducers occurring in the presence of high salt concentrations were investigated for selected transducer residues. Residue A122R1 was also investigated in terms of changes upon the use of acidic pH (pH 3.4) and 30% (w/v) PEG3350. Third, three doubly labeled transducer variants were analyzed to reveal the relative position of the two amphipathic sequences AS1 and AS2. Eventually, in order to elucidate the relative arrangement of the transducer molecules with respect to the tightly bound receptor molecules, inter-spin distances between spin labels introduced at transducer and receptor sites were obtained. The effect of detergent solubilization on the relative orientation of the transducer with respect to the receptor was also performed for selected doubly labeled mutants. The pulse experiments presented here were carried out and analyzed in collaboration with Julia Holterhues and Enrica Bordignon. In order to prevent, or at least attenuate possibly occurring lipid-induced effects in terms of inter-complex interactions, a compromise between sample concentration and lipid content was chosen. We used a 200-fold molar excess of lipids per NpSRII/NpHtrII complex (instead of 40- or 400-fold molar excess).

5.7.1 Salt- and lipid-induced effects on singly labeled NpHtrII mutants

Results of pulse EPR experiments in terms of a 4-pulse double electron-electron resonance (DEER) analysis of various singly labeled NpHtrII variants are presented in figures 5.33 (residues A80, A88, S89, T92 to R99 in the AS1) and 5.34 (residues M100, G101 in the AS1, L105 and D115 in the connector region and residues G118, D119 and A122 in the AS2). The corresponding experimentally obtained traces representing the normalized dipolar evolution functions $V(t)/V(0)$ are depicted in figure A.1 in the supplementary data part in the appendix. Therein, the backgrounds fit with an exponential function $(B(t))$ due to a homogeneous three-dimensional distribution of the spins in the sample is superimposed to the traces. In figure 5.33 and 5.34 the normalized form factors $F(t)/F(0)$ $(F(t) = V(t)/B(t))$ obtained by Tikhonov regularization ($\alpha = 1000$) are presented in the left panel. Although oscillations in the traces obtained for transducer variants in the presence of 150 mM KCl

Measurements for residues A88, S89, T92, A94 to R99, G101, D115 and A122 in the presence of 150 mM KCl were performed in the laboratory of G. Jeschke at the MPI for Polymer Research in Mainz, Germany (present address ETH Zürich, Zürich, Switzerland)
5.7 Inter-spin distance analysis

(see black traces in figures 5.33 and 5.34) are visible, showing the presence of dipolar interactions, they are not well defined and damp in most of the cases within the first microsecond.

More defined distance distributions, leading to better defined oscillations in the dipolar function could only be observed for residues S89, M100, G118 and A122 all exhibiting distances between 1.9 and 2.5 nm. Contrarily, all mutants investigated in the amphipathic sequence 1 (A88R1, T92R1 to R99R1) exhibit very broad distance distributions. The distance data obtained for residues A80 located in helix TM2 in the lipid bilayer and D119 in the N-terminal end of the AS2 is also characterized by very broad distributions. Since the oscillations visible in the form factor traces possess significantly larger and stronger decaying amplitudes during their first half-period compared to the following times, a reliable separation of the oscillatory and purely decaying parts needs signal detection for at least two periods [Jeschke et al. 2004a]. In this case, the mean distance as well as its standard deviation extracted from the analysis of the oscillations and their decay in the experimental traces are of reliable nature. In the case only one period of the oscillation is visible in the traces recorded, only a rough estimate about the mean distance can be obtained, whereas reliable information on the width of the distance distribution is not obtainable. For the consideration of pairs of nitroxides the period of a dipolar oscillation in terms of the distance accounts for [Jeschke et al. 2004a]

\[ t_{\text{dip}}(r) = r^3 \frac{4\pi\hbar}{\mu_0 g^2 \mu_B^2} \approx r^3 \times 0.0192 \, \mu s \, nm^{-3}, \]  

with \( r \) as the distance between the two nitroxides (in nm). In order to clarify the feasibility of the distance distributions presented in figures 5.33 and 5.34, boxes of different colors are inserted into the \( P(r) \) versus distance (\( r \)) plots. Therein, white boxes (arranged on the left) describe data defining reliable mean distances and standard deviations (two periods of oscillation), light yellow boxes indicate regions of the plots only giving rough estimates about the mean distance, but no information about the distributions (one period of oscillation), and for data presented within the gray boxes (arranged on the right) no distance interpretation is possible ((less than one period of oscillation)). One further aspect worth accounting for in the interpretation of the distance data derived is the average number of spins per nanoobject \( <n> \) (here per NpSR1I/NpHtrII157 complex in its 2:2 stoichiometry) obtained from the modulation depth in the input data. Although differences in the mean distances and distributions are not caused directly by low spin label efficiencies, but indirectly via a worse signal-to-noise ratio in the singly labeled mutants, consideration of the spin labeling efficiencies is helpful, particularly if parts of the distance distributions obtained are out of the resolvable distance range (e.g. below 1.6 nm). Reliable spin numbers can give information on the spin label fraction exhibiting distances beyond the range resolved. Considering singly labeled transducer moieties in complex with NpSR1I reconstituted in PML, yields a spin number of \( <n> = 2 \) in the case of 100% spin labeling efficiency. In this regard, most of
Figure 5.33: 4-pulse DEER data (part I) obtained for various NpHtrII mutants designated by the 1-letter amino acid code and the residue number. Calculated spin numbers per complex ($<n>$) are depicted. Left: Background corrected, normalized experimental data $F(t)/F(0)$, fit by the Tikhonov regularization with a regularization parameter $\alpha = 1000$ (dotted lines). Right: Distance distributions $P(r)$ normalized to unity. Trace colors: 150 mM KCl, black; 2 M KCl + 20% (v/v) glycerol, olive. Colored boxes show the reliability of the distance data: white, reliable distance and distribution; light yellow, approximate distance; gray, no predictions possible.
5.7 Inter-spin distance analysis

Figure 5.34: 4-pulse DEER data (part II) obtained for various NpHtrII_{157} mutants designated by the 1-letter amino acid code and the residue number. Calculated spin numbers per complex ($<n>$) are depicted. Left: Background corrected, normalized experimental data $F(t)/F(0)$, fit by the Tikhonov regularization with a regularization parameter $\alpha = 1000$ (dotted lines). Right: Distance distributions $P(r)$ normalized to unity. Trace colors: 150 mM KCl, black; 2 M KCl + 20% (v/v) glycerol, olive; pH 3.4, 150 mM KCl, blue; 30% (w/v) PEG3350, magenta. Colored boxes show the reliability of the distance data: white, reliable distance and distribution; light yellow, approximate distance; gray, no predictions possible.
the data obtained under low salt conditions exhibits similarly high spin numbers, describing
typical spin labeling efficiencies of 70 to 90%. In the case of high spin numbers as observed
for residues A88, R99, G101, G118, A122, generally no large additional distributions are
expected outside the resolvable distance range. Nonetheless, as indicated in the distance
distributions obtained for residues A95, A97, M100, D115 and D119, fractions below the
detection limit of DEER cannot be excluded. Contrary, in extreme cases as detected for
positions 93 and 105, where very high spin numbers (1.9 and 2.0, respectively) are present
together with distance distributions indicating fractions below the detection limit, the re-
liability of the data obtained has to be questioned. On the other hand, low spin labeling
efficiencies (below 70 %), resulting in low modulation depths of the dipolar evolution time
traces, point to the existence of distances below the 1.6 \( \text{nm} \) border. If the distance distri-
butions also indicate the presence of shorter distances, as e.g. obtainable for residues A80,
S89, T92, K96 and S98, such considerations are supported. However, to understand the
whole distance distribution, including distances shorter than 1.6 \( \text{nm} \), continuous wave EPR
experiments are indispensable.

Increasing the salt concentration to 2 \( M \) KCl (plus 20% (v/v) glycerol, see olive traces
in figures 5.33 and 5.34) for six of the HAMP residues investigated leads in half of the
cases (residues A88, R99 and M100) to a remarkable decrease in the width of the distance
distributions and the associated prominence of one of the distances resolved in the 150 \( mM \)
KCl distributions. No changes are on the contrary observable for residues G118 and A122.
Under the assumption of a “two-state” equilibrium between the \( \text{cHAMP} \) and \( \text{dHAMP} \)
conformation one expects that at low temperature the equilibrium is shifted towards the
compact \( \text{cHAMP} \) conformation, i.e. the prevalent conformation at high salt concentrations.
Indeed, minor changes are detectable in the presence of high salt concentrations, indicating
the high-salt low-temperature structure of the HAMP domain is slightly more compact than
the low-salt low-temperature analog, and/or that the freezing procedure traps a greater
population of the \( \text{cHAMP} \) when high salt concentrations are present. The spectra obtained
under low salt conditions could then be slightly contaminated by a small fraction of proteins
in the \( \text{dHAMP} \) conformation.

Due to the fact that acidic pH (pH 3.4) as well as the addition of 30% (w/v) PEG3350
was shown (see section 5.1.2) to induce similar changes in the room temperature cw X-band
EPR spectra as high salt concentrations, the inter-spin distances for residue A122R1 at
the N-terminal end of the AS2 was checked under these conditions (see \( F(t) \) and \( P(r) \) in
figure 5.34). Apparently, the main peaks in the resulting distance distributions (at about
2.5 \( \text{nm} \)) do not change within the experimental errors with respect to the low and high salt
data. An additional peak emerges at about 3.4 \( \text{nm} \) in both cases. Because no changes in
the spin number \( < n > \) could be observed compared to the high-salt results, a collapse of
the structure, leading to additional inter-spin distances by virtue of protein aggregation, is
not expected. To what extent the additionally resolved, longer distances can be ascribed to
5.7 Inter-spin distance analysis

a conformational change cannot be judged, but a more inhomogeneous sample concerning the transducer conformations can be suggested. However, these measurements showed that the HAMP domain remains stable even under non-physiological conditions.

Here, it is worth recalling that the breadth of some of the distance distributions may also originate in the fact that the MTSSL side chain is assumed to possess different conformers/rotamers, which might induce deviations from the observation of a single maximum in the distance distribution. Generally, as can be seen nicely in the distance distributions for residues exhibiting a single maximum (see e.g. G118 in figure 5.34), the distributions are relatively large, showing the general flexible nature of the nitroxide side chain used in this study.

The mean distances obtained from the 4-pulse DEER experiments (where possible) were extracted from the distributions given in figures 5.33 and 5.34, and compared to those derived from the fitting of simulated to experimental cw EPR spectra obtained at 160 K. Cw and pulse EPR techniques are complementary due their different accessible distance ranges (≈0.8 to 2.0 nm for cw EPR, ≈1.6 to 6 (in very favorable case up to 8) nm for pulse EPR). The resulting distance pattern is presented in figure 5.35. Included in the pattern are distances derived from a first set of cw EPR spectra obtained at low salt concentrations (open squares [DOEBBER 2005, BORDIGNON et al. 2005]), from a new set of spectra recorded at low and high salt concentrations (black and olive squares), and from the pulse-EPR low and high salt measurements (black and olive stars). The corresponding experimentally obtained cw X-band EPR spectra are presented in figure A.2 in the supplementary data (appendix). No dipolar broadening was visible in the cw spectra of residues A88 to S89 and their counterparts in the second transducer. For the following three positions minor dipolar interactions in the spectra became apparent, resulting in distances of 2.0 nm (residue L90) and 1.9 nm (residues S91 and T92) at the border of the cw EPR distance resolution. Stronger interactions were detected between the spin labels attached to positions 93 (1.7 nm) to 97 (1.8 nm for positions 94 to 97) showing the strongest proximity of the two transducer molecules in this region. This finding is in agreement with the accessibility data in figure 5.20 and the polarity data in figure 5.25, both showing decreased values in the L93/A94 region due to the predicted increased secondary/tertiary interactions. For the remaining part of the C-terminal end of the AS1, comprising residues S98 to G101, small interactions (1.9 nm) could be detected between the transducer variants, with the spin labeled residue M100 exhibiting a shorter mean inter-spin distance of 1.7 nm. These findings are fully in line with the accessibility data obtained in the presence of high salt concentrations, where a remarkable decrease in the water accessibility is detectable for residue M100 (see figures 5.21 and 5.23). Thus, the inter-spin distances support the shift of the “two-state” equilibrium towards the cHAMP component at low temperatures. Selected residues investigated in the connector (L105) and the AS2 (A122) show no (> 2.0 nm) and minor (1.9 nm) dipolar interactions, respectively, indicating that the connector regions of the two HAMP domains are not in
Figure 5.35: Inter-spin distances obtained from pulse (stars) and cw (squares) EPR methods on singly labeled NpHtrII_{157} mutants in complex with NpSRII reconstituted in PML. Distances obtained from recent measurements performed at low (150 mM, black) and high salt concentrations (2 M KCl + 20% (v/v) glycerol, olive) are compared to previous low salt data (open symbols) [DOEBBER 2005, BORDIGNON et al. 2005]. Squares with orange arrows represent a lower distance limit determined from cw EPR spectra. For residues possessing two mean distances in the $P(r)$ distribution of the pulse EPR experiments both values are depicted. For the sake of clarity, superimposed symbols are decreased to half width. Errors in the mean distances derived from cw EPR were set to ±0.2 nm, errors for data derived from pulse EPR were estimated from the individual standard deviations given in figures 5.33 and 5.34. The gray area indicates the expected inter-spin distances, $d_{SL}$, based on the structure of the HAMP domain from A. fulgidus [HULKO et al. 2006] with primary sequences alignment according to figure 2.8. The distance data was obtained from $(d_{Cbeta} - 0.25 \text{ nm}) \leq d_{SL} \leq (d_{Cbeta} + 1.25 \text{ nm})$ according to [ALEXANDER et al. 2008]. The connector between the two amphipathic sequences is highlighted by the box in dotted lines. Note the axis-break between residue numbers 106 and 117.

close contact, whereas the amphipathic sequences 2 and 2’ are thought to be involved in secondary/tertiary interactions with each other.  

Comparison of the entire distance data presented in figure 5.35 first of all reveals deviations between several cw-EPR and pulse-EPR derived distances (see figure 5.35 squares and stars, respectively). It is obvious that the cw-EPR obtained values are biased towards shorter distances in the case the overall distribution is close to or exceeds 2 nm. This means e.g. that in the case a nitroxide pair exhibits a mean inter-spin distance above 2.0 nm with a quite broad distribution, part of the distribution (flank of the distribution) might be sufficient to cause a dipolar broadening in the cw EPR spectrum and thus cause a “wrong” mean distance. However, since all cw EPR experiments are exposed to the same conditions concerning this issue, the cw-EPR derived values can well be compared among each other. As already observed in the polarity data extracted from the low temperature
(160 \textit{K}) measurements, only minor deviations are observable for residues investigated under low and high salt conditions. This absence of the salt-induced effects at low-temperature, which is in agreement with the temperature dependence of the mobility data, enlightens once more the similarities of the high salt and low temperature structures of the HAMP domain.

In order to compare the distances obtained for the HAMP domain from \textit{N. pharaonis} to the earlier introduced HAMP domain from the hyperthermophile archaeon \textit{A. fulgidus} [Hulko et al. 2006] (see figures 2.8 and 5.22), the expected values of inter-spin distances obtainable for spin labeled residues in the NMR HAMP model are inserted in figure 5.35. In [Alexander et al. 2008] distances between spin labels (\(d_{SL}\)) are converted into distance ranges between the \(\beta\) carbons (\(d_{C\beta}\)). The analysis presented by Meiler \textit{et al.} showed that (\(d_{C\beta} - 0.25 \text{ nm}\) ≤ \(d_{SL}\) ≤ (\(d_{C\beta} + 1.25 \text{ nm}\)). Hence, it was possible to determine the expected inter-spin distance distribution of the Af1503 HAMP domain from \textit{A. fulgidus} for the residues in the amino acid sequence corresponding to those investigated in the \textit{N. pharaonis} HAMP. The gray area in figure 5.35 represents this distribution. It is immediately evident that the theoretically determined pattern can nicely be described by the distance data obtained experimentally in this study. Particularly the values extracted from pulse EPR measurements (see stars in figure 5.35) follow the trend of the simulated motif.

Despite the already increased amount of lipids used for the distance analysis presented, cw distance measurements at 160 \textit{K} were performed for corresponding residues with 400-fold molar excess of lipids per NpSRII/NpHtrII\textsubscript{157} complex. Moreover, in order to exclude possible viscosity-induced distance changes, several NpHtrII\textsubscript{157} variants were investigated in the presence of 40\% (w/v) sucrose. The results showed no deviations from the data presented in figure 5.35 in both cases.

Altogether, the data presented in the last paragraphs strongly supports the consistency of the \textit{cHAMP} conformation prevalent at high salt concentrations and the conformation present at low temperatures. Furthermore, the \textit{cHAMP} structure investigated here shows striking similarities to the NMR-derived structure of the HAMP domain from the hyperthermophile archaeon \textit{A. fulgidus} [Hulko et al. 2006].

One more aspect is worth accounting for in analyzing pulse EPR data yielding distance distributions with significant contributions in the 1.6 to 2.0 \textit{nm} range as applies here. The reliability of the results for such short distances cannot be compared with that observed for higher distances. This is due to the fact that the modulation depth (depth of dipolar modulation) of the experimental data (dipolar evolution function) becomes distance-dependent when the strength of the dipolar couplings are comparable to the excitation bandwidth of the microwave pulses [Jeschke and Polyhach 2007]. A typical excitation bandwidths of 16 \textit{MHz} can be obtained for a 4-pulse DEER experiment with 32 \textit{ns} observer pulses.
and a 12 ns pump pulse, and causes artificial attenuation of the distances below 2.0 nm in the distribution of distances [MARYASOV and TSVETKOV 2000]. Jeschke et al. found out that the dependence of the modulation depth on the dipolar frequency can be approximated by a Gaussian function, where the standard deviation is described by the excitation bandwidth. Incorporating this relation into the kernel matrix \( K(t, r) \) used in the Tikhonov regularization for the determination of the distance distribution \( P(r) \) with the simulated time-domain signal \( S(t) \) (see equation 4.19) \( S(t) = K(t, r) \cdot P(r) \) compensates the mentioned attenuation of the short distances [JESCHKE and POLYHACH 2007]. Due to the fact that the use of the idealized kernel function in terms of the excitation bandwidth correction (EBC) leads to a considerable increase in the time needed for a simulation, using excitation bandwidth correction is not the default setting in the DEERAnalysis2006 program, but has to be activated. However, as many of the transducer single mutants investigated exhibit distances nearby or below 2.0 nm, the fitting procedure of the experimentally obtained dipolar evolution traces was repeated with activated excitation bandwidth correction. The comparison of the resulting distributions of distances with and without EBC can be found in the supplementary data part in the appendix (see figure A.3). Although nearly all of the distance distributions obtained for the transducer mutants show deviations using the EBC, only part of them was found to show relevant changes of the mean distances with respect to the cw EPR-derived distances shown in figure 5.35. Please note that the changes in the distance distributions are always connected with increased spin numbers \( <n> \) upon the use of excitation bandwidth correction (see numbers in each graph: top, without EBC; bottom, with EBC) as one might already have expected from the distance distribution changes. The root mean square (r.m.s.) deviations of the fittings to the experimental data on the other hand did not change at all. It is furthermore worth mentioning that very strong dipolar interactions corresponding to distances even below the 1.6 nm border might cause a DEER signal that can be misinterpreted in terms of longer distances in the distance distribution output \( P(r) \) [JESCHKE and POLYHACH 2007]. Therefore, additional investigation of inter spin distances with the cw EPR method is advisable in each case.

5.7.2 Doubly labeled \( \text{NpHtrII}_{157} \) mutants

Doubly labeled variants in each transducer were also investigated in order to gain information on the AS1 to AS2 relative distance under different conditions. The residues chosen for the doubly labeled transducer mutants investigated are depicted in figure 5.36, left. Each of the two residues mutated in the amphipathic sequence 1 (L93 and M100) was combined with the spin labeled position 118 in the amphipathic sequence 2, yielding the transducer double mutants NpSRII/NpHtrII_{157}-L93R1-G118R1 and NpSRII/NpHtrII_{157}-M100R1-G118R1, which were reconstituted in PML to form dimeric complexes (2:2 stoichiometry). By virtue of the presumably similar structures between the high-salt \( N.\)
pharaonis and the A. fulgidus [HULKO et al. 2006] HAMP domain, analogous positions are depicted in the latter one as well (see figure 5.36 right).

![Figure 5.36](image)

**Figure 5.36:** Schematic model of the N. pharaonis HAMP domain (left, see figure 5.1) and ribbon diagram representation of the A. fulgidus HAMP domain (right, see figure 2.8) showing the positions of the residues mutated in N. pharaonis for inspection of intra-transducer distances. The residues investigated in this study are highlighted by the colored balls designated by the 1-letter amino-acid code followed by the amino acid’s number in the protein sequence on the left. For comparison, analogous residues in Af1503 are highlighted and colored accordingly on the right. In order to clearly recognize the residues’ locations, the NMR-redived structure shown on the right was rotated with respect to the previous presentations (see figures 2.8) and 5.22.

Distances estimated between the $C_\beta$ atoms of the double mutants in case the HAMP domain is engaged in a structure characterized by a linear prolongation of the transmembrane transducer helix TM2, and extracted from the Af1503 PDB structure (2ASW [HULKO et al. 2006]) amount to $\approx 6.0$ nm (L93-G118) and $\approx 4.5$ nm (M100-G118) in case of the linear extension, and $\approx 0.9$ nm (L93-G118) and $\approx 1.6$ nm (M100-G118) in case of the four-helix bundle structure from A. fulgidus. According to the previously described comparison of inter-spin label distances and those obtainable for the corresponding $\beta$ carbons of the residues investigated, error margins can be accounted for $(d_{C\beta} - 0.25 \, \text{nm}) \leq d_{SL} \leq (d_{C\beta} + 1.25 \, \text{nm})$ [ALEXANDER et al. 2008]. Please note that the complexes investigated here always exhibit the 2:2 stoichiometry comprising two receptor and two transducer molecules. Accordingly, in the distance distributions obtained also the cross-interactions between the spin label side chains (e.g. besides L93-G118 (L93′-G118′) also L93-L93′, L93-G118′ (L93′-G118) and G118-G118′) have to be accounted for.
The experimentally obtained 4-pulse DEER traces for the two double mutants NpSRII/NpHtrII$_{157}$-L93R1-G118R1 and NpSRII/NpHtrII$_{157}$-M100R1-G118R1, the background corrected data as well as the resulting distributions of distances are presented in figure 5.37. The distance distributions of the NpHtrII$_{157}$ single mutants L93R1 and G118R1 already introduced in figure 5.33 and 5.34 are characterized by very broad peaks possessing their maxima below or at the edge of the distance range resolvable (see cw EPR data L93), and a more or less distinct peak with its maximum at about 2.1 nm, respectively (see figure 5.37A.III). Both variants exhibit expected high spin labeling efficiencies, i.e. 1.9 and 1.8. Thus, no major fractions of the distance distribution are expected outside the detection range of DEER. Simulating the shape of the resulting distance distribution $P(r)$ by a simple sum of Gaussians (single Gaussians depicted by dashed lines, sum depicted by dotted lines in figure 5.37) represents the original data well in all cases. Three main distances at 2.1, 2.8 and 3.7 nm are thereby obtainable in the distribution of distances observed for the L93-G118 mutants under high salt conditions (olive profile in figure 5.37A.III). The 2.1 nm distance value coincides with that obtained for the G118 single mutant. However, the complexity of the distribution impedes the identification of the desired L93-G118 distance. In this regard, it is noteworthy that the cross-interactions (e.g. for the L93-G118 double mutant, the L93-G118' and the L93'-G118 interactions) are assumed to deviate only slightly from the intra-transtducer analog, and are thus not distinguishable. Concerning the spin numbers of doubly labeled transducer mutants, a spin labeling efficiency (SLE) of 100% would yield $<n> = 4$. Measurements performed on several singly labeled detergent-solubilized NpSRII/NpHtrII$_{157}$ variants in the HAMP domain of N. pharaonis (data not shown), have mostly shown labeling efficiencies of 80% and higher (see also spin numbers depicted for the single mutants: $<n> = 2 = 100$% SLE). The consequentially decreased spin number per complex containing doubly labeled transducers would amount to 3.2. However, the spin number obtained for the fully labeled L93-G118 double mutant is 1.9, indicating either a fraction of distances resolved below the detection limit, very low spin labeling efficiencies, or impaired formations of the 2:2 complexes upon the introduction of the spin labels. In order to identify the distances describing the L93-G188 interaction, a sample exhibiting 30% of fully labeled NpSRII/NpHtrII$_{157}$ complexes and 70% of wild type complexes (purple data in figure 5.37A.III) was investigated. Here, the fractions of all distances in the distribution except those defining the L93-G118 interaction should be decreased considerably. Decreasing the amount of labeled transducers to 30% results in a statistical distribution of the labeled complexes in the sample in such a way that about 10% of the protein sample exhibit all four residues (L93, L93', G118 and G118') spin labeled, about 40% exhibit exclu-

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$d$The numbers and the positions of the centers of the Gaussians needed to best fit the distance distributions was identified by testing different compositions in each case.

$e$Note that although the wild type transducer exhibits a native cystein at position 173, the transducer is still truncated at position 157 and thus exhibits no spin labeling sites.
5.7 Inter-spin distance analysis

Figure 5.37: Results of 4-pulse DEER measurements on the singly and doubly labeled NpSRII/NpHtrII<sub>157</sub>-L93R1-G118R1 (A.I - A.III) and NpSRII/NpHtrII<sub>157</sub>-M100R1-G118R1 (B.I - B.III) mutants reconstituted in PML. Measurements were performed on the singly labeled NpHtrII<sub>157</sub> variants (black and red) with fully labeled double mutants (olive), with 30% of fully labeled double mutants in combination with 70% of either NpSRII/NpHtrII<sub>157</sub> wild type complexes (purple), or NpSRII/NpHtrII<sub>157</sub> complexes carrying diamagnetic spin labels of comparable architecture to MTSSL at the analogous mutated positions (dark blue), in the presence of 2 M KCl + 20% (v/v) glycerol. A.I/B.I) Normalized dipolar evolution functions $V(t)/V(0)$ (solid traces) and exponentially decaying background (dotted lines) fitted by DeerAnalysis2006 [Jeschke et al. 2006]. A.II/B.II) Background corrected, normalized experimental data $F(t)/F(0)$ (solid traces), fit by the Tikhonov regularization ($\alpha = 100/1000$) (dotted lines). Calculated spin numbers per complex ($<n>$) are depicted. C) Distance distributions $P(r)$ normalized to unity (solid lines, two distributions are compared in each graph). Gaussians fitted to $P(r)$ mimicking the single distances (dashed lines) as well as the resulting simulations of $P(r)$ (dotted lines) are shown. Colored boxes superimposed by the legends show the reliability of the distance data: white, reliable distance and distribution; light yellow, approximate distance; gray, no predictions possible.
sively one labeled transducer and thus solely the L93-G118 (L93'-G118') distance, and about 50% do not contribute at all to the distance distribution since they are completely free of attached spin labels. About 90% of the resulting distance distribution describes the desired L93-G118 (L93'-G118') interaction and the remaining 10% all the other distances obtainable. Due to minor deviations from a 100% spin labeling efficiency (SLE) in the 30% fully labeled transducers, variations in the theoretical contributions may arise. For comparison, in a NpSRII/NpHtrII\textsubscript{157}-L93R1-G118R1 sample, containing exclusively 100% labeled complexes, about 34% of the dipolar interactions present can be ascribed to the inter-transducer interactions (17% L93-L93' and 17% G118-G118'), and about 66% to the intra-transducer analogs including the cross-interactions (L93-G118, L93'-G118', L93'-G118, L93-G118' each of which about 17%), provided that all interactions yield distances within the distance range resolvable. Differences with respect to the 100% labeled double mutant are clearly visible in the distance distribution of the 70% wt transducers containing sample, although also three Gaussians with mean distances (1.9, 2.7 and 3.9 nm) similar to that obtained for the 100% labeled complexes were found to represent the distribution quite well. Major changes in the fractions of the three Gaussians are observable in the 1.6 to 3 nm range, indicating the resolution of the intra-transducer distance in this range. Interestingly, the expected average spin number per complex of 2 for the statistical spin label distribution described above could be obtained with only minor deviations (<n> = 1.8). Thus, all interactions present in the spin diluted NpSRII/NpHtrII complex are assumed to be resolved. However, since the aspired spin dilution could not be attained in the way it was intended, more precise predictions about the intra-transducer distance cannot be done.

Analogous experiments were carried out for the NpSRII/NpHtrII\textsubscript{157}-M100R1-G118R1 variant. The distance distributions of the singly labeled transducers in complex with NpSRII, possessing more or less the same mean distances (M100: 2.0 nm, G118: 2.1 nm) and moderate standard deviations, are depicted in figure 5.37B.III. The corresponding spin numbers calculated from the modulation depth of the experimentally obtained traces with values of 1.6 and 1.8 point to the expected good spin labeling efficiency of both residues. Compared to the distance distributions obtained for the previously investigated L93-G118 double mutant, the shape of \( P(r) \) even increases in complexity. The distance distribution observable for the 100% labeled complexes (olive lines in figure 5.37B.III) obviously comprises contributions from four different dipolar interactions that can nicely be simulated with four Gaussians centered at 2.1, 2.9, 3.5 and 4.4 nm. It is noteworthy that distance distributions exhibiting relatively small standard deviations of the mean distances cannot unambiguously be calculated from dipolar evolution traces possessing very broad and undefined modulations. However, at least part of the distribution centered at 2.1 \( nm \) can be assigned to the interactions between M100 and M100' as well as between G118 and G118', whereas the nature of the remaining contributions is completely unknown. An unexpected low spin number of 2.2 was obtained analogously to the L93-G118 double mutant 100%
Inter-spin distance analysis

labeled. In order to illuminate the intra-transducer distances, again a spin diluted (30% of fully MTS-labeled complexes) sample was investigated. Since \( P(r) \) for the spin-diluted L93-G118 double mutant has shown to be very broad, concern about the asymmetry between the transducers carrying two spin labels and the label-free wt transducers arose. In this regard, transducer dimers containing both species might possess impaired structural features, leading to wrong distance distributions. Therefore, NpSRII/NpHtrI\(_{157}\)-M100R1-G118R1 complexes, where 30% were fully labeled with the commonly used MTSSL and 70% with a diamagnetic compound giving no EPR signal, but exhibiting a similar topology to that of MTSSL, was investigated as well. Nonetheless, the four mean distances found for the 100% labeled analogs are retrievable in the spin-diluted sample (see blue lines in figure 5.37B.III). This points to the fact that an asymmetry of the transducers induced by the combination of labeled and non-labeled transducer molecules does not evoke an impaired structure of the HAMP domain. Also in this case the fractions of the different Gaussian contributions changed after spin-dilution, where all peaks are decreasing in amplitude except the one centered at 1.9 nm, showing an increased amplitude. The latter one quite well resembles the corresponding intra-transducer distance calculated for the four-helix bundle structure of the HAMP domain from \( A. \text{fulgidus} \) (\( \approx 1.6 \) nm). Though the direction of the changes is in line with an intra-transducer (M100-G118) distance of about 1.9 nm, the extent of the changes is not as big as calculated above. Moreover, the peak centered at 4.4 ns in both distributions of the doubly labeled variant can neither be assigned to interactions between the singly labeled transducers (M100R1, G118R1), nor to cross-interactions (e.g. M100-G118') due to the assumed similarities between the intra- and inter-transducer distances. This peak might, however, be due to i) a minor fraction of the \( d\text{HAMP} \) conformation trapped at low temperatures or induced by a slight shift of the “two-state” equilibrium towards the \( d\text{HAMP} \) conformation caused by the double mutation, ii) inter-complex interactions (e.g. inter-dimer interactions within possible trimers of dimers that were found to exist for the closely related chemoreceptors), or iii) an artifact as the peak is resolved at the border of the reliability (see colored boxes). However, the possibility that the double mutation causes an allover structural change can also not be excluded at this point.

A slightly higher spin number (2.1) than expected for a spin-diluted sample was obtained, within the experimental errors still being feasible. Nonetheless, it is worth mentioning that the comparatively low spin numbers obtained for the 100% labeled NpSRII/NpHtrII complexes can also be brought in line with the comparatively high numbers obtained for the spin-diluted samples, when exclusively homogeneous NpHtrII dimers were formed. This means that the MTS-labeled transducers interact with each other, and those carrying the diamagnetic compound as well.

The effort to decrease the width of the distance distributions with the assumed HAMP stabilizers, i.e. acidic pH and 30% (w/v) PEG3350, did not yield enhanced resolutions of the distances in the HAMP domain. Additionally performed cw X-band EPR mea-
surements on the double mutants presented (data not shown) did also not yield distances in the range accessible (0.8 to 2.0 nm) besides those detectable for the transducer-transducer interactions. Thereby, the presence of very short intra-transducer distances can be excluded. The effect mentioned in terms of the excitation bandwidth correction of the 4-pulse DEER data, where dipolar interactions corresponding to very short distances (below 1.6 nm) might cause a DEER signal that can be misinterpreted as longer distances [Jeschke and Polyhach 2007], can thus be excluded as well.

Altogether, the investigation of the double mutants did not reveal definite intra-transducer distances. Estimations could be done for the two transducer variants (L93-G118, M100-G118) in that way that the distances are expected to be within the 1.6 to 3 nm range in both cases. In terms of the two models of the HAMP domain (linear prolongation vs. four helical bundle), the findings are rather indicative for the presence of the compact composition of the HAMP according to the NMR-derived parallel, four-helix bundle.

5.7.3 Receptor-transducer interactions

To unravel the relative distance between transducer and receptor, a series of doubly labeled mutants was engineered, carrying one spin label in the receptor helices and one in the transducer. Due to the 2:2 stoichiometric ratio of the NpSRII/NpHtrII\textsubscript{157} complex, a double mutation involving receptor and transducer residues may result in several inter spin interactions all influencing the shape of the resulting spectrum in terms of dipolar broadenings. Thus, distances obtained by the fitting of simulated powder spectra to the experimental ones have to be separated into fractions originating from transducer-transducer and from receptor-transducer interactions. Receptor-receptor dipolar interactions as well as cross-interactions possibly present between NpSRII and the second NpHtrII\textsubscript{157}' are expected to be beyond the detectable range of cw X-band EPR distance analysis and thus negligible.

The residues chosen in the receptor molecule are located in the E-F-loop (S154, at the cytoplasmic edge of the receptor helix F) and one helical turn towards the lipid bilayer at the N-terminal end of helix F (K157, S158), whereas the transducer residues A88 to A95 are located in the center of the predicted α-helical amphipathic sequence 1 comprising two helical turns (see figure 5.38).

As the resulting distance data was already discussed in [Doebber 2005, Bordignon et al. 2005] only the outcomes of the analysis in terms of the inter spin distances are depicted in table 5.5 (experimental data shown in figure A.4 in the supplementary data part of the appendix). For comparison with the transducer-transducer interactions obtained earlier, the reader is referred to figure 5.35.

Strong dipolar interactions between the transducer and the receptor molecules are only observable for the receptor positions 157 and 158 with residues A88 to L90 in the transducer
HAMP domain, yielding distances ranging from 1.4 to 1.7 nm. Interactions of the NpSRII-K157 and -S158 variants with the transducer positions 91 to 94, located one to two helical turns up compared to residue A88, are on the contrary very weak. As these interactions do not exceed the analogous obtained for the singly labeled NpHtrII_{157}, the results point

<table>
<thead>
<tr>
<th>NpHtrII_{157} variant</th>
<th>distance to NpSRII-S154R1 /nm</th>
<th>distance to NpSRII-K157R1 /nm</th>
<th>distance to NpSRII-S158R1 /nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>A88R1</td>
<td>1.6 [1.6]</td>
<td>1.7 [1.7]</td>
<td></td>
</tr>
<tr>
<td>S89R1</td>
<td>1.5 [1.5]</td>
<td>1.6 [1.6]</td>
<td></td>
</tr>
<tr>
<td>L90R1</td>
<td>1.4 [1.4]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S91R1</td>
<td>≈ 2.0 [&gt; 2.0]</td>
<td>1.9 [&gt; 2.0]</td>
<td>1.8 [&gt; 2.0]</td>
</tr>
<tr>
<td>T92R1</td>
<td>≈ 2.0 [&gt; 2.0]</td>
<td>&gt; 2.0 [&gt; 2.0]</td>
<td></td>
</tr>
<tr>
<td>L93R1</td>
<td>1.7 [&gt; 2.0]</td>
<td>1.8 [&gt; 2.0]</td>
<td></td>
</tr>
<tr>
<td>A94R1</td>
<td>≈ 2.0 [&gt; 2.0]</td>
<td>1.7 [&gt; 2.0]</td>
<td></td>
</tr>
<tr>
<td>A95R1</td>
<td>1.8 [&gt; 2.0]</td>
<td></td>
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</tr>
</tbody>
</table>

Table 5.5: Transducer-receptor inter-spin distances in the NpSRII/NpHtrII_{157} 2:2 complex reconstituted in PML. On the left the spin labeled transducer residues (A88 to A95) are depicted. The three columns on the right show their distances to the respective receptor residues investigated (S154, K157, S158). The error is estimated to ±0.1 nm for all distances given. The distances determined from the fitting of simulated spectra to the experimental ones are given. Since these distances also include possible transducer-transducer interactions (see figure 5.35) the estimated extracted receptor-transducer distances are shown in parenthesis. Measurements for the double mutants NpSRII-K157R1/NpHtrII_{157}-A88R1, -S89R1, and -L90R1 were carried out by J. P. Klare, member of the group of H.-J. Steinhoff.
to inter spin distances above 2.0 nm for these double mutants. The analysis suggests a linear prolongation of the AS1 with respect to the transmembrane transducer helix TM2 as well as α-helical structure of at least the central part of the AS1. A linear extension of the transducer AS1 into the bulk water is moreover expected to yield interactions between the receptor residue S154, located one helical turn towards the cytoplasm, and the corresponding transducer variants S91R1 to A95R1 comparable to that observed for NpSRII positions 157 and 158 with the transducer positions A88 to L90. Contrarily, inter spin interactions determined do not exceed those obtained for the singly labeled transducers in complex with NpSRII, and are thus estimated to be above 2.0 nm (see table 5.5). These results do not support further linear prolongation of the transducer molecules in this part of the HAMP domain.

5.7.4 Micelle vs. lipid bilayer

In order to specify the changes observed in the mobility and accessibility experiments upon solubilization of the NpSRII/NpHtrII<sub>157</sub> complex under investigation, a cw EPR distance analysis with selected residues was carried out. As shown in the previous section, only minor proximities are suggested to exist between the region of the receptor possessing position S154, and the part of the transducer HAMP domain comprising residues S91 to A95 (AS1) in the PML-reconstituted samples. Cw X-band EPR spectra obtained at low temperatures (160 K) from two singly labeled transducer variants (L93R1 and A95R1) and the corresponding doubly labeled mutants (NpSRII-S154R1/NpHtrII<sub>157</sub>-L93R1, -A95R1) are depicted in figure 5.39 for the DDM-solubilized (red lines) and the PML-reconstituted (black lines) complexes. The spectra obtained for the singly labeled transducer variants in complex with NpSRII in the solubilized states were used as reference spectra. In fact, in the 1:1 stoichiometric ratio no dipolar broadening is expected due to neighboring transducers. The spectra of the doubly labeled receptor-transducer mutants in the reconstituted states, given on the right of figure 5.39, show no relevant differences with respect to the single mutants as pointed out in the previous section. This indicates the absence of close interaction (< 2.0 nm) between the receptor and the transducer when reconstituted in PML. Inspecting the experimental data recorded for the solubilized NpSRII-S154R1/NpHtrII<sub>157</sub>-L93R1 variant yields no relevant changes in the dipolar interactions with respect to the reconstituted complexes. An increase in the signal amplitude of the central resonance line that is due to the absence of the second transducer in the 1:1 (solubilized) complex, and the corresponding absence of the transducer-transducer dipolar interaction present in the 2:2 complex, is visible. An opposite behavior upon solubilization (disruption of the 2:2 complex) is evident from the spectrum recorded for the NpSRII-S154R1/NpHtrII<sub>157</sub>-A95R1 variant, showing the appearance of dipolar broadening. The interaction between the receptor and the transducer in the 1:1 complex responsible for this dipolar broadening is visible as a decrease
5.7 Inter-spin distance analysis

Figure 5.39: Comparison of spin normalized low temperature (160 K) cw X-band EPR spectra from singly and doubly labeled NpSRII/NpHtrII\textsubscript{157} complexes in the lipid-reconstituted (black lines) and detergent-solubilized (red lines) states. On the left, the spectra obtained for the singly labeled NpHtrII\textsubscript{157} variants L93R1 and A95R1 located in the AS1 of the HAMP domain (see figure 5.38) are depicted, on the right, the corresponding spectra from the doubly labeled variants comprising residue S154 located at the C-terminal end of helix F in the receptor (see figure 5.38). To better visualize the differences between the single and double mutants investigated in the solubilized complexes, gray boxes with heights adapted to the EPR signals’ amplitudes are inserted.

in the central resonance line’s signal amplitude compared to the solubilized (highlighted by gray box in figure 5.39, bottom). Fitting of a simulated spectrum to the experimentally one revealed a distance of 1.5 ± 0.2 nm. By virtue of the presence of the monomeric NpSRII/NpHtrII\textsubscript{157} complexes in the solubilized samples, this distance can unambiguously be ascribed to the interaction between the transducer residue A95 and the receptor residue S154. This finding clearly supports the assumption that at least the central part of the AS1 of the transducer HAMP domain is in closer contact with the region of the receptor carrying position 154 in the solubilized 1:1 than in the reconstituted 2:2 complex. A major rearrangement of HAMP domain is thus suggested to occur upon solubilization.
CHAPTER 6

DISCUSSION

In this work, two different sample preparations were used for the investigation of the structural and functional features of the NpSRII/NpHtrII complex. Detergent-solubilized complexes, showing a 1:1 stoichiometric ratio between NpSRII and NpHtrII, were examined to unveil possible structural differences with respect to the lipid-reconstituted complexes, where a dimeric (2:2 stoichiometry) complex formation is observable. The structural properties of the latter composition in the physiological lipid environment were investigated by virtue of several environmental changes (e.g., salt concentration, temperature).

The 1:1 stoichiometry observed between NpSRII and NpHtrII after solubilization is a clear indication for the impaired interaction between the transducer molecules. Intact transducer-transducer interaction is only present in the native NpSRII/NpHtrII 2:2 complexes in lipid environments. In the purple membrane, dimerization of the transducers is also performed in the absence of the receptor molecules. Due to the high homologies with chemoreceptors, which are known to form dimers and higher-order complexes, full functionality of the complexes investigated here is thought to require the presence of at least the dimeric forms. The topology of the solubilized NpSRII/NpHtrII_{157} complexes was found not to be the same as that of the reconstituted analogs.

As apparent from the features of the cw RT X-band EPR spectra, showing two components as in the case of the reconstituted analogs, we generally found some similarities in the DDM-solubilized complexes with respect to the reconstituted analogs. However, specific positions show a different spectral shape. Thus, a structural rearrangement in the membrane-proximal region of the HAMP domain is suggested to occur upon solubilization. The different EPR-derived parameters determined here unveil the structural properties of the transducer HAMP domain in the solubilized NpSRII/NpHtrII_{157} complexes. The increased signal amplitudes in the dynamic component 2 visible for the NpHtrII_{157} residues up to A94 and for M100 and G101 are in agreement with the absence of the second transducer molecule. Mobility and polarity data suggest an $\alpha$-helical structure of the transducer up to position A94. Most strikingly, distance measurements performed for doubly labeled receptor-transducer complexes reveal stronger tertiary interactions between the two entities in the solubilized form. A distance of $1.5 \pm 0.2 \text{ nm}$ was determined between receptor posi-
tion 154 located in the E-F loop and transducer position 95 (center of AS1). On the other hand, no interactions could be observed between receptor position 154 and a residue near A95 in the transducer, i.e. L93, showing the distinct topology of the transducer HAMP domain in this region. Results of the mobility, accessibility and polarity analyses performed for the residues at the C-terminal end of the AS1 show increased secondary/tertiary interactions, when compared to the data obtained for the reconstituted variants, in line with the distance data. Additionally, a lack of periodicity can be observed in the data obtained for this region, pointing to the absence of defined secondary structural elements. The EPR data analysis leads to the schematic representation of the detergent-solubilized complex in its monomeric form as depicted in figure 6.1. The model shows the α-helical structure of the membrane-adjacent region of the transducer molecule up to residue A94, the lack of defined secondary structure for the following region of the AS1, and the tight interaction

![Figure 6.1: Model representation of the membrane-adjacent part of the transducer HAMP domain of NpHtrII in complex with NpSRII in the DDM-solubilized form. The side view of the monomeric (1:1) NpSRII/NpHtrII complex including the ribbon diagram of the crystal structure of the transmembrane part of NpSRII (blue) and NpHtrII (cyan) (PDB 1H2S) is depicted up to residue G103 of the transducer. Below residue A94, the AS1 is modeled as α-helical, linear prolongation of the transmembrane transducer helix TM2 (gray). The following region comprising residues A95 to G103 is depicted without defined secondary structure due to the ambiguous nature of the EPR data obtained for this region. For the sake of clarity, the β carbons of residues L93, A95 and G101 in the transducer as well as S154 and K157 in the receptor are highlighted by the balls. The part including positions 95 to 101 is colored according to the CrOx accessibility obtained (see scale at bottom).](image-url)
between the transducer HAMP around residue A95 and the receptor E-F loop harboring residue S154. Topological details on the connector and the AS2 are not depicted due to the sparse data obtained for these regions. It is worth mentioning that the results of Spudich et al. [Yang et al. 2004] on the membrane-proximal region of HtrII from N. pharaonis as determined by FRET (Förster resonance energy transfer) and cystein cross-linking studies. Their resulting model suggests a close proximity of the E-F cytoplasmic loop residue S154 in NpSRII and residues S91 to A95 in the HAMP domain from NpHtrII, in line with the data presented here on the solubilized complex.

To which extent the structural differences observed with the EPR parameters, describing the topology of the complexes upon solubilization, impair the functionality of the NpSRII/NpHtrII complex, was investigated in terms of light-induced absorption changes of NpSRII alone and in complex with NpHtrII157 in the DDM-solubilized and the PML-reconstituted form [Klare et al. 2006]. Experiments were performed on two different photocycle intermediates (M and O state) as well as on the ground state at ambient temperatures. Although the absorption maxima of NpSRII are not affected by solubilization [Chizhov et al. 1998], differences were particularly observed in the decays of the M and the ground state, yielding accelerated responses for the reconstituted samples. Moreover, for all three states investigated, considerable effects on the photocycles could be observed after binding of the transducer to the receptor only in the solubilized samples, supporting the stronger interaction between the two entities in their detergent-solubilized rather than in their lipid-reconstituted forms. The EPR analysis of the NpSRII/NpHtrII157 complex in its detergent-solubilized form elucidated that non-negligible changes in the structural elements of the membrane-adjacent region of the transducer HAMP domain and in the receptor-transducer interactions occur upon solubilization. Hence, membrane-proximal domains of complexes such as the one investigated here are considered to sensitively react on changes in the surrounding conditions. The structural rearrangements upon solubilization identified for the NpSRII/NpHtrII157 complex in this study are correspondingly assignable to both the absence of the second transducer and the differences between the headgroups of polar lipids and detergents. This is e.g. supported by Kamo and coworkers [Sudo et al. 2003], who found via absorption spectroscopy and flash photolysis that the presence of both the (truncated) NpHtrII159 and the native membrane increase the thermal stability of the receptor NpSRII. That exchange of the native lipid-bilayer with the non-native micellar envelope can impair the structural and functional features of a protein was also demonstrated by Saito et al. [Tanio et al. 1998] for bacteriorhodopsin. In that study, the enhanced flexibility in the loop regions as well as conformational changes within the α-helical transmembrane elements upon stepwise delipidation and solubilization of the proteins was revealed via 13C NMR. These changes were accompanied by the identification of the monomeric instead of the naturally found trimeric form of bacteriorhodopsin.
In general, solubilization of membrane proteins or protein complexes can be used in order to facilitate the application of several biophysical methods, where membrane proteins in their native lipid environment cannot be examined. Nonetheless, the present study shows that in some cases the solubilization of membrane proteins can result in considerable structural rearrangements and corresponding impaired functionalities of the complexes of interest. Particularly the membrane-proximal region at the cytoplasmic side of the NpSRII/NpHtrII complex investigated here shows to be sensitive towards solubilization. Hence, data obtained from solubilized membrane proteins has to be handled with care when reliable information is to be extracted for the native complexes.

The main discovery of the EPR analysis carried out for the PML-reconstituted NpSRII/NpHtrII complexes, exhibiting a 2:2 stoichiometry between the transducer and the receptor, is the presence of two components in the cw X-band EPR spectra of the HAMP residues investigated, which are defined by different reorientational correlation times of the spin label side chains. The two spectral components, describing i) one protein subpopulation of very high dynamicity (dHAMP conformation) and ii) one characterized by high compactness (cHAMP conformation), are identified and explained in terms of a “two-state” equilibrium. Since both components are observable in several cw X-band EPR spectra, they do not simply represent an artifact due to the incorporation of the nitrooxide spin label.

Changing the salt concentration of the solvent shows to affect the ratio of the two spectral components. Component 1 is the prevailing one under physiological salt conditions (3.5 M KCl), and is thus assumed to be structurally and functionally relevant. Contrarily, component 2 is found to be more pronounced in the presence of non-native, low salt concentrations such as 150 mM KCl. Indeed, the transducer constructs used in this study lack several components comprising the functional signaling arrays found in cells, such as CheA and CheW, what may induce destabilization of the HAMP domain in a fraction of the NpSRII/NpHtrII complexes. Moreover, the EPR experiments were carried out with either transducer molecules missing the methylation sites responsible for adaptation or fully demethylated analogs, which could shift the adaptation bias towards full intrinsic deactivation or even towards a non-native attractant signaling state. However, due to the presence of the second component in nearly all spectra recorded, and by virtue of the fact that using C-terminal truncated transducer entities as well as the full length variant shows not to affect the ”two-state” equilibrium, both the cHAMP and the dHAMP conformation are accordingly concluded to represent different, but intact conformational states of the NpHtrII linker region.

In this regard, it is worth mentioning that SDSL EPR shows to facilitate the separate investigation of the different protein conformations present. We observe that i) the mobility values are biased towards the dHAMP in the presence of 150 mM KCl, and biased towards the cHAMP at 3.5 M KCl, ii) the water accessibilities obtained under low salt conditions
at 3 mM NiEDDA rather define the dHAMP conformation, those obtained at 20 mM NiEDDA rather the cHAMP conformation, iii) at low temperatures (here 160/80 K) the cHAMP conformation is the prevailing one, thus EPR distance, EPR polarity and ENDOR data describe the compact HAMP. From fitting simulated to experimental cw RT X-band EPR spectra, moreover, the reorientational correlation times $\tau_c$ of the nitroxides in the respective conformations as well as their spectral fractions can be obtained.

6.1 cHAMP - The Compact HAMP Conformation

The cHAMP conformation described by the spectral component predominant at more native salt concentrations (3.5 M KCl), is found to be characterized by high compactness. The spectral component of this conformation shows contributions of two different rotameric states of the nitroxide side chains, typical for secondary/tertiary interaction sites, for at least each NpHtrII157 HAMP mutant investigated in terms of temperature-dependent measurements. Predictions about the presence of the two rotameric contributions in the remaining EPR spectra of the transducer mutants can hardly be done due to the difficult identification of the rotamers in the RT spectra. The spectral compositions obtained under high salt conditions are shown to be reproducible for different C-terminal transducer truncations as well as for the full length NpHtrII. That the cHAMP conformation does not solely originate from a viscosity-induced effect is moreover verified by reference measurements in the presence of 40 % (w/v) sucrose, mimicking a solvent viscosity, which is much higher than that of the high-salt induced one. Concerning the mobility and accessibility data obtained for the cHAMP up to residue S91 in the AS1, $\alpha$-helical behavior and side chain interactions with secondary/tertiary structure can be identified. The low mobility values obtained for the remaining positions (except residues R99 and E116) examined in the cytoplasmic end of the AS1, the connector and the AS2 indicates interactions of the nitroxide side chains with proximal residues. The accessibility data shows increasing water accessibilities of the residues following S91 towards the cytoplasm, pointing to the protrusion of the side chains into a medium containing more water molecules. Despite this increase, which is in line with the mobility values, the water accessibilities are very low. In case of exchange rates between the nitroxides and the paramagnetic quencher NiEDDA (20 mM) obtained in the presence of 3.5 M KCl, most of the values are too low for side chains completely exposed to the water phase, but rather typical for residues involved in strong tertiary interactions, what is confirmed by the corresponding oxygen accessibilities. Periodical patterns in the accessibility profiles clearly show the $\alpha$-helical features of the C-terminal end of the AS1. In case of the connector residues, much higher NiEDDA encounter rates were to be expected if no interactions with neighboring residues were present. In this regard it is noteworthy that lowered values for the side chain mobilities, accessibilities towards paramagnetic quenchers, environmental polarity and the amount of protons from the bulk water in ENDOR measure-
ments might be caused indirectly by the presence of higher salt concentrations. Although not fully understood so far, it has been proposed that kosmotropes enhance the structure of water surrounding the ions [Frank and Franks 1968, Franks 2002], thereby increasing hydrophobic interactions present in the protein. A hydrophobic ring compound as present in the MTSSL used here protruding into the bulk water would disturb the highly ordered structure and is thus avoided by the system. The spin label side chain is accordingly considered to tightly adsorb to the protein surface, particularly at high salt concentrations.

The results obtained from EPR polarity and ENDOR measurements are in agreement with the mobility and accessibility data discussed for the high salt measurements. The data supports secondary/tertiary interactions of the side chains in the AS1 and the AS2, their contact with the water phase (except M100 in ENDOR) as well as the finding that the connector, though exposed to the water phase, is expected to be involved in interactions with structural elements. α-helical structure of the C-terminal end of the AS1 is furthermore supported by the periodical pattern observable in the polarity profile, in line with the accessibility profile. The most interesting finding in the ENDOR analysis is that residue M100, located at the AS1-connector transition, does not interact with considerable amounts of exchangeable protons (i.e. water) in the spin label’s microenvironment. Thus, shielding interaction with structural elements are expected. The fact that the inter-transducer distances obtained by cw and pulse EPR for residue M100 show values about 1.7 nm is on the one hand in line with the transducers’ proximity in this region. On the other hand, a very tight transducer-transducer interaction, excluding water molecules from this residue’s location, is expected to yield distances that are even shorter. Thus, additional structural elements have to be present, shielding position 100 from the environmental solvent. The remaining distances obtained for the cHAMP conformation show the transducers’ proximity in all HAMP subdomains. However, it is worth mentioning that in case the HAMP domain was a simple straight prolongation of the transmembrane helices TM2 and TM2’, the inter-transducer distances obtained for residues at the C-terminal end of the AS1 would have been decisively longer as the distances of those at the N-terminal end of the AS1. As this is apparently not the case, neither for the cw nor the pulse EPR distance patterns, an inclination of the AS1 and the AS1’ towards each other is concluded to be present. Such a distortion in the helical axis might be feasible by virtue of the two putative helix-breaking residues G83 and G84 in the AS1 allowing the predicted kink. In this regard it is worth noting that cysteine substitution at position 83 has shown to eliminate the phototaxis without impairing the affinity of NpHtrII to bind to NpSRII, whereas cystein substitution at position 84 did not yield a functional loss [Yang and Spudich 2001]. Helix-breaking elements as glycines or prolines can also be found in the N-terminal half of the AS1 in HAMP domains from other organisms (see e.g. figure 2.6).

Cw distance data obtained from doubly labeled receptor-transducer mutants further supports the inclination of the transducers towards each other. Although no distinct distances
could be extracted from the distance distributions of the pulse EPR measurements carried out for doubly labeled transducer mutants, an approximated range of distances covering 1.6 to 3 nm can be assumed, indicative for the presence of a parallel, four-helix bundle. That the cHAMP conformation resembles quite well that of the solution NMR-derived structure of the isolated HAMP domain from the hyperthermophilic archaeon *A. fulgidus* [Hulko et al. 2006] is moreover supported by i) the periodical pattern in the mobility and accessibility data (AS1 and AS2), ii) the consistency of the *in silico* solvent accessibilities of the HAMP from *A. fulgidus* [Swain and Falke 2007] and the exchange frequencies $W_{ex}$ obtained from NiEDDA accessibility measurements, and, though no clear-cut evidence, iii) the agreement of the *in silico* distance ranges, which would be expected when SDSL EPR was applied to the Af1503 HAMP (determined according to [Alexander et al. 2008]), and the experimentally obtained distance distributions from the NpHtrII HAMP. Moreover, the hydrophobic residues L90, L93, A97 and M100 in the heptad repeat pattern of the NpHtrII AS1, which are expected to point into the hydrophobic core of the predicted coiled-coil domain, are considered to be perfectly arranged that way. For the cHAMP very low polarity values for residues L90 and A97, low side chain mobilities and water accessibilities for residues A97 and M100, short inter-transducer distances for residues L93 and M100 are obtainable. From ENDOR measurements, moreover, the conclusion can be made that residue M100 has to be located at a protein site excluding water molecules, where the side chain strongly interacts with tertiary structure. For the sake of clarity, the corresponding (also hydrophobic) residues in the HAMP domain from *A. fulgidus* are depicted in figure 6.2. One hydrophobic residue (Y121) investigated at the N-terminal end of the AS2 of the *N. pharaonis* HAMP does not show such a clear evidence. As apparent from figure 6.2, the corresponding spin label side chain is neither supposed to point directly towards the water phase nor to be oriented towards the bundle core. This equivocation can be retrieved in the data collected from different EPR measurements. On the one hand, the $A_{zz}$ values and the water accessibilities show values typical for nitroxide side chains being not or only slightly in contact with water molecules, indicating a buried position. On the other hand, the ENDOR measurements definitely show the presence of water in the side chain’s microenvironment, pointing to a water accessible location. The fact that from the ENDOR experiments tertiary interactions of the spin label side chain with proximal residues are obtainable as well, supports the bimodal nature of residue Y121, and thus confirms the bundle formation in the cHAMP. Interestingly, the residues proximal to Y121 (L120, A122, A123, F124) also exhibit hydrophobic character. However, it is noteworthy that a typical coiled-coil structure that would be described by a knobs-to-holes packing of the hydrophobic core residues, could not be identified for the HAMP domain from *A. fulgidus* but rather an unusual knobs-to-knobs packing. Nonetheless, the two kinds of residual core packings were predicted to be nearly isoenergetic and could be interconverted by a 26° rotation in all four helices [Hulko et al. 2006].
Figure 6.2: Characteristic features of selected NpHtrII HAMP residues adapted to the Af1503 HAMP domain. The ribbon diagram representation of the NMR-derived HAMP domain from *A. fulgidus* [Hulko et al. 2006] according to figure 2.8 is shown in terms of a side view (left) and a top view (right). The $C_{\beta}$ atoms of hydrophobic residues from the NpHtrII AS1 (L90, L93, A97 and M100) and AS2 (Y121) are highlighted by light yellow balls at the corresponding Af1503 HAMP sites (I284, L287, A291, I294 and A316). In the connector region selected amino acids are colored according to their hydrophobic (light yellow) or charged/polar (dark orange) nature as derived from the *N. pharaonis* HAMP.

The excellent agreement between the *N. pharaonis* and the *A. fulgidus* HAMP obtainable for the structural properties of the AS1 and the AS2 is not apparent for the connector, linking the two HAMP subdomains. Obviously, one reason is the sparseness of the EPR data collected for the respective residues. Moreover, the predicted flexible structure of the connector might cause deviations in the connector’s structural arrangement relative to the amphipathic sequences in different organisms. The possible flexibility is underlined by the presence of two glycines at the AS1-connector transition (G103) and the connector-AS2 transition (G118), what seems to be an intrinsic feature of HAMP domains as evident from the sequence alignment given in figure 2.6. Glycines are the smallest of the amino acids and thus optimally placed at protein sites where structural flexibility is needed or even rearrangements occur. It is moreover worth mentioning that most of the residues located in the connector region are charged (most of them negatively) as highlighted by the dark orange regions in figure 6.2 (residues D102, D104, D106, E108, E110, R112, R113, E114, D115 and E116 in NpHtrII; although residue T111 in NpHtrII is polar, it is colored in the same way), showing the connector’s water binding capacity. Hydrophobic residues can only be found in minor amounts (in NpHtrII L105, V107 and L109). Nonetheless, the structural information obtainable on residue L105 coincides with the connector’s interaction with tertiary structural elements as it is the case in the four helical bundle. To which extent the hydrophobic residues present in the connector region are crucial for the stability of the four helical bundle, e.g. by means of their interactions with hydrophobic residues in the bundle’s core,
cannot be predicted so far. Contrary, the fact that negatively charged residues that preferentially exist on water-exposed sites of the protein [BANDYOPADHYAY and SONAWAT 2000], cannot be found in the AS1, but in higher amounts in the connector (D102, D104, E106, E110, E114, D115, E116), and also in the AS2 (D119, D125, E126), supports the formation of the compact bundle. Altogether, the four-helical-bundle structure obtained by NMR on the unusual archaeal membrane protein of unknown function [HULKO et al. 2006], seems to well represent the \( c \)HAMP conformation of the transducer from \textit{Natromonomas pharaonas}.

### 6.2 dHAMP - THE DYNAMIC HAMP CONFORMATION

The mobility and accessibility patterns obtained from EPR measurements performed in the presence of 150 mM KCl are in line with a stepwise protrusion of the nitroxide side chains of the dHAMP into the bulk water up to residue A95 in the AS1. Periodical patterns in the mobility and accessibility plots suggest \( \alpha \)-helical structure of this region of the HAMP. Contrary to the \( c \)HAMP data, mobility and water accessibility values determined for dHAMP residues located in the following parts of the linker are typical for side chains fully exposed to the water phase. Particularly the mobility values obtained for the cytoplasmic end of the AS1, the few residues investigated in the connector and the AS2 exceed those typical for helix surface sites and in most cases also for loop regions. They are rather characteristic for nitroxides attached to C-terminal ends of proteins or partially unfolded structures, both cases where backbone motions play a major role in the determination of the overall reorientational correlation times \( \tau_c \) of the R1 side chain. Thus, a definite distinction between very dynamic helical conformations and partially unfolded structures cannot be done for the C-terminal end of the AS1, the connector and the AS2, whereas strong interactions of the side chains with secondary/tertiary structural elements of the transducer and the receptor can be excluded. The test measurements performed in the presence of 6 \( M \) urea moreover support the very dynamic nature of the dHAMP conformation. Remarkable changes upon the addition of the denaturant cannot be observed for the NpHtrII\textsubscript{157} variants investigated under low salt conditions, showing its already destabilized/partially unfolded character. Therefore, a major rearrangement between the cHAMP and the dHAMP conformation can be expected to take place, where the latter one is considered to be characterized by loosened secondary structural features compared to the former.

### 6.3 A ”TWO-STATE” EQUILIBRIUM - PHYSIOLOGICAL RELEVANCE?

According to the structural features identifiable for the cHAMP and the dHAMP conformation, figure 6.3 represents the two conformations of the NpSRII/NpHtrII complex. The cHAMP topology (left) is modeled as a combination of the transmembrane part of the NpSRII/NpHtrII complex resolved by X-ray crystallography (see figure 2.3) and the NMR-
derived structure of the HAMP domain from *A. fulgidus* (see figure 2.8). The structural model of the dHAMP conformation is shown on the right of figure 6.3. Due to the high dynamicities obtained, its conformation is visualized by the cHAMP topology with loosened structural features, where the previously well-defined α-helical structure of the AS1 and AS2 is exchanged by very dynamic helical regions from the C-terminal end of the AS1 further on towards the cytoplasmic side of the HAMP.

![Figure 6.3: Models of the proposed structures of the compact (cHAMP, left) and the dynamic (dHAMP, right) conformation of the NpHtrII HAMP domain. For modeling of the cHAMP, the NMR structure of the Af1503 HAMP domain (PDB 2ASW [Hulko et al. 2006]) was attached to the crystal structure of the NpSRII/NpHtrII transmembrane domain (PDB 1H2S [Gordeliy et al. 2002]). The dHAMP conformation was modeled based on the cHAMP model taking into account the increased dynamics observed for this conformation. For the sake of clarity, the complexes were inserted into schematic lipid-bilayers. The equilibrium between the two conformation is indicated by the arrows at the bottom of the figure.](image)

In literature, the prototype function of the solution-NMR-derived four-helix-bundle structure of the isolated Af1503 HAMP has already been discussed extensively. *In vivo* cross-linking studies carried out for the cytoplasm-facing *E. coli* aerotaxis receptor, i.e. Aer, where a PAS (Per-ARNT-Sim) and a HAMP domain function together as signal input-output module, identified the four helical bundle structure of the Aer HAMP, though the N-terminal ends of the AS1 were found to possess a slightly more dynamic nature [Taylor 2007, Watts et al. 2008]. It was also suggested that although the HAMP helices may rotate during signal transduction, as it has been observed for the HAMP from *A. fulgidus* [Hulko et al. 2006], a rotation-output from the signaling domain is unlikely.
Moreover, *in silico* studies even predicted the importance of the bundle formation for Aer stability or maturation [Taylor 2007, Watts et al. 2008].

In this regard, one aspect is noteworthy with respect to the halophilic protein complex investigated here, which is natively not exposed to extremely high but only moderate temperatures (318 to 323 K [Rodriguez-Valera 1993]). Slightly different structural features between thermophilic (*T* ≈ 318 to 353 K) and mesophilic (*T* ≈ 303 to 313 K) organisms were found, where the former are supposed to counterbalance the higher temperatures via increased structural stability [Chakravarty and Varadarajan 2002]. In that study, preferentially the residues located at exposed sites have been shown to be arranged in order to optimize the protein’s interactions. Therefore, in thermophiles an approximately 1% higher occurrence of helical content and a corresponding lower loop content is considered. Comparison between a HAMP domain from a hyperthermophilic organism (*T* ≈ 353 to 393 K) as *Archaeoglobus fulgidus* and that from a non-hyperthermophilic counterpart as *Natronomonas pharaonis* or *Escherichia coli* might consequently result in equivalent differences. Thus, as obtained for the N-terminal ends of the AS1 by Taylor *et al.* [Taylor 2007, Watts et al. 2008], minor deviations in the helix-to-loop ratio are still in line with the four-helical bundle revealed.

Swain and Falke furthermore investigated the topology of the HAMP domain from the *E. coli* aspartate chemoreceptor Tar by means of cystein-reactivity and cystein-scanning/disulfide-mapping studies on the full-length, membrane-bound receptor [Swain and Falke 2007]. Their results were found to be highly correlated with the formation of the parallel, four-helix bundle, where both the signaling ON as well as the OFF state closely resemble the NMR-derived HAMP motif. Only minor conformational rearrangements between the two states were obtained. Interestingly, the possibility of the deviating connector arrangements in HAMP regions from different organisms mentioned above, is supported by these studies since detectable differences in the structural packing was observed for the connector and thus concluded to be not conserved throughout the linker regions existing [Swain and Falke 2007].

The parallel, four helical bundle indentified for the membrane-proximal region from Af1503 seems to indeed depict a HAMP prototype structure. Nevertheless, some concern about its viability still exists due to its unknown function and the fact that it is *in vivo* tethered to a transmembrane, but not to a signaling domain as applies for other HAMPs. Moreover, the NMR model was obtained well below the living temperature of the hyperthermophile *A. fulgidus*, what could have trapped the HAMP domain in one of its most stable conformations as it was shown for the NpHtrII linker region in the present study. The fact that the Af1503 HAMP was found to be highly destabilized under more physiological conditions (temperatures close to 350 K), as revealed by CD measurements [Hulko et al. 2006], questions its integrity. On the other hand, chimera studies, where the Af1503 was introduced in an *E. coli* system, performed at 310 K, showed that the HAMP was still
functional [Hulko et al. 2006]. Consequently, the additional existence of a more dynamic Af1503 HAMP might be possible as well. Nonetheless, how the fusion of a HAMP domain from a hyperthermophile with an *E. coli* chemoreceptor can result in a common output signal, remains to be determined. It might be considered that HAMP domains have adapted to a variety of different signaling mechanisms by virtue of the several thousand HAMP domains existing.

In literature, evidences for a dynamic conformation being an intrinsic feature of HAMP domains have been described already. CD experiments performed by Inouye and coworkers [Kishii et al. 2007] on the isolated HAMP domain from the *E. coli* osmosensing histidine kinase EnvZ, revealed the HAMP´s inability to form a stable structure in the isolated construct. The authors mentioned the possibility that such a structural fragility might be of major importance in the signal transduction process. In this regard, the “two-state” equilibrium revealed in the present study between the cHAMP and the dHAMP conformation of the *N. pharaonis* transducer molecule present both at low (150 mM KCl) and high (3.5 M KCl) salt condition becomes of utmost relevance. Its existence is unambiguously shown by the performance of cw EPR X-band measurements with different concentrations of the paramagnetic quencher NiEDDA and the reducing agent ascorbic acid. The equilibrium of the two conformational states, exhibiting different dynamic properties, can at least be shifted by salt concentration, pH and temperature towards one of them. That particularly the salt concentration of the solvent medium plays a decisive role in halophilic and extremely halophilic proteins and protein complexes has already been published oftentimes. As also investigated here, the known Hofmeister series [Hofmeister 1888] of stabilizing and destabilizing cations and anions applies also for the HAMP domain from *N. pharaonis*. According to [Chitra and Smith 2001, Shimizu et al. 2006, Dér et al. 2007, Dér 2008], structural alterations between a protein´s stable conformational states induced by the use of different cations (at different ranks of the Hofmeister series), are thought to result from the accompanied changing properties in the solvent water structure, and thus the protein/water interface. Besides the nature of the cations/anions present in the molecular environment, their concentrations are of major significance since halophilic organisms not only tolerate high environmental salt concentrations but rather require them for functional integrity and viability [Lanyi 1974]. That the presence of salt concentrations below the physiological ones leads to structural destabilization or even denaturation, where e.g. the disruption of α-helical structure occurs, is assumed to describe an intrinsic property of halophilic proteins [Pundak et al. 1981]. Depending on the range of the salt concentration used, different interactions are considered to be responsible. At low salt concentrations (below \( \approx 1 \ M \)), electrostatic repulsion is thought to be the desicive aspect for the loosened/destabilized protein structure (see for one of the first reviews [Lanyi 1974]). This can easily be explained by the generally high occurence of acidic residues, such as aspartate and glutamate, on surface exposed sites of halophilic proteins/protein complexes, which in case
of the investigated HAMP domain from *N. pharaonis* applies at least for the connector. The negative charges are neutralized by the anions of the salt present in the surrounding water and thus shielded from each other by this extensive solvation (as hydrated ions are thought to be bound [Shimizu et al. 2006]). Electrostatic repulsion between the respective, negatively charged residues accordingly does not take place. Thereby, the proper, stable protein conformation can be maintained in the presence of sufficient amounts of cations. In the case this amount becomes too low, charge shielding can no longer be preserved. Electrostatic repulsion destabilizes the protein structure. However, Lanyi [Lanyi 1974] considered that the stabilizing/destabilizing effects observable over a wide range up to physiological salt concentrations of halophilic organisms cannot be explained exclusively by electrostatic charge shielding. The charge-shielding effect was supposed to be already maximal at salt concentrations such as 0.1 to 0.5 M. Therefore, it has been stated that a further prominent feature of halophilic proteins and protein complexes, namely the minor occurrence of hydrophobic residues, has to be regarded as well. A low content of hydrophobic residues results in weak hydrophobic interactions, whereby high salt concentrations are needed to maintain them [Lanyi 1974]. Yet, it is obvious from the sequence alignment shown in figure 2.6 that the two amphipathic sequences of the *N. pharaonis* HAMP do not exhibit low amounts of hydrophobic residues but rather high amounts. On the contrary, in the connector that is considered to be exposed to the solvent and thus somehow shielding the α-helical parts of the HAMP from the bulk water, hydrophobic residues can hardly be found. Hence, the moderate occurrence of hydrophobic residues in the AS1 and the AS2 might accordingly not be sufficient to keep the stabilized structure of the protein complex even under low salt conditions, resulting in a destabilized or denatured conformation. It is moreover worth mentioning that the increased hydrophobic interactions at high salt concentrations responsible for the tight, stable packing of halophiles can also be regarded as the main reason for protein aggregation. The presence of the acidic residues at surface sites on the other hand avoid such aggregation by virtue of the electrostatic repulsions present when the complexes approach [Elcock and McCammon 1998]. Altogether, neither electrostatic nor hydrophobic interactions alone are thought to determine the structural arrangement of an halophilic complex, both aspects have to be regarded.

The phenomenon that higher salt concentrations induce structural stability of the NpSR1II/NpHtrII complexes is also demonstrated in the present work. The stability towards urea denaturation is unambiguously enhanced in the presence of 3.5 M KCl when compared to the presence of 150 mM KCl. A final urea concentration of 6 M is not sufficient to fully unfold the transducer molecules, where this effect is shown to be more pronounced under high salt concentrations. The same enhanced stability of halophilic proteins to urea denaturation has been observed by Gloss and coworkers [Wright et al. 2002]. Cw X-band EPR experiments performed here at different temperatures support the findings since the HAMP domain is evidently less sensitive towards changes in the temperature under high
salt compared to low salt concentrations. That the effects induced by changes in the temperature and the salt concentration are correlated, has already been demonstrated previously [Poland and Scheraga 1970, Mullakhanbhai and Larsen 1975] since structural stabilization at lower temperatures and the requirement of higher salt concentrations for stabilization at higher temperatures for halophiles could be revealed. Eventually, stabilizing effects on the structural features of the NpHtrII HAMP comparable to that observed for high salt conditions are obtainable from EPR experiments carried out in the presence of acidic pH (pH 3.4) and 30 % (w/v) PEG3350 in the present study. The reasons for the more or less comparable stabilization induced by the two protein environmental conditions are of different nature. Acidic pH is known to mainly disturb the electrostatic interactions since acidic residues protonate and the electrostatic repulsions, destabilizing halophiles under low salt conditions, consequently decrease [Elcock and McCammon 1998]. The structural arrangement is thus shifted towards a stabilized state. In this regard it is noteworthy that the two main chemoreceptor systems in *E. coli*, i.e. Tar and Tsr, both comprising HAMP domains, were found to exhibit a short region within the linker domain that is critical for signaling during pH sensing, although their responses have shown to be of opposite nature [Umemura et al. 2002]. Though the signaling units from chemoreceptors are meant to be triggered by changes in their chemical environments, contrary to phototactic systems such as from the NpSRII/NpHtrII complex, HAMP domains might generally be constructed to adapt to several external stimuli. Hence, the compact HAMP structure induced by acidic pH might represent the cHAMP conformation or a conformation of similar topology. Nonetheless, lowering the pH can also disrupt salt-bridges, what in some cases may cause destabilization of intact protein structures [Elcock and McCammon 1998]. The effect on the NpHtrII HAMP domain induced by PEG3350, possessing the same direction as that of acidic pH in terms of shifting the “two-state” equilibrium towards the more compact state, might be regarded as stabilization effect as well.

Altogether, the sensitivity of the HAMP domain investigated here towards changes in the environmental salt concentration, temperature, pH and osmolyte condition is demonstrated in detail in terms of modulating the “two-state” equilibrium between the cHAMP and the dHAMP conformation in the NpSRII/NpHtrII complex. Furthermore, such a behavior is not unique in the pool of HAMP domains existing.

In order to exclude the possibility that the conformational changes discussed are of indirect nature, namely induced by major rearrangements of other compounds interacting with the NpHtrII HAMP domain, experiments on the lipid-bilayer and on the receptor NpSRII were carried out as well. As evident from the minor salt-induced spectral changes observable for three NpSRII variants, the receptor unit is found not to be the source of the major rearrangements taking place in the transducer HAMP domain but *vice versa* rather influenced by the latter. Concerning the purple membrane, only minor changes in the lipid-bilayer composition can be identified here upon the use of increased salt concen-
trations as was also reported previously [TENCHOV et al. 2006]. The authors found that
the lipid-bilayer of halophiles, containing 50 to 80% of the diacidic phospholipid PGP-Me
(see e.g. figure 2.10), was resistant against aggregation and leakage in the whole range from
0 to 4 M NaCl, whereas membranes from non-halophiles became leaky and aggregated at
higher salt concentrations. The effects observed were assigned mainly to steric repulsion
due to the large PGP-Me head groups rather than to electrostatic repulsion by means of
the head group’s doubly charged character [TENCHOV et al. 2006]. In the present study,
it is moreover demonstrated that not only the presence of the PGP-Me lipids influences
the stability of the lipid-bilayer but also the presence of the NpSRII/NpHtrII complex.
Changing the temperature from ambient (298 K) to higher temperatures (343 K) evidently
destabilizes the isolated membrane-bilayer composition as observable from the increased
dynamics of the 5DSA label inserted in the bilayer. Contrarily, no effect is observable upon
increased temperature when the NpSRII/NpHtrII complex is present, pointing to the stabiliz-
ing function of the protein complex. The fact that measurements performed with higher
lipid contents (200- or 400-fold instead of 40-fold molar excess of lipids per 1:1 complex)
do not yield considerable differences neither in the mobility, accessibility, distance, polar-
ity nor in the temperature data unambiguously excludes lipid-induced effects. This also
tells us that possible trimer formations found in chemoreceptor arrays (see for recent pub-
lications [VAKNIN and BERG 2007, TAYLOR 2007, SWAIN and FALKE 2007]) that are also
thought to exist in the receptor/transducer complex investigated here (trimers of dimers,
recent EPR experiments on the cytoplasmic tip of the NpHtrII transducer, personal commu-
nication with J. P. Klare, group member), cannot be resolved in the experimental data pre-
sented. This finding may be due to the fact that i) trimers of dimers of the NpSRII/NpHtrII
complexes always exist during the experiments carried out, ii) never exist here or iii) that
the trimer formation does not have any effect on the structure of the dHAMP and cHAMP
conformation.

To which extent the shiftable nature of the “two-state” equilibrium can further be assigned
to a signal transduction mechanism is not yet characterized. The thermodynamic data
extracted from temperature-dependent cw EPR measurements, first, corroborates the equili-
brium’s existence via the similar Gibbs energy changes (ΔG; the amount of energy available
for chemical work [WINZOR and JACKSON 2006]) found for all residues investigated under
low and high salt conditions, respectively. Second, by virtue of the differences between
the low-salt and the high-salt ΔG values, the different nature of the dHAMP predominant
at 150 mM KCl, and the eHAMP prevalent at 3.5 M KCl, is evaluated. Nonetheless,
it is noteworthy that according to Jackson and coworkers [WINZOR and JACKSON 2006]
caution has to be exercised when analysing the outcomes of thermodynamic investigations.
Particular caution was advised when from thermodynamic parameters as standard enthalpy
and entropy molecular information is to be extracted, where energy changes are quantified
and used for the interpretation of molecular processes. The comparisons presented here have to be treated accordingly.

The average Δ\(G\) values determined from the temperature dependent measurements of several NpHtrI\textsubscript{157} variants in complex with NpSRII reconstituted into PML account for \(\Delta G_{\text{low}} = 3 \pm 2\ \text{kJ/mol}\) and \(\Delta G_{\text{high}} = 9 \pm 8\ \text{kJ/mol}\) for the low (150 mM KCl) and high (3.5 M KCl) salt measurements, respectively, at ambient temperature (298 K). That values for the changes in Gibbs energy are considered to linearly increase with increasing solvent salt concentrations, has amongst others been shown by Gloss and coworkers, Gupta and Ahmad [Wright et al. 2002, Gupta and Ahmad 1999]. They determined the unfolding free energies (\(\Delta G\)) from urea denaturation data obtained from absorption, fluorescence and CD spectroscopy carried out for mesophilic and halophilic organisms. In both studies the linear correlation between \(\Delta G\) and the salt concentration was limited to such organisms, where the native protein contains charge-charge interactions and/or anion binding sites. In this regard, the urea denaturation measurements on the NpSRII/NpHtrII complex discussed above, where the stabilizing function of higher salt concentrations on halophiles is described, become relevant again. It is evident that enhanced structural stability leads to retarded denaturation and thereby to increased values for \(\Delta G\). However, the assignment of the small energetic difference obtainable between \(\Delta G_{\text{low}}\) and \(\Delta G_{\text{high}}\) (at 298 K approximately \(6 \pm 5\ \text{kJ/mol}\)) cannot easily be done. In literature, a “two-state” equilibrium existing between two conformations of \emph{stratum corneum} (SC), namely a strongly and a weakly immobilized one, was investigated in terms of thermodynamic parameters by EPR spectroscopy [Alonso et al. 2001]. An apparent energetic gain for the nitroxide spin label attached to form a hydrogen bond with the protein backbone, describing the strongly immobilized state, rather than to be dissolved in the aqueous environment, describing the weakly immobilized state, was \(\approx 42\) and \(\approx 25\ \text{kJ/mol}\) in the 275 to 303 K and 303 to 343 K range, respectively. Since the energetic difference calculated from the \(\Delta G\) values obtained in this study is evidently lower, the structural rearrangements between the dHAMP and the cHAMP are presumed not to change the H-bond network between the nitroxides and the transducer. The HAMP domain thus seems to be in a subtle balance between the various physical interactions, where conformational rearrangements resulting in enhanced residual dynamicity are expected to take place. The diminutiveness of the processes taking place in terms of energetic differences is also obvious when e.g. the complete unfolding (except helices G and F) of the integral membrane protein bacteriorhodopsin, accounting for \(\Delta G \approx 1070\ \text{kJ/mol}\), or the average unfolding per transmembrane helix (from BR), accounting for \(\Delta G \approx 130\ \text{kJ/mol}\) [Preiner et al. 2007], are regarded. Eventually, the similarity of the \(\Delta G\) value determined from temperature-dependent experiments performed for the ColA-A105R1 (\(\Delta G = 4 \pm 3\ \text{kJ/mol}\)) variant and those obtained for the \emph{N. pharaonis} dHAMP conformation is worth mentioning. Due to the fact that colicin A is known to use a molten-globule-like structure to penetrate membranes [Pulagam 2007], it might well be
that such an extremely dynamic conformation resembles the dHAMP structure. Moreover, the finding that the temperature-dependent measurements performed for the *N. pharaonis* receptor and BR are considered to solely yield a temperature-induced shift in the ratio of spin label rotamer contributions and not between different protein conformations clearly indicates that the HAMP conformational changes are not directly correlated to changes in the transmembrane part. Contrarily, the changes observed for the NpSRII residue might rather be induced indirectly by interactions with the transducer HAMP.

The significance of the interplay between conformational states exhibiting different degrees of dynamics could not be illuminated so far. Experimental evidences clarifying the activation mechanism for negative phototaxis away from repellents or positive phototaxis towards attractants do not exist. Nonetheless, the hypothesis of a "frozen-dynamic" model for the activation/deactivation of chemoreceptors was put forward, where the interplay between a dynamic state and one occupying smaller conformational space of the signaling domain was assumed to be the decisive point [Kim 1994]. In a recent publication on bacterial chemoreceptors, it was also noted that a "two-state" equilibrium between protein populations with different degrees of dynamics can be the language of the activation/deactivation of the cytoplasmic domain [Hazelbauer et al. 2007]. In that study, the relevance of higher order structures and multiple levels of molecular interactions was noted as well. In [Taylor 2007], besides the already noted finding that the NMR-derived four-helix bundle also represents the *E. coli*-Aer-chemoreceptor structure, the authors also mentioned the presence of a fraction of partially unfolded variants. This might describe the dynamic conformation, too. Most interestingly, recent results on *E. coli* chemoreceptor arrays indicate that signals can be transmitted *via* the disruption of protein-protein interactions in the signaling assembly [Borrok et al. 2008]. There, evidence was found that attractant binding perturbs such interactions, whereas repellents stabilize larger arrays. In a very recent state-of-the-art cryo-electron-microscopy experiment of the role of HAMP domains in signaling units, Subramaniam and coworkers [Khursigara et al. 2008] also identified the co-existence of two conformations of the *E. coli* serine chemoreceptor Tsr, i.e. an "expanded" and a "compact" conformation, in line with the present study. They showed that the distribution of the chemoreceptors between the two conformations could be altered by ligand binding and methylation. Moreover, they proposed that the "compact" conformation represents the "kinase-on" (repellent chemotaxis), and the "expanded" analog the "kinase-off" (attractant chemotaxis) states of the chemoreceptor trimers. To what extent these considerations can be adopted to assign the cHAMP to the signaling ON and the dHAMP to the OFF state remains to be determined. Nonetheless, first indications that the signaling ON state (active state) of the NpSRII/NpHtrII complex is characterized by MTS side chain immobilizations of residues in the AS1, pointing to a more compact topology, were found by SDSL EPR (see [Holterhues 2009]). Such major rearrangements in the HAMP domain during signaling are on the other hand not in line with the signaling mechanism suggested for the linker
region from *A. fulgidus*, where the HAMP domain solely alternates between the described knobs-to-knobs and knobs-to-holes (canonical coiled coil) conformation *via* concerted helix rotations [Hulko et al. 2006]. However, the rotation of the transducer transmembrane helix TM2, taking place upon light activation of the receptor NpSRII, shown by EPR and X-ray crystallography [Wegener et al. 2001, Moukhametzianov et al. 2006], might well trigger a rotation in the HAMP helices, what in turn could shift the equilibrium between the two HAMP conformations towards the ON or OFF state.

### 6.4 Outlook

It has been shown that the “two-state” equilibrium existing between a highly dynamic (*dHAMP*) and a very compact (*cHAMP*) protein conformational state can be shifted towards one of them by several external stimuli. However, the relevance with respect to the signal transduction processes taking place in order to lead the cell towards favored environmental conditions could not be resolved so far. Furthermore, the nature of the two spectral components identified throughout the *Natronomonas pharaonis* HAMP domain has to be studied in more detail. In this regard, it would be interesting to follow the salt- and temperature-induced spectral changes observable with a spin label species exhibiting side chain dynamics different from that of the R1 label used in this study. Freed and coworkers investigated e.g. the differences in the motional behavior of the R1 and the R2 label, where in the latter case at the 4-position of the nitroxide-ring compound the proton is substituted by an additional methyl group, thereby increasing the bulkiness of the side chain and decreasing its conformational freedom [Tombolato et al. 2006a]. The authors pointed out that the R2 spin species appears to be more suitable for the investigation of protein dynamics, contrary to R1, that is more sensitive towards environmental changes. Additionally, even more restricted side chains (as e.g. the iodacetamide label) could help revealing the unsolved questions. Distributions of distances obtained by cw and pulse EPR methods would become more distinct, increasing the quality of the data, particularly when several distance distributions are resolved and overlapping in one data set. Although the nature of the different components observable in the cw X-band EPR spectra could be assigned to protein conformational subpopulations and spin label side chain rotameric states, pressure-dependent experiments are considered to enlighten further details. In this regard, it would not only be interesting to follow the changes of the system occurring upon a stepwise pressure increase but also upon pressure jumps to resolve the kinetics of the *cHAMP-dHAMP* reaction. Eventually, in order to gain further insight into the spatial arrangement of the amphipathic sequences towards each other, and due to predicted AS1-AS2’ interactions [Swain and Falke 2007], distance measurements could be carried out. To do so, dimeric NpSRII/NpHtrII complexes should be investigated, where one transducer carries a spin label in the AS1, and the second transducer a spin label in the AS2. Comparison of
the resulting distance distributions with those already obtained for doubly labeled NpHtrII variants presented in this study, is moreover thought to deepen the understanding of the distances resolved. We are quite confident that future SDSL EPR experiments in combination with complementary techniques, such as e.g. NMR, will unravel the functional and molecular details of the transition between the different dynamic states of HAMP domains.
Chapter 7

Synopsis

The photosensitive unit triggering the negative phototaxis in the extremely halophilic archaeon Natronomonas pharaonis is formed by the receptor sensory rhodopsin II (NpSRII) and its cognate transducer (NpHtrII) in a 2:2 stoichiometry. Upon light excitation, a structural rearrangement in the receptor initiates a displacement/rotation of the transmembrane transducer helix 2 (TM2), which can be considered as starting event for the signal transduction. This signal is further transmitted in an unknown mechanism to the cytoplasmic signaling domain through the signal transduction unit comprising two HAMP domains that are highly conserved signal transduction modules in a variety of protein families.

Structural information in terms of X-ray crystallographic data already exists for the transmembrane part of the NpSRII/NpHtrII complex as well as for the rod shaped cytoplasmic part of the transducer due to its high homologies with chemoreceptors. Moreover, the solution NMR structure of the isolated HAMP domain from the hyperthermophilic archaeon Archaeoglobus fulgidus recently obtained shows a homodimeric, parallel, four-helical coiled coil with an unusual interhelical packing, which is thought to propagate a signal by virtue of concerted helix rotations.

Here, a site-directed spin labeling electron paramagnetic resonance (SDSL EPR) investigation on the N. pharaonis HAMP domain was carried out for structural and functional elucidation, particularly with respect to conformational changes induced by environmental agents such as salt and temperature. A series of spin labeled mutants was engineered in NpHtrII157, a truncated analog containing only the first HAMP domain following the TM2. This truncated transducer is shown to be a valid model system for a signal transduction domain. The NpHtrII157 variants complexed with NpSRII were investigated in the detergent-solubilized and the lipid-reconstituted states.

In the room temperature cw X-band EPR spectra of singly labeled transducer variants reconstituted in purple membranes two spectral components are consistently present, which are characterized by different degrees of dynamics of the spin label’s side chains. The two spin label subpopulations can be assigned to protein fractions possessing highly dynamic (dHAMP) and very compact HAMP (cHAMP) domain structures, respectively. Changing the salt concentration of the solvent shows that under low salt conditions (150 mM KCl) the
fraction of the dynamic conformation (component 2), and under high salt conditions (3.5 M KCl) that of the compact analog (component 1) is prevalent. The salt-induced spectral changes show to follow the Hofmeister series of stabilizing and destabilizing anions/cations, and that higher salt concentrations lead to the suppression of the fraction of the spectral component 2. A characteristic feature of component 1, namely two spectral contributions arising from different rotameric states of the spin label side chains, is also recognizable in the room temperature EPR spectra.

Mobility and accessibility analyses (section 5.1.1 and 5.3.1) indicate a lack of fixed structure of the dHAMP domain from the center of the amphipathic sequence 1 on. Contrarily, defined secondary structural elements and tertiary interactions of the spin label side chains with neighboring residues are observable in all cHAMP subdomains (AS1, connector, AS2). ENDOR (electron nuclear double resonance) measurements carried out on analogous NpSRII/NpHtrII157 mutants (section 5.6) support the mobility and accessibility data obtained under high salt conditions, where residue M100 defines a key position, pointing to strong secondary/tertiary interactions of this region of the transducer. Acidic pH (pH 3.4) of the solvent or the presence of 30% (w/v) PEG3350 (polyethylene glycol 3350) (section 5.1.2) are shown to mimic the high salt environment in terms of the suppression of the dHAMP conformation at least at room temperature. Cw X-band EPR experiments on NpSRII mutants and 5-doxylstearic acid (5DSA) enriched lipid-bilayers performed under different salt conditions and temperatures (section 5.1.1) reveal the HAMP-specific character of the salt effect, and the stabilized nature of the lipid-bilayer in the presence of the NpSRII/NpHtrII157 complex.

In order to further investigate and verify the origin of the two spectral components, titration experiments with the paramagnetic quencher NiEDDA (Ni(II)ethylenediamine diacetate) and incubation experiments with the reducing agent ascorbic acid were carried out. Specific effects of NiEDDA, decreasing the nitroxide’s relaxation times of water-exposed spin labels, and ascorbate, irreversibly reducing water-exposed nitroxides, on the two spectral moieties prove the presence of a “two-state” equilibrium between the dHAMP and the cHAMP conformation. Temperature-dependent (283 to 343 K) cw X-band EPR measurements (section 5.2) show that increasing the salt concentration and lowering the temperature have the same effect on the two spectral components, both shifting the “two-state” equilibrium towards the cHAMP conformation. Moreover, from the determination of the fractions of the two spectral components at different temperatures, the thermodynamic properties (ΔH, ΔS, ΔG) of the dHAMP-cHAMP equilibrium can be derived. Equal values for ΔG obtainable for all transducer variants investigated under low and high salt conditions, respectively, corroborate the presence of the “two-state” equilibrium. Results from analogous temperature-dependent test measurements performed for three mutants of different entities (section 5.2), i.e. NpSRII, bacteriorhodopsin (BR) and colicin A (ColA), underline the HAMP-specific character of the “two-state” equilibrium. That this equilibrium can be
shifted towards the compact conformation by lowering the temperature is also supported by polarity and cw/pulse EPR distance data obtained for several NpHtrII_{157} mutants in complex with NpSRII reconstituted in PML measured at 160 and 50 K, respectively (section 5.4.1 and 5.7.1). Only minor changes are detectable between the polarity values and the distances defining the transducer-transducer interactions under high and low salt data.

Inter-spin distance measurements on doubly labeled NpSRII/NpHtrII variants are investigated in order to unravel the relative distance between transducer and receptor (section 5.7.3). Relevant dipolar interactions between residues A88 to L90 in the transducer AS1 and residues S154, K157 and S158 in the receptor are observable, indicating the spatial proximity of the two protein regions. The cw inter-spin distance analysis also reveals the fact that the HAMP domain is not engaged in a structure characterized by a simple linear prolongation of the transmembrane transducer helix TM2, but that the transducer moieties are rather slightly inclined towards each other.

To test the significance of the solution NMR-derived HAMP structure from *A. fulgidus*, where a parallel, four-helix bundle was found, comparisons between EPR data obtained in this study and different data determined for the Af1503 HAMP is done. The water accessibility data obtained for spin labeled transducer variants under high salt conditions is in full agreement with the solvent accessibilities calculated for the HAMP domain from *A. fulgidus* (section 5.3.1). This indicates the coincidence of the *N. pharaonis* cHAMP with the four helical bundle from *A. fulgidus*. Furthermore, the distances obtained from cw and pulse EPR measurements carried out for singly labeled NpHtrII_{157} variants in complex with NpSRII reconstituted in PML are within the experimental errors in line with analogous distances calculated in silico for the Af1503 HAMP (section 5.7.1). Therefore, also doubly labeled transducer variants, with one spin labeled site in the AS1 and one in the AS2, are examined for the illumination of the intra-transducer interactions. Though not a clear-cut evidence, the dipolar interactions resolved in the EPR experiments are rather indicative for a four helical bundle than for a simple linear prolongation of the HAMP with respect to the transmembrane transducer helix 2.

EPR mobility, accessibility, polarity and inter-spin distance data obtained for the NpHtrII_{157} mutants investigated with high lipid contents (400-fold instead of 40-fold molar excess per NpSRII/NpHtrII_{157} complex) exclude the presence of lipid-induced effects. Possible formations of dimers within trimers similar to that observed for chemoreceptors do not play a decisive role in the investigations presented here.

A set of EPR experiments was carried out for DDM-solubilized NpSRII/NpHtrII_{157} complexes (1:1 stoichiometric ratio) analogous to the analysis of the lipid-reconstituted samples. The values obtained for mobility, accessibility, and polarity parameters as well as for inter-spin distances yield significant structural differences with respect to the reconstituted complexes. Strong tertiary interaction between the transducer (residue A95) and the receptor (residue S154) is found in the absence of the second transducer, pointing to a major
conformational rearrangement in the HAMP domain. This finding is in line with impaired functionalities observed upon solubilization in previous studies.

In summary, solubilization of the NpSRII/NpHtrII\textsubscript{157} shows to considerably affect the structural and functional features of the \textit{N. pharaonis} HAMP with respect to the lipid-reconstituted analog. The HAMP domain is found to be engaged in a “two-state” equilibrium between a highly dynamic (\textit{dHAMP}) and a more compact (\textit{cHAMP}) conformation when reconstituted in membranes that might be relevant for the cellular signaling mechanism. The structural properties of the \textit{cHAMP} are in agreement with the NMR-derived parallel, four-helix bundle of the HAMP domain from the hyperthermophile \textit{Archaeoglobus fulgidus}. 
APPENDIX A

SUPPLEMENTARY DATA

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Table A.1: Part I: Reorientational correlation times $\tau_c$ for the two spectral components (c1: compact, c2: dynamic), and the respective fraction of component 1 $f_1$ ($f_2 = 1 - f_1$) extracted from fitting simulated to experimental spectra. Experimentally obtained spectra were recorded at different temperatures for selected NpHtrII157 variants in complex with NpSRII reconstituted into PML. For the sake of clarity, the low salt (150 mM KCl) data is depicted in the left part, the high salt (3.5 M KCl) data in the right.
Table A.2: Part II, for description see caption of table A.1. Experimentally obtained spectra were recorded at different temperatures for selected NpHtrII\textsubscript{157} variants in complex with NpSRII reconstituted into PML, and one spin labeled amino acid in colicin A (ColA-A105R1).

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Figure A.1: Results of 4-pulse DEER measurements on various NpHtrII variants in complex with NpSRII reconstituted into PML exhibiting a 2:2 stoichiometry. The normalized dipolar evolution functions $V(t)/V(0)$ (solid lines) and exponentially decaying background arising from a three-dimensional distribution of surrounding spins (dotted lines) fitted by DeerAnalysis2006 [Jeschke et al. 2006]. Measurements were performed in the presence of 150 mM KCl (black), 2 M KCl + 20% (v/v) glycerol (olive), pH 3.4 (150 mM KCl, blue) and 30% (w/v) PEG3350 (150 mM KCl, magenta). The spectra are designated by the 1-letter amino acid code followed by the amino acid’s number in the sequence and the R1 spin label. For comparison, spectra obtained for one residue are superimposed to the respective low salt data. They are normalized to fit the slope of the background fit (for residue R99R1 not feasible). Traces detected for longer times $t$ are shifted to the back.
Figure A.2: Comparison of spin normalized low temperature (160 K) cw X-band EPR spectra of singly labeled NpHtrII variants. Spectra recorded for NpHtrII variants in complex with NpSRII reconstituted into PML in the presence of 150 mM KCl (black lines, previously published data [Doebber 2005, Bordignon et al. 2005]; gray lines recent data) and 2 M KCl (plus 20% (v/v) glycerol, olive lines) are superimposed to the respective reference spectrum without dipolar broadening (dotted lines) from the solubilized complexes. The strength of the dipolar interaction and the resulting broadening are visible in the differences of the central amplitudes of the spin normalized spectra to that of the reference spectrum.
Figure A.3: Comparison of pulse EPR-derived distance distributions $P(r)$ for the residues investigated in section 5.7.1 determined with (dashed lines) and without (solid lines, corresponding to those depicted in figures 5.33 and 5.34) excitation bandwidth correction (EBC). The distributions are designated by the 1-letter amino acid code followed by the residue’s number in the amino acid sequence (top right in each graph). Below the spin numbers per nanoobject $< n >$ are given (without EBC: top; with EBC: bottom).
Figure A.4: Comparison of spin normalized low temperature (160 K) cw X-band EPR spectra of doubly labeled NpSRII/NpHtrII157 variants. Spectra obtained in the reconstituted 2:2 complexes for a double mutant carrying one spin label at one of the receptor positions selected (S154R1, K157R1, S158R1) and one in the amphipathic sequence 1 of the transducer HAMP domain (A88R1 to A95R1) are depicted by solid lines. In order to estimate the dipolar interaction present in some of the experimental data shown, the respective singly labeled NpHtrII157 variants in the 2:2 complex with NpSRII reconstituted into PML are superimposed (dashed lines).
Bibliography


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DECLARATION

I hereby declare that this submission is my own work, and that, to the best of my knowledge and belief, it contains no material previously published, or written by another person, nor material, which to a substantial extent has been accepted for the award of any other degree or diploma of the university, or other institute of higher learning, except where due acknowledgment has been made in the text.

Osnabrueck, Germany
January 16, 2009

Meike Döbber