Structural analysis of colicin A:

*in vitro, in vivo and in silico studies*
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Dissertation

Presented to the Department of Biology/Chemistry,
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for the degree of 'Doctor Rerum Naturalium'

by

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Abstract

Organisms inhabiting the same ecological niche compete with each other for nutrients and space. In case of microorganisms, this is an electro-chemical war and in most of the cases, the weapons are pore-forming toxins, which affect an electrical gradient across the cell membrane. The pore forming toxins are generally secreted as water-soluble proteins and then insert into membranes for their cytotoxicity. In order to study the properties of these pore forming toxins concerning their membrane insertion pathway and membrane bound conformations, we have chosen “colicin A” as a model system.

Colicin A is a water-soluble toxin that forms a voltage-gated channel in the cytoplasmic membrane of target bacteria. In the present thesis, we aimed at studying the closed channel state, the membrane insertion mechanism, the acidic pH induced molten globule state and the interaction of colicin A in living E. coli cells. For that, we used Electron Paramagnetic Resonance (EPR) spectroscopy in combination with site-directed spin labeling (SDSL) method to explore the structural details of colicin A.

The EPR studies of the membrane-bound colicin A (reconstituted into proteoliposomes) suggest the transmembrane orientation of the hydrophobic hairpin in the closed channel state. The pH dependent membrane insertion studies indicate that the membrane binding efficiency is significantly enhanced at pH < 3. Moreover, in the presence of a membrane potential, the pH induced membrane-bound state is able to open channels in the liposomes. The membrane-bound conformation (induced by acidic pH) is similar to the conformation of reconstituted colicin A which support the umbrella model for the closed channel state of colicin A. The studies on pH dependent conformational changes suggest that colicin A forms a molten globule at pH 2. The molecular details of pH induced conformational changes were analyzed by molecular dynamic simulations. The results of the MD simulations agree with the EPR results. Conformational changes of colicin A upon interaction with living E. coli cells could also be followed. Comparison between colicin A in wild type (WT) cells and tolB knock-out mutants suggest that the observed conformational changes originate from colicin A which has been already translocated to the inner membrane.
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# Abbreviations

<table>
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<th>Description</th>
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<tbody>
<tr>
<td>ANS</td>
<td>1-anilinonaphthalene-8-sulphonate</td>
</tr>
<tr>
<td>cell-ColA</td>
<td>Colicin A that bound to the <em>E. coli</em> cells</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-Dithio-DL-threitol</td>
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<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ED</td>
<td>Essential dynamics</td>
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<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
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<td>Ev</td>
<td>Eigenvector</td>
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<td>Fig</td>
<td>Figure</td>
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<tr>
<td>GDHCl</td>
<td>Guanidinium Hydrochloride</td>
</tr>
<tr>
<td>GRASP</td>
<td>Graphical representation and analysis of structural properties</td>
</tr>
<tr>
<td>LMV</td>
<td>Large multilamellar vesicles</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
<tr>
<td>MG</td>
<td>Molten globule</td>
</tr>
<tr>
<td>MTS</td>
<td>Methanethiosulphonate</td>
</tr>
<tr>
<td>Ni-EDDA</td>
<td>Nickel ethylene diamine diacetic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>O₂</td>
<td>Molecular oxygen</td>
</tr>
<tr>
<td>pH-mem-ColA-</td>
<td>Acidic pH induced membrane-bound colicin A</td>
</tr>
<tr>
<td>rec-mem-ColA-</td>
<td>Reconstituted membrane-bound colicin A</td>
</tr>
<tr>
<td>R1</td>
<td>Spin-labeled side chain</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td>RMSIP</td>
<td>Root mean square inner product</td>
</tr>
<tr>
<td>SDSL</td>
<td>Site-directed spin labeling</td>
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<tr>
<td>sol-ColA</td>
<td>Water soluble colicin A</td>
</tr>
<tr>
<td><em>tolB</em></td>
<td>Knockout of <em>tolB</em> gene</td>
</tr>
<tr>
<td>VMD</td>
<td>Visual molecular dynamics</td>
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<tr>
<td>WT</td>
<td>Wild type cells</td>
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1. Introduction

1.1 Motivation

The formation of pores in target cell membranes is a mechanism of cytotoxicity found in organisms ranging from bacteria to humans. Pore-forming toxins comprise a special class of proteins, which are secreted as water-soluble proteins and insert into the target cell membranes to open pores [1-3]. These pores are often regulated by a number of chemical and physical factors, e.g. ligands and a membrane potential. Some of these pores are selective for certain ions, generally called ion channels (e.g. K⁺ channel), whereas others are less selective and they can also pass small organic molecules along with ions (e.g. colicin channels) [1].

The ability of pore-forming proteins to create regulated pores into a cell is an extremely interesting prospect with direct relevance to both therapeutic and biosensor applications. The following are few examples for pore-forming toxins [2]:

**Alpha haemolysin**: This toxin is produced from *Staphylococcus aureus* and forms non-regulatory pores in human cells. Alpha haemolysin with built-in triggers and regulatory switches has been engineered and is presently being exploited as a tool for application to biotechnology and medicine [2].

**Aerolysin**: *Aeromonas hydrophila*, a Gram-negative bacterium produces this toxin, which creates voltage-dependent channels. Recent studies have shown that aerolysin is capable of neutralizing HIV-1 in a dose-dependent manner, demonstrating the potential use of aerolysins as antiviral agents [2].

**Colicins**: Enterobacteriaceae family members produce colicins which form voltage-gated pores in the target bacterial plasma membranes [4, 5]. Study of toxins like colicins that selectively kill microorganisms while causing no harm to the host strain, will provide a better understanding of the biological principles underlying microbial defense mechanisms and provide useful information for designing antibiotics [2]. Smarda et al. and Lancaster et al. reported that colicins can selectively kill tumor cells [6-8].

**Anthrax toxin**: *Bacillus anthracis* produces anthrax toxin, which is known to insert into membranes at acidic pH and forms voltage-gated channels. Engineered anthrax toxins, which are capable of delivering large macromolecules and cause cytolysis of target cells
have been designed and tested. Such toxins may have potent application in cancer treatment [2].

**Diphtheria toxin**: *Corynebacterium diphtheriae* produces diphtheria toxin which is also known to insert in membranes at acidic pH and form voltage-dependent channels [9]. The engineered diphtheria toxin can target and kill specific tumor cells [2].

The pore-forming proteins play key roles in many biological functions [10], however the translocation of proteins across lipid bilayers and formation of pores is still poorly understood. In order to understand the biological principles lying behind the function of these toxins, one should know the structural and functional details of this special class of proteins. The study of these proteins might give the answers for the following questions:

1. How does a water-soluble protein insert into a membrane?
2. What are the conformational changes involved in the unfolding and refolding of a polypeptide chain during and after its translocation and/or insertion?
3. What is the mechanism involved in the opening and closing of the channel?

To answer these questions we have chosen colicin A as a model system. The advantages to choose colicins as a representative of pore-forming toxins are: (i) colicins are produced in large amounts and are generally released into the external medium, (ii) it is now possible to purify these 30-70KD proteins in large amounts allowing many biochemical, biophysical and structural investigations, (iii) the pore-forming domain is highly resistant to proteases and active in vitro conditions, and (iv) they are not active against human beings.

### 1.2 Colicins

Colicins are plasmid-encoded bacteriocins that are produced by various *E. coli* strains and are active against other *E. coli* strains [3, 4]. Up to date, almost 34 colicins have been studied, e.g., colicin A, B, Ia, E1-E9, K, N, and colicin U. Colicins kill their targets either by nuclease activity or by forming pores in the cytoplasmic membrane of the target cell. In the present study, we focused on “colicin A”, a pore-forming protein.

Colicins are encoded by the so called Col plasmids, which vary widely in size and properties. For example, pColE1 and pColA are the representative plasmids for colicin E1 and colicin A, respectively. In pColA, the following three genes are
important for colicin A mechanism [4]: (i) \textit{caa} - gene for colicin A production, (ii) \textit{cal} - gene for colicin A secretion, and (iii) \textit{cai} - gene for immunity against to colicin A.

\textbf{Figure 1.1}: pColA plasmid. The arrangement of \textit{caa}, \textit{cal} and \textit{cai} genes is illustrated.

Colicin A is the product of gene \textit{caa}. Under normal conditions, the expression of \textit{caa} gene is repressed by a repressor protein, LexA and only induced by the ‘SOS’ response which is associated with the repair of damaged DNA [3, 4]. In bacterial cultures, colicin production can be induced by DNA damaging mutagens like U.V light or mitomycin C [4, 5]. Between these two mutagens, mitomycin C is the most efficient to induce large amounts of colicin production for biochemical and biophysical studies. Once induced, colicin A reaches 50% of the total cell protein due to the combination of a strong promoter and multiple copies of the plasmid. Once produced, lysis protein, a \textit{cal} gene product plays an important role in the secretion of colicin A [3]. Immunity protein that is encoded by a gene \textit{cai}, is a transmembrane protein and protects the cells against the same type of colicins specifically [11]. The immunity proteins are constitutively expressed at low levels (\textit{10}^2 to \textit{10}^3 molecules per cell) when compared to the number of colicin A molecules (\textit{10}^7 molecules per cell) [12].

\textbf{1.2.1 Structural details of colicins}

X-ray crystallographic models for complete protein of the pore-forming colicins are available for colicin B [13], colicin N [14], and colicin Ia [15]. X-ray models for only the pore-forming domain are available for colicin A [16] and colicin E1 [17]. Many colicins have three functional domains: (i) Central domain or receptor binding domain (R-domain), (ii) N- terminal domain or translocation domain (T-domain), (iii) C-terminal domain or pore-forming domain (P-domain). To give an example, the structure of colicin Ia that shows three domains is presented in Fig 1.2. The receptor binding domain binds to the receptor [1, 3, 4] and then the translocation domain helps to
translocate the pore-forming domain from the outer membrane to the inner membrane [3-5, 18]. The pore-forming domain is mainly involved in the cytotoxic function of the toxin and carries the whole property of pore formation.

**Figure 1.2:** Domains architecture of colicins. Ribbon structure of colicin Ia (1CII.pdb) [15] is shown to represent pore-forming colicins that are organized into three domains. The three individual domains are colored and named separately.

The X-ray crystal structures of the water-soluble pore-forming domains of colicin A, B, E1, IA and N have the same topology and they have structural similarity with the T domain of diphtheria toxin. Figure 1.3 shows the structure of water-soluble pore-forming domain of colicin A.

**Figure 1.3:** Structure of pore-forming domain of colicin A (1COL.pdb). The pore-forming domain is cartoon rendered and the number of helices is given. The amphipathic helices are colored in green and the hydrophobic hairpin (helices 8 and 9) is colored in magenta.
The water-soluble pore-forming domain of colicin A is globular and comprises of ten helices which are arranged into three layers [16]. Hydrophobic helices 8 and 9 are located in the core of the protein and are surrounded by a ring of the remaining eight amphipathic helices. The hydrophobic surfaces of the amphipathic helices face towards the central hydrophobic core and the hydrophilic faces are exposed to the aqueous environment, making the protein soluble in water.

1.2.2 Action of colicin A on a target cell

*E. coli* cells, which are the targets for pore-forming colicins have an outer membrane and an inner membrane (plasma membrane or cytoplasmic membrane). Since the pore-forming colicins form pores in the inner membrane, colicins must across a formidable physical barrier to reach their destination. Hence, colicins require specific receptors and a protein machinery to reach the plasma membrane.

The action of colicin A on a target cell includes mainly three phases:

1. Binding to the receptor of the outer membrane,
2. Translocation from the outer membrane to the inner membrane,
3. Formation of a channel in the inner membrane.

![Figure 1.4: A schematic representation of the colicin A mechanism. (For details see text)
1.2.2.1 Binding to the receptor

Colicins parasitize the metabolite receptors for their binding and transport into the target cell. The receptor for colicin A is BtuB, an outer membrane protein that imports Vitamin B12. At low concentration of B12, there are 200-250 copies of BtuB in the outer membrane [3, 4]. Although colicins are considered as single hit killers, it requires more than one bound colicin to kill a cell. It appears therefore that the majority of colicins, which bind to their receptors, do not succeed in penetrating to the cytoplasmic membrane. There are two reason for this: most of the proteins are degraded by cell surface proteases before they can enter the cell and only a fraction of the total number of receptor sites are competent for later translocation. Binding of colicins to BtuB is a typical protein-protein interaction, which requires only appropriate ionic conditions, permitting an active conformation of both proteins.

1.2.2.2 Translocation from the outer to the inner membrane

The outer membrane contains small pores (10-20 Å in diameter) which are made up of special proteins called porins, involving in nutrient uptake by two different mechanisms. Small molecules (Mr < 700 Da) are imported into the cell by diffusing across the membrane through pores, known as passive transport. Bulky nutrients such as siderophores and vitamin B12 that exceed the diffusion limit of the outer membrane porins are imported across the outer membrane by energy dependent ligand-gated translocation pathways. This energy dependent pathway is also termed active transport.

Both the energy dependent and energy independent transport pathways have been parasitized by colicins. Moreover, genetic studies have shown that in addition to the porins, the Tol (for Tolerant) group of proteins is required for the energy independent import of colicins, whereas the TonB proteins are needed for the energy dependent import. Thus, to translocate from the outer membrane, colicins use either the TonB or the Tol system [3, 19, 20]. Depending on these two pathways, colicins are classified into two groups [3].

1. Group A colicins which translocate through the TOL system  
e.g., colicin A, E1-E9, K, L, N, bacteriocin 28b and colicin DF13.
2. Group B colicins, which translocate through the Ton system.  
e.g., colicins B, D, Ia, Ib, M and V.

**TonB pathway:** This is an energy dependent pathway. TonB is required for many molecular uptake pathways including iron and vitamin B12. Additionally, TonB
requires ExbB and ExbD that are homologous to the TolR and TolQ of the Tol system. TonB dependent processes mediate the energy of the electrochemical potential from the inner membrane to the outer membrane.

**Tol-Pal pathway**: The natural function of the Tol system in a cell is still under debate but the Tol mutant cells are leaky to periplasmic proteins and hypersensitive to drugs and detergents. The Tol system comprises TolA, TolB, TolQ and TolR proteins. In addition to these proteins, an outer membrane lipoprotein Pal (Peptidoglycan associated lipoprotein) is also known to be involved in this process. TolA and TolR anchor the inner membrane with one transmembrane helix, TolQ is an integral protein of the inner membrane and TolB is a periplasmic protein that associates with an ATP dependent Pal protein. These Tol proteins are preferentially located at contact sites between the inner and the outer membranes. Their concentration is increased by a factor of two when the cells are treated with colicin A.

Colicins, which use the Tol system for translocation, use OmpF or TolC for translocating from the outer membrane. Transfer of colicin across the outer membrane may require unfolding of the toxin in order to thread through the lumen of the outer membrane receptor (e.g. OmpF) [21]. At least 12 Tol dependent colicins require two outer membrane receptors for cytotoxicity. One exception is colicin N that uses only OmpF. It is curious that dual receptor requirement is not reported for TonB dependent colicins. Colicin A utilizes two outer membrane proteins, BtuB and OmpF.

### 1.2.2.3 Pore formation

The pore-forming domain of the colicins inserts into the cytoplasmic membrane and forms a channel that opens with a trans-negative membrane potential. Colicin channels are non-specific which allow passing of all ions including small organic molecules like glutathione and NAD+. The efflux of ions through the channel cause the membrane depolarization [4, 22], thereby causing cell death.

Colicins can kill a cell through a single hit mechanism that implies that a single molecule is sufficient to depolarize the membrane. Colicin A channels can decrease the membrane potential from -165 to -85 mV [22]. The depolarization is not primarily responsible for cell death but it can affect the proton-motive force, which is needed for ATP synthesis. The reduced proton-motive force and the depleted phosphate pools rapidly depletes the ATP levels of the cell which ultimately leads to the death of the cell [1]. Colicin channels also cause the depletion of the potassium levels. In the absence of
potassium, colicins also decrease the respiration activity of the cell. Respiring bacteria in high potassium medium should retain their main metabolic functions [1]. This would explain the rescue of colicin effected cells in a medium with high levels of potassium. This implies that the decrease in intracellular ATP or potassium increases the probability of cell death.

From the colicin functional mechanism the following topics are still a matter of debate:

1. What is the topology of the closed channel state (the umbrella model or the penknife model)?
2. What is the topology of the open channel state?
3. What is the insertion mechanism when a stable water-soluble protein inserts into membranes?
4. What is the translocation mechanism?
5. Does the topology of the channel in model membranes differ from that in live \textit{E.coli} cells?

Answering these questions may give new insights into the functional mechanism of colicins and also into many general properties of other pore-forming proteins. In the present work, we aimed to answer questions 1, 3 and 5 by using the strategy of site-directed spin labeling (SDSL) in combination with electron paramagnetic spectroscopy (EPR).

\textbf{1.3 SDSL-EPR}

An understanding of many biological functions requires the knowledge of molecular structures of proteins and/or nucleic acids that are involved in the respective biological mechanisms. X-ray crystallography and NMR spectroscopy are general conventional methods to derive structural information of proteins. The limitations of these methods are the amount of protein needed for X-ray crystallography and the molecular weight, which should be \(< 200\) KDa for NMR. Though X-ray crystal structures are available for some of the membrane proteins, it is still very difficult to crystallize membrane proteins. The large structures of the membranes do not allow the application of solution NMR spectroscopy. Alternative methods such as solid-state NMR, EPR spectroscopy and cryo-electron microscopy are available to study the structural properties and topologies of membrane proteins. Among these methods, the advantage of using EPR is that the structural details of a protein can be studied in its
physiological environment. However, the disadvantage of EPR is that the proteins need to be mutated to have a single cysteine for spin labeling.

With the exception of some metallo-proteins, most of the proteins that do not have unpaired electrons cannot be directly studied by the EPR method. This situation is changed after the pioneering work of Wayne Hubbell who developed a method called site-directed spin-labeling (SDSL) [23] to introduce nitroxide spin labels (carrying a stable radical) at any desired site in a protein.

In SDSL, the natural amino acid at the selected position is replaced by a cysteine, which can be specifically labeled with a nitroxide spin label. For this, native cysteines of a protein should be replaced by suitable non-reactive amino acids. The spin labeling might cause perturbation in the protein structure and might affect the function of a protein. Therefore, the function of the spin-labeled proteins should be checked prior to study by EPR. The most commonly used spin label is the methanethiosulphonate (MTS) spin label that generates a side chain designated as R1 (Fig 1.5).

Figure 1.5: Site-directed spin labeling. The native amino acid (e.g. glycine) is mutated to cysteine and then cysteine is labeled with methanethiosulphonate spin label, which is designated as R1 side chain. The protein backbone is rendered in ribbon style (grey color).
SDSL combined with EPR has emerged as a powerful tool for monitoring the structure and dynamics of both soluble and membrane proteins. The strategy of SDSL-EPR has been extensively used to study the structure and topology of bacteriorhodopsin [24], T4 lysozyme [25], annexin 12 [26, 27], K⁺ channel [28] and colicin E1 [29]. Due to the tremendous advantages of EPR, we have chosen this technique to explore the structural details of colicin A. In the present work, for the first time we studied the dynamics of spin labeled proteins in living cells by using SDSL-EPR.

1.4 Description of the chapters

The contents of the following chapters are briefly described here.

Chapter 2: The EPR theory and its applications (mobility, accessibility and polarity) are described.

Chapter 3: Water-soluble colicin A and the membrane bound closed channel state are studied in detail by applying EPR mobility, accessibility and polarity methods. The detergent mediated reconstitution method is introduced to apply for pore-forming proteins. The results support the umbrella model.

Chapter 4: The pH dependent membrane insertion mechanism is studied in detail. A method to study the colicin A functionality in liposomes is introduced. The results also support the umbrella model for the closed channel state.

Chapter 5: This chapter will address pH dependent conformational changes of colicin A (pH 7-2) and answer the question at which pH colicin A forms a molten globule. In this chapter, we show how to use the EPR method to study unordered protein structures like molten globules, which are very difficult to study by conventional methods.

Chapter 6: Colicin A conformational changes upon interaction with the living E. coli cells are studied by EPR. The conformational changes of colicin A upon interaction with the wild type strain are compared with those determined for tolb knock out strain, suggesting that the observed conformational changes result from colicin A that was translocated from the outer to the inner membrane.

Chapter 7: Molecular dynamics simulations are applied to study the molecular details of the pH induced conformational changes. The MD trajectories are analyzed by essential dynamics analysis to obtain the concerted motions. The results suggest that colicin A at pH 2 is very dynamic and shows large displacements from the native structure. MD results are in close agreement with the EPR results discussed in chapter 5.
Colicin A conformational changes are compared with the conformational changes of colicin Ia and colicin N.

Chapter 8: Summary and outlook

1.5 References


Todd, A.P., et al., *Site-directed mutagenesis of colicin E1 provides specific attachment sites for spin labels whose spectra are sensitive to local conformation.* Proteins, 1989. 6(3): p. 294-305.
2. EPR theory and applications

2.1 Theory

The nitroxide spin label has a free radical, which occupies the 2p orbital of the N-O group. The paramagnetic properties of nitroxide radicals are due to the intrinsic angular momentum of the unpaired electron (spin). In a constant magnetic field, electron spins orient themselves in two directions, namely along the field and against the field (the Zeeman effect). Since, these orientations interact differently with the magnetic field, two Zeeman energy levels with different populations arise in the system. The energies of states of a nitroxide are described by the simple spin Hamiltonian, \( H_{\text{nitroxide}} \), neglecting the nuclear Zeeman term and the spin-spin interaction term:

\[
H_{\text{nitroxide}} = H_{\text{Electron-Zeeman}} + H_{\text{Hyperfine}}
\]

\[
= g \beta_e B S + S A I
\]

1. The first term (\( H_{\text{Electron-Zeeman}} \)) describes the interaction of the unpaired electron with a strong externally applied stationary magnetic field, \( B \). In general, this interaction is anisotropic as indicated by the \( g \) tensor. The constant \( \beta_e \) is the Bohr magneton and \( S \) is the electron spin operator.

2. The second term (\( H_{\text{Hyperfine}} \)) gives the hyperfine interaction of the unpaired electron with the \(^{14}\text{N}\) nucleus (nuclear spin \( I=1 \)). This interaction is described by a hyperfine tensor, \( A \). The nuclear spin operator is \( I \).

The term hyperfine interaction is obtained by summing the Fermi contact interaction and the anisotropic (i.e. dipolar) interaction \[1\].

\[
S AI = a_{\text{iso}} SI + S \hat{A} I
\]

Where \( a_{\text{iso}} \) is the isotropic coupling constant and \( \hat{A} \) is the magnetic dipole tensor. In solution, the anisotropic \( \hat{A} \) averages to zero and the observed hyperfine splitting gives the isotropic hyperfine coupling constant directly \[2\].

In the isotropic case, solving this Hamiltonian yields the magnetic energies and assuming that a constant magnetic field \( B \) is applied parallel to the \( Z \) direction, the spin vectors \( S \) and \( I \) are replaced by the magnetic quantum numbers \( M_S \) and \( M_I \).

\[
E_{\text{nitroxide}} = g_{\text{iso}} \beta_e B M_S + a_{\text{iso}} M_S M_I
\]
The allowed EPR transitions are those in which $\Delta M_S = \pm 1$ and $\Delta M_I = 0$. The corresponding three resonance frequencies for the allowed transitions are

$$\Delta E = g_{\text{iso}} \beta_e B + a_{\text{iso}} M_I \{+1,0,-1\} \quad (4)$$

When $M_I = 1$, 

$$\Delta E_1 = g_{\text{iso}} \beta_e B + a_{\text{iso}} \quad (5)$$

When $M_I = 0$, 

$$\Delta E_0 = g_{\text{iso}} \beta_e B \quad (6)$$

When $M_I = -1$, 

$$\Delta E_{-1} = g_{\text{iso}} \beta_e B - a_{\text{iso}} \quad (7)$$

The above equations 4, 5 and 6 explain the three-line pattern for the nitroxide. Fig. 2.1 depicts the energy states as function of B field and shows the three allowed transitions leading to a three-line first derivative EPR spectrum of a nitroxide in solution.

![Figure 2.1](image)

**Figure 2.1:** Energy levels of nitroxide ($S = \frac{1}{2}$ and $I = 1$) as function magnetic field. There are three resonance fields for three transitions. The resulting first derivative EPR spectrum is shown.

The success and importance of spin labels to study the structure and dynamics of proteins is based on the fact that the $g$ and $A$ tensors are anisotropic making the EPR spectra critically dependent on the rate and amplitude of the reorientational motion of
spin label. For a nitroxide in low viscosity solution, the anisotropic parts of the $g$ and $A$ tensors are averaged out, hence $a_{\text{iso}}$ and $g_{\text{iso}}$ are observed as shown in Fig. 2.2A.

In frozen solution, the nitroxides are completely immobilized and randomly oriented. The resulting powder spectrum, shown in Fig. 2.2B, is the envelope of the spectra of nitroxides in all possible orientations with respect to the applied magnetic field. When the symmetry is lower than axial, all three principal values of hyperfine tensor may be different. They are designated as $A_{XX}$, $A_{YY}$ and $A_{ZZ}$. As $A_{ZZ}$ is considerably greater than $A_{XX}$ or $A_{YY}$ for a nitroxide, it is possible to obtain a reasonably accurate value for $A_{ZZ}$ from such a powder spectrum from the separation between the low and high field peaks.

**Figure 2.2:** (A) First derivative EPR spectrum of a rapidly tumbling nitroxide spin label (in solution) (B) First derivative EPR spectrum of an immobilized and randomly oriented nitroxide spin label (in frozen solution).

### 2.2 Applications

The SDSL-EPR method is applied extensively to study the structure and function of proteins that are labeled with nitroxide spin labels (R1 side chains) [3, 4]. The fundamental types of information that can be obtained by this method are:

1. dynamics (mobility) of the nitroxide side chain,
2. accessibility of the nitroxide side chain towards paramagnetic quenchers soluble either in water or in lipid environments,
3. polarity of the nitroxide microenvironment,
4. distance between two nitroxides (not used in this work).
2.2.1 Dynamics of the side chain (Mobility)

The dynamics of a spin-labeled side chain can be characterized in terms of its “mobility”, which is used here in a general sense that includes effects due to the rate, amplitude and geometry of reorientational motion of the nitroxide ring [4]. The reorientational motion of the nitroxide side chain is contributed from: (i) internal dynamics of the side chain, (ii) local backbone fluctuations, and (iii) rotational diffusion of the protein. The rotational diffusion of proteins with molecular weight $>$50KD exceeds 30ns. Thus, it is negligible in the EPR time scale, when compared with rotational diffusion of the nitroxide side chain (~1ns) [5]. Consequently, the EPR spectra shown in the present thesis are considered to reflect mainly the internal dynamics of the R1 side chain and the local backbone fluctuations. The mobility of the spin-labeled side chain can be qualitatively analyzed by inspection of the EPR spectral line shapes and quantitatively assessed by the inverse of the line width of the central line ($\Delta H_0^{-1}$) and inverse of the second moment ($\langle \Delta H^2 \rangle^{-1}$) [3].

The dynamics of protein side chains primarily depend on their interactions with different structural elements of the protein. For example, the side chains in the protein interior are more restricted than the side chains in loop regions [6]. Hence, the degree of motional restriction of a nitroxide spin label depends on the secondary and tertiary structures of the spin-labeled site and its microenvironment. Fig. 2.3 shows the location of a spin label in a protein and the resulting EPR spectra.

Residues of helix surface and loop regions show a high degree of mobility due to weak interactions with the rest of the protein. In this case, the R1 side chain yields the EPR line shape with large amplitudes, small hyperfine splitting (narrow lines) and small line widths (Fig. 2.3A). In contrast, residues that are buried in the interior of the protein and those with strong interactions with other protein elements show a low degree of mobility. In this case, the R1 side chain is relatively immobilized, resulting in EPR spectra with large apparent hyperfine splitting, smaller amplitudes (due to spectral broadening), and large line widths (Fig. 2.3B).
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Figure 2.3: Mobility of the R1 side chain. The relationship between the topology of the spin-labeled side chains and EPR spectral shape is illustrated. Proteins that are spin-labeled at different positions are shown on the left side and the corresponding first derivative EPR spectra are given on the right side. The backbone of protein is rendered in ribbon style, the amino acids in stick and the spin label is in ball and stick style. (A) The spin label is located in a loop region. Since the loop regions are very dynamic, the spin label is very mobile as reflected in the EPR spectrum with small apparent hyperfine splitting and small line widths. (B) The spin label that is located in the protein interior where its motion is restricted by strong helical contacts is reflected in an EPR spectrum with large apparent hyperfine splitting and with large line widths. The dotted lines highlight the outer hyperfine splittings and the yellow bar is used to indicate the line widths.

Figure 2.4 shows a plot of $\Delta H_0^{-1}$ vs. $\langle \Delta H^2 \rangle^{-1}$ that represents the mobility of the nitroxide side chains of bacteriorhodopsin [7]. Sites from a given topographical region cluster together in specific regions of mobility in the plot. Thus, all buried sites (157, 160 and 167-171) are highly immobilized due to dense packing in the interior of the protein, whereas sites in loops (161 and 162) are highly mobile, presumably reflecting the backbone fluctuations (Fig. 2.4). Therefore, these semi-empirical parameters have been used to analyze the structure and dynamics of the protein structural elements.
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2.2.2 Accessibility to paramagnetic quenchers

Solvent accessibility of a side chain can be determined from the collision frequency of the nitroxide with freely diffusible paramagnetic quenchers (exchange reagents) that are soluble either in water or in lipid phase. Ni-EDDA (Nickel ethylenediamine-diacetic acid) or Crox (chromium oxalate) are used as probes for aqueous environment and molecular oxygen (O\textsubscript{2}) is used as a probe for membrane environment. Figure 2.5 illustrates the accessibility method.

In a membrane/water system, the nonpolar O\textsubscript{2} preferentially concentrates in the hydrophobic region of the membrane with a well defined intra-membranous concentration profile [8], whereas Ni-EDDA is restricted to the aqueous environment being excluded from the core of the lipid bilayer [4, 8]. This behavior can be applied to determine the localization of the spin-labeled side chains in a protein [9]. For example, the nitroxide side chain located at a protein surface is highly accessible to Ni-EDDA and O\textsubscript{2}. In contrast, the nitroxide side chain that is located in the protein interior is accessible neither to Ni-EDDA nor to O\textsubscript{2}. The nitroxide side chain, which is located in the membrane bilayer, shows accessibilities higher to O\textsubscript{2} and negligible to Ni-EDDA. Accordingly, this method of EPR is very useful to study the topology of membrane proteins.
Figure 2.5: Accessibility of the R1 sites. (A) Schematic illustration of the accessibility of the R1 side chain to the paramagnetic quenchers, O₂ and Ni-EDDA. The molecular oxygen concentration is higher in the membrane due to its hydrophobic nature whereas the Ni-EDDA concentration is higher in the aqueous compartment. (B) Plots of the saturation curves showing the central peak-peak amplitudes vs. increasing microwave power. From the saturation plots, the accessibility in terms of exchange rate ($W_{ex}$) was determined as described in the text.

Collisions between spin labels and exchange reagents (O₂ and Ni-EDDA) lead to Heisenberg spin exchange, which requires a direct contact interaction in an encounter complex [10]. Therefore, the Heisenberg exchange rate ($W_{ex}$) can be used as a measure of exposure of the nitroxide to the solvent, which contains the exchange reagents. When the longitudinal relaxation time, $T_1$ of an exchange reagent is shorter than the encounter complex life time, the Heisenberg exchange leads to changes in $T_1$ and $T_2$ of spin labels which lead to a resistance in saturation [10].

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

(8)

Changes in relaxation times of the nitroxide are reflected in changes in the values of $P_{1/2}$, a parameter that can be obtained from the continuous wave saturation curves.

$$P_{1/2} = \frac{(2^{2/3} - 1)}{\Lambda^2 \gamma^2 T_1 T_2}$$

(9)

Where $\Lambda$ = is the resonator efficiency and $\gamma = g\beta 2\pi / h$.

Saturation curves (Fig. 2.5) are determined from peak-peak amplitudes ($I_{pp}$) of the central resonance line ($H_0$) of the first derivative EPR spectra, which are measured at different incident microwave power levels. $P_{1/2}$ is defined as the power level of the incident radiation at which the amplitude of the saturated line is half of the amplitude in
the absence of saturation. Values of $P_{1/2}$ are calculated from fitting of the following function to the experimental amplitudes.

$$I_{pp} = \frac{F \sqrt{P}}{1 + \left(2^{1/2} - 1\right)P/P_{1/2}}$$  \hspace{1cm} (10)

$F$, $P_{1/2}$ and $\varepsilon$ are adjustable parameters. $F$ is a scaling factor, $P_{1/2}$ is the power where the first derivative amplitude is reduced to half of its unsaturated value, and $\varepsilon$ is the measure of the homogeneity of the resonance line broadening. For the homogeneous and inhomogeneous broadening limits, $\varepsilon = 1.5$ and $\varepsilon = 0.5$, respectively. In the present experiments, under nitrogen and at relatively low collision frequencies, $\varepsilon$ is close to 1.5, indicating homogeneous broadening. A homemade program was used to fit the saturation curves and to determine the $P_{1/2}$ values.

For the common case where $W_{ex} << 1/T_2$, $T_2$ may be taken as a constant \[10\], and

$$\Delta P_{1/2} = P_{1/2} - P_{1/2} (N_2)$$  \hspace{1cm} (11)

$$= \left(\frac{2^{2/3} - 1}{\Lambda^2 \gamma^2 T_2}\right)(W_{ex})$$  \hspace{1cm} (12)

Where $P_{1/2}$ is determined in the presence of exchange reagent and $P_{1/2} (N_2)$ is determined in the absence of exchange reagent. Although $\Delta P_{1/2}$ is proportional to $W_{ex}$, it depends on the motion of the nitroxide through $T_2$ and on properties of the resonator through $\Lambda$. To reduce or eliminate these dependencies, a dimensionless quantity $\Pi$, a widely used accessibility parameter is defined as

$$\Pi (O_2) = \frac{P_{1/2} (O_2)}{\Delta H_0 (O_2)} - \frac{P_{1/2} (N_2)}{\Delta H_0 (N_2)}$$  \hspace{1cm} (12)

$$\Pi (Ni-EDDA) = \frac{P_{1/2} (Ni - EDDA)}{\Delta H_0 (Ni - EDDA)} - \frac{P_{1/2} (N_2)}{\Delta H_0 (N_2)}$$  \hspace{1cm} (13)

$P_{1/2}$ values were divided by peak-peak line width of the central line ($\Delta H_0$) to avoid the contribution of spin-spin relaxation effects while comparing saturation curves of spin labels with different line shapes. (Note: Here, the $\Pi$ values are not normalized by the corresponding value of DPPH).
The Heisenberg exchange rates which are proportional to the collision rates are related to the accessibility parameter $\Pi$ by

$$W_{ex} = \frac{\Pi}{\alpha}$$  \hspace{1cm} (14)

Where $\alpha$ represents a cavity dependent normalization factor. For the used cavity type, $\alpha = 2.001$ (derived by M. Döber, personal communication).

### 2.2.3 Polarity

The principal values of the hyperfine tensor of a nitroxide are dependent on its polarity of its environment [4]. The hyperfine interaction depends in fact on the localization of the electron density, which is influenced by the surrounding polarity and the effect is shown in Fig. 2.6.

![Figure 2.6: Localization of the electron density on the nitroxide group. The unpaired electron is shown as arrow between 2p orbitals of the nitrogen and oxygen. (A) If the oxygen forms a hydrogen bond, the electron density shifts towards the nitrogen atom. This is shown by the large 2p orbital of the nitrogen. (B) In hydrophobic environment, the electron density in the 2p orbital of the nitrogen is decreased.](image)

In a nonpolar environment, the unpaired electron density is shifted towards the oxygen atom resulting in minimal hyperfine interaction. In case of polar environment, the electron density is shifted towards the nitrogen atom of the nitroxide group (Fig. 2.6), resulting in enhanced hyperfine interaction. This enhanced hyperfine interaction is reflected in an increased hyperfine splitting that is measured by the $A_{ZZ}$ parameter, shown in Fig. 2.7A. Larger $A_{ZZ}$ values indicate a polar environment around the
nitroxide side chain and, in contrast, smaller $A_{ZZ}$ values indicate a nonpolar environment (Fig. 2.7A). For example, the $A_{ZZ}$ values for several labeled positions of the bacteriorhodopsin F helix [4] are shown in Fig. 2.7B. According to the crystal structure, the residues 162 and 168 are located at the end of transmembrane helix. They show high $A_{ZZ}$ values (> 3.6 mT) while the residues being located in the middle of the transmembrane helix show low $A_{ZZ}$ values (~ 3.35 mT). Especially, these polarity profiles are very useful to study the location of the nitroxide side chains in the membrane proteins or to determine the topology of a membrane protein.

Figure 2.7: Polarity of the nitroxide microenvironment. EPR spectra of MTS spin label in nonpolar (continuous line) and polar (dotted line) environments. X-band spectra of bacteriorhodopsin variants L93R1 (protein interior, continuous line) and K129R1 (extracellular surface, dotted line). (B) The polarity parameter, $A_{ZZ}$ of nitroxides located on helix F of bacteriorhodopsin spanning the transmembrane bilayer. (This figure was adopted from Savitsky et al. [4]).

### 2.2.4 Inter-nitroxide distance

The inter-residual distances can be obtained by labeling the protein with spin labels at two desired positions and analyses of the spin-spin interaction between these two spin labels which is composed of dipolar interaction and exchange interaction [4]. In the present work, inter nitroxide distances are not measured.
2.3 References

2. L.Vos, W., Distance constraints from site-directed spectroscopy as a tool to study membrane protein structure. 2006, Wagwningen University.
3. Conformation of the closed channel state of colicin A in proteoliposomes: An umbrella model.

**Abstract:** Colicin A is a water-soluble toxin, which forms a voltage-gated channel in the cytoplasmic membrane of *E. coli* [1]. Until now, two models were proposed for the closed channel state: the umbrella model and the penknife model. In order to study the membrane-bound closed channel state, mutants of colicin A, each containing one cysteine were labeled with a nitroxide (MTS) spin label, reconstituted into liposomes and studied by EPR. The spin-labeled colicin A in solution and in liposomes was analyzed in terms of nitroxide mobility, accessibility to paramagnetic reagents and polarity. The nitroxide mobility and accessibility data of soluble colicin A are in excellent agreement with the crystal structure of the colicin A pore-forming domain. Moreover, the EPR studies suggest that colicin A has a completely different conformation in liposomes when compared with its water-soluble conformation. The residues that are buried in the soluble colicin A are exposed to the lipid environment in the reconstituted colicin A. The investigated residues that belong to H8 and H9 helices show accessibilities significant to O2 and negligible to Ni-EDDA, indicating their location inside the membrane. These results are also supported by the low polarity values (Azz) of the residues 176, 181 and 183. Additionally, the accessibility and polarity data suggest that the spin-labeled side chains of the amphipathic helices (H1-H7 and H10) are located at the membrane water interface. The EPR results support the umbrella model for the closed channel state of colicin A.

3.1 Introduction

The pore-forming domain of colicin A penetrates into the membrane to perform its function and has three possible conformations: a water-soluble state, a closed channel state and an open channel state. The X-ray crystal structure of the water-soluble pore-forming domain of colicin A with 2.4 Å resolution was reported by Parker et al [2]. It is a globular structure with a bundle of ten α-helices (H1-H10) that are arranged into three layers. Among these ten, H8 and H9 helices (hydrophobic hairpin) are completely hydrophobic and tightly packed by the remaining eight amphipathic helices, which protect the hydrophobic hairpin from the surrounding aqueous environment.
Since colicin channels are regulated by a voltage gating mechanism, the membrane-bound colicin exists in an open and closed channel states [3, 4]. Although the x-ray crystal structure is available for the water-soluble pore forming domain of colicin A, there are no clear structural details available for the membrane-bound states. A few studies reported that a trans negative potential drives the insertion of amphipathic hairpin 5-6 into the membrane to open the channel [5]. Colicin channels are closed when there is no trans negative potential [5, 6] and the amphipathic helices move back from the trans-membrane orientation and stay close to the surface of the membrane bilayer [7]. The involvement of the hydrophobic hairpin in the membrane insertion has been proven [6] but its orientation with respect to the membrane plane is still ambiguous. Hence, in the present study, we aimed to elucidate the structure and topology of the closed channel state of colicin A. This closed channel state has been studied extensively in vitro and two models were proposed: the umbrella model and the penknife model (Fig. 3.1).

Figure 3.1: Models for the closed channel state of colicin A. Colicin A pore-forming domain is rendered in cartoon style. The amphipathic helices are colored in green and the hydrophobic hairpin (H8 and H9) is colored in magenta.

In the umbrella model [2], the hydrophobic hairpin (H8 and H9) is arranged in a transmembrane orientation and the amphipathic helices are located on the surface of the membrane. In the penknife model [8, 9], all helices are arranged parallel to the plane of the membrane. The arrangement of helices H3-H10 remains similar both in the water-
soluble and in the penknife model but with looser packing compared to that in solution. The separation of H1 and H2 from the bundle may expose the hydrophobic faces of H8 and H9, which bind to, but do not penetrate the membrane phase. H1 and H2 lie with their hydrophobic faces exposed to the membrane just as in the umbrella model [8, 10].

Solid-state NMR [11], FRET [12], FTIR, EPR [13] and other biochemical experiments for colicin E1 and the planar bilayer experiments for colicin Ia supported the umbrella model for the closed channel state. However, in the case of colicin A, the topology of the closed channel state is still unclear. Parker et al. [2] proposed the umbrella model for colicin A which is also supported by Gonzalez-Manas et al. [14]. In contrast to that, Lakey et al. [9] proposed the penknife for colicin A in which the hydrophobic hairpin is orientated parallel to the plane of the membrane. The penknife model for colicin A was also supported by fluorescence experiments [9], proteolysis [15], neutron scattering [16], disulphide bond engineering [17], and by EPR [18] experiments. Consequently, the question arises whether the closed channel state of colicin A is different when compared with that of E1 and Ia. Nevertheless, the conformation of the closed state of colicin A is still controversial. Therefore, we investigated the closed state of colicin A, which might clarify the ambiguity of the closed channel conformations of many water-soluble toxins, as the colicins have structural similarity with other toxins like diphtheria toxin, anthrax, tetanus, haemolysin, and equinatoxin. Additionally, the structural elucidation of the closed state might provide new insights to understand the conformational changes between closed and open channel states.

In the present work, SDSL-EPR [18, 19] was used to explore the structural details of the membrane-bound colicin A. The present work also demonstrates the validity of the detergent-mediated reconstitution method for water-soluble proteins, addressing specific protocols to separate the unbound protein from the proteoliposomes.

### 3.2 Methods

#### 3.2.1 Preparation and purification of Colicin A

**Bacterial strains and growth conditions:** *Escherichia coli* K-12 C600 mutants, each containing single cysteine mutations in *cau* gene at positions 26, 33, 42, 62, 91, 105, 115, 154, 166, 169, 176, 181, 183, 184 and 192 were used for protein preparation. Dr. Denis Duchê (CNRI, Marseille, France) kindly provided these strains, which were
mainly designed for FRET experiments. Bacteria were grown at 37°C in LB (Luria-Bertani) medium that contains 10 g/L of peptone, 5 g/L of yeast extract and 10 g/L of NaCl. For colicin production, bacterial growth was carried out with rotative shaking (Aerotron shaker, Infors) for aeration during incubation.

**Inoculation:** *E. coli* cells were grown overnight in LB medium containing ampicillin (100 mg/L). Four liters of fresh LB medium with ampicillin (100 mg/L) were inoculated with 40 ml of the overnight culture per liter to obtain an OD₆₀₀ = 0.1 and grown at 37°C. **Induction:** At OD₆₀₀ = 0.7-1.0, the protein expression was induced by mitomycin C (300 ng/ml) and grown for further 4 hours.

**Separation of the protein fraction:** The culture medium was centrifuged at 4500 rpm, 4°C for 20 minutes using a Sorvall centrifuge equipped with a SLA 3000 rotor. The supernatant was collected and the proteins were precipitated with ammonium sulphate (243 g/L) at 4°C. After one hour, the solution was centrifuged at 4200 rpm, 4°C for 45 minutes in the same rotor. The pellet was resuspended in 10 mM sodium phosphate buffer, pH 6.8 and the solution was dialyzed overnight against 10 L of 10 mM sodium phosphate buffer, pH 6.8 to remove the remaining ammonium sulphate. The buffer was exchanged twice.

**Fast Protein Liquid Chromatography (FPLC):** The dialyzed solution was centrifuged at 15000 rpm, 4°C for 45 minutes using a Sorvall centrifuge equipped with a SLA 34 rotor. The supernatant was loaded onto a Hitrap SP FF column at 4°C using FPLC system (Pharmacia). The column was equilibrated with 10 mM sodium potassium buffer, pH 6.8 with a flow rate of 4 ml/minute. The protein that bound to the column was eluted by a 0 - 400 mM NaCl gradient. The elute was monitored at 280nm by an UV detector. Fractions of 5ml were collected and the corresponding fractions to the peak given from UV detection were collected and checked for colicin A presence using SDS-PAGE electrophoresis.

**SDS-PAGE Electrophoresis:** The stacking gel was prepared with 1.25 ml of Tris-HCl buffer, 25 µl of 20% (w/v) SDS, 3.75 ml of an acrylamide solution, 3.70 ml of bidistilled water, 25 µl of 10% (w/v) ammonium persulphate and 5µl of TEMED. The resolving gel was prepared with 2.5 ml of Tris-HCl buffer, 50 µl of 20% (w/v) SDS, 0.49 ml of an acrylamide solution, 3.2 ml of bidistilled water, 25 µl of 10% (w/v) ammonium persulphate and 5µl of TEMED. The gels with loaded samples were run for 30 - 45 minutes. Subsequently, the gels were stained with Comassie Blue for visualization of the protein bands (Fig. 3.2).
3.2.2 Spin labeling of colicin A

3.2.2.1 Spin labeling of colicin A with water exposed cysteine

Colicin A bearing a unique cysteine was spin-labeled with the methane-thiosulphonate (MTS) spin label. Freshly prepared 1M DTT stock solution was added (10 mM final conc.) to the pooled colicin A fractions and incubated overnight at 4°C. DTT was removed by exchanging the buffer 5 times (1:5 dilution each) with 10 mM potassium phosphate buffer (pH 6.8) by using an Amicon stirred cell (Millipore) at 3-4 bars pressure under argon. The MTS spin label stock solution (100 mM) in acetonitrile was added to the concentrated protein solutions (1mM final conc. of MTS) and incubated overnight at 4°C. The unbound free spin label was removed by overnight dialysis against 10L phosphate buffer (pH 6.8) and the spin-labeled colicin A was concentrated to 10 mg/ml by using PEG 20000.

3.2.2.2 Spin labeling of colicin A with buried cysteine

The freshly prepared DTT stock solution was added to the pooled colicin A fractions (10 mM final conc.) and incubated overnight at 4°C. DTT was removed by exchanging the buffer three times (1:5 dilution) with potassium phosphate buffer (pH 6.8) using an Amicon cell at 3-4 bars pressure under argon. The buffer was exchanged
twice with 10 mM potassium phosphate buffer containing 6M guanidinium hydrochloride. The MTS spin label stock solution (100 mM) in acetonitrile was added to the concentrated protein solutions (1 mM final conc. of MTS spin label) and incubated overnight. The unfolded and labeled protein was folded back by the pulse dilution method. In pulse dilution, the unfolded protein was added to one liter of buffer drop by drop while stirring thoroughly. After a while, the resulting solution was centrifuged at 4200 rpm, 4°C for 45 minutes using a Sorvall centrifuge equipped with the SLA 3000 rotor. The supernatant was collected with care and the buffer was exchanged by continuous flow dilution and then concentrated to 1 mg/ml by using a Masterplex tangential flow system. The spin-labeled protein samples were further concentrated to 5-7 mg/ml by using a 50 ml stirred cell (Millipore). The protein was further concentrated to 10 -15 mg/ml using PEG 2000. Finally, the remaining free spin label was removed by overnight dialysis if necessary and the spin-labeled protein was stored at -80°C.

3.2.3 Preparation of Liposomes

3.2.3.1 Preparation of large multilamellar vesicles (LMV)

*E. coli* natural lipids dissolved in chloroform (purchased from Avanti Polar Lipids, Inc.) were taken in a glass beaker and then the solvent was evaporated with a stream of nitrogen for 2 hours. The dry lipid film on the bottom of the glass beaker was hydrated with 50 mM potassium phosphate buffer (pH ~ 7.5) that was bubbled with argon gas and stirred vigorously until the lipid film was completely hydrated. The resulting suspension of large multilamellar vesicles (LMV) with a lipid concentration of 20 mg/ml was stored under liquid nitrogen. Extensive care was taken throughout the preparation process to protect the lipids from light and air.

3.2.3.2 Preparation of large unilamellar vesicles (LUV)

To prepare LUV, the stable LMV suspension was extruded using a Mini Extruder (Avanti Polar Lipids, Inc.). Lipid extrusion is a technique in which the lipid suspension is forced through a polycarbonate filter with a defined pore size to yield liposomes with the size of the pore. The LMV suspension was disrupted by five freeze-thaw cycles and then extruded through a 400 nm pore filter to obtain large unilamellar vesicles with an average diameter of 400 nm. The extrusion was performed several times until the lipid suspension became transparent.
3.2.4 Detergent-mediated reconstitution

The method which is familiar to reconstitute membrane proteins [20] was modified and optimized to reconstitute the membrane-bound conformation of water-soluble toxins in liposomes. A schematic representation of detergent-mediated reconstitution of colicin A into proteoliposomes is shown in Fig. 3.3.

Zwittergent 3-10 or n-decyl -N, N- dimethyl -3-amino-1-propane sulphonate (purchased from Calbiochem) was used for reconstitution of colicin A into liposomes. Spin-labeled colicin A (2 mg/ 200 µl) and LUVs (15 mg/ 1 ml) were solubilized with Zwittergent 3-10 at 40 mM final concentration. The combined mixture of the detergent solubilized protein and LUVs was shaken vigorously for 10 minutes. Afterwards, the detergent was removed step-wise using bio-beads. Bio-beads (5 times of the detergent weight) were added to the solubilized protein-LUV mixture and shaken vigorously for one hour at room temperature. The bio-beads were then exchanged by fresh bio-beads.

**Figure 3.3:** Schematic representation of the Zwittergent-mediated reconstitution method. (a) Large unilamellar vesicles (b) Spin-labeled colicin A (c) Detergent destabilized liposomes (d) Detergent solubilized protein. The detergent is removed by bio-beads. (e) Resulting proteoliposomes.
and shaken well. This process was repeated three times and finally the double amount of bio-beads was added and shaken vigorously overnight at 4°C. After removing the bio-beads, the resulting proteoliposomes were dialyzed for 3 hours against one liter of 50 mM potassium phosphate buffer (pH ~ 7.5) by exchanging the buffer for three times. The resulting solution contains proteoliposomes, liposomes and unbound spin-labeled colicin A. This method has been proven to work not only with colicin A but also with other water-soluble proteins that insert into membranes (data not shown).

### 3.2.5 Separation of the unbound protein from the proteoliposomes

Unbound protein was separated from proteoliposomes by using the nycodenz gradient flotation method. A suspension (1 ml) of proteoliposomes was mixed with 1ml of 20% nycodenz and loaded at the bottom of a centrifugation tube. This was followed by 3 ml of 5% nycodenz and finally 1ml of 50 mM potassium phosphate buffer, pH 7.5. The appearance of the gradient before and after centrifugation is shown in Fig. 3.4.

![Figure 3.4: Separation of proteoliposomes from the unbound protein using a Nycodenz density gradient floatation method.](image)

The gradient tube was centrifuged at 200,000×g, 10°C for 30 minutes by using a Sorvall ultracentrifuge with a S80-AT3 rotor. The light yellowish band (formed between the 5% nycodenz and phosphate buffer) contains proteoliposomes without unbound protein. This band was collected and then washed in 5 ml of 50 mM phosphate buffer (pH 7.5) and centrifuged again at 233,000×g for 20 minutes. The sedimented proteo-
liposomes were resuspended in 50 µl potassium buffer (pH 7.5) and used for further EPR analysis.

### 3.2.6 EPR measurements

EPR experiments were performed with the spin-labeled colicin A mutants in solution (10 mM potassium phosphate buffer, pH 7.4) and in liposomes (50 mM potassium phosphate buffer, pH 7.4).

**Mobility**: Room temperature EPR spectra were obtained using a Varian model E-101 X-band spectrometer fitted with a Bruker dielectric resonator. All spectra were obtained at 0.5 mW incident microwave power and a field modulation of 0.15 mT. Glass capillaries with 1 mm diameter were used to load 10 µl of the sample. The inverse of the central line width ($\Delta H_0^{-1}$) was determined manually and the inverse of the second moment ($\langle H^2 \rangle^{-1}$) was determined using Unispec07 program (C.Beier).

**Accessibility**: The collision frequencies of spin labels with paramagnetic quenchers (exchange reagents) were determined by the EPR (CW) power saturation method. The paramagnetic quenchers used in the present study are $O_2$ and Ni-EDDA (Nickel-ethylene diamine diacetic acid). Samples were loaded into gas permeable TPX capillaries and EPR spectra were recorded on a home made X-band EPR spectrometer fitted with a loop-gap resonator. In order to determine the saturation behavior of spin labels in the presence of $O_2$, the sample in the TPX capillary was equilibrated with air while recording the spectra. To determine the saturation behavior in the absence of any exchange reagents, the sample in the TPX capillary was equilibrated with $N_2$. To determine the saturation behavior in the presence of Ni-EDDA, 25 mM final conc. of Ni-EDDA was added to the sample. The program Powerfit (M.Kühn) was used to obtain $P^{1/2}$ values and $W_{ex}$ were calculated as described in chapter 2.

**Polarity**: 40 µl of the sample was loaded into quartz capillary and spectra were obtained at 160°K from a home made X-band EPR spectrometer. The spectra were fitted with Dipfit program [21] to obtain $A_{zz}$ values.

### 3.3 Results

Cysteine replaced residues (each at one time) at positions 26, 33, 42, 91, 105, 115, 154, 166, 169, 176, 181, 183, 184 and 192 were labeled with MTS spin label, which is designated as R1 [19]. These positions in the crystal structure of colicin A [2] are shown in Fig. 3.5.
Figure 3.5: Spin-labeled positions. (A) X-ray crystal structure of water-soluble pore-forming domain of colicin A (1COL.pdb [2]) with the mutated cysteine positions. The backbone is displayed in ribbon style and the sites where R1 was substituted are identified as spheres on the Cα atom positions (violet color). The amphipathic helices are colored in green and the hydrophobic hairpin is colored in magenta. (B) Amino acid sequence of the colicin A pore forming domain. The spin-labeled positions are marked by the small MTS structures.
According to the crystal structure, residues 26(H1), 33(H2), 42(H2), 62(H3), 91(H5), 105(H5a), 115(H6) and 192(H10) are located within the amphipathic helices whereas residues 154(H8), 169(H9), 176(H9), 181(H9), 183(H9) and 184(H9) are located within the hydrophobic hairpin (Fig. 3.5). Residue 166 is located in the H8/H9 loop region. Residues 26, 42, 105, 192 and those located at H8 and H9 are buried in the interior of the protein. In contrast to that, residues 33, 62 and 115 are located on the surface of the helices. Residues 33, 91 and 169 form the first \( \alpha \) helical turn (N-terminal end of the helix) of helices H2, H5 and H9, respectively.

In the present work, the conformation of the water-soluble colicin A (sol-ColA) and of the reconstituted colicin A (rec-mem-ColA) were analyzed by means of three EPR parameters [18]:

1. mobility of the R1 side chain,
2. accessibility of the R1 side chain to O\(_2\) and Ni-EDDA, and
3. polarity of the R1 side chain in its microenvironment.

**Mobility of the R1 sites:** The EPR spectra of the investigated R1 sites of the sol-ColA and the rec-mem-ColA are shown and compared in Fig. 3.6. In sol-ColA, residues 26R1(H1), 42R1(H2), 105R1(H5a), 154R1(H8), 176R1(H9), 183R1(H9) and 192R1(H10) show large apparent hyperfine splitting (Fig. 3.6), indicating that these residues are strongly immobilized. This is a characteristic feature of R1 side chains that are buried in the protein interior [18, 19]. In contrast to that, residues 33R1(H2) and 166R1(H8/H9 loop) show sharp and narrow lines (Fig. 3.6), revealing their high mobility which is a characteristic feature for residues that are located in the loops regions. Residues 62R1(H3), 115R1(H6) and 181R1(H9) show a small apparent hyperfine splitting (Fig. 3.6), characteristic for residues in helix-surface regions. Residues 91R1(H5), 169R1(H9) and 184R1(H9) have both prominent immobile and mobile components, reflecting two spin populations with a different rotamer of the R1 side chain. This is an evidence that these residues are subjected to tertiary contacts.

In case of the R1 sites of rec-mem-ColA, the spectra do not show powder spectra like behavior, suggesting lack of strong tertiary interactions, which is in contrast to the several sites of the sol-ColA. However, almost all spectra from rec-mem-ColA show two resolved components, a mobile and an immobile one. A similar behavior was reported previously for residue 165R1 of bacteriorhodopsin [22], which was shown to exhibit an equilibrium between two populations with different mobilities. Similar to
this, all investigated side chains of rec-mem-ColA (except 26R1) are characterized by the presence of two motional states with a small difference in the mobility. The narrow lines observed for 26R1(H1) indicate high mobility of the nitroxide attached to this site.

Figure 3.6: Comparison between EPR spectra of R1 labeled sol-ColA and rec-mem-ColA. The spin-normalized spectra of R1 sites of sol-ColA (pH 7.4) are colored black. Spectra of R1 sites of rec-mem-ColA (pH 7.4) are colored red. The liposomes were purified by gradient centrifugation to avoid the unbound protein. Spectra are overlaid to show the changes in amplitudes and line shapes. Spectra were acquired at room temperature over a scan width of 13mT. Residue number is given on the left side for each spectrum. Bars are placed at the low field spectral line to visualize the mobile and immobile components.
In addition to the line shape analysis, the mobility of the R1 side chain can be assessed quantitatively by two semi-empirical mobility parameters, the inverse of the central line width ($\Delta H_0^{-1}$), and the inverse of the second moment ($\langle H^2 \rangle^{-1}$). A plot of mobility ($\Delta H_0^{-1}$) values for the R1 sites of both sol-ColA and rec-mem-ColA is shown in Fig. 3.7.

As evident in Fig. 3.7, in sol-ColA, residues 33R1(H2), 62R1(H3), 115R1(H6) and 166R1(H8/H9 loop) show high mobility (values of $\Delta H_0^{-1}$), suggesting their location either on the helix-surface or in the loop regions (Fig. 3.7). The lowest mobility, characteristic of buried residues is found for 42R1(H2), 105R1(H5a), 154R1(H8), 176R1(H9) and 183R1(H9). The residues 26R1(H1), 91R1(H5), 181R1(H9), 184R1(H9) and 192R1(H10) show intermediate mobility, presumably arising from tertiary interactions (Fig. 3.7). In rec-mem-ColA, residue 26R1 shows significant mobility, residues 33R1, 42R1, 62R1, 91R1, 105R1, 115R1, 154R1, 184R1, 166R1, 183R1, 192R1 show intermediate mobility and 169R1, 176R1, 181R1 show less mobility (Fig. 3.7).

The inverse of the second moment ($\langle H^2 \rangle^{-1}$), another measure of R1 mobility is sensitive to the changes in apparent hyperfine splitting. A plot of $\Delta H_0^{-1}$ versus $\langle H^2 \rangle^{-1}$
was used in the previous studies [23, 24] to map the side chains from different topographical regions of the protein fold. Thus buried, surface and loop residues each cluster together in regions of low, intermediate and high mobility, respectively. Fig. 3.8 shows such a mobility map for sol-ColA and rec-mem-ColA. The mobility map clearly shows the differences in mobilities of the R1 sites between sol-ColA and rec-mem-ColA. In the mobility plot, residues (except 26R1) that belong to surface and buried regions in sol-ColA are moved to helix/contact regions in rec-mem-ColA. In contrast to other residues, 26R1 belongs to the loop/surface region in rec-mem-ColA.

**Figure 3.8:** Mobility map ($\Delta H^{-1}$ vs. $\langle H^2 \rangle^{-1}$) for sol-ColA (black squares) and rec-mem-ColA (red circles).

**Accessibility of R1 sites of sol-ColA:** The structural information provided by the mobility analysis can be supplemented with the patterns of the residue accessibility towards paramagnetic exchange reagents like Ni-EDDA or O$_2$. The accessibility can be deduced from the Heisenberg exchange rates ($W_{ex}$) of the nitroxide with the exchange reagents. The information provided by the accessibility data enable to clarify whether the R1 sites are exposed either to the lipid phase, to the aqueous phase or to the protein interior. O$_2$ preferentially partitions into the hydrophobic core of the lipid bilayer due to its hydrophobic nature, whereas Ni-EDDA preferentially partitions in the aqueous phase and is excluded from the core of the membrane bilayers. Consequently, the membrane-
exposed residues exhibit high accessibility to O₂ and negligible to Ni-EDDA. Fig. 3.9A shows the accessibility patterns of the R1 sites of sol-ColA.

**Figure 3.9:** Accessibility parameter. (A) Exchange rates ($W_{ex}$) of R1 sites of the sol-ColA for O₂ and Ni-EDDA. For a comparison, the mobility values are shown (grey trace) (B) Exchange rates ($W_{ex}$) of R1 side chains of the rec-mem-ColA for O₂ and Ni-EDDA. The R1 sites with significant accessibility to O₂ and negligible accessibility to Ni-EDDA indicate the residues location in the membrane, they are marked with a dot-lined square. The $W_{ex}$ values were determined in the presence of 25 mM Ni-EDDA or in aqueous solution equilibrated with air.
The overall exchange rates for O\(_2\) are lower than the exchange rates for Ni-EDDA, because O\(_2\) is less soluble in a polar environment. Residues 26R1(H1), 33R1(H2), 62R1(H3), 115R1(H6) 166R1 (H8/H9) show high accessibility to both Ni-EDDA and O\(_2\) (Fig. 3.9A), indicating their exposure to the aqueous environment. The partial accessibility of 91R1 to Ni-EDDA suggests that this residue is just partially exposed due to tertiary interactions. Residues 42R1(H2), 105R1(H5a), 154R1(H8), 169R1(H9), 176R1(H9), 181R1(H9), 183R1(H9), 184R1(H9) and 192R1(H10) are neither significantly accessible to Ni-EDDA nor to O\(_2\), characteristic for residues that are buried in a protein. However, these buried residues (176R1) are slightly accessible to O\(_2\) when compared to Ni-EDDA, due to size exclusion effects that limit the diffusion of larger Ni-EDDA complex into the protein interior. Comparison of the accessibility data with the data of mobility parameter (Fig 3.9A) shows that the residues that are located in helix/surface and loop regions show higher accessibility to Ni-EDDA and higher mobility whereas the buried residues shows negligible accessibility to Ni-EDDA and low mobility. From the above results, we can conclude that the conformation of sol-ColA is a tightly packed globular structure. The structural information derived from the EPR data is in agreement with the X-ray crystal structure (1COL.pdb).

The accessibilities of the R1 sites of rec-mem-ColA are shown in Fig. 3.9B. The overall accessibility to O\(_2\) is higher than the accessibility to Ni-EDDA, suggesting the incorporation of colicin A in the lipid bilayer of the proteoliposomes. According to their accessibility patterns (Fig. 3.9B), the R1 sites of the rec-mem-ColA can be clustered into two groups:

1. The residues of helices H1-H7 (33R1, 42R1, 62R1, 91R1, 105R1, 115R1), the C-terminal end of H9 (184R1) and H10 (192R1) show considerable accessibility to O\(_2\) and lower accessibility to Ni-EDDA (Fig. 3.9B), suggesting that these residues are located at the membrane-water interface. All these residues, except 184R1 belong to the amphipathic helices.

2. Residues 166R1 (H8/H9 loop) and 169R1(H9) show high accessibility to O\(_2\) while 154R1(H8), 176R1(H9), 181R1(H9), 183R1(H9) show considerable accessibility to O\(_2\) like the residues of amphipathic helices. Furthermore, all residues that belong to H8 and H9 (except 184R1) show negligible accessibility to Ni-EDDA, suggesting that these residues are located deeply embedded in the membrane.
To summarize the characteristics of the investigated R1 side chains, the values of $W_{ex}$ for $O_2$ are plotted against those for Ni-EDDA and shown in Fig. 3.10.

![Figure 3.10](image)

**Figure 3.10:** Accessibility map of R1 side chains of sol-ColA (black squares) and of rec-mem-ColA (red circles). The values were determined in the presence of 25mM Ni-EDDA and equilibrated with air. The two-dimensional space of the plot is divided into three areas: protein internal or buried region (white), aqueous phase (blue) and lipid phase (yellow). The dotted line indicates where the ratio of $W_{ex}$ for Ni-EDDA and $O_2$ is one. This represents the accessibility close to the head group region of the lipids.

This plot facilitates to discriminate the nitroxide groups that are located in the protein interior, at the protein-lipid interface, in the lipid or in the aqueous phases [25]. Residues located in the protein interior are characterized by low accessibility for both $O_2$ and Ni-EDDA, due to fact that the buried residues are not accessible for direct collisions with paramagnetic reagents. Residues exposed to the lipid phase are characterized by high accessibility to $O_2$ and negligible accessibility to Ni-EDDA. Residues exposed to the aqueous phase have maximum accessibility to Ni-EDDA due to high concentration of Ni-EDDA in the aqueous phase. As can be seen in Fig. 3.10, all the R1 sites of rec-mem-ColA are located in the lipid phase whereas the R1 sites of sol-ColA are located in the aqueous phase or in the protein interior. Furthermore, in rec-mem-ColA, the R1 sites that are located within the amphipathic helices are located at the lipid/aqueous phase region of the plot.

To better characterize the topology of lipid-embedded proteins, the immersion depth of the nitrooxides in lipid bilayers can be estimated using an immersion depth
parameter ($\Phi$), which was calculated from the nitroxide accessibility to O$_2$ and Ni-EDDA. The immersion depth parameter is a linear function of depth in the membrane and is not strongly dependent on the lipid composition of membrane bilayer [26]. As reported for bacteriorhodopsin [26], residues with $\Phi \sim 0$ are located approximately at 5 Å from the phospholipid head groups and those with $\Phi \sim 4$ are located approximately at 22.5 Å from the lipid-water interface i.e. close to the center of the bilayer (Fig. 3.11B). When the immersion depth values of rec-mem-ColA are compared with the values of bacteriorhodopsin, it is apparent from the Fig. 3.11 that the hydrophobic hairpin (H8 and H9) is located deeply in the lipid bilayer while the amphipathic helices are located close to the lipid-water interface.

**Figure 3.11:** Immersion depth parameter ($\Phi$) (A) $\Phi$ for rec-mem-ColA. The depth parameter was determined from the residues accessibilities. ($\Phi = \ln(\Pi(O_2)) / \Pi(Ni-EDDA)$). The values of $\Pi$ were determined in the presence of 25 mM Ni-EDDA and in aqueous solution equilibrated with air. (B) $\Phi$ for D helix of bacteriorhodopsin [26], which was adopted to show as reference. The depth parameter was determined from ($\ln(\Pi(O_2)) / \Pi(NiAA)$). The values of $\Pi$ were determined in the presence of 20 mM NiAA and in aqueous solution equilibrated with air (~0.27 mM oxygen). Negative values indicate location of the R1 sites in the bulk water phase whereas positive values indicate locations of the R1 sites in the membrane phase.

**Polarity profiles:** The polarity varies across the membrane due to the water gradient from the head group region to the center of the lipid bilayer. Hence, the center of the bilayer is less polar and the polarity increases towards the head groups. EPR provides valuable tools to derive polarity profiles due to the sensitivity of the hyperfine component ($A_{ZZ}$) of the nitroxide. The $A_{ZZ}$ value for nitroxides is expected to be large in a polar (~3.6-3.7 mT) and small in a nonpolar environment (~3.3-3.4 mT). Therefore, the $A_{ZZ}$ value can be utilized to obtain the location of the membrane associated spin-
labeled protein domains with respect to the membrane environment. To determine $\Delta_{ZZ}$ values, measurements need to be performed at temperatures below 200K to freeze the reorientational motion of the R1 side chain. The low temperature spectra of both sol-ColA and rec-mem-ColA are shown in Fig. 3.12. When comparing the spectra of sol-ColA to rec-mem-ColA, a clear shift of the $2\Delta_{ZZ}$ is observed, indicating the changed polarities from sol-ColA to rec-mem-ColA.

![Figure 3.12: Low temperature (160K) EPR spectra of colicin A. Spectra of sol-ColA are colored in black and rec-mem-ColA are colored in red. The residue number is given on the left side and the $\Delta_{ZZ}$ values obtained from fitting of the spectra are given on the right side.](image)

The $\Delta_{ZZ}$ values that were determined from the low temperature spectra (Fig. 3.12) are presented in Fig. 3.13. The polarity values are closely related to the mobility and accessibility data. In sol-ColA, the residues with higher mobility and higher water accessibility (26R1, 33R1, 62R1, 91R1, 115R1 and 166R1) show also higher polarity values (larger $\Delta_{ZZ}$ values), confirming the exposure of these residues to the water phase. On the other hand, the residues with low mobility and low water accessibility (42R1, 105R1, 154R1, 176R1 and 183R1) show low polarity values (smaller $\Delta_{ZZ}$ values),
confirming their location in the interior of the protein. Although the residues (169R1, 181R1, 184R1 and 192R1) show negligible accessibility to Ni-EDDA, they show high polarity values.

![Figure 3.13: Plot of AZZ values vs. residue number. The AZZ values are determined for R1 sites of both sol-ColA (black) and rec-mem-ColA (red) from the EPR spectra presented in Fig. 3.12. Lower AZZ values indicate nonpolar and the higher AZZ values indicate polar environment. The AZZ value of free spin label in aqueous solution is approximately 3.7 mT.](image)

The AZZ values determined for bacteriorhodopsin [18] whose crystal structure has been well characterized, are taken as reference (Fig. 2.7) for interpretation of the AZZ values for rec-mem-ColA. Accordingly, the AZZ values obtained for 26R1(H1), 33R1(H2), 42R1(H2), 62R1(H3), 91R1(H5), 105R1(H5a), 115R1(H6), 169R1(H9), 184R1(H9) and 192R1(H10) indicate that these residues of rec-mem-ColA are located within 10-15 Å distance from the phospholipid head groups. A very low AZZ value of 176R1 (H9) indicates the location of this residue in the center of the bilayer. The AZZ values of 154R1(H8), 166R1(H8/H9), 181R1(H9) and 183R1(H9) suggest that these positions are located close to the center of the bilayer. According to these results, helices H8 and H9 of rec-mem-ColA are located inside the membrane whereas the amphipathic helices are located peripheral to the membrane bilayer.
3.4 Discussion

3.4.1 From the water-soluble to the membrane-bound state of colicin A

The differences in the EPR spectral line shapes between sol-ColA and rec-mem-ColA confirm a drastic conformational change occurring upon membrane binding of colicin A (Fig. 3.6). The mobility and accessibility data of spin-labeled sol-ColA reveals that some of the examined R1 sites are buried in the protein core and others are exposed on the surface of the protein. These are the characteristic features of a globular structure with tightly packed residues undergoing tertiary interactions, thus the EPR data of sol-ColA is consistent with the crystal structure of the colicin A pore-forming domain.

The line shape analysis reveals that the inter-helical contacts, which are visible in sol-ColA do not exist anymore in rec-mem-ColA. However, the \( \Delta H_0 \) values are comparatively low for R1 sites of rec-mem-ColA. The overall higher \( O_2 \) accessibilities suggest that the lower \( \Delta H_0 \) values are not due to the immobilization of R1 side chains by tertiary interactions. There might be two reasons for the low mobility values for R1 sites of the rec-mem-ColA: (i) the strength of the hydrogen bond is relatively high in the trans-membrane helices due to nonpolar nature of the membrane. Therefore, the strengthening of hydrogen bonds might restrict the segmental backbone fluctuations of the helices which are embedded in the membrane. (ii) the rotational diffusion is restricted for membrane proteins when compared to water-soluble proteins. Thus, the contribution of the rotational diffusion to the overall mobility of R1 is significantly less for membrane proteins. When compared to Ni-EDDA, significant accessibilities are observed to \( O_2 \) for the R1 sites of rec-mem-ColA, suggesting that the scanned R1 sites of rec-mem-ColA are located in the membrane bilayer. The overall decreased polarity values for rec-mem-ColA also strongly support this finding.

3.4.2 The closed channel state

The arrangement of helices in the membrane bilayer can be elucidated with the help of accessibility and polarity data. In rec-mem-ColA, the R1 residues that are located in the hydrophobic hairpin (H8 and H9) are accessible from the lipid phase (\( O_2 \)) and inaccessible to Ni-EDDA, a characteristic feature of residues located in transmembrane helices. This suggests that H8 and H9 are transmembrane helices in rec-mem-ColA. In rec-mem-ColA, residue 176R1, which is located in the middle of H9, shows a polarity value (~3.3mT) similar to that of 180R1 (~3.35mT) of bacterio-
rhodopsin [18]. It is known from the crystal structure of bacteriorhodopsin that residue 180R1 is located at the center of the lipid bilayer (Fig. 2.7). Furthermore, the polarity values increase from 176R1 to either side of H9 (169R1, 181R1, 183R1, 184R1). This clearly indicates that the helix H9 is a transmembrane helix, which supports the umbrella model.

In case of the penknife model, the residue 176R1(H9) should be located in a slightly polar environment and all residues of colicin A should show intermediate accessibility to Ni-EDDA [18]. This is in contrast to our present results, which completely precludes the possibility of the penknife model for rec-mem-ColA. The accessibility and polarity data favor an umbrella model for the closed state of the channel with the hydrophobic hairpin oriented perpendicularly to the membrane bilayer, as shown in Fig. 3.14. This result agree with the streptavidin binding analysis of the biotinylated colicin A in the planar lipid bilayers [5].

![Figure 3.14: Proposed model for the closed channel state of colicin A: Based on the EPR data, the orientation of the hydrophobic hairpin (magenta colored) is transmembrane and the amphipathic helices (green colored) are located just below the phospholipid head groups. Helix H1 is located closer to the surface of the bilayer when compared to the other amphipathic helices. Residue 166R1(H8/H9 loop) is located in a structured environment in the lipid phase.](image)

### 3.4.3 Location of the amphipathic helices

Concerning the location of the amphipathic helices in the umbrella model of colicins, several published data [6, 11, 12, 27, 28] suggest them to be on the surface of the membrane bilayer. The detailed accessibility analysis performed for several positions on the amphipathic helices allows estimating the depth of the helices in the membrane bilayer. The limited accessibility to Ni-EDDA supports the idea that the R1
side chains of the amphipathic helices are not completely exposed to the aqueous phase. Furthermore, the values of immersion depth and polarity reveal that the amphipathic helices are located at the membrane-water interface, but not on the surface of the membrane. This result agrees with FRET data which had been determined for colicin E1 [29].

3.4.4 Umbrella model or penknife model?

The two models of the closed channel state have been studied extensively by many groups using a variety of techniques. Most of the groups suggest the umbrella model for colicin E1 [11-13, 28], colicin Ia [6] and the penknife model for colicin A [9, 18, 30, 31]. A few published results, however, support the umbrella model for colicin A. The present work might help to clarify these disputes about the membrane-bound closed channel state of colicin A.

In the case of colicin E1 and colicin Ia, the authors used the combination of neutral and negatively charged lipids at pH 4 [11-13, 28]. However, in the case of colicin A, the authors who suggested the penknife model used 100% negatively charged lipids at pH 5 [8, 9, 30, 32, 33], to examine the closed state. Moreover, they checked also the in vitro activity of colicin A in asolectin vesicles but not in 100 % anionic vesicles which were used to study the closed channel state [30]. S. Zakharov et al. reported that colicin E1 could not open channels in 100% negatively charged lipids [34].

In the case of 100% negatively charged lipids, the positively charged amino acids tend to bind just to the negative surface of the lipids due to the strong electrostatic interactions. The interaction between the negatively charged head groups and the positively charge amino acids does not allow the helices to move and prevents the insertion of the hydrophobic hairpin into the membrane. This would specify the reason for the association of the hydrophobic hairpin with helices 3-7 in the penknife model. In addition to this, 100% anionic lipid composition is not physiological in E. coli and colicins were shown to be non-functional in the liposomes of 100% negatively charged lipids [34, 35]. Therefore, in the present work we used the E. coli natural lipids and confirmed the results with three different methods of EPR technique (mobility, accessibility and polarity). Under in vitro conditions, the spontaneous membrane insertion of colicin A at low pH results in a mixture of inserted and adsorbed colicins (for details see chapter 4). Therefore, to avoid this possible mixture of different
conformations, the membrane-bound conformation of colicin A was reconstituted into proteoliposomes by a detergent mediated reconstitution method, which was improved and optimized to study colicin A. Furthermore, the unbound protein was also separated from the proteoliposomes. However, we could analyze the closed channel state by reconstituting the membrane-bound conformation, but we could not study the membrane insertion mechanism and conditions required for membrane insertion. These will be described in chapter 4.

3.5 Conclusions

To investigate the topology of the closed channel state, colicin A was spin-labeled at different sites and its water-soluble and membrane-bound states were studied by EPR spectroscopy. The structural information which is derived for the water-soluble state is in agreement with the x-ray crystal structure. Conformational changes occur during the transition from the soluble to the membrane-bound state. The accessibility and polarity data suggest the umbrella model to be valid for the closed channel state of colicin A. This finding corresponds to that of others for colicin E1 and colicin Ia.

3.6 References


4. Acidic pH induced membrane insertion and the closed channel state of colicin A

**Abstract:** The investigation of transitions of pore-forming toxins from a soluble to a membrane-bound state offers a promising strategy for evaluating the mechanism involved in the process of membrane insertion. Colicin A is a water-soluble toxin that inserts into the membrane of the target bacteria and forms a voltage-gated channel. The membrane insertion mechanism and the structural details of acidic pH induced membrane-bound colicin A were investigated. The ability of colicin A to insert at different pH (2-7) into the liposomes composed of E. coli natural lipids (~75% POPE and 20% POPG) was examined and the activity of colicin A in the liposomes was determined. The EPR spectra revealed that colicin A poorly binds to the membrane at pH 4-7 and efficiently inserts at pH 2-3. The detailed analysis of the acidic pH (~2.2) induced membrane-bound state of colicin A suggests that the hydrophobic hairpin is arranged across the membrane bilayer whereas the remaining amphipathic helices are arranged peripherally to the plane of the membrane. The EPR data support the umbrella model for the closed channel state and also reveal that 5-10% of the colicin A remains bound to the surface of the membrane. In the presence of a membrane potential (created by valinomycin), the acidic pH induced membrane-bound colicin A is shown to be functional in the liposomes, as demonstrated by the efflux of tempophosphate spin label from the lumen of the liposomes.

4.1 Introduction

Many pore-forming toxins are secreted as water-soluble proteins and insert into the membranes of target cells to exert their lethal activity. Some examples of these channel-forming proteins are colicins, diphtheria toxin, tetanus toxin, equinatoxin, aerolysin etc. The knowledge of the structural and functional details of their soluble and membrane-bound conformations should enable us to understand many biological aspects of these toxins.

Moreover, the information about how these toxins transit from the water-soluble to the membrane-bound state should enable a better understanding of the general mechanism of protein insertion into membranes. The most common feature of these
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toxins is that in the water-soluble conformation the proteins are tightly packed in globular structures with a central hydrophobic core. For example, colicins, diphtheria and Bcl-XL proteins comprise two central hydrophobic helices (hydrophobic hairpin) that are enclosed by several amphipathic helices [1]. The hydrophobic faces of the amphipathic helices make tight helical contacts with the hydrophobic hairpin to prevent the entry of water molecules into the hydrophobic core. The hydrophilic faces of the amphipathic helices are surface-exposed to make the protein soluble in water. In order to insert into the membranes, the water-soluble toxin has to undergo drastic conformational changes. The event that triggers these conformational changes in vivo is still matter of debate. Before inserting into the membrane, the soluble proteins attain a translocation competent state that favors the membrane insertion. In vitro this competent state can be induced by acidic pH, high temperature and detergents [2]. Bychkova et al. [2] hypothesized that water-soluble proteins form molten globule states at acidic pH prior to membrane insertion. The diphtheria toxin is known to be exposed to the acidic pH before it inserts into membranes [3]. Davidson et al. [4] reported that acidic pH enhances the membrane penetration of colicins in vitro. Studies on colicins and other water-soluble toxins suggest that binding of the pore-forming domain to membranes involves an initial electrostatic interaction between the positively charged protein and the negatively charged lipids followed by non-electrostatic binding (hydrophobic interactions) [5].

Acidic pH (~ 4-5) has been used to initiate the in vitro binding of colicins into liposomes. In earlier studies, pH 4 was used to insert colicin E1 and colicin Ia into membranes composed of 70% neutral lipids and 30% anionic lipids [6-9]. In the case of colicin A, pH 5 was used in combination with liposomes containing 100% negatively charged lipids [10-14]. The authors who used 100% anionic lipids reported that anionic lipids lower the surface pH (by less than 1 unit) compared to the bulk pH. Now the question arises, unlike other colicins, why does colicin A require 100% anionic lipids to insert into membranes in vitro. Is the membrane insertion mechanism different among different colicins, although they share structural and functional similarities?

In order to answer to these questions, the membrane insertion mechanism of colicin A was studied at different pH values (2-7). Additionally, the pH induced membrane-bound conformation was also studied in detail.
4.2 Methods

4.2.1 Preparation and spin labeling of colicin A

Colicin A was prepared and labeled as described in sections 3.2.1-3.2.2.

4.2.2 Preparation of liposomes

Liposomes were prepared as described in section 3.2.3.

4.2.3 Membrane insertion of the colicin A pore-forming domain

The spin-labeled colicin A (10 mg/ml, 10 mM potassium phosphate, pH 7.4) of 80 µl was added to 140 µl of the respective buffer (50 mM phosphate buffer for pH 7, 2 and 50 mM citrate buffer for pH 6 to 3). Colicin A at different pH (pH 2-7) was added to 1ml of the liposome suspension (20 mg/ml, 50mM potassium phosphate buffer, pH 7.5) and mixed thoroughly. The pH of the resulting protein-liposomes mixture was 7.3. The proteoliposomes were separated from the unbound colicin A as described in section 3.2.5. The resulting proteoliposomes (50 mM phosphate buffer, pH 7.5) were used for further EPR analysis.

4.2.4 In vitro assay of the channel activity in liposomes

Liposomes that enclose 5 mM tempophosphate (4-Phosphophenoxy tempo hydrate), 50 mM potassium phosphate buffer (pH 7.5) and 100 mM KCl were prepared. Wild type colicin A at pH 2.5 was added to the liposomes and mixed thoroughly (as described in section 4.2.3). The proteoliposomes were separated from the unbound proteins as described in section 3.2.5. The resulting proteoliposomes were resuspended in 50 mM sodium phosphate and 80 mM NaCl. Valinomycin (25nM final concentration) was then added to an aliquot of the proteoliposomes to induce a diffusion potential. The proteoliposomes in the presence and absence of valinomycin were further analyzed by EPR to check the efflux of tempophosphate. The accessibility of tempophosphate to Ni-EDDA, which was added to the extra vesicular buffer was checked by saturation experiments. As a control experiment, the liposomes without colicin A incorporated were also analyzed similarly as described above.

4.2.5 EPR

EPR measurements were performed as described in section 3.2.6. To obtain the concentration of the spin-labeled proteins in a sample, the second integral of the EPR
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spectrum, which is proportional to the spin concentration was calculated and compared to the value obtained for a 100 µM solution of the MTS spin label.

4.3 Results

4.3.1 Activity of membrane-bound Colicin A in liposomes

Colicin channels are known to permit the flow of small organic molecules like NAD$^+$ and glutathione along with the other ions [15]. Thus, the ability of colicin A to form a channel was investigated using liposomes that enclose the tempophosphate, a membrane impermeable spin-labeled ion that is smaller than NAD$^+$ and glutathione. The efflux of tempophosphate from the interior (trans) to the exterior (cis) environment of the liposomes can be considered as an indicator for the opening of the colicin A channel. Presence of tempophosphate in the exterior compartment can be monitored by determining its accessibility towards a membrane impermeable Ni-EDDA complex, which was added later to the exterior compartment. Therefore, the activity of colicin A in liposomes could be correlated to the efflux of the membrane impermeable spin-labeled ions.

The channel of colicin A is opened only in the presence of a trans negative membrane potential. The K$^+$ ionophore valinomycin can create a trans negative potential if $[K^+]_{int}/[K^+]_{ext} > 1$ and a trans positive potential if $[K^+]_{int}/[K^+]_{ext} < 1$ [16]. Thus, liposomes were prepared with tempophosphate, K$^+$ and Cl$^-$ ions in their interior (trans) and Na$^+$ and Cl$^-$ ions in their exterior (cis) compartments. Fig. 4.1 illustrates the basic idea of this experiment. The incorporation of valinomycin creates a diffusion potential across the membrane due to the diffusion of K$^+$ along the concentration gradient, inducing the opening of the colicin A channel. After a while, the channel closes again due to the membrane depolarization caused by both colicin A activity and loss of K$^+$ concentration gradient ($[K^+]_{int}/[K^+]_{out} \sim 1$) due to valinomycin activity.

The efflux of tempophosphate into the exterior (cis) compartment of the liposomes containing colicin A was investigated in the presence and absence of valinomycin. For control measurements, liposomes without colicin A were also used. In order to check the efflux of tempophosphate from the lumen of the liposomes, its accessibility to Ni-EDDA was determined by means of CW power saturation measurements (see methods 3.2.6). Table 4.1 shows the measured accessibility of tempophosphate to Ni-EDDA.
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Figure 4.1: *In vitro* activity of colicin A. (A) The lumen of the proteoliposomes contain tempophosphate, 100mM KCl, 50mM potassium phosphate buffer (pH 7.5) and the exterior compartment contains 80 mM NaCl and 50 mM sodium phosphate buffer (pH 7.5). (B) valinomycin (red circles) creates a membrane potential by diffusing K⁺ ions from higher to lower concentration. In the presence of a potential, colicin A opens the channel and allows the efflux of the lumen contents including tempophosphate. Arrow marks indicate the movement of ions (C) when the K⁺ concentration of both *trans* and *cis* compartments of the liposomes reaches its equilibrium, valinomycin diffuses K⁺ ion from *cis* to *trans* and from *trans* to *cis*. After a while, colicin A channel is closed due to the membrane depolarization, which is caused, by both the valinomycin and the colicin A channel. The presence of tempophosphate in the extravesicular region (*cis*) of the liposomes was checked by its accessibility to Ni-EDDA.
Table 4.1. Collision frequency of extra vesicular tempophosphate to Ni-EDDA

<table>
<thead>
<tr>
<th></th>
<th>$W_e$ for Ni-EDDA/MHz</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>- valinomycin</td>
</tr>
<tr>
<td>Liposomes (without colicin A)</td>
<td>0± 0.2</td>
</tr>
<tr>
<td>with tempophosphate</td>
<td></td>
</tr>
<tr>
<td>Proteoliposomes (with colicin A)</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>with tempophosphate</td>
<td></td>
</tr>
<tr>
<td>Tempophosphate (1 mM) in</td>
<td>19.0 ± 1.9</td>
</tr>
<tr>
<td>solution</td>
<td></td>
</tr>
</tbody>
</table>

The accessibility data suggested that:

1. In the absence of both colicin A and valinomycin, tempophosphate is inaccessible to Ni-EDDA, indicating that the liposomes are not leaky for tempophosphate.
2. In the absence of colicin A and presence of valinomycin, tempophosphate is inaccessible to Ni-EDDA, suggesting that the efflux of tempophosphate cannot be facilitated by the valinomycin.
3. In the presence of both colicin A and valinomycin, tempophosphate is considerably accessible to Ni-EDDA, indicating that the colicin A channel is opened in the liposomes under the investigated conditions.
4. In the presence of colicin A and absence of valinomycin, tempophosphate is inaccessible to Ni-EDDA, suggesting that the colicin A channel is closed in the absence of a membrane potential.

From the above observations, we can conclude that acidic pH induced membrane-bound colicin A in liposomes is able to open the voltage-dependent channels.

4.3.2 pH dependent membrane insertion of colicin A

As mentioned in the introduction, colicins are known to insert into membranes at acidic pH. Therefore, the influence of pH on the membrane insertion was investigated using colicin A, which is spin-labeled at position 176. The same liposome preparation and similar conditions were used throughout this experiment. The spectra of membrane-bound colicin A where the membrane insertion is induced by different pH ranging from 7 to 2 are shown in Fig. 4.2 and Fig. 4.3.
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Figure 4.2: pH dependent binding of colicin A to the liposomes. (A) the room temperature EPR spectra of residue 176R1 that bound to membranes. The pH indicated (left side of the graph) is the pH of colicin A when added to the liposomes (pH 7.5). The pH of the EPR samples was pH 7.3. (B) Concentration and signal intensities were determined from the spectra shown in Fig. 4.2A. The concentration of the spin-labeled protein was calculated as described in methods 4.2.5. The signal intensities were obtained from the intensity of the central resonance line of the spectra.

A striking difference is observed in the signal intensities of the EPR spectra (Fig. 4.2A). The signal intensity at pH 7 is relatively low and slightly increases at pH 6, 5, 4. This indicates a poor binding of colicin A to the liposomes at pH 7, whereas the binding ability is slightly enhanced by lowering the pH from 7 to 4. The signal intensity is significantly increased at pH 3 and then drastically at pH 2, suggesting that colicin A binds efficiently only at pH ≤ 3.

Figure 4.3: pH dependent binding of colicin A to liposomes. Room temperature EPR spectra of colicin A 176R1 bound to membranes. The pH indicated is the pH of colicin A when added to the liposomes (pH 7.5). The pH of the EPR samples was pH 7.3. For clarity, all the spectra were normalized to their central peak maximum.
The small differences observed in the EPR spectral shapes (Fig. 4.3) reveal that the conformation of colicin A might slightly change from pH 7 to pH 2. The above results reveal that colicin A binds more efficiently at pH 2 when compared to pH 7, suggesting that pH < 3 is required for efficient penetration of colicin A into the membranes under in vitro conditions. In the following, pH 2.3 ± 0.2 for colicin A prior to adding to the liposomes was chosen as to induce the membrane insertion.

### 4.3.3 Conformation of low pH induced membrane-bound colicin A

Conformational changes that occur during the transition from the water-soluble colicin A (sol-ColA) to the acidic pH induced membrane-bound state (pH-mem-ColA) were analyzed in terms of mobility, accessibility and polarity of the nitroxide spin label attached at different sites. The spectra of the investigated positions in sol-ColA and pH-mem-ColA are presented in Fig. 4.4.

**Figure 4.4:** First derivative EPR spectra of R1 labeled sol-ColA (black) and pH-mem-ColA (green). Spectra were acquired over a scan width of 13mT. All spectra are normalized to their central peak maximum. Residue numbers at which the spin label is attached are given.
As evident in Fig. 4.4, the EPR spectra of pH-mem-ColA are notably different from the spectra of sol-ColA. Since the EPR spectral line shapes are very sensitive to the changes in protein tertiary and secondary interactions, the changes in spectra indicate changes in conformation occurring during the transition from the sol-ColA to the pH-mem-ColA. The EPR analysis of sol-ColA was described in detail in chapter 3. In pH-mem-ColA, the spectra of R1 sites (33R1, 42R1, 62R1, 115R1 and 166R1) that are surface-exposed in sol-ColA show reduced amplitudes with respect to sol-ColA, indicating a significant reduction in the overall nitroxide motion. In sol-ColA, the residues 42R1(H2), 105R1(H5a), 154R1(H8), 176R1(H9), 183R1(H9) that are buried in the protein interior show large apparent hyperfine splitting. The corresponding spectra of pH-mem-ColA do not show large apparent hyperfine splitting, suggesting the loss of specific tight packing interactions between helices. This reveals that the hydrophobic core in the membrane-bound state does not have any more strong helical contacts with the other amphipathic helices. Furthermore, the narrow line of 26R1(H1) indicates high mobility whereas almost all other R1 sites (pH-mem-ColA) show both mobile and immobile components, indicating intermediate mobility. The presence of two components in the spectra can be explained by a complex anisotropic motion of the nitroxide or by the presence of two populations with different mobilities. The values of $\Delta H_0^{-1}$ that indicate mobility of the R1 sites are shown in Fig. 4.5.

![Figure 4.5](image)

**Figure 4.5:** Mobility of sol-ColA (black) and pH-mem-ColA (green). The inverse of the central line width ($\Delta H_0^{-1}$) for the investigated R1 sites was determined from the EPR spectra that are shown in Fig. 4.4.
The mobility of pH-mem-ColA for residues 33R1, 42R1, 91R1, 115R1, 166R1, 169R1, 181R1, 184R1 and 192R1 is decreased compared to sol-ColA whereas the mobility is increased for 26R1, 42R1, 105R1, 154R1, 176R1 and 183R1, which are buried in sol-ColA (Fig. 4.5). The most mobile site in pH-mem-ColA is 26R1 and its mobility is similar to that of surface-exposed residues (33R1, 62R1 and 115R1) of sol-ColA. Residues 105R1, 169R1, 176R1 and 181R1 of pH-mem-ColA show low mobility. This is similar to that of sol-ColA residues (42R1, 105R1, 176R1 and 183R1) which are characterized by inter-helical contacts. The remaining residues show intermediate mobility in pH-mem-ColA.

To obtain additional information on the protein topology, we determined the accessibilities of each R1 site to the paramagnetic quenchers O2 and Ni-EDDA. Fig. 4.6 shows the exchange frequencies (Wex) for R1 sites of sol-ColA and pH-mem-ColA.

![Figure 4.6: Accessibility. (A) Exchange rates (Wex) for O2 and Ni-EDDA for the indicated R1 sites of sol-ColA. The dotted lines indicate the observed minimum value for both O2 and Ni-EDDA. (B) exchange rates for O2 and Ni-EDDA for the R1 sites of pH-mem-ColA. For clarity, the plot is rescaled and shown in the inset. The Wex values were determined in presence of 25 mM Ni-EDDA and in aqueous solution equilibrated with air.](image)

The accessibility of R1 sites in sol-ColA was discussed in detail in chapter 3. Briefly, the residues that are located on helix surfaces (26R1, 33R1, 62R1 and 115R1) and loop regions (166R1) are accessible to Ni-EDDA, whereas the residues (42R1, 105R1, 154R1, 169R1, 176R1, 181R1, 183R1, 184R1 and 192R1) that are buried in the protein interior are neither accessible to Ni-EDDA nor to O2. The structural properties of sol-ColA determined from accessibility profiles are in agreement with the X-ray crystal structure.
In pH-mem-ColA, the overall accessibility of the R1 sites to Ni-EDDA is significantly less when compared to their accessibilities in sol-ColA (Fig. 4.6A and B). On the other hand, the accessibility to O₂ is significant.

Residues (26R1, 42R1, 33R1, 62R1, 91R1, 115R1, 166R1 and 192R1) that are located within amphipathic helices show almost similar accessibility to O₂ and Ni-EDDA, however the accessibility to Ni-EDDA is significantly less when compared to their accessibility in sol-ColA. This indicates that the amphipathic helices of the pH-mem-ColA are located at the lipid-water phase (Fig. 4.6B). Furthermore, residues (154R1, 166R1, 169R1, 176R1, 181R1, and 183R1) that are located in the hydrophobic hairpin show considerable accessibility to O₂ in pH-mem-ColA when compared to sol-ColA. Additionally, with the exception of 166R1, these residues show negligible accessibility to Ni-EDDA in pH-mem-ColA. This is characteristic for the residues that are located in the transmembrane helices, suggesting that H8 and H9 helices of pH-mem-ColA are transmembrane helices, which supports the umbrella model. However, this statement contradicts with the water accessibility of 166R1, which is located in the H8/H9 loop (Fig. 4.6B).

If we assume that the hydrophobic hairpin is located at the membrane-water interface, the R1 sites of the hydrophobic hairpin should show small but significant accessibility to Ni-EDDA. However, Fig. 4.6 shows clearly that this is not the case. The accessibility data suggest rather that H8 and H9 are transmembrane helices in pH-mem-ColA. However, residue 166R1 which is located at the H8/H9 loop region seems to be accessible to Ni-EDDA, with a $W_{ex}$ value much smaller than that of sol-ColA. If the umbrella model would be the correct model for the membrane-bound state, 166R1 should be inaccessible to Ni-EDDA.

In order to clarify this ambiguity, the accessibility data were supplemented with the information of the polarity values for the R1 sites, which are shown in Fig. 4.7. In pH-mem-ColA, the $A_{zz}$ values for the residues of the amphipathic helices vary between 3.4 and 3.5 mT, indicating intermediate polarity. This indicates that the R1 sites are located at the membrane-water interface. Residue 176R1 shows a very low $A_{zz}$ (3.3 mT) value, which is characteristic for the residues located in the core of the membrane. As evident in Fig. 4.7, 166R1 is located in a nonpolar environment, indicating its location in the membrane. The polarity values suggest the transmembrane arrangement for the hydrophobic hairpin, which supports the umbrella model.
4.4 Discussion

4.4.1 Membrane insertion of colicin A

Present results reveal that colicin A poorly binds to the membrane at pH ranging from 7 to 4. However, its binding efficiency is enhanced significantly at low pH (< 3), suggesting the requirement of acidic pH for colicin A to insert into membranes under in vitro conditions. The membrane insertion of colicin A occurs mainly in two steps: binding to the membrane (electrostatic interactions) and insertion (hydrophobic interaction) into the membrane. The electrostatic interactions lead to the binding of a positively charged protein to the negatively charged lipids. This is followed by the hydrophobic interactions that favor the insertion of the hydrophobic hairpin into the membrane. Thus, the important factor that plays a key role in this mechanism is the positive charge density on the surface of colicins. However, colicin A has a net negative charge on its surface. On the other hand, the hydrophobic hairpin cannot insert into the membrane unless the interactions between the hydrophobic hairpin and the amphipathic helices are disturbed. A net positive charge distribution of a protein can be facilitated by protonation of acidic residues (negatively charged). A schematic model for this membrane insertion mechanism is shown in Fig. 4.8.
Figure 4.8: Schematic representation of membrane insertion mechanism of colicin A. (A) water-soluble pore-forming domain of colicin A at neutral pH (B) Negatively charged amino acids are protonated at pH <3, resulting in net positive charge on the surface of colicin A. (C) The positively charged and energetically unstable colicin A binds to the membrane bilayer which contains negatively charged lipids. (D) The hydrophobic interactions between the hydrophobic hairpin and the lipid bilayer favor the insertion of the hairpin into the bilayer.
The protonation of some or all acidic residues disturb the tight packing interactions due to loss of ionic interactions, and disturbances in the hydrogen bond network of a protein. This state of a protein is known as a molten globule or a translocation competent state (*For details see chapter 5*). *In vitro*, this molten globule state can be triggered by acidic pH. Hence, water-soluble proteins require low pH to insert into membranes [2]. Thus, the acidic pH enhances the insertion of colicin A into the membranes by triggering the molten globule state for colicin A. Since, the protonation of residues strongly depends on their pKa, the pH value that triggers the molten globule state varies in different proteins. For example, we found that colicin A forms molten globule states at pH < 3 (chapter 5) whereas colicin E1 forms a molten globule at pH ~ 4 [17]. This would explain why colicin A, E1 and Ia bind to lipids at different pH, although they have similar structure and function.

### 4.4.2 Conformation of the membrane-bound state (acidic pH induced)

Significant changes were observed in the pore-forming domain of colicin A upon the transition from the water-soluble to the membrane-bound state (low pH induced). It is apparent from the data that the changes are not localized to a few sites within the colicin A but they are global. From the polarity (particularly for residue 176R1) and the accessibility data of the R1 sites that are located at helices H8 and H9, we can conclude that these helices are evidently oriented perpendicular to the plane of the membrane in pH-mem-ColA, which supports the umbrella model. If this is the case, 166R1 (H8/H9 loop) should not be accessible to Ni-EDDA. However, 166R1 in pH-mem-ColA is slightly accessible to Ni-EDDA, which might indicate a fraction of molecules with position 166 located at the surface of the membrane.

To better understand the topology of pH-mem-ColA, the present results are compared to the reconstituted membrane-bound colicin A (rec-mem-ColA) and water-soluble colicin A (sol-ColA). In chapter 3, the conformations of both sol-ColA and rec-mem-ColA were discussed in detail. As evident in Fig. 4.9, the EPR spectra of pH-mem-ColA are similar to the spectra of rec-mem-ColA, indicating similar conformation in both reconstituted and low pH induced membrane-bound colicin A.
Figure 4.9: Comparison between the EPR spectra of sol-ColA, rec-mem-ColA and pH-mem-ColA. The spin normalized EPR spectra of the R1 sites measured for sol-ColA (black), rec-mem-ColA (red), pH-mem-ColA (green) are overlaid to show the changes in amplitudes and line shapes. The spectral shapes of rec-mem-ColA and pH-mem-ColA are nearly identical.

Fig. 4.10 shows the mobility, accessibility and polarity values of sol-ColA, rec-mem-ColA and pH-mem-ColA. The mobility and accessibility values for the R1 sites of both pH-mem-ColA and rec-mem-ColA are changed in the same direction from the values of sol-ColA, however the changes are not exactly identical. The plots reveal that the values of pH-mem-ColA are deviated slightly towards the values of sol-ColA (Fig. 4.10A, C and D).
Figure 4.10: Mobility, accessibility and polarity for the R1 sites of sol-ColA (black), rec-mem-ColA (red) and pH-mem-ColA (green). (A) The mobility values \(\Delta H_0\) of indicated R1 sites were determined from room temperature EPR spectra shown in Fig. 4.9. (B) The polarity values \(A_{zz}\) for investigated R1 sites. (C) Accessibility in terms of exchange rates \(W_{ex}\) of R1 sites to O$_2$. \(W_{ex}\) values were determined in the presence in aqueous solution equilibrated with air (~0.27mM oxygen). (D) Accessibility of R1 sites to Ni-EDDA. \(W_{ex}\) values were determined in the presence of 25 mM Ni-EDDA.

Fig. 4.10B shows the polarity values for sol-ColA, rec-mem-ColA and pH-mem-ColA. The similarity observed in the polarity values (except for 33R1) of both pH-mem-ColA and rec-mem-ColA suggest similar conformations. Similar EPR line shapes (Fig. 4.9) and polarity values (Fig. 4.10B) suggest that both pH-mem-ColA and rec-mem-ColA might have similar membrane-bound conformation, which supports the umbrella model.

The comparison between rec-mem-ColA and pH-mem-ColA suggest that a major fraction of pH-mem-ColA exists in a conformation as described by the umbrella model, but a minor fraction (5-10%) of pH-mem-ColA is bound at the surface of the
membrane. This surface bound fraction might adopt either a penknife model or a conformation similar to sol-ColA. In case of the penknife model, the hydrophobic hairpin is located at the membrane-water interface, so that the residues of the hydrophobic hairpin should show increased accessibility to Ni-EDDA. In the present case, hydrophobic hairpin residues of pH-mem-ColA are not accessible to Ni-EDDA. Additionally, R1 sites of H1 and H2 (which are supposed to be membrane exposed in penknife model) also show deviations towards sol-ColA. Therefore, we can exclude the possibility of penknife model for the minor fraction that is bound to the surface of the membrane. Consequently, the deviations of mobility and accessibility values towards sol-ColA (Fig. 4.10) might suggest that the conformation of 5-10% surface bound pH-mem-ColA is similar to the conformation of sol-ColA. This 5-10% of sol-ColA like state cannot be a contamination from an unbound protein, because the unbound protein was separated prior to EPR measurements. Therefore, the resulting conformation that is similar to sol-ColA might arise from the colicin A bound to the surface of the liposomes where it cannot insert into the membranes.

Our results do not support the existence of an equilibrium between the penknife and the umbrella model [9], because we have seen only one conformation in rec-mem-ColA samples. In pH-mem-ColA samples, the existence of a second population might be due to the competition between proteins to bind to the lipid bilayer. When colicins are embedded in the membrane bilayer, the surface negative charge of the lipids is still attractive for new colicins that have a positive charge. The conformation of a second population that binds on the surface close to embedded colicins might be similar the sol-ColA conformation. M. Lindeberg et al. [18] reported that colicin E1 did not open or unfold on the surface of lipids if the hairpin could not insert into membranes due to the rigidity of the head groups. This statement supports our hypothesis.

From our results, we can conclude that colicin A at pH 2.2 can insert efficiently into membranes and the results support the umbrella model. Furthermore, 5-10% of colicin A may be bound to the surface of the lipids in the soluble conformation as shown in Fig. 4.11. We can also exclude the possibility of reversible binding of colicin A because we did not see this reversibility in rec-mem-ColA samples.
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Figure 4.11: Model of pH-mem-ColA in liposomes. 90-95% of the pH-mem-ColA is inserted into membrane as in the umbrella model whereas 5-10% of the protein is still bound to the surface of liposomes. The conformation of the surface-bound fraction is closer to that of the water-soluble colicin A.

4.4.3 The closed channel state of colicin A: Umbrella model or penknife model

It has been already mentioned in the introduction that the umbrella model was very well proven for colicin E1 and colicin Ia with biochemical and spectroscopic experiments where the combination of neutral lipids (70%) with negatively charged lipids (30%) were used and incubated with colicins at pH 4 [6, 9, 19, 20]. A new model known as penknife model was proposed for colicin A by Lakey et al. [10]. The unique feature of all experiments that supported the penknife model is the usage of 100% negatively charged lipids and incubated with colicin A at pH 5 [10, 13, 21]. However, in *E. coli*, the plasma membrane comprises approximately 70-75% POPE, 20-25% POPG and 5-10% of cardiolipin. Hence, in the present work we used lipids that are derived from *E. coli*.

The authors who used 100% anionic lipids and supported the penknife model could not show the functionality of colicin A. However they used asolectin lipids to show the functionality [13]. Zakharov et al., reported that colicin E1 could not form a
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channel with 100% negatively charged lipids [8]. The same authors who proposed the penknife model reported the trans-membrane orientation of the hydrophobic hairpin (umbrella model) in planar bilayer experiments [22], suggesting that the penknife model is valid only because of the choice of 100% anionic lipids as experimental model. In the present study, we have also shown that colicin A is functional in the liposomes. We have shown that colicin A also binds at pH 7-4, but we could not analyze its topology in detail due to its low binding efficiency. However, the difference in line shapes reveals that the conformation of colicin A inserted at pH 7 may not be similar to that of colicin A inserted at pH 2.

In the present study, we used *E.coli* natural lipids to maintain physiological conditions, and pH 2.2 is used to trigger the molten globule state of colicin A. Unlike previous studies of colicin E1, Ia and A [10], we added colicin A at pH 2.2 to the liposomes at pH ~7.5 in buffer. After addition of colicin A to the liposome suspension, the bulk pH is still close to the neutral pH (pH 7). Another main difference from previous studies of colicin A, E1 and Ia is that the unbound protein was separated from proteoliposomes to avoid a mixture of conformations. The experimental procedure that we used to study the closed channel state and protein insertion leads to a functional system, which opens a channel in the presence of a membrane potential.

4.5 Conclusions

Colicin A poorly binds to the membranes at neutral pH whereas the binding efficiency is significantly enhanced at acidic pH. The results show that the conformation of acidic pH induced membrane-bound colicin A is consistent with that of the reconstituted colicin A (chapter 3). In the membrane-bound state, helices H8 and H9 are oriented as trans-membrane helices while the remaining helices are oriented peripheral to the membrane. This supports the umbrella model for the closed channel state of colicin A. However, a minor fraction (5-10%) of colicin A is still bound to the membrane surface.

4.6 References


5. Structural analysis of the acidic pH induced molten globule state of colicin A

Abstract: Water-soluble pore-forming proteins that are functional in membranes are known to form molten globule states before they insert into the membrane. The pH (~7-2) induced conformational changes of colicin A pore-forming domain were investigated with SDSL-EPR. Residues 26, 33, 42, 62, 91, 105, 115, 166, 169, 176, 192 in the pore-forming domain were spin-labeled and used to deduce the molten globule properties at acidic pH. The EPR analysis indicated that the conformation of the colicin A pore-forming domain is relatively stable in the pH range of 7 to 4, but it undergoes drastic conformational changes at pH < 4. The EPR spectra at pH 2 revealed that the side chains of the amphipathic helices (except for residues 115 and 192) become highly flexible whereas the side chains of the hydrophobic core become more immobilized. Furthermore, the increased polarity of residue 176R1 (H9) indicates that the hydrophobic core is slightly hydrated at pH 2. Residue 42R1 which is buried at pH 7 becomes accessible to water at pH 2 whereas residue 166R1 which is accessible to water at pH 7 is buried at pH 2. The results suggest that the molten globule properties are evident at pH 2 and are also supported by the fluorescence measurements with ANS. The pKa of the molten globule transition is estimated from the results, pKa ~3. The EPR method has been proven a suitable technique to study the structural details of colicin A in the molten globule form.

5.1 Introduction

Protein function strictly depends on the protein structure or tertiary fold, which is organized by several non-covalent forces. Disturbances in these non-covalent interactions lead to a pre-native structure, called molten globule (MG) state. MG states are folding intermediates that play an important role in many cellular processes [1]. A MG state can be simply described as a compact globule with substantial secondary structure but with lack of tertiary interactions [1, 2].

Water-soluble proteins that insert into or cross membranes for their activity typically consist of a central hydrophobic core surrounded by amphipathic helices with
surface exposed polar residues (Fig. 5.1). Apart from the functional role, the hydrophobic core stabilizes the structure in membranes and the hydrophilic residues stabilize the protein in aqueous environment. In order to penetrate into the bilayer, these proteins necessarily undergo drastic conformational changes to expose their hydrophobic parts from the interior of the protein [3]. This can be achieved by loosening the tertiary interactions. The state of a protein without proper tertiary interactions is nothing but a MG state. Thus, many water-soluble proteins essentially undergo transitions into MG states before they insert into the membrane [1].

![Figure 5.1](image)

**Figure 5.1**: Water-soluble pore-forming domain of colicin A. Molecular surface is displayed to show the tightly packed hydrophobic hairpin surrounded by amphipathic helices. Amphipathic helices are colored in green and the hydrophobic hairpin is colored in magenta.

Some water-soluble membrane bound proteins possess native MG states (e.g. SecA), while some proteins need an external induction for the MG transition. Various experimental conditions like acidic pH, detergents, urea, temperature etc. can trigger the MG states in stable native proteins [1]. Among these conditions, the acidic pH is the most probable trigger for many proteins in vivo. For instance, acidic pH induces MG in diphtheria toxin, a water-soluble toxin that travel across the acidic organelles (endosomes) before inserting into the membrane [4]. In vitro, many water-soluble proteins are also known for their ability to insert into the membrane at acidic pH [1, 4, 5]. However, their membrane insertion mechanism in vivo is poorly understood. Therefore, conformational studies on a MG in vitro might give more insights into the mechanism of membrane insertion of soluble proteins and into details of the protein folding.

Previous studies suggested the requirement of acidic pH for the membrane insertion of colicin A, B, E1 and Ia [5]. Evans et al. showed that colicin A forms a MG below pH 4
However, the structural details of the acidic pH induced conformational changes of colicin A have not yet been studied. The main goal of the present chapter is to investigate structural details of the conformational changes occurring in the transition from the native to the MG state by lowering the pH.

Because of the flexible nature of the MG states, it is very difficult to obtain structural details in high resolution by conventional methods like X-ray crystallography or NMR spectroscopy. Hence, many scientific groups previously characterized the MG states by using low-resolution methods. Dolgikh et al. [2] described the MG state by far and near-UV CD spectroscopy as a compact globule, native-like secondary structure with slowly fluctuating tertiary structure. Kataoka et al. [7] showed by using small angle solution X-ray scattering that the MG state is a compact (not as compact as native state) and globular structure but swollen and expanded. They also reported that the MG state has a definite tertiary fold, which may not be similar to the native state. Z.M Bu et al. [8] showed by incoherent quasielastic neutron scattering that the side chains in the MG states are significantly more mobile than those in the native protein, and explore a larger length scale in shorter time. 1D NMR hydrogen/deuterium exchange measurements performed by Morozova et al. [9] suggested clustering of protected residues in the core of the protein. Wormald et al. [10] used pulsed NMR techniques and reported that the mobility increased for hydrophilic side chains, although no increase in the mobility of the core hydrophobic residues was observed. From the conclusions of the above-mentioned scientific groups, we can describe the MG state as a compact structure with substantial amount of native-like secondary structure but lack of native-like tertiary packing, flexible hydrophilic side chains, slightly increased hydrodynamic radius and entry of water into the hydrophobic core.

In the present chapter, the SDSL-EPR method was used to study the tertiary interactions of the MG state of colicin A, the presence of secondary structure, the exposure of residues to the environment, the entry of water into the hydrophobic core and the flexibility of the side chains. The EPR results are corroborated by fluorescence experiments detecting the binding of ANS probe to the hydrophobic regions. This investigation will provide more insights into the protein insertion mechanism and required conditions. This
will also introduce EPR as a powerful method to study the MG states in addition to the native ordered structures.

5.2 Methods

5.2.1 Protein preparation and spin labeling

Colicin A was prepared, purified and spin-labeled as described in section 3.2.1.

5.2.2 Sample preparation

The phosphate buffer (pH 7.4) of the spin-labeled colicin A (10 mg/ml) was exchanged with a specific buffer to attain the required pH. Phosphate buffer was used for pH 7 and 2. Citrate buffers were used for pH 6, 5, 4 and 3. The buffer was exchanged twice with 5 times dilution using 10 KDa Centricons (centrifugal concentrators).

5.2.3 EPR Measurements

The EPR measurements were performed as described in section 3.2.6.

5.2.4 ANS Fluorescence measurements

All emission fluorescence measurements were performed in a home made fluorescence spectrometer using a 1 cm path length quartz cuvette. An excitation wavelength of 372 nm was used and the emission spectra were recorded in the range 400-600 with scan rate of 1.9 nm/sec. Fluorescence spectra of ANS at 10 µM in solution with 1mg/ml of colicin A.

5.3 Results

5.3.1 Side chain flexibility

The EPR spectra obtained for R1 at each site of colicin A indicating the conformational changes occurring from pH 7 to 2 are presented in Fig. 5.2 and 5.3. To understand the variations in side chain mobility, the spectra are also superimposed as shown in Fig. 5.2.

pH 7: The EPR spectra of residues 42R1(H2), 105R1(H5a), 176R1(H9) and 192(H10) show smaller amplitudes (Fig. 5.2) with a large apparent hyperfine splitting (Fig. 5.3),
indicating that the residues are strongly immobilized due to strong tertiary interactions or buried in the interior of the protein. Residues 62R1 (H3) and 115R1 (H6) show relatively higher amplitudes (Fig. 5.2) with a smaller apparent hyperfine splitting (Fig. 5.3), characteristic for residues located on the surface of a helix. Residues 33R1 (H2) and 166R1 (H8/H9) show narrow peaks (Fig. 5.2 and 5.3), typically observed for residues located in the loop regions. A more detailed EPR analysis of the water-soluble colicin A pore-forming domain at pH 7.4 was described in chapter 3. The data obtained were shown to be in agreement with the x-ray crystal structure.

Figure 5.2: Spin normalized room temperature EPR spectra from each R1 site recorded at pH 7 to pH 2. Since all spectra are spin-normalized, larger spectral amplitudes indicate high mobility and smaller amplitudes indicate low mobility. Residue numbers are given on the left side of the spectra. For the convenience of presentation, the amplitudes of some spectra were multiplied by a scaling factor shown to the right side of the spectra.
Figure 5.3: Spin normalized room temperature EPR spectra of colicin A containing the R1 nitroxide side chain at indicated sites. The spectra were recorded in solution at pH ranging from 7 to 2. For the convenience of presentation, the amplitudes of some spectra were multiplied by a scaling factor shown to the right side of the spectra. The residue number is given on the top of each group of spectra.
pH 6: Almost no changes are observed from pH 7 to pH 6 (Fig. 5.2).

pH 5: Almost no changes are observed except for residue 62R1 (H3) which shows an increase in the immobile component (Fig. 5.3).

pH 4: Residues 26R1 (H1), 105R1 (H5a) show increased mobility and 62R1 shows a more distinct differentiation between the mobile and immobile components.

pH 3: Residues 33R1, 42R1, 62R1, 91R1, 105R1, 115R1 and 166R1 show two well separated spectral components of both immobile and mobile fractions (Fig. 5.3), typically due to tertiary interactions at contact sites, which apparently arise from an equilibrium between different rotamers of the R1 side chain. Residues 169R1 (H9) and 176R1 (H9) are relatively more immobilized when compared to the mobility at pH 7. The spectrum of residue 26R1 (H1) shows very narrow peaks, indicating a higher mobility when compared to 26R1 at pH 7 (Fig. 5.2 and 5.3).

pH 2: The spectra of residues 26R1, 33R1, 42R1, 62R1, 91R1, and 105R1 show very sharp lines (Fig. 5.2 and 5.3), which reflect a high degree of side chain flexibility. Additionally, a small immobile component in the spectra (Fig. 5.3) of the above-mentioned positions except for 26R1 is observed, indicating that one of the rotamers of the side chain undergoes tertiary contacts with neighboring residues. The R1 side chains 115R1, 166R1 and 192R1 show smaller amplitudes (Fig. 5.2) with two spectral components, a prominent immobile one and a small fraction of a mobile component (Fig. 5.3). The dominant immobile component indicates strong tertiary interactions of these residues at pH2. Residues 169R1(H9), 176R1(H9) are relatively more immobilized when compared to their mobility at pH 7, indicating that the mobility of side chains of the hydrophobic hairpin is more restricted at pH 2 (Fig. 5.2 and Fig. 5.3).

A quantitative evaluation of the mobility can be assessed by a semi-empirical parameter, the inverse line width ($\Delta H_0^{-1}$). The values of $\Delta H_0^{-1}$ as a function of pH are shown in Fig. 5.4. To follow up the discussion easily, the residues are clustered based on their similar behavior. Upon changing the pH from 7 to 2: (i) the mobility increases for
residues 26R1, 33R1, 42R1, 62R1, 91R1 and 105R1 (Fig. 5.4A) (ii) In contrast, the mobility decreases for residues 115R1, 169R1, 176R1 and 192R1 (Fig. 5.4B). The plot (Fig. 5.4) reveals that except 115R1 and 192R1, the residues located at amphipathic helices become more mobile (located at loop/surface regions) whereas the residues of the hydrophobic hairpin become more immobilized (located at helix/buried region).

Figure 5.4: The values of $\Delta H_0^{-1}$ as function of pH. For the convenience of discussion, the residues are clustered into two groups depending on their mobility change. (A) Residues showing increasing mobility from pH 7 to pH 2 (B) Residues showing decreasing mobility from pH 7 to pH 2. In the case of two distinct spectral components can be resolved, the major component is given.

A plot of inverse second moment ($\langle \Delta H^2 \rangle^{-1}$) vs. inverse line width ($\Delta H_0^{-1}$) is shown in Fig. 5.5. This plot characterizes principle topographic regions of protein fold (see chapter 2). As evident in Fig. 5.5, at pH 2, residues 26R1, 33R1, 42R1, 91R1 and 105R1 are identified at loop/surface regions in classified topographic regions of the plot. In contrast, residues 115R1, 166R1, 169R1, 176R1 and 192R1 are identified in the helix/buried regions at pH2. This plot clearly shows the changes in topographical regions of colicin A upon changing the pH from 7 to 2 (Fig. 5.5).
Figure 5.5: Mobility map. The values of $\langle H^2 \rangle^{-1}$ (inverse of the second moment) vs. $\Delta H_0^{-1}$ (inverse of the central line width) were obtained from the EPR spectra that are shown in Fig. 5.3. Residues number is given at the top left corner of each plot. According to Isas et al. [11] and Mchaourab et al. [12], the topological regions of a protein are indicated by grey colored boxes.
5.3.2 *Comparison between the molten globule and the unfolded state*

To verify whether the increased side chain flexibilities at pH 2 for residues 26R1, 33R1, 42R1, 62R1, 91R1 and 105R1 are due to the presence of unfolded regions, the EPR spectra of 42R1 at pH 2 is compared with the EPR spectrum of 42R1 in 5M Guanidinium hydrochloride (GdHCl). Fig. 5.6 reveals a distinguished difference in spectral amplitudes and line shapes, indicating that the conformation of 42R1 at pH 2 is completely different when compared with the unfolded protein. The smaller spectral amplitude of 42R1 at pH2 suggests that the side chain mobility and backbone fluctuations at pH 2 are not as high as in the unfolded protein (Fig. 5.7A). This result indicates that the increased side chain mobility at pH 2 is due to the loose packing of residues but not due to an unfolding of the protein.

![Figure 5.6: Comparison between the room temperature spectra of 42R1 in GdHCl and 42R1 at pH 2. (A) spectra are spin normalized to show the difference in amplitudes. (B) For better visualization of line shapes, spectra are normalized to their central peak.](image)

5.3.3 *Water accessibility of side chains*

The water accessibility of the R1 side chain can be estimated by determining the Heisenberg exchange rates ($W_{ex}$) of Ni-EDDA with the nitroxide of the R1 side chain [13]. The continuous wave EPR saturation method was used to determine the values of $W_{ex}$ (see chapter 2). In order to check the residue accessibilities, 42 R1 (showing increased mobility from pH 7 to pH 2) and 166R1 (showing decreased mobility from pH 7 to pH 2) were
selected. Upon changing the pH from 7 to 2, residue 42R1 shows increased water accessibility whereas residue 166R1 shows decreased water accessibility (Fig. 5.7). However, the water accessibility of 42R1 at pH 2 is not as high as that of surface exposed residues like 166R1 at pH 7. This again clearly proves that the protein has still a compact conformation and is not completely unfolded.

Figure 5.7: Solvent accessibility of 42R1 and 166R1 in the pH range from 2 to 7. $W_{ex}$ values were determined in the presence of 16 mM Ni-EDDA. Higher values of $W_{ex}$ indicate exposure of side chain to the bulk aqueous phase and smaller values indicate that the side chain is buried into the protein interior.

5.3.4 Hydration of the hydrophobic core

In the MG state, water may enter into the hydrophobic core due to the loosely packed tertiary fold. The presence of water in the hydrophobic core can be estimated by hyperfine splitting parameter, $A_{zz}$, which is very sensitive to the polarity of the R1 microenvironment. Residue 176R1 (H9), located in the center of hydrophobic core was selected to check the polarity changes induced by acidic pH. Fig. 5.8 shows the polarity of 176R1 in the pH range from 7 to 2. The hyperfine splitting (Fig. 5.8A) and the $A_{zz}$ values (Fig. 5.8B) increase at pH 3 and pH 2 when compared to pH > 3, indicating that the polarity considerably increases from pH 7 to pH 2. The increased polarity at pH 2 (3.47 mT) might suggest a certain degree of water penetration into the hydrophobic core of
colicin A. This value is however not as high as expected for a side chain in the bulk water phase (3.7mT).

**Figure 5.8:** Polarity analysis of 176R1: (A) EPR spectra of 176R1 (pH 7-2) were recorded at 160K. The spectra were colored in pink for pH 7, cyan for pH 6, blue for pH 5, green for pH 4, red for pH 3 and black for pH 2. (B) $A_{zz}$ values extracted from the spectra shown in Fig. 5.8A. An increase in $A_{zz}$ values indicates an increase in polarity.

### 5.3.5 ANS Emission Fluorescence

To support the EPR analysis of the MG state of colicin A, the ANS binding ability to colicin A was assessed in the pH range from 7 to 2. ANS has been used to study the MG states of several proteins [14, 15]. ANS is a hydrophobic probe that tends to bind to exposed hydrophobic parts of the protein. ANS molecules bind neither to the natively folded protein nor to the denatured protein. The binding of the ANS probe to the exposed hydrophobic regions can be characterized by an enhanced fluorescence intensity and by a shift in the wavelength of the maximum emission. Fig. 5.9 shows the fluorescence intensities of ANS added to colicin A in the pH range from 7 to 2. The fluorescence intensity increases from pH 7 to pH 2 (Fig. 5.9A and Fig. 5.10). The fluorescence intensity of ANS is negligible at pH 7 (Fig. 5.9A) and in 6M GdHCl (Fig. 5.9B). Fig. 5.10 reveals that ANS exhibits the highest fluorescence intensity at pH 2. A shift in wavelength emission maximum at pH 3 and pH 2 indicates binding of ANS to largely exposed hydrophobic regions of the protein, a characteristic feature of the MG state. This result supports that colicin A forms a MG state at pH 2 with largely exposed hydrophobic surface
and lack of tertiary structure with tight packing of residues. These results are in agreement with the EPR results.

Figure 5.9: ANS fluorescence intensity as a function of wavelength (A) Fluorescence intensity of colicin A with ANS probe was recorded at room temperature in solution at pH 7-2. (B) The fluorescence intensity of colicin A at pH 2 is compared with that of colicin A in 6M GdHCl.

Figure 5.10: The fluorescence intensity (black) and emission maximum (red) which were determined from the spectra of colicin A presented in Fig. 5.9. Shifting of the wavelength of maximum emission indicate the binding of ANS to the hydrophobic surfaces of the protein.
5.4 Discussion

Water-soluble toxins that insert into membranes for their function require a transition to a MG state. External agents like extreme pH, high temperatures and mild denaturing agents can induce MG states [7, 14]. The unique 3D structure of globular proteins is stabilized by non-covalent forces like electrostatic and hydrophobic interactions. These interactions determine protein tertiary folding and its side chain topology. Complete loss of these interactions results in protein unfolding, inducing a random coiled structure. Partial loss of these interactions results in MG states.

Acidic pH mainly disturbs the electrostatic interactions that are very important for the protein tertiary packing. Hydrogen bonds and ionic interactions between positively (Lys/Arg) and negatively (Glu/Asp) charged residues play a key role to keep the protein properly folded. These amino acids are protonated at a pH lower than their pKa. Lys and Arg are protonated at physiological pH whereas Glu and Asp are protonated at acidic pH. Hence, at acidic pH, the protonation of Asp and Glu results in neutralization of charges on these residues thereby breaking electrostatic interactions, causing a loss of the protein tertiary packing which is a characteristic feature of a MG state.

Here, we used the SDSL-EPR method to characterize the pH induced transition of colicin A to the MG state. We have taken advantage of the EPR technique, which enables us to study the side chain flexibility, the hydration of the hydrophobic core and the compactness of the MG.

The EPR spectra have been shown to be very sensitive to the local conformations of colicin A in the pH range from 7 to 2 (Fig. 5.2 and 5.3). The changes in the EPR spectra are initiated at pH < 4 and large changes are apparent at pH 2. The differences in the EPR spectra determined at pH 7 and 2 suggest differences in R1 mobility, which are primarily due to the differences in tertiary interactions. The EPR spectra of R1 being involved in tertiary interactions are expected to be extremely sensitive to changes in the relative positions of the secondary structures. Therefore, the spectral changes observed can be correlated to the change in conformation of colicin A at pH 2.

With the exception of 169R1 (H9) and 176R1(H9), the spectra of the R1 sites reflect two well-resolved components, indicating the presence of two spin label
populations with different mobilities (lower and higher mobility). The ratio between the mobile and the immobile spin label populations is drastically increased from pH 3 to pH 2 except for 115R1, 166R1 and 191R1. The two spectral components might arise from an equilibrium between two conformations of colicin A or from the presence of two different rotamers of the side chain located at interfacial sites.

At pH 2, the side chains of most of the R1 sites at the amphipathic helices are highly dynamic (except 115R1(H6) and 192R1(H10)). The side chain mobility is determined by the motion of side-chain itself and segmental backbone fluctuations. The side chain flexibility is strictly restricted in native proteins due to tertiary interactions that also include electrostatic, hydrophobic and van der Waals interactions. At pH 2 the protonation of negatively charged residues results in loss of electrostatic interactions, which makes the backbone and the side chains more flexible. Similarities between line shapes of 26R1(H1), 33R1(H2), 42R1(H2), 62R1(H3), 91R1(H4) and 105R1(H5a) suggest similar behavior of almost all amphipathic helices. The strong immobilization of residues located in the hydrophobic hairpin (169R1 and 176R1) suggests that the hydrophobic core becomes more compact at pH 2.

The comparison between the spectra of 42R1 at pH2 and in GdHCl, reveal that this side chain at pH 2 is not as dynamic as in the unfolded state. GdHCl is a chaotropic agent, which weakens the electrostatic interactions of both backbone and side chains, inducing protein unfolding. However, at pH 2 the protonation of acidic residues causes loss of side chain electrostatic interactions. Therefore, the MG state is still compact due to remaining hydrophobic interactions and ionic bond interactions (from the non-protonated negatively charged amino acids whose pKa is \( \leq 2 \)). The above results suggest that colicin A is not unfolded at pH 2, hence the increased side chain flexibility cannot be due to a random coil state.

The water accessibility of residues 42R1 and 166R1 is consistent with the mobility data. In fact, residue 42R1 shows increased mobility and accessibility while residue 166R1 shows decreased mobility and accessibility upon changing the pH from 7 to 2. These results suggest that residues with higher mobility are more exposed to the aqueous environment and the immobilized residues are oriented towards the interior of the protein. A very low solvent accessibility was detected for residue 166R1 at pH 2. This suggests that
colicin A is still compact at low pH. The increased solvent accessibility of 42R1, which is buried in the native state (at pH 7), supports the results of NMR pulse-labeling experiments of colicin E1 [10] where it was shown that some buried tryptophans become solvent accessible upon induction of the MG state.

The increased polarity in the microenvironment of residue 176R1 supports another postulated feature of the MG state, namely the hydrated hydrophobic core [16, 17]. The perturbation in the tightly packed tertiary interactions allows the water to penetrate into the hydrophobic core. Nevertheless, the water penetration is minimal when compared to the bulk water phase.

A significant amount of an \( \alpha \)-helical secondary structure and exposed residues are a prerequisite for ANS binding. Hence, the increased ANS fluorescence intensity at pH 2 suggests that colicin A at pH 2 is compact with a prominent secondary structure. Furthermore, the blue shift of the maximum of the fluorescence intensity indicates that the ANS is bound at hydrophobic sites [14], confirming that the globular structure is opened at least at some positions.

From the above results, colicin A at pH 2 can be described as a compact structure with prominent secondary structure, reduced tertiary interactions, highly mobile side chains within the amphipathic helices, less mobile hydrophobic hairpin residues, frequent non-native contacts, and a hydrated hydrophobic core. The MG properties, which were previously determined by different techniques, are compared to the present results. Immobilized residues at positions 115R1, 166R1, 169R1, 176R1 and 192R1, lack of solvent accessibility for 166R1 at pH 2, and the low solvent accessibility for 42R1 with respect to the loop regions, suggest that colicin A at pH 2 is compact. This is supported by viscometry and x-ray scattering results [2, 7]. CD spectroscopy data [2, 18] revealed that MG states have a pronounced secondary structure and lack of tertiary interactions. Incoherent quasielastic neutron scattering [8] showed that the side chains are more mobile in the MG state. 1D NMR coupled with hydrogen-deuterium exchange experiments of colicin E1 [10] revealed that the mobility of the more hydrophilic side chains is increased while there is no increase of the mobility in the hydrophobic core.

The above-mentioned techniques give a generalized view of the side chain flexibility for the entire protein. In the present study, we found that the change of the side
Chapter 5: Structural analysis of molten globule

Chain flexibility is specific for different positions. The residues of the hydrophobic hairpin become more immobilized (169R1 and 176R1) with respect to the native state. The buried residues at pH 7 (42R1, 91R1, 105R1) become more flexible, and a few residues with high mobility at pH 7 are buried (115R1, 166R1). The increased accessibility of the natively buried residue 42R1 is in agreement with the NMR pulse labeling experiments [19] and proteolytic experiments of the MG state where buried sites become accessible to proteases [10]. Finally, the EPR analysis of colicin A at pH 2 uncovers all the characteristic features of the MG state, hence we can conclude that colicin A forms a MG at pH \( \approx 2 \). This is in agreement with the CD spectroscopic data of Evans et al. who reported that colicin A forms a MG between pH 3 and pH 2 [6].

Colicin A, E1 and Ia have been extensively studied by many groups to understand the closed and open channel states of the membrane bound conformations. Although the pore-forming colicins have functional and structural similarities, they bind to the membrane at different pH. Colicin E1 and Ia are proven to bind at pH 4. Wormald et al [10] reported that for colicin E1 most of the changes occurred between pH 4.5 and 6.0. These findings together with our EPR data suggest that the pH for the transition from the native state to the MG is different for different colicins.

5.5 Conclusions

EPR studies of colicin A in the pH range from 7 to 2 revealed that it forms a MG at pH 2. At this pH, most of the spin-labeled side chains of the amphipathic helices become highly mobile while those located at the hydrophobic hairpin become highly immobilized. The MG is compact with pronounced secondary structure and the hydrophobic core is hydrated. The observed side chain flexibility is not due to the unfolded protein. Residues being buried in the native colicin A are clearly exposed at pH 2 and some residues being exposed in the native state are buried at pH 2. The EPR method has been proven to be a powerful technique to study the topology of the MG state, which cannot be studied by the conventional methods like X-ray crystallography and NMR spectroscopy.
5.6 References


6. Conformational changes of colicin A in living *E. coli* cells

**Abstract:** The conformational changes of colicin A upon interaction with living *E. coli* cells were investigated using SDSL-EPR. For majority of the spin-labeled sites, the observed changes in the EPR spectra from the water-soluble to the cell-bound colicin A suggest conformational changes upon interaction of colicin A with the living *E. coli* cells. Moreover, the experiments in *E. coli* mutants (*tolB*) reveal no changes in the EPR spectra of residues 115R1(H6) and 192R1(H10), suggesting that the pore-forming domain does not undergo conformational changes before it enters into the periplasm. Therefore, the observed local conformational changes of colicin A in WT cells originate from the cell-bound colicin A that has been already translocated from the outer membrane to the inner membrane. In the present work, the rate of signal decay due to the cell anti-oxidants was limited by using potassium ferricyanide (1mM) and Origami cells. The fluorescent image of *E. coli* cells and the viability test of the EPR cell samples suggest that the observed conformational changes are from the cell-bound colicin A. This research work reveals that the strategy of SDSL-EPR spectroscopy is a promising tool to study the “in-cell” protein dynamics.

6.1 Introduction

Proteins are essential parts of all living organisms and play an important role in almost all processes of the cell. Ongoing developments in molecular biology, biochemistry and biophysics have led to elucidate the structural and functional details of proteins *in vitro* under defined experimental conditions, which do not necessarily reflect the situation of those proteins in living cells. Since the proteins work in a much more complex environment that involves many other intracellular molecular components in living cells it is important to know the properties of these proteins in the spatial environment of the cell. Hence, an important challenge in biology is to extend our *in vitro* knowledge of protein structure and function to the level of protein interactions in living cells.

Investigating the protein dynamics of living systems is difficult, as it requires techniques that minimally perturb normal physiological activities. Advances in the last decade led to realize the dream of biologists to watch the dynamics of proteins in living
cells. Today, we can explore protein dynamics within living cells with light microscopy [1], NMR [2, 3], FRET [4] and other methods. The protein localization in living cells can be studied properly with GFP-fusion proteins [5] or other fluorescent labeled proteins. However, this method is limited in studies of conformational changes of proteins. Quantum dots (fluorescent semiconductor nanocrystals) have been used to study the diffusion of glycine receptors in living neuron cells. FRET has been utilized to study the *in vivo* conformational changes of proteins, but this technique has problems of background signals and photo-bleaching [6]. The mean molecular dynamics of cell proteins *in vivo* have been studied by neutron scattering [7]. *In vivo* NMR has been widely used for identification of small molecules and has recently reported conformational changes of small proteins like apocytochrome b5 and calmodulin [2, 3], but the disadvantage of *in vivo* NMR is starvation of cells because experiments need longer time periods. These methods have both advantages and disadvantages (explained in the discussion section) based on the type and extent of perturbation, sensitivity, and spatial and temporal resolution, as well as specificity. In the present study, we extend the spectrum of methods for *in vivo* studies on proteins by applying EPR spectroscopy [8-10] to study the conformational dynamics of colicin A in living *E. coli* cells. A few studies have been published which report the application of EPR and spin labeling in living cells. Previously, spin-labeled lipids were used to study membrane phases in plant cells [11] and in Chinese hamster ovary cells [12]. Spin-labeled ligands were used to report the gating of FepA in living bacteria [13]. For the first time, we applied here the EPR technique to study spin-labeled proteins in living *E. coli* cells. For that purpose, we have chosen colicin A that is imported from the outer membrane to the inner membrane.

6.2 Methods

6.2.1 Strains

Colicin A single cysteine mutants and *E. coli* C600 (WT) were kindly provided by Dr. Denis Duchè, CNRI, France. The *E. coli* Origami strain (*gor* and *trxB*) was kindly provided by Prof. Dr. Jung, university of Munich. The *E. coli tolB* mutants were kindly provided by the Nara institute of technology, Japan.

6.2.2 Protein preparation and spin labeling

Colicin A was purified and spin labeled as described in section 3.2.1
6.2.3 Sample preparation

Cells were inoculated \( \text{OD}_{600} = 0.1 \) with an overnight culture and grown in LB medium at 37°C. The cells \( \text{OD}_{600} = 1.0 \) were harvested by centrifugation at 4500 rpm, 20°C, for 20 minutes using a Sorvall centrifuge with a SLA 3000 rotor, suspended in buffer containing 100 mM sodium phosphate buffer (pH 7.0), 0.2% glucose and 0.3 mM KCl and incubated for 30 minutes. The cells were harvested again by centrifugation and suspended in the same buffer. The cell suspension (1 ml) was taken and pelleted down using an Eppendorf centrifuge, at 8000 rpm, at room temperature for one minute. 200 µl of spin-labeled colicin A (10 mg/ml) was added to the cells. In case of \( E. coli \) WT and \( \text{tolB}^- \) cells, potassium ferricyanide \( (K_3[Fe(CN)_6]) \) was added to a final concentration of 1 mM. After one minute, the suspension was centrifuged again at 8000 rpm, at room temperature for one minute, and the cell pellet was washed with 1 ml of 10 mM potassium phosphate buffer, pH 7.4. The washing process was repeated and the cell pellet was resuspended in 20 µl of phosphate buffer, pH 7.4. The cells were loaded into glass capillaries for EPR measurements. The same procedure was applied for treating the cells with met-hemoglobin, which is used as control.

6.2.4 Sample preparation for measuring nitroxide reduction

Cells were harvested and suspended as described in 6.2.3. Cells were pelleted from 500 µl of the cell suspension \( \text{OD}_{600} = 0.4 \) for 100 times diluted). The cell pellet was suspended in 50 µl of MTS spin label solution (100 µM) and the resulting cell suspension was immediately loaded into glass capillaries for EPR measurements. For delaying the rate of signal reduction, potassium ferricyanide was added to the sample (1 mM of final concentration).

6.2.5 Viability test

The samples were prepared (as described in 6.2.3) with and without colicin A. The cells (from EPR sample tubes) were suspended in 1 ml of 10 mM potassium buffer, pH 7.4. Ten fold serial dilutions were made from the cells suspension. 10 µl from each dilution was placed on a nutrient agar plate and spread with glass spreader. The plates were incubated overnight (18 hrs) at 37°C and colony-forming units (CFUs) were counted.
6.2.6 Fluorescent labeling and imaging

Colicin A bearing a cysteine at position 166 was labeled with Alexa fluor 488 C₅-maleimide. Freshly prepared 1M DTT stock solution was added to the pooled colicin A fractions (10 mM final conc.) and incubated overnight at 4°C. DTT was removed by exchanging the buffer 5 times (1:5 dilution) with 10 mM potassium phosphate buffer (pH 6.8) by using an Amicon stirred cell (Millipore) at 3-4 bar pressure under argon. An Alexa 488 stock solution (10 mM) was added to the concentrated protein solutions (1mM final conc. of Alexa 488) and incubated overnight. The unbound fluorophore was removed by ion-exchange chromatography as described in section 3.1. The protein-containing fractions were pooled and the buffer was exchanged with 10 mM potassium phosphate buffer (pH 7).

E. coli cells treated with fluoro-labeled colicin A (see section 6.2.3) were analyzed with a laser scanning microscope (Leica).

6.3 Results

6.3.1 Decrease of the EPR signal intensity

Free thiols of the surface and the periplasm of a cell readily reduce the nitroxide of the spin label. However, the rate of the EPR signal decrease can be delayed for a certain time by conducting the experiments in a less reducing environment. The rate of signal-decrease using free MTS spin label was investigated in WT (C600) and in Origami cells (carry mutations in the thioredoxin reductase (trxB) and the glutathione reductase (gor)).

Fig. 6.1 shows the EPR signal decay of free MTS spin label after incubation with untreated WT, potassium ferricyanide treated WT and with Origami cells. The rate of signal-decrease is faster in untreated WT than in origami cells and then in potassium ferricyanide treated WT cells. In case of the untreated WT cells, the signal decreases with a half-time of less than one minute, whereas in the potassium ferricyanide treated WT cells the signal decreases with a half-time of 14 minutes (Fig. 6.1). At this concentration of potassium ferricyanide (1mM) the viability of E. coli cells (was checked by plating method) was not affected. In case of the untreated Origami cells, the signal decreases with a half-time of 5 minutes (Fig. 6.1). This is attributed to their lack of gor and trx.
Figure 6.1: Free MTS spin label signal reduction upon incubation with living cells. The signal intensities were derived from the amplitude of the central resonance line. The curves were normalized in relation to each maximal value.

6.3.2 Binding of colicin A to E. coli cells

In order to check whether colicin A interacts with E. coli cells under the conditions used for the EPR measurements, experiments were performed by testing the viability of EPR cell samples and additionally colicin A localization was determined by replacing the spin-labeled colicin A with the fluorophore Alexa 488 labeled colicin A.

In parallel, E. coli cells (prepared according to the protocol for EPR samples) were treated with and without colicin A and then were examined for their viability by a plating method. Fig. 6.2 shows the agar plates with bacterial colonies after 18hrs incubation. In case of the E. coli cells without colicin A (control), bacterial lawns are observed up to $10^5$ dilution, whereas numerous isolated colonies are observed from $10^6$ dilution on (Fig. 6.2). In contrast, in case of the colicin A treated E. coli cells, isolated colonies are found even at $10^0$ and $10^1$ dilution and then no colonies could be observed in further dilutions. The viability test reveals that about 99% of the colicin A treated cells did not survive when compared to the control, indicating that colicin A interacts with the cells of the EPR samples.
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Figure 6.2: Viability of the cells without and with colicin A (62R1). The cells were taken from EPR samples. The dilution factor of the cells is given. Control plates initially show bacterial lawns (due to large number of bacteria) whereas isolated colonies can be observed at $10^6$ dilution. No colony forming units (CFUs) were observed from colicin A treated cells after $10^2$ dilution.

To localize the colicin A upon interaction with the *E. coli* cells, cells were treated with Alexa 488 labeled colicin A and then examined in a confocal laser scanning microscope. The corresponding image of the cells is shown in Fig. 6.3. The image reveals that fluorescence appeared only at the membrane of the treated cells but not in the cytoplasm or in the medium. This result indicates that colicin A binds effectively to the *E. coli* cells.

Figure 6.3: Laser scanning image of *E. coli* cells. Cells were treated with colicin, A which was labeled with Alexa fluor 488 at position 166.
Control experiments were performed to check whether the EPR spectra (shown in Fig. 6.7-Fig. 6.8) obtained from the cell samples is from the cell-bound colicin A or from the unbound colicin A. For this control experiment, the spin-labeled met-hemoglobin, which is unable to bind or to import into E. coli cells, is used as a control. Cells were treated individually with, without spin-labeled colicin A, or with spin-labeled met-hemoglobin (Fig. 6.4).

![Figure 6.4: Comparison between colicin A and met-hemoglobin treated cells. Room temperature EPR spectra of cells treated without any protein, with spin-labeled colicin A and with spin-labeled met-hemoglobin. Concentration of the met-hemoglobin is 100 µM and concentration of colicin A is 94 µM.](image)

As can be seen in Fig. 6.4, the EPR signal from the met-hemoglobin treated cells is negligible when compared with the signal from the colicin A treated cells. On the other side, it is almost similar to the cells that are treated without any protein (baseline). This experiment suggests that the procedure for sample preparation, which is developed for the present work, does not leave any significant amount of unbound protein in the EPR samples. Therefore, it can be concluded that the observed EPR spectra (Fig. 6.7-6.8) originate from the spin-labeled colicin A that interacts with the E. coli cells.

### 6.3.3 Optimum concentration of colicin A required for In-cell EPR

The required protein concentration has to be considered when studying the conformational changes of a protein in vivo. Binding of colicin A to the E. coli cells mainly depends on the number of receptors available and the time used to incubate the
cells with the colicin A. Approximately 200-300 BtuB receptors are present per a cell [14]. Colicin A was incubated with the cells only for 30-60 sec due to the signal decaying problems. Therefore, from an initially added amount of colicin A, only a fraction interacts with the cells and the rest remains as an unbound fraction.

To determine the required initial added amount of colicin A for obtaining a significant EPR signal, serial dilutions (2 fold) of spin-labeled colicin A (94.5 µM) were used. The EPR signal detected from the washed cell-pellet gives the fraction of cell-bound colicin A whereas the signal from the supernatant reflects the fraction of unbound colicin A. Fig. 6.5 shows the EPR signal derived from the cell-pellet and from the supernatant.

![Figure 6.5: Colicin A that bound and unbound to the cells. (A) Absorption EPR spectra obtained from washed cell-pellet, a fraction of cell-bound colicin A (62R1). (B) Absorption EPR spectra obtained from the supernatant, a fraction of unbound colicin A (62R1). The initial amount of protein added to the cells is indicated on the left side of the graph. Colicin A with respective concentration was incubated with 1.5×10^{12} cells in 200 µl volume. The area under the absorption spectra is proportional to the number of spins.](image)

As revealed from Fig. 6.5A, the signal intensity decreases with decreasing initial amount of colicin A, indicating that higher protein concentration enhances the binding efficiency of colicin A to the receptors. However, the amount of unbound fraction also increases with increasing protein concentration. For a better understanding, the areas under the absorption spectra of both the cells and supernatant (Fig. 6.5) were calculated and shown in Fig. 6.6. The area under the absorption spectra is related directly to the number of spins (in this case spin-labeled colicin A). At very low concentration of initially added colicin A (6 µM), the fraction of unbound protein is lower than the fraction of cell-bound colicin A. At the concentrations of 12-23 µM, the fraction of both
bound and unbound fraction are almost similar. Above 47 µM concentration of initially added protein, the ratio of unbound to bound protein is drastically increased.

**Figure 6.6:** Concentrations of unbound colicin A and of colicin A that bound to the cells. The concentrations were calculated from the areas under the absorption spectra that are shown in Fig. 6.5. Note: Area under the absorption spectrum gives the number of spins present in the corresponding EPR sample.

This indicates that above 50µM concentration of colicin A most of the receptors are blocked by colicin A. According to our estimations from the resulting EPR spectra, approximately 200 colicins are bound to each cell. Calculation of spin concentrations (described in section 4.2.7) revealed that when colicin A (94µM) was initially added, 64 % of the initial amount was unbound whereas 26.5 % was bound to the cells and 10 % of the protein was either washed out or reduced by the cell thiols. For the present study, we used 160-200 µM concentration of the colicin A.

### 6.3.4 Conformational changes of colicin A in living E. coli cells

R1 side chains that are in the slow motional limit are readily identified by the so called powder spectrum with large apparent hyperfine splitting whose separation depends on solvent polarity. At the other extreme, the R1 residues that are in the fast motional limit are readily identified by a narrow overall breadth and sharp lines in the EPR spectrum. In a protein, the mobility of an amino acid side chain depends on secondary and tertiary interactions. For instance, the residues that are located in the protein interior are minimally allowed to move because of the tightly packed protein
interior. On the other side, the residues that are located on the surface of the protein and loop regions are highly mobile due to weak interactions with neighbor residues. Therefore, the mobility of R1 side chain in its vicinity provides the information about the local conformational changes of a protein.

The changes observed in the EPR spectra for the transition from water-soluble colicin A (sol-ColA) to cell-bound colicin A (cell-ColA) indicate conformational changes upon the interaction of sol-ColA with living *E. coli* cells. As shown in Fig. 6.7 and 6.8, the spectra of residues 62R1 and 115R1 of sol-ColA show small hyperfine splitting with small line widths, suggesting that these residues are mobile. Additionally, the accessibility data (see chapter 3) support that these two positions are located on the surfaces of the helices. The spectra of residue 62R1 (cell-ColA) are complex, reflecting two resolved populations characterized by different mobilities (Fig. 6.7), one of which is relatively immobilized, suggesting that 62R1 (cell-ColA) either has contacts with another protein or is subjected to increased tertiary interactions. The spectra of 115R1 (cell-ColA), show an increased mobile component (Fig. 6.7), suggesting an increased mobility for this residue in cells. The spectra of residue 91R1 (sol-ColA) shows two component spectra, indicating tertiary interactions with nearby groups of the protein which is also confirmed by its partial accessibility to the paramagnetic quenchers (chapter 3). The spectra of residue 91R1 (cell-ColA) show an increase in the mobile and a decrease in the immobile fractions (Fig. 6.7), indicating that tertiary interactions became weaker in cell-ColA.

The EPR spectra of residues 42R1 (Fig. 6.7), 105R1 (Fig. 6.7), 176R1(Fig. 6.8) and 192R1 (Fig. 6.8) of sol-ColA show a large apparent hyperfine splitting, characteristic of the nitroxide side chains in very slow motional regimes. The mobility and accessibility data (chapter 3) suggested that these sites are buried in the protein interior. In living cells, the spectra of 42R1 and 105R1 show an increase in the mobility with time as clearly seen from the shift of the low field peak to higher B-fields (Fig. 6.7). These data suggest that 42R1 and 105R1 are not buried in the protein interior in cell-ColA. The mobility of residue 176R1 did not change much from sol-ColA to cell-ColA. Residue 169R1 (cell-ColA) shows a prominent immobile component (Fig. 6.8), indicating that the mobility of residue 169R1 is restricted, characteristic for residues with tertiary contacts. Residue 192R1 (cell-ColA) shows two nitroxide populations with an increased overall mobility when compared with sol-ColA (Fig. 6.8).
Figure 6.7: Room temperature EPR spectra of 42R1, 62R1, 91R1 and 105R1 during interaction with *E. coli* cells. The spectra of sol-ColA are colored in black. The spectra of cell-ColA are colored in red. The time from the first scan of EPR measurements is shown on the left side (sample preparation takes less than 5 minutes). For clarity, the outer hyperfine splittings are indicated with dashed lines. The spectra are normalized to their central peak maximum.
Figure 6.8: Room temperature EPR spectra of 115R1, 169R1, 176R1 and 192R1 during interaction with *E. coli* cells. EPR spectra of sol-ColA are colored in black. The spectra of cell-ColA are colored in red. The time from the first scan of EPR measurements is shown on the left side (sample preparation takes less than 5 minutes). For clarity, the outer hyperfine splittings are indicated with dashed lines. The spectra are normalized to their central peak maximum.
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The dynamics of the side chain can be assessed by a semi-empirical parameter, the inverse of the central line width ($\Delta H_0^{-1}$). Highly mobile R1 sites (e.g. R1 at loop regions) are characterized by high $\Delta H_0^{-1}$ values whereas immobilized R1 sites (e.g. R1 at buried positions) have lower $\Delta H_0^{-1}$ values. Fig. 6.9 shows the change in mobility values of 105R1 upon interaction with the cells. Fig. 6.9 reveals that residue 105R1 is buried in sol-ColA whereas the side chain motion is increased in cell-ColA. The data indicate that 105R1 is then located in cell-ColA at a helix contact or a helix surface site. The significant changes in the EPR line shapes and the line widths of 105R1 suggest that colicin A undergoes a significant conformational change upon transition from sol-ColA to cell-ColA.

![Figure 6.9](image)

**Figure 6.9:** The inverse of the central line width ($\Delta H_0^{-1}$) of cell-bound 105R1 vs. time. The data points are fitted with an exponential function. Colicin A (160 µM) was incubated with $1.5 \times 10^{12}$ E. coli cells in 200µl volume. The range of $\Delta H_0^{-1}$ values representing different protein topologies are marked on the right side of the plot according to Isas et al [15] and McHaourab et al [16].

In the present study, we used potassium ferricyanide (1 mM final concentration) to avoid the signal decay while working with WT cells. It was reported previously that 70 mM ferricyanide effects the respiration and at high concentrations it can also effect the cell permeability [17]. Hence, to avoid any side effects from the usage of potassium ferricyanide, we used an alternative E. coli strain called Origami (trxB- and gor-) which are sensitive to colicin A. Fig. 6.10 shows the EPR spectra of both WT cells and Origami cells for 115R1 and 192R1.
Figure 6.10: Room temperature EPR spectra of 115R1(H6) and 192R1(H10) in wild type cells (WT) and Origami cells (gor' and trxB'). EPR spectra of sol-ColA are colored in black. The spectra of cell-ColA are colored in red. The time from the first scan of EPR measurements is shown on the left side (sample preparation takes less than 5 minutes). For clarity, the outer hyperfine splittings are indicated with dashed lines. The spectra are normalized to their central peak maximum.
Comparison of the EPR spectra of cell-ColA in WT cells and Origami cells reveals that the EPR line shapes are similar in both cell systems (Fig. 6.10), suggesting similar conformational changes. This indicates that potassium ferricyanide does not have any effect at the concentration of 1 mM on *E. coli* cells. Moreover, the viability of potassium ferricyanide treated WT cells is similar to the viability of untreated WT cells. Therefore, using potassium ferricyanide does not cause any changes in the interaction of colicin A with the *E. coli* cells.

### 6.3.5 Conformational changes colicin A in tolB knockout *E. coli* mutants

TolB is a periplasmic protein that is involved in the import of colicin A from the outer membrane to the inner membrane [18]. *tolB* mutants are resistant to the toxicity of colicin A (Activity tested by plating method). Since TolB is essential for the import of colicin A, in *tolB* mutants colicin A remains bound to the outer membrane. Hence, we used *tolB* mutants to localize where the conformational changes of cell-ColA happen in the cell. Fig. 6.11 shows the EPR spectra of residues 62R1, 115R1 and 192R1 of colicin A in *tolB* knockout cells.

![Figure 6.11: Room temperature EPR spectra of 62R1(H3), 115R1(H6) and 192R1(H10) interacting with *tolB* cells. EPR spectra of sol-ColA are colored in black and the spectra of cell-ColA are colored in red. The time from the first scan of EPR measurements is shown on the left side (sample preparation takes less than 5 minutes). For clarity, the immobile component of the low field line is indicated with dotted line. The spectra were normalized to their central peak maximum.](image)
The spectral line shapes of 115R1 and 192R1 are almost identical to the line shapes of sol-ColA. Interestingly, 62R1 shows complex spectra with two components of different mobilities. The immobile component indicates that 62R1 has tertiary contacts either with the outer membrane proteins or within colicin A itself upon interaction with *E. coli* cells.

Fig. 6.12 shows the EPR spectra of both WT cells and *tolB* mutant cells. Unlike the results for WT, 62R1 in *tolB* mutant cells shows a resolved component, characteristic for an immobile R1 side chain. However, residues 115R1 and 192R1 show increased mobility in WT cells whereas they do not show any changes in *tolB* cells.

**Figure 6.12:** Room temperature EPR spectra of 62R1(H3), 115R1(H6) and 192R1(H10) interacting with WT cells and mutant (*tolB*) cells. The spectra of sol-ColA are colored in black and the spectra of cell-ColA are colored in red. The spectra were normalized to their central peak maximum.
6.4 Discussion

Protein conformations and dynamics strongly depend on the solvent conditions and on the interactions with their partners in vivo. Thus, it became very interesting for biologists to study protein function and dynamics in vivo. Thus, we intended to study the conformational changes of colicin A upon interaction with E. coli cells. The signal-decay, caused by the reduction of the nitroxide is the main limiting factor of SDSL-EPR to study the protein dynamics in living cells. In fact, the reducing environment of the cell thiols readily reduces the nitroxide radicals attached to the protein. Reduction of a nitroxide leads to a diamagnetic species with no EPR signal. The signal intensity thus decays with time. To overcome this limitation, we used oxidizing agent such as potassium ferricyanide that reduces the rate of signal decay (Fig. 6.1). Although, E. coli are not affected by potassium ferricyanide at lower concentration [17], it is important to check whether potassium ferricyanide causes any effect on the colicin A conformation and its interaction with the E. coli cells. Therefore, we used the E. coli Origami strain, which is a mutant for thioredoxin reductase (trxB) and glutathione reductase (gor). The conformational changes of colicin A are found to be similar in WT cells (using potassium ferricyanide) and in origami cells (Fig. 6.10). Hence, potassium ferricyanide and cells with less reducing cytoplasm can be used to overcome the signal reduction problem of EPR spectroscopy application to in-cell studies.

The experiments with the fluorophore-labeled colicin A in living cells and the viability test confirm that colicin A is bound to the E. coli cells. The EPR spectra obtained from E. coli cells clearly show the presence of different conformations. Especially, the spectral features, which are characteristic for the soluble colicin A can be seen at least initially in the spectra from cells. Moreover, a fraction of colicin A bound to the inner membrane and a fraction that still bound to the outer membrane are constitutively present in the spectra. The immediate K⁺ efflux from the cells (data not shown) suggest that colicin A immediately forms open channels. The number of colicin A molecules that translocated from outer membrane to inner membrane increases with time.

Previous studies on colicin kinetics reported that one colicin molecule is enough to kill the cell. The main reason for cell death is the depletion of K⁺ intracellular levels [19]. However, cells can be rescued from death in the presence of K⁺ ions and ATP [19].
Hence, we used potassium phosphate as a buffer system for EPR measurements to increase the ratio between membrane inserted and receptor bound colicin A.

The changes in the EPR line shapes (Fig. 6.7 - 6.8) suggest local conformational changes in colicin A upon interaction with the living cells. With the exception of 169R1, the increased mobility suggests that the tightly packed globular colicin A partially unfolds in living cells. This is clearly visible from the increased mobility of residues 42R1, 105R1 and 192R1, which are buried in water-soluble colicin A.

In order to hit the target, colicin A first binds to the receptor of the outer membrane and then translocates from the outer membrane to the inner membrane using OmpF, TolB, TolA, TolQ and TolR (Fig. 6.13). Since, tol knockout strains are resistant to colicin A toxicity, Tol proteins are considered as essential for the action of colicin A [20]. Previous studies of radioactive colicin A suggest that colicin A remains bound to the outer membrane in tolA- mutants [20]. In the present study, tolB- mutants were used to further investigate whether the observed conformational changes (Fig. 6.7-6.8) occur upon interaction of colicin A with the inner membrane or with the outer membrane.

![Figure 6.13](image.png)

**Figure 6.13:** A schematic representation of the import of colicin A in both WT and mutant (tolB') cells. Since TolB protein is essential for the translocation, colicin A cannot be translocated from the outer membrane to the inner membrane. Mutant (tolB') cells were tolerant against colicin A toxicity (tested by plating method).
TolB is a periplasmic protein and its maturation and localization is known to be required for colicin import [18]. Comparison of the EPR spectra indicates that the spectral changes observed in WT cells are not identical to the spectral changes observed in \textit{tolB} strains. Moreover, in \textit{tolB} cells, spectra of residues 115R1 and 192R1 did not show any difference to the respective sol-ColA samples. In previous reviews on colicin A, it was assumed that the pore forming domain threads before it translocates through the OmpF [21]. Our results in \textit{tolB} mutants are in contrast to this statement and suggest that colicin A did not undergo drastic conformational changes while interacting with the proteins (BtuB or OmpF) of the outer membrane. Residue 62R1(H3) in \textit{tolB} mutants shows restricted mobility which might be due to the steric interaction with the OmpF porin. This would also explain the reason why this immobile component is more intense initially in wild type cells. This result suggests that colicin A helix 3 probably is in contact with OmpF. The results obtained for \textit{tolB} mutants also preclude the possibility of conformational changes induced in the pore-forming domain of colicins either by BtuB or by OmpF [21]. These results also revealed that the conformation of the pore-forming domain is almost identical to the water-soluble structure before it translocates from the outer membrane. This suggests that the conformational changes observed in WT cells originate from colicin A that is translocated from the outer membrane to the inner membrane.

The conformation for the closed channel state of colicin A in liposomes is described in detail in chapter 3 and 4. To understand the conformation of colicin A in living \textit{E. coli} cells, we compared the spectra in Fig. 6.14. Although the spectra are not exactly identical, the tendency of the mobility changes is comparable with those obtained in artificial membranes especially for residues 42R1, 62R1, 91R1, 105R1, 169R1 and 192R1. The differences are mainly due to the presence of different conformations (closed and open channel states and outer membrane bound conformation etc.) of colicin A in living cells whereas this is not the case in liposomes.
Figure 6.14: Comparison of the EPR spectra of colicin A in liposomes (reconstituted) and in *E. coli* cells. The spectra of water-soluble colicin A are colored in black and the spectra of colicin A in liposomes or upon interaction with *E. coli* cells are colored in red. The spectra were normalized to their central peak maximum.
6.5 Conclusions

Our results reveal local conformational changes of spin-labeled colicin A which bound to *E. coli* cells. Experiments in *tolB* mutants suggest that the pore-forming domain of colicin A does not undergo significant conformational changes upon interaction with the outer membrane. The results indicate that the conformational changes observed in WT cells originate mainly from colicin A that bound to the inner membrane of the *E. coli* cells. Present work introduces the application of SDSL-EPR to investigate the dynamics of spin-labeled proteins in living cells.

6.6 References


7. MD simulations: Acidic pH induced conformational changes of colicin A, Ia and N.

Abstract: For a more detailed understanding of the underlying processes of pH dependent conformational scenarios, a series of molecular dynamics (MD) computer simulations were performed on three different colicins, colicin A, Ia and N at different pH values (pH 7, 4, 3 and 2). The resulting MD trajectories were analyzed to obtain structural and dynamic properties, which were analyzed by the essential dynamics method. The results suggest that colicin A is very stable at neutral pH and very dynamic at low pH. Colicin Ia also shows distortions at acidic pH but the regions that are involved in the conformational changes are different with respect to colicin A. Colicin N is very stable at all pH values. The calculated surface electrostatic potentials suggest that the surface positive charge increases with decreasing pH, disclosing the reason why colicins bind to lipids efficiently at acidic pH and require negatively charged lipids. The results of these simulations are in quantitative agreement with the fluorescence and EPR studies and the dynamic properties determined by MD simulations allow for an expanded description of the molten globule state of colicin.

7.1 Introduction

Proteins are linear chains of amino acids that fold into thermodynamically stable structures to perform their biological function. The conformation of a protein at which the protein is functional is known as ‘native’ state. The folding process from the linear chains to the native state occurs mainly in two steps: establishment of secondary structures (e.g. helices and beta sheets) and folding of the tertiary structure. The tertiary fold is primarily stabilized by hydrophobic and electrostatic interactions. The electrostatic interactions, i.e. the interactions between charged groups, depend on the protonation state of each charged residue, which in turn, depends on the pH of the surrounding solvent. As a consequence, the pH of the solvent influences the electrostatic interactions that stabilize the native state of a protein. Therefore, changes in pH or shifts in pKa’s affect the amino acid protonation states that ultimately change the protein conformation. For example, low pH induces conformational changes in trypsinogen, exposing sites for protease activity, which convert trypsinogen into a functional trypsin.
Furthermore, water-soluble proteins that insert into membranes are known to undergo a low pH induced conformational change leading to a translocation competent state or a molten globule (MG) state before they insert into membranes [1]. Therefore, the study of pH induced conformational changes might give new insights into membrane insertion, protein folding and details of the molten globule states.

Previous studies on colicins suggested that colicin A and E1 insert into the membrane efficiently at acidic pH. Acidic pH induced molten globule formation in colicins has been reported for colicin A [2, 3], colicin B and colicin E1 [4]. Additionally, acidic pH induced structural changes have been shown previously for colicin E1 [5] and in the present work for colicin A (chapter 5).

However, the characterization of acidic pH induced protein states (or molten globules) at the molecular level remains difficult due to their dynamic nature and their heterogeneous distribution of conformational states. High-resolution techniques like X-ray crystallography and NMR allow for structural characterization of well-ordered structures like native states at atomic resolution. Nevertheless, the dynamic structures like molten globules and unfolded states can be studied qualitatively by lower resolution methods such as circular dichroism, fluorescence spectroscopy, ultraviolet spectroscopy, SAXS, 1D NMR, EPR (chapter 6) etc. Even though the global properties of pH induced conformational changes have been provided by such low resolution techniques, the atomic details of these protein states are still not characterized. As up to now no experimental technique is able to provide information at the atomic level for such dynamic structures, in the present study, we used molecular dynamics simulations to study the pH induced conformational changes in different colicins.

The method of molecular dynamics (MD) simulations calculates the time dependent behavior of a molecule according to Newton’s law of motion. The advantage of using MD is that an all-atom representation of each state as a function of time can be obtained. Therefore, MD simulations have been successfully applied to answer many biological questions. The major limitation of MD simulations is the accessible time scale during realistic solution simulations (ps to ns range). Particularly, it is not known how fast the pH induced conformational changes in colicins take place. If the time scale is in the microsecond/millisecond range, it is not accessible to MD simulation. In the present work we do not expect our simulations equilibrate to the molten globule state. Instead, the work is intended to provide insight into how the structural and dynamical
properties of the native state initially change when the protein is introduced into an environment of low pH.

![Energy Landscape of a Protein](image)

**Figure 7.1:** The energy landscape of a protein. The iso-energetic minima are separated from each other by energy barriers of various heights. Each of these minima corresponds to one conformational sub state of a protein. The conformational transitions are barrier crossings (arrows) and the transition rate is determined by the height of the barrier. (Source: Prof. Dr. Grubmüller home page)

Protein dynamics include a variety of vibrations (fast motions), local fluctuations and structural rearrangements (slow motions) which are generally known as conformational transitions (Fig. 7.1). As the protein function is often intimately linked to its conformational dynamics, it is not very straightforward to extract the functionally relevant motions from simulated trajectories (see movie-1 in the CD attached to this thesis). Thus, in order to provide the information about collective or concerted motions, suitable tools like principal component analysis (PCA) or essential dynamics (ED) [6] analysis methods were used. These methods can efficiently filter global, collective (often slow) motions from local, fast motions. The basic idea of the ED method has been found to be useful for revealing functionally significant fluctuations in various protein systems [6, 7].

Previously, the structural properties of the molten globule state have been studied by MD simulations at high temperature (to accelerate the unfolding process) [8, 9] and at pH 2 [10]. In the above-mentioned publications, all charged residues were protonated to attain pH 2. But, in the present study the residues were protonated according to the pKa values obtained from pKa calculations.

The acidic pH (pH < 3) induced membrane binding efficiency (chapter 4) and the acidic pH induced conformational changes of colicin A (chapter 5) were studied by
EPR. In the present study, we are mainly interested in the pH induced conformational changes of colicin A, but we also intended to compare the behavior of colicin A with colicin Ia and colicin N. These colicins although they all have similar structure (see Fig. 7.2) and function, they insert into the membranes (*in vitro*) at different pH values and are differently translocated. Colicin A and colicin N use the Tol system for translocation whereas colicin Ia uses the TonB system. On the other hand, colicin A and colicin N use different receptor systems.

**Figure 7.2:** Structural comparison of colicin A, Ia and N. (A) Structurally aligned colicin A (green) and colicin Ia (lavender) (B) Structurally aligned colicin A (green) and colicin N (Orange). The structural alignment was done by the Stamp module of the multiseq which is implemented in VMD program.

### 7.2 Methods

#### 7.2.1 Initial coordinates

Starting coordinates for the MD simulations and pKa calculations were obtained from the x-ray crystal structures database (www.rcsb.org/pdb). Colicin A (pdb entry-1COL), Colicin N (1A87), Colicin Ia (1CII) with resolutions of 2.4, 2.5, 3.1 and 3.0 Å respectively, were used for the present study.

#### 7.2.2 pKa calculations

pKa calculations were performed by the Multi-Conformation Continuum Electrostatics (MCCE) method [11]. MCCE is a simulation program that combines electrostatics and molecular mechanics. In this program, the protein side chain motions are simulated explicitly while the dielectric effect of the solvent and the bulk protein material is modeled by continuum electrostatics (by using Delphi). The pKa calculations were performed from initial pH 0.0 with successive increments of one pH unit up to pH 15. Titratable groups included in the pKa calculation were the N-terminus, ASP,
GLU, HIS, LYS, ARG and the C-terminus. The following parameters were used: interior dielectric constant 4; exterior dielectric constant, 80; solvent probe radius, 1.4Å; ionic radius, 2.0Å and ionic strength, 150mM.

### 7.2.3 Molecular Dynamics simulations

MD simulations were performed with the GROMACS simulation package V3.3.1 [12]. The OPLSAA force-field was used and the titratable amino acids were protonated according to the pKa calculations. The TIP4p water model was used as an explicit solvent. The protein was solvated with water in a periodic rectangular box, which was filled by solvent on all sides, and the distance between the box walls and the solute was 0.6 nm. To make the system neutral, counter ions Cl⁻ and Na⁺ were added by replacing water molecules at the most appropriate electrical potential. The systems were subsequently energy minimized with a steepest descent method for 5000 steps or until it reaches convergence. In all simulations the temperature was maintained close to the intended value (300K) by weak coupling to an external temperature bath with a coupling constant of 1 fs. The LINKS algorithm was used to constrain all bonds. A twin range cut-off was used for the calculation of the non-bonded interactions. Interactions within the short range cutoff were updated every time step whereas interactions within the long range cutoff were updated every 5 time steps together with the pair-list. The Particle Mesh Ewalds (PME) method was used for the calculation of the long-range interactions. All atoms were given an initial velocity obtained from a Maxwellian distribution at the desired initial temperature. All simulations, starting from the crystallographic structure, were equilibrated for 200 ps at a temperature of 300K, with position restraints on the protein to allow relaxation of the solvent molecules. These first equilibration runs were followed by additional 50 ps runs at 50 K without position restraints on the protein. The temperature was gradually increased from 50 K to 300 K by performing short runs of 50 ps each for every 50 K temperature increase. Simulations of 10 ns at 300K were performed for colicin A (colA), colicin N (colN), colicin Ia (colla) at pH 7, pH 4, pH 3 and pH 2.

### 7.2.4 Essential dynamics or principal component analysis

Principal component analysis (PCA) is a means to reduce the data set. PCA of MD trajectory data, often called essential dynamics (ED) is frequently used to separate large-scale correlated motions from local harmonic fluctuations [13].
ED analysis constructs a new orthogonal basis set for the atomic coordinates in a trajectory, such that the greatest variance occurs along the first vector, with decreasing variance along successive vectors. These vectors are often called principal components or eigenvectors. The eigenvalues represent the relative amount of molecular motion that occurs along each eigenvector.

**Theory**

1. Fitting to a reference structure: Since only the internal motions are usually of interest in the study of protein dynamics, the overall translational and rotational motions were removed by fitting the MD trajectory at each time step to a reference structure. The internal motion is now described by a trajectory $x(t)$, where $x$ is a $3N$-dimensional vector of all atomic coordinates, represented by a column vector.

2. Construction of the covariance matrix: The symmetric correlation between atomic motions can be expressed in a $3N \times 3N$ covariance matrix ($C$) of the atomic positional displacements.

$$C = \text{cov}(x) = \left\langle (x - \langle x \rangle) (x - \langle x \rangle^T) \right\rangle$$

Where the $T$ superscript represents the transpose and an angle bracket $\langle \rangle$ represents an average position of the positions $x_i$ ($i = 1, ..., N$) over time (whole trajectory). Atoms moving in a similar direction yield positive correlations, atoms moving in opposite direction yield negative correlations and atoms moving randomly yield no correlation.

3. Diagonalization of the covariance matrix: The symmetric matrix $C$ can be diagonalized by an orthogonal coordinate transformation matrix $T$

$$TCT^T = \text{diag}(\lambda_1, \lambda_2, \lambda_3, ..., \lambda_{3N})$$

Diagonalization of the covariance matrix yields a set of eigenvalues and eigenvectors (ev). The $i^{th}$ column of $T$ represents an eigenvector or principal or essential mode belonging to the eigenvalue, $\lambda_i$. The eigenvalues are the positional mean square fluctuations, i.e. the amplitude of the correlated motions, along the direction of the corresponding eigenvectors. The eigenvectors indicate
directions in the total configuration space, representing correlated displacements of groups of atoms in the system.

Eigenvectors are sorted by the size of this displacement, i.e. the first eigenvector is the eigenvector with the largest eigenvalue. Hence, the first eigenvectors are those collective motions that best approximate the sum of fluctuations and the last eigenvectors correspond to the most constrained degrees of freedom.

The characteristics of collective motions can be studied by projecting the trajectory on to the corresponding eigenvector and translating these projections to 3D space to visualize the atomic displacements connected with that eigenvector. Projection on to selected eigenvectors filters out the thermal noise and the position of each individual structure along an eigenvector can be obtained by the projection.

$$P_i = (x - \langle x \rangle) \cdot T_i$$

Here, $x$ is a structure (a vector in configurational space), $\langle x \rangle$ is the average structure, $T_i$ is an eigenvector and $P_i$ is the displacement of the structure along the $i^{th}$ eigenvector with respect to the average structure. The three dimensional structure corresponding to a displacement along a single eigenvector can be obtained by:

$$x = P_i \cdot T_i + \langle x \rangle$$

One can also examine the meaning of a single eigenvector by generating the trajectory with atomic positions interpolated between extreme projections on the selected eigenvector. Essential dynamics analysis was performed by g_covar and g_anaeig programs of Gromacs package 3.3.1.

### 7.2.5 Combined essential dynamics analysis

Combined essential dynamics [6] is a tool to evaluate similarities and differences between the essential motions in different trajectories of similar proteins. In this method, first, the MD trajectories of similar systems are concatenated to form one big trajectory and the covariance matrix is constructed by fitting to the same reference structure. The resulting eigenvectors now indicate concerted motions of atoms which are common in the separate trajectories [14]. The individual trajectories are then projected onto the combined eigenvectors which are calculated from the combined trajectory. This method provides information about the difference in the “equilibrium structure” and “fluctuations” along the different eigenvectors calculated from combined trajectory.
1. The difference in the average projections of two separate trajectories on a
specific eigenvector from the combined eigenvector trajectory indicates that the
equilibrium structures along that direction are different (static shift).

2. Large differences in the positional fluctuations around the average positions of
these projections indicate differences in the dynamics along this direction.

In the present study, this method is applied to compare the dynamics of colicin A
and colicin N in different protonation states.

### 7.2.6 Root mean square inner product

The overlap between the essential subspaces of two sets of simulations can be
obtained by Root Mean Square Inner Product (RMSIP). Hence, RMSIP can be used to
compare the essential subspace of two proteins.

\[
\text{RMSIP} = \left( \frac{1}{10} \sum_{i=1}^{10} \sum_{j=1}^{10} (\eta_i \cdot \nu_j)^2 \right)^{\frac{1}{2}}
\]

Here, \( \eta_i \) and \( \nu_j \) are the eigenvectors from each eigenvector set. In the present work, the
subspace that is contributed from the first ten eigenvectors is considered as essential
subspace.

### 7.2.7 Electrostatic potentials

The Grasp program [15] was used to calculate electrostatic potentials for
qualitative analysis. The resulted coordinates from MD simulations and the atomic
charges that were used for MD simulations were used for GRASP calculations. The
molecular surface was calculated for each structure and the calculated electrostatic fields
were displayed on the molecular surface.

### 7.3 Results and discussion

#### 7.3.1 pKa calculations

In order to obtain the protonation states of amino acids at specific pH, pKa
values were calculated for each charged residue. The calculated pKa values are within
an error of 0.5 to 1.0 with the experimental results from other proteins [11]. Fig. 7.3A
reveals the pKa values of colicins. The pH at which 75% of a residue is protonated, is
considered as the protonated state of that residue. An example for titration curves of
acidic charged residues that are located at H1 helix of colA are shown in Fig. 7.3B. All
residues are protonated at pH 2 for colN and colla but two Asp residues in colA are still not protonated at pH 2 due to their low pKa values. The pKa value of each residue was given in Appendix.

![Figure 7.3: pKa curves. (A) Plot of net charge vs pH, calculated at a step width of 1.0 pH units. (B) Titration curves of ASP and GLU that are located at H1 of colA. The cutoff for the protonation state is indicated with a black line. For example, ASP27 was not considered as protonated at pH 2.](image)

### 7.3.2 Analysis of MD simulations

MD simulation were performed for colicin A (colA), colicin N (colN), colicin Ia (colla) at pH 7, pH 4, pH 3 and pH 2. In the following sections, these will be abbreviated as colA-pH7, colA-pH4, colA-pH3, colA-pH2, colN-pH7, colN-pH4, colN-pH3, colN-pH2, colla-pH7, colla-pH4, colla-pH3, and colla-pH2. The results of MD simulations were analyzed concerning two aspects: structural properties and dynamic properties.

#### 7.3.2.1 Structural properties

In order to examine the stability of the simulations, the root mean square deviation (RMSD) of all Cα atoms from the crystal structure was calculated. Fig. 7.4A shows the RMSD deviation as a function of time, suggesting that the systems are not much deviated from the crystal structure. However, these simulations might give an idea about the initial affect of low pH on colicins with respect to neutral pH. In the present work, the trajectories from 5ns to 10ns were considered for the analysis. From the histogram of the RMSD distribution (Fig. 7.4B), it is evident that colA at pH 2, pH 3 and pH 4 explores two conformers while colA at pH 7 is more homogeneous and
explores only one conformation. In Table 7.1, a summary of the average values of different structural data calculated from the simulation are reported.

![Figure 7.4: Root mean square deviation (RMSD). (A) RMSD of Ca- atoms of colicin A from crystal structure as a function of time. (B) Histograms of RMSD distribution for colA at pH 2 (black), pH 3 (red), pH 4 (green) and pH 7 (blue).](image)

The radius of gyration (Rg) represents the size and shape of the molecule. It is found to be very stable from pH 7 to 2 (see Table 7.1), suggesting that colA, colIa and colN at pH 2 are initially as compact as their native structures at pH 7. Hydrogen bonds (H-bonds) play an important role in stabilizing the protein tertiary fold. Thus, a decreasing number of H-bonds at acidic pH makes the protein unstable due to disturbances in the tertiary fold. Therefore, in order to check whether the protein tertiary fold is disturbed, the number of H-bonds was calculated at each time step. The number of H-bonds decreases for colA and colIa upon changing the pH from 7 to 2 (Table 7.1). Fig. 7.5 shows the frequency of occurrence of hydrogen bonds (H-bonds) in colA, also indicating that the number of H-bonds is significantly decreased from pH 7 to pH 2. However, a significant number of hydrogen bonds remain stable at pH 2. Furthermore, the number of H-bonds in colN is only slightly reduced at pH 2.
Table 7.1. Structural properties of colA, colN and colIa

<table>
<thead>
<tr>
<th></th>
<th>Rg (Å)</th>
<th>SASA (nm²)</th>
<th>SAS(_{\text{phobic}}) (nm²)</th>
<th>SAS(_{\text{phillic}}) (nm²)</th>
<th>Number of H-bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>colA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2</td>
<td>15.7 ± 0.04</td>
<td>104.6±1.5</td>
<td>53.2±1.0</td>
<td>51.3±1.0</td>
<td>129±6</td>
</tr>
<tr>
<td>pH 3</td>
<td>15.7 ± 0.05</td>
<td>102.4±1.5</td>
<td>50.8±1.1</td>
<td>51.5±0.9</td>
<td>137±6</td>
</tr>
<tr>
<td>pH 4</td>
<td>15.69±0.04</td>
<td>102.8±1.6</td>
<td>49.7±1.2</td>
<td>53.1±0.9</td>
<td>144±6</td>
</tr>
<tr>
<td>pH 7</td>
<td>15.65±0.04</td>
<td>102.6±1.4</td>
<td>49.9±1.0</td>
<td>52.7±0.9</td>
<td>145±6</td>
</tr>
<tr>
<td>colIa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2</td>
<td>14.9±0.05</td>
<td>93.5±1.4</td>
<td>49.7±1.0</td>
<td>43.8±0.9</td>
<td>122±5</td>
</tr>
<tr>
<td>pH 3</td>
<td>15.0±0.04</td>
<td>95.7±1.5</td>
<td>51.5±1.0</td>
<td>44.1±0.9</td>
<td>129±5</td>
</tr>
<tr>
<td>pH 4</td>
<td>14.9±0.04</td>
<td>91.2±1.4</td>
<td>47.3±1.1</td>
<td>43.9±1.4</td>
<td>135±5</td>
</tr>
<tr>
<td>pH 7</td>
<td>15.0±0.04</td>
<td>93.0±1.2</td>
<td>47.7±1.0</td>
<td>45.3±0.8</td>
<td>138±5</td>
</tr>
<tr>
<td>colN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2</td>
<td>16.2±0.04</td>
<td>114.1±1.7</td>
<td>59.2±1.1</td>
<td>54.9±1.1</td>
<td>137±5</td>
</tr>
<tr>
<td>pH 3</td>
<td>16.2±0.04</td>
<td>111.7±1.5</td>
<td>56.7±1.1</td>
<td>54.9±0.9</td>
<td>143±5</td>
</tr>
<tr>
<td>pH 4</td>
<td>16.1±0.05</td>
<td>110.8±1.5</td>
<td>55.6±0.9</td>
<td>55.6±0.9</td>
<td>149±5</td>
</tr>
<tr>
<td>pH 7</td>
<td>16.2±0.04</td>
<td>111.5±1.6</td>
<td>55.0±1.1</td>
<td>56.4±0.9</td>
<td>146±5</td>
</tr>
</tbody>
</table>

Rg - Radius of gyration  
SASA - Solvent accessible surface area  
SAS\(_{\text{phobic}}\) - Solvent accessible surface area of hydrophobic residues  
SAS\(_{\text{phillic}}\) – solvent accessible surface area of hydrophilic residues  
H-bonds – hydrogen bonds

Figure 7.5: Histograms of number hydrogen bonds (H-bonds) for colA-pH 7 (blue), colA-pH 4 (green), colA-pH 3 (red) and colA-pH 2 (black)
Solvent accessible surface area (SASA) can be divided into SASA for hydrophobic residues ($S_{\text{phobic}}$) and hydrophilic residues ($S_{\text{phillic}}$). $S_{\text{phobic}}$ is slightly increased for all colicins from pH 7 to pH 2, indicating the exposure of a few hydrophobic core residues to the solvent. $S_{\text{phillic}}$ is slightly decreased, indicating that some hydrophilic residues that are exposed at pH 7 are buried at pH 2.

The number of residues involved in secondary structure is another measure of protein folding and unfolding, is shown in table 7.2. The helical content is slightly decreased from pH 7 to pH 2 in colA and colIa, indicating the presence of substantial secondary structure at acidic pH but loose their secondary structure at a few regions. In case of colN, the helical content is very stable from pH 7 to pH 2, suggesting that colN is very stable also at acidic pH.

Table 7.2. Acidic pH induced changes in secondary structure properties

<table>
<thead>
<tr>
<th></th>
<th>Number of residues that are involved in secondary structure</th>
<th>Number of residues that are involved in helices</th>
</tr>
</thead>
<tbody>
<tr>
<td>colA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2</td>
<td>148.6±3.8</td>
<td>118±4.0</td>
</tr>
<tr>
<td>pH 3</td>
<td>155.1±3.3</td>
<td>126.3</td>
</tr>
<tr>
<td>pH 4</td>
<td>152.6±3.4</td>
<td>127.3±4.2</td>
</tr>
<tr>
<td>pH 7</td>
<td>155.2±</td>
<td>128.7±4.7</td>
</tr>
<tr>
<td>colIa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2</td>
<td>131.6±4.1</td>
<td>101.4±4.6</td>
</tr>
<tr>
<td>pH 3</td>
<td>135.4±3.0</td>
<td>108.6±4.4</td>
</tr>
<tr>
<td>pH 4</td>
<td>132.7±</td>
<td>108.3±3.3</td>
</tr>
<tr>
<td>pH 7</td>
<td>137±4.1</td>
<td>110±4.0</td>
</tr>
<tr>
<td>colN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2</td>
<td>161.9±3.9</td>
<td>128.8±4.9</td>
</tr>
<tr>
<td>pH 3</td>
<td>160±4</td>
<td>129±5.2</td>
</tr>
<tr>
<td>pH 4</td>
<td>159±4.4</td>
<td>126.7±5.2</td>
</tr>
<tr>
<td>pH 6</td>
<td>159.4±4.2</td>
<td>128±5.1</td>
</tr>
</tbody>
</table>
7.3.2.2 Dynamics properties

In order to examine the collective motions, essential dynamics analysis was performed on the trajectories. The Ca atoms, all protein atoms and all protein atoms without hydrogens (protein-H) were analyzed separately. The root mean square fluctuations that are shown in Fig. 7.6 indicate that most of the molecular motions can be described by displacements along the first few eigenvectors. This is also shown by sharply peaked first few eigenvalues which are presented in Fig. 7.7A for Ca-atoms and in 7.7B for protein-H atoms.

**Figure 7.6:** Root mean square fluctuations along eigenvectors (ev) which are sorted according to the largest eigenvalues.

**Figure 7.7:** Eigenvalues (in decreasing order of magnitude) obtained from ED analysis of colA (A) Ca atoms and (B) protein-H atoms. Eigenvalues corresponding to the first ten eigenvectors are only shown. For clarity, the two graphs are scaled differently.

The first few eigenvalues for colA-pH2 are larger than the values found for colA-pH 3, colA-pH 4 and colA-pH 7, indicating that at pH 2 colA has large mean square fluctuations. In addition, for pH 7 (Fig. 7.7A and 7.7B) a steeper decrease in the
eigenvalues than for pH 2, 3 and 4 is revealed, suggesting that more constrained motions due to strong tertiary interactions are present at pH 7. Acidic pH mainly weakens the electrostatic interactions. The first eigenvalues from the analysis of protein-H (Fig. 7.7B) are larger than the eigenvalues from Cα atoms, because the number of atoms involved in these displacements is approximately 10 times larger than the number of Cα atoms [16]. The summed contribution of all individual eigenvectors to the total fluctuation of the molecule can be explained by relative subspace positional fluctuations, shown in Fig 7.8.

![Figure 7.8](image)

**Figure 7.8:** Relative positional fluctuations with respect to the total fluctuations. (A) protein-H atoms (B) Cα atoms. For better visualization, the positional fluctuations for the first 50 eigenvectors are shown in the inset. A line is drawn at eigenvector 10 to illustrate the percentage of fluctuations contributed from the first ten eigenvectors.
The relative positional fluctuations for the first 10 eigenvectors i.e. the most collective motions describe approx. 94% (colA-pH2), 90% (colA-pH3), 90% (colA-pH4), and 84% (colA-pH7) of the total fluctuations (Fig. 7.8). Therefore, the three dimensional subspace defined by the first 10 eigenvectors can describe almost all conformational states. It is also evident from Fig. 7.8B that the first 10 eigenvectors contribute to only 84% of the fluctuations in colA-pH7, indicating that the backbone of colA-pH7 is more rigid than the backbone of the other systems. The degrees of freedom (the essential vectors) largely depend on the interactions between residues of the protein. Since, colA-pH 2 shows larger fluctuations due to fewer interactions.

The essential subspace that is defined by the first ten eigenvectors is similar for those systems which have similar dynamic properties. The root mean square inner products (RMSIP) between two eigenvector sets can be used to describe the similarities between the essential subspaces of two trajectories. The RMSIP value is 1 for identical systems whereas it yields 0 for completely dissimilar systems. It is evident from Table 7.3(A) that the RMSIP values are slightly different for colA whereas almost similar for colN at pH 7, 4, 3 and 2. The RMSIP values suggest that colA has different dynamics properties at acidic pH with respect to its native state (pH 7) whereas colN has similar dynamic properties in the pH range from 7 to 2.

Table 7.3. Root mean square inner product (RMSIP) between the first 10 eigenvectors of (A) colA and (B) colN. The diagonal values represent the RMSIP between first and second halves of the trajectory.

<table>
<thead>
<tr>
<th></th>
<th>pH2</th>
<th>pH3</th>
<th>pH4</th>
<th>pH7</th>
<th>(B)</th>
<th>pH2</th>
<th>pH3</th>
<th>pH4</th>
<th>pH7</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH2</td>
<td>0.67</td>
<td>0.58</td>
<td>0.54</td>
<td>0.56</td>
<td>pH2</td>
<td>0.67</td>
<td>0.66</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td>pH3</td>
<td></td>
<td>0.69</td>
<td>0.61</td>
<td>0.63</td>
<td>pH3</td>
<td>0.64</td>
<td>0.64</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>pH4</td>
<td></td>
<td></td>
<td>0.66</td>
<td>0.59</td>
<td>pH4</td>
<td>0.68</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH7</td>
<td></td>
<td></td>
<td></td>
<td>0.72</td>
<td>pH7</td>
<td></td>
<td></td>
<td></td>
<td>0.65</td>
</tr>
</tbody>
</table>

The 3D structures obtained by projecting the trajectory on eigenvector 1 reveal that the structure of colA-pH 7 is almost similar to the crystal structure while colA-pH 2 shows largest structural displacements and helix bending motions. Fig. 7.9 shows the porcupine plots which are used to indicate the direction and amplitude of motion of the atoms along that eigenvector. These plots show that the helices of colA move along a proper direction at pH 2 while they show random fluctuations at pH 7. These results
suggest that the protonation of residues at acidic pH initiates specific movements in helices that might be essential for the hairpin to form a molten globule. However, the porcupine plots in Fig. 7.9 represent the movements along the first eigenvector of colA at pH 7 and pH 2, which are not the same eigenvectors.

**Figure 7.9:** Porcupine plot of principal motions along eigenvector 1 for colA-pH 7 and colA-pH 2. The protein backbone is displayed in ribbon style. Each blue arrow represents a Cα atom and indicates the direction of movement. The length of the arrow indicates the amplitude of motion.

With the essential dynamics analysis one could analyze the collective motions along corresponding eigenvectors but this method is not suitable to compare different simulations of a protein along the same eigenvector. For this reason, we performed a combined essential dynamics analysis (see section 7.2.5) to compare the displacements of simulations performed at pH 2, pH 3, pH 4 and pH 7 along the same eigenvector.

**Figure 7.10:** Eigenvalues for the first 10 eigenvectors of the combined trajectory (Protein-H) and of the individual trajectories (Protein-H).
The eigenvalues of the combined trajectory and of the individual trajectories are shown in Fig. 7.10. The plot reveals that the eigenvalues of the concatenated trajectory are different from the eigenvalues of the single trajectories, indicating that the collective motions described by the essential eigenvectors coming from the four single trajectories are different. The differences in the trajectories were studied by projecting the individual trajectories onto the combined eigenvectors. Fig 7.11 shows the average projections and mean square fluctuations for colA and colN.

**Figure 7.11:** (A) Average projections for colA. (B) Mean square fluctuations for colA. (C) Average projections of colN. (D) Mean square fluctuations of colN. Difference in average projections indicates difference in equilibrium structures. Large differences in the fluctuations indicate the change in dynamic properties.

The average projections along eigenvector 1 (ev1) for colA and colN at pH 2, 3, 4 and 7 (Fig. 7.11) are similar, indicating that the equilibrated structures are similar along the direction of ev1. Small mean square fluctuations along ev1 indicate that the structures are less dynamic. Along ev1, the structure has the highest variance due to the displacement of the whole molecule from the crystal structure. This can be avoided by
fitting to the average structure instead to the crystal structure while calculating the covariance matrix.

Along ev2, the averaged projection for colA-pH2 is largely different from that at other pH values, suggesting that the equilibrated structure at pH 2 is different with respect to the structures at other pH values. Moreover, colA-pH 2 shows large mean square fluctuations along ev2 (Fig. 7.11B) followed by those of colA-pH 3, pH 4 and pH 7, indicating that the overall motions increase from pH 7 to pH 2. In case of colN, the overall fluctuations are very low when compared to that of colA and there is not much difference in the fluctuations of different protonation states of colN (Fig. 7.11D).

In order to show the dynamic differences between different pH states in terms of conformational space sampling, the projections along eigenvectors that show higher fluctuations are derived from combined essential dynamic analysis. Fig. 7.12 shows the 2D plot of projections along ev2 and ev3 for colA, revealing that colA-pH2, colA-pH3 and colA-pH4 sample different conformational spaces when compared to its starting crystal structure. However, the conformational space of colA-pH7 fluctuates around the starting structure.

**Figure 7.12:** 2D-projections of colA (Cα atoms). The projections were obtained from the combined analysis of Cα atoms from complete trajectories.

Fig. 7.13 shows the 2D projections plots which are derived from trajectories for colA and colN. The structure of colA-pH7 is highly constrained and sampled only one conformation, suggesting that colA is very stable at pH 7 (Fig. 7.13A). In the simulations of colA at pH 3 and pH 2, the structures explore large conformational space
due to reduced stability, which is caused by decreased electrostatic interactions at low pH. On the other hand, colN uniformly samples the conformational subspace, indicating that colN is very stable although the electrostatic interactions are decreased (Fig. 7.13B). The 2D-projection plots reveal that a change in electrostatic interactions results in different conformational states for colicin A, but not for colicin N (Fig. 7.13A and 7.13B).

The 3D representation of these displacements can be obtained by projecting the trajectory on the corresponding eigenvector. Fig. 7.14-Fig. 7.22 show the 3D structures obtained by projecting the individual trajectories on combined eigenvectors. Fig. 7.14 shows the extreme structural displacements along the eigenvectors that show maximum fluctuations (Fig. 7.11). In the case of colA, structural displacements are increasing upon changing the pH from 7 to 2. This is consistent with the traces of the covariance matrix (Cα atoms) that account for the overall amount of motion. The traces are 2.5 (colA-pH7), 4.1 (colA-pH4), 3.9 (colA-pH3) and 6.5 (colA-pH2). Especially, in colA-pH 2, the fluctuations are higher in loop regions of H1/H2, H3/H4, H7/H8, H8/H9 and in helices H5, H3, H6. Analysis of colN at different pH reveals that colN fluctuates almost similar at all protonation states although it has a different equilibrium structure along ev2 (Fig. 7.14).
Figure 7.14: Superimposed 10 structures of colA and colN between maximum and minimum projections along ev2 that show significant fluctuations in Fig 7.11. The structure with minimum projection is colored blue and maximum projection is colored red.
The superimposed structures of colA at pH 7, 4, 3 and 2 are presented in Fig. 7.15. The conformation of colA-pH7 is very close to the crystal structure. The detailed analysis of colA-pH2 reveals that the top halves of H1, H2, H3, H6, H7, H10 move into one direction with larger displacements while the bottom halves move to the opposite side with smaller displacements (Fig. 7.16). The movements of H4, H5 and H5a include a complete displacement of the helices towards only one side. It is also worth to mention that these helices are located around the hairpin. Furthermore, helices H8 and H9 move into opposite directions. In colA-pH2, the internal structure of all helices are very stable except H3 and the end of H1.

Figure 7.15: Cartoon rendered structures of colA-pH2 (black), colA-pH3 (red), colA-pH4 (green), colA-pH7 (blue) along ev2 are superimposed. The arrows indicate the direction of helix movements. Yellow arrows indicate that top and bottom of the helices move in opposite directions. Pink color arrows indicate that the complete helix moves into the same direction.

A closer look from the top allows to divide the movements in three segments (black circles in Fig. 7.16C). The segments, which are located on either side of the hydrophobic hairpin, move into opposite directions. The remaining third segment (black circle), that includes H5 and H5a, moves down towards the hairpin. From these results, we conclude that the hairpin moves out from the center of protein core either towards H5 or towards H10 (as represented by the black arrows in Fig. 7.16C). This is also supported by the instability of H3.
Figure 7.16: (A) & (B) Displacements of helices in colA-pH 2 (black) with respect to colA-pH 7 (blue) from two different points of view. Cartoon rendered colA-pH2 position is superimposed on colA-pH 7. Yellow arrows indicate the direction of helix movements. (B) Structures displayed from top view. Black circles indicate the segments that move together along the same direction. Yellow and pink arrows indicate the direction of movement. Black arrows indicate the assumed possible directions for the hydrophobic hairpin (H8 & H9) to come out from the protein core before it inserts into the membrane.

Some of the triggering events that cause the helix displacements are highlighted in Fig. 7.17. The protonation of one acidic residue on H10 reflects the breakage of an ionic interaction between H3 and H2 (yellow circle in Fig. 7.17A), causing the movement of the lower part of H3 away from H10 and H9.

In particular, the most significant conformational changes are represented by the dynamic H1/H2 loop moving towards H9 (Fig. 7.17B) together with a displacement of the N-terminal H1 towards H2 (right side of H1). The movement of a lysine and a histidine of H1 moves towards the H8/H9 loop and the H8 helix respectively, causing the displacement of the loop towards H4 and ultimately reflects the movement of H4 away from the H8/H9 loop. Another important conformational change is initiated by a protonated acidic residue of H1, causing the displacement of H8 towards H7. The top half of H7 moves towards H6, resulting a movement of H6 (Fig. 7.17C) towards H5. The broken ionic interaction between H2/H3 loop and H7 also facilitates the movement of H7 towards H6. Finally, H6 moves H5 along with H5a downwards. These changes in the electrostatic interactions induce the displacements of helices in a programmed manner.
Figure 7.17: Origin of motions in colA-pH2: structure at initial position (left) and extreme position (right) of motion. Basic residues are colored blue and acidic residues are colored red. The backbone is rendered in cartoon style and residues are rendered in CPK style (A) H3 moves further due to the protonation of an acidic residue, Glu-198 (B) Lys-25 of H1 pushes the H8/H9 loop which further causes the movement of H4 helix. (C) An acidic residue Asp-7 which is located at H1 causes the displacement H8 towards H7, reflecting the movement of H7 towards H6. This moves down the H5 towards the H8/H9 loop.

To see the difference in conformations along ev2, ev3 and ev4, the superimposed structures are shown in Fig. 7.18. ColA-pH7 and colA-pH4 have almost similar conformations along ev2, ev3, ev4, suggesting that colA-pH7 and colA-pH4 explore
only one conformation. However, colA-pH3 and colA-pH2 show significant structural
difference along ev1, ev2 and ev3, indicating that colA-pH3 and colA-pH2 sample
different conformational states. Nevertheless, this can be checked again with prolonged
simulations. This result is also in agreement with EPR where colicin A at pH 2 and 3
shows two conformations (mobile and immobile spectral components).

\[\text{Figure 7.18: 3D structures that move along eg2 (blue), eg3 (pink), eg4 (green). The structures}
\text{are superimposed and rendered in ribbon style. Each state is displayed in two views (top and}
\text{bottom).} \]

Conformations at different pH are compared between different colicins. The
superimposed structures of colA, colN and colla are shown in Fig. 7.19.

\[\text{Figure 7.19: The structural displacements along ev2 of colA, colN and colla at pH2}
\text{(black), pH3 (red), pH4 (green), and pH 7 (blue).} \]
With decreasing pH, structures of colA and colla are distorted with respect to their native structure. In contrast to that, colN is comparatively more stable with decreasing pH. Although the amplitudes of fluctuations along the first few principal components are different between colA and colN, the overall conformational changes occur in the same regions (Fig. 7.19). Conversely, the structural elements that are involved in the conformational changes of colla are entirely different with respect to colA and colN. In colla, conformational changes mainly occur in the H1/H2 loop, the H3/H4 loop, the H4/H5 loop and in helices H2, H4 and H5 (Fig. 7.20).

**Figure 7.20:** Superimposed structures of colla-pH2 (black), colla-pH3 (red), colla-pH4 (green) and colla-pH7 (blue). The regions that are involved in large displacements are marked with black circle.

The calculated electrostatic potentials revealed that the charge density on the surface of colA changes with decreasing pH (Fig. 7.21). At neutral pH, colA has a negative charge (-3) whereas colN (+8) and colla (+7) have total positive charge on their surface. The surface positive charge density is increased with decreasing pH. This would explain why different colicins efficiently bind to lipids at different pH and why colicins require negatively charged lipids to insert into membranes. It is known from previous studies that colicin A binds to lipids at pH < 3 (chapter 6), colicin 1a binds at pH 4 and colicin N binds at neutral pH. These observations can therefore easily be explained by the electrostatic potentials calculated in this study.
7.4 Conclusions

Molecular dynamic simulations of colicin A from pH 7 to pH 2 revealed that colicin A undergoes conformational changes at acidic pH. Hydrogen bonds and the secondary structure content are decreased with decreasing pH. Additionally, the dynamic properties suggest that colicin A may become very dynamic at pH 2 and is more stable at pH 7. This is in agreement with the EPR results of chapter 5. Colicin N is almost stable even at acidic pH when compared with colicin A.
7.5 References

8. Summary and Outlook

8.1 Summary

Colicin A is a plasmid encoded water-soluble bacterial toxin that kills sensitive bacteria by forming voltage-gated channels in the cytoplasmic membrane of the target bacteria. Until now, two models were proposed for the closed channel state: an umbrella model and a penknife model. Although, the umbrella model has been proven for colicin E1 and Ia, the closed channel state and the membrane insertion mechanism of colicin A is still unclear and controversial. The main goal of this thesis is to explore the closed channel state and the principles underlying the membrane insertion mechanism of colicin A. In the present work, different aspects of colicin A were studied in vitro, in vivo and in silico.

Figure 8.1: A schematic overview of the present thesis.
**In vitro**

This section is mainly focused on the closed channel state, the membrane insertion mechanism and the conformational changes that occur prior to insertion into the membrane.

Mutants of colicin A, each containing one cysteine were labeled with a nitroxide (MTS) spin label and its membrane-bound state was reconstituted into liposomes by a detergent-mediated reconstitution method (chapter 3). The spin-labeled colicin A in solution and in liposomes was analyzed by using three applications of SDSL-EPR. The nitroxide mobility and accessibility data of soluble colicin A are in excellent agreement with the crystal structure of the colicin A pore-forming domain. In the membrane-bound state, the residues that are located in the hydrophobic hairpin are exposed to the lipid phase and inaccessible to the water phase. Furthermore, the polarity values of these residues also suggested their location within the core of the lipid bilayer. The residues, which are located within the amphipathic helices showed intermediate accessibility to both the lipid phase and the water phase, indicating their location in the membrane-water interface. The mobility, accessibility and polarity values suggested that the arrangement of the hydrophobic hairpin is perpendicular and the arrangement of the amphipathic helices is parallel to the plane of the membrane, supporting the umbrella model for the closed channel state of colicin A.

In the previous studies, 75% neutral and 25% acidic charged lipids in combination with pH 4 were used to induce the membrane insertion of colicin E1 and Ia, which were supported by the umbrella model. In the case of colicin A, 100% acidic charged lipids in combination with pH 5 were used. In previous EPR experiments, 100% acidic charged lipids in combination with pH 8 and freeze-thaw cycles were used to induce the membrane insertion of colicin A. In both cases, the results supported the penknife model for the closed channel state, however the activity of colicin A in 100% acidic charged lipids was not proven.

Therefore, we aimed at studying the membrane insertion mechanism under more physiological conditions. Additionally, the acidic pH induced membrane-bound state was also analyzed in detail (chapter 4). The ability of colicin A to insert at different pH (2-7) into liposomes composed of *E. coli* natural lipids (~ 75% neutral and 25% acidic lipids) was examined and the activity of colicin A was checked in liposomes. The EPR spectra revealed that colicin A bound to the membrane poorly at neutral pH and efficiently at pH < 3. The
detailed study of the acidic pH (~ 2) induced membrane-bound state suggested a similar conformation as observed for the reconstituted membrane-bound colicin A, which supported the umbrella model for the closed channel state. The EPR data also revealed that 5-10% of colicin A remains bound to the surface of the membrane.

The activity of membrane-bound colicin A in liposomes was studied using the tempophosphate spin label, which was enclosed in liposomes (chapter 4). The efflux of the tempophosphate in the presence of a membrane potential (created by valinomycin) indicated the opening of the channel in liposomes.

The pH induced conformational changes in the water-soluble colicin A revealed that colicin A is stable in the pH range from 7 to 4, but it is converted to a molten globule state at pH < 3 (chapter 5). The typical molten globule properties, e.g., the side chain flexibility, an immobilized and hydrated hydrophobic core, the presence of non-native contacts were observed. The formation of a molten globule at low pH was additionally confirmed by fluorescence experiments with ANS.

**In vivo**

The interaction of colicin A with living *E. coli* cells was studied (chapter 6) and considerable conformational changes were detected upon binding to the cells. Comparison of the changes observed in wild type cells with the changes observed in mutant (*tolB*) cells suggested that the conformation observed in the wild type cells corresponds to colicin A that had been translocated from the outer membrane to the inner membrane. In the present work, the rate of signal decay due to the cell anti-oxidants was limited by using potassium ferricyanide (1 mM) and Origami cells (*gor*, *trxB*).

**In silico**

To understand the molecular details of pH induced conformational changes, a series of MD simulations were performed (chapter 7). The essential dynamics analysis of MD trajectories suggested that colicin A is almost stable in the pH range from 7 to 4, but very dynamic at pH 2, in agreement with the experimental results obtained with EPR (chapter 5). Compared to colicin A, colicin Ia undergoes pH dependent conformational changes, but colicin N is almost stable from neutral pH to acidic pH. The calculated surface electrostatic potentials suggested that the surface positive charge increases with decreasing pH,
disclosing the reason why colicins require negatively charged lipids and bind to lipids efficiently at acidic pH.

8.2 Successes and limitations

- The data obtained from the EPR analysis of water-soluble colicin A is in agreement with the X-ray crystal structure.
- The protocol was optimized to reconstitute the membrane-bound conformation of colicin A into proteoliposomes (chapter 3) and the unbound protein was separated from the proteoliposomes.
- The new membrane insertion protocol for colicin A was introduced (chapter 4). In the previous studies 100% negatively charged lipids (pH 5) were used to induce the membrane insertion. In contrast to that, we used *E. coli* natural polar lipids (75% neutral charged and 25% negatively charged lipids) and the membrane insertion was induced by colicin A, which is incubated at pH 2 (the final pH of proteoliposomes is 7.5). Unlike to the previous studies of colicins, we did not use bulk acidic pH for the liposome-protein mixture. The limitation for this method is a small fraction (5-10%) of colicin A still bound to the surface of the membrane.
- The activity of colicin A was examined in liposomes by detecting the efflux of tempophosphate in the presence of a membrane potential.
- SDSL-EPR was successfully used to study the molten globule properties like side chain flexibility, hydration of the hydrophobic core and solvent accessibility.
- For the first time, we introduced the conformational studies of spin-labeled proteins in living cells. However, mixtures of different conformations were observed. We still have to improve this protocol in order to reduce the rate of signal decay in living cells.
- Since we performed MD simulations only for 10ns time scale, we do not expect our simulations equilibrate to the molten globule state. Instead, the work is intended to provide insight into how the structural and dynamical properties of the native state initially change when the protein is introduced into an environment of low pH.
8.3 Outlook

The results obtained in this thesis and the methodological developments achieved and described herein give rise for further studies to clarify the structural and mechanistic details of the action of colicins and related channel-forming toxins.

1. Distances between helices of the closed channel state: In chapter 3, the closed channel state was described depending on the location of the helices in the membrane. In order to know the arrangement of helices with respect to the other helices, distances can be measured using double spin-labeled colicin A.

2. Influence of the lipid composition on membrane insertion: In the present study, influence of pH on the membrane insertion mechanism was studied. In addition to this, the influence of the lipid composition will give new insights into the membrane insertion mechanism of many water-soluble proteins.

3. Conformational studies of colicin A in living cells: In chapter 6, we presented the first results of conformational changes of spin-labeled colicin A in living cells. Performing these experiments in tol, ompF and btuB knockout strains will give more insights into the toxin import mechanisms.

4. pH induced changes in other colicins: Although colicins A, B, E1, Ia and N share structural and functional similarities, they bind to the membrane at different pH. In addition to our studies for colicin A, Ia and N, continuation of these studies for colicin E1 and B will provide more details about the molecular details of the pH induced conformational changes and the molecular basis of colicin action.
Appendix

**Calculated pKa values**

Table: pKa values that were calculated by MCCE method for colicin A, N and Ia are given in the following table. These values are used in the chapter 7.

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### pKa curves for charged residues of colicin A

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**Figure:** The pKa curves determined for colicin A.
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