CHEMBIOCHEM

DOI: 10.1002/cbic.200700507

Specific Isotope Labeling of Colicin E1 and B Channel Domains For Membrane Topological Analysis by Oriented Solid-State NMR Spectroscopy

Christopher Aisenbrey,[a, b, c] Monica Cusan,[b] Stephan Lambotte,[b] Pieter Jasperse,[b] Julia Georgescu,[a, b] Ulrike Harzer,[b] and Burkhard Bechinger*[a, b]

An approach is presented to selectively label the methionines of the colicin E1 and B channel domains, each about 200 residues in size, and use them for oriented solid-state NMR investigations. By combining site-directed mutagenesis, bacterial overexpression in a methionine auxotroph E. coli *strain and biochemical purification, quantitative amounts of the proteins for NMR structural investigations were obtained. The proteins were selectively labeled with ^{15}N at only one, or at a few, selected sites. Multidimensional heteronuclear correlation high-resolution NMR spectroscopy and mass spectrometry were used to monitor the quality of isotopic labeling. Thereafter the proteins were reconstituted into oriented phospholipid bilayers and investigated by proton-decoupled ^{15}N solid-state NMR spectroscopy. The colicin E1 thermolytic fragment that carries a single ^{15}N methionine within its hydrophobic helix 9 region exhibited ^{15}N resonances that are characteristic of helices that are oriented predominantly parallel to the membrane surface at low temperature, and a variety of alignments and conformations at room temperature. This suggests that the protein can adopt both umbrella and pen-knife conformations.*

Colicins are plasmid-encoded bacterial toxins that are released into the environment under stress situations. Several members of the family are membrane-active and carry a pore-forming domain at their C terminus.[1–3] The C-terminal domains of colicins have been shown to insert into bilayers and to form voltage-gated channels in black lipid membranes, a process that involves significant structural transitions of the protein.[4,5] To better understand membrane insertion and permeation, several colicins and their channel-forming domains have been studied by X-ray crystallography, and the structures of the soluble states have been determined (e.g., refs. [3,6]). The three-dimensional folds of the channel domains closely resemble each other, and in addition they share structural similarities with the Bcl-2 family of proteins that are involved in apoptosis, and other bacterial toxins such as proteins that are secreted by diphtheria, tetanus, and botulinum, as well as the *Bacillus thuringiensis* δ toxin. The colicins therefore provide interesting model systems to study the structures as well as the biophysical interactions that govern protein insertion and pore formation.

In aqueous solution or in the crystals, the pore-forming colicin domains adopt globular folds that are characterized by ten α-helices that are arranged in a three-layered structure.[3,6] The hydrophobic core consists of a hairpin of helices 8 and 9, which is surrounded by amphipathic and hydrophilic regions. To match the interfacial properties of the lipid bilayers, membrane insertion is associated with pronounced changes in the tertiary arrangement of the colicin channel domains, and two models have been suggested to describe the membrane-associated structures of the closed colicin channels. The first is the umbrella model in which the two hydrophobic helices are arranged in a transmembrane orientation, and the amphipathic helices intercalate into the interface;[7] this model was later refined to be a dynamic array of loosely connected helices, in which the large protein surface allows close interactions with the lipid environment.[8] The second model is the pen-knife model in which the helices all orient approximately parallel to the surface and form tightly packed structures.[9] Whereas experimental evidence has been collected in support of the umbrella model for colicin E1[3] or colicin Ia,[10] data in support of the pen-knife model exist for the colicin A channel,[2] which shares close sequence homology to the colicin B C-terminal domain (Figure 1). Both membrane structural arrangements of the protein helices agree with a model in which the lipids are part of the channel lining,[8] which is analogous to the propositions that have been made for antibiotic peptides.[11] Interestingly, during voltage gating the colicin A or colicin Ia channel domains are capable of translocating large hydrophilic protein domains across the membrane.[12] To understand the mechanisms of pore formation and immunity, a description of the topological arrangements and their dynamic properties in membranes is necessary. For example, the question remains

[a] *Dr. C. Aisenbrey, Dr. J. Georgescu, Prof. Dr. B. Bechinger*
Institut de Chimie
Université Louis Pasteur Strasbourg—CNRS, UMR 7177
4, Rue Blaise Pascal, 67000 Strasbourg (France)
Fax: (+33) 3-90-24-51-51
E-mail: bechinger@chimie.u-strasbg.fr

[b] *Dr. C. Aisenbrey, M. Cusan, Dr. S. Lambotte, Dr. P. Jasperse, Dr. J. Georgescu, Dr. U. Harzer, Prof. Dr. B. Bechinger*
Max-Planck-Institut für Biochemie
Am Klopferspitz 18A, 82152 Martinsried (Germany)

[c] *Dr. C. Aisenbrey*
Present address: Biofysikalisk Kemi, Umeå Universitet
90187 Umeå (Sweden)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

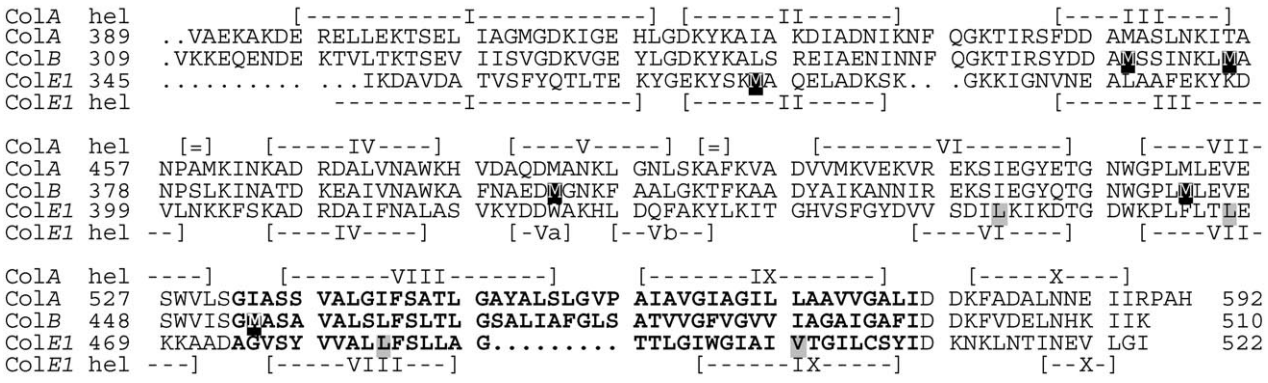

Figure 1. The alignment of the C-terminal sequences of colicin A, B and E1. The numbers represent the amino acid positions in the full-length protein sequence. α-Helical structures of colicin A[68] and colicin E1[69] are indicated by the bars above or below the protein sequences, respectively. The 3_{10}-helical structures in colicin A are indicated by a double bar (=) and the extended hydrophobic regions around helices 8 and 9 are shown in bold. Whereas the colicin E1 sites that were modified by site-directed mutagenesis and labeled with ^{15}N methionine are indicated by a gray background, methionine residues in the wild-type sequences of colicin E1 and B are shown in white on black. For the constructs shown in this paper, M370 of colicin E1 has been replaced by leucine. The wild-type (5 Met) and the M354L mutant (4 Met) of colicin B have been investigated.

whether, despite their functional relationship and the similarity of their structures in solution, the various colicins indeed adopt different topologies in membrane environments, such as those that are described by the umbrella and pen-knife models.

Solid-state NMR spectroscopy has proven to be a valuable method for the investigation of membrane-associated polypeptides (for reviews see refs. [13–15]). By using this technique, the first structures of bilayer-inserted peptides[16] or of membrane receptor-associated peptides have been determined.[17] Furthermore the tilt angles of the helices with respect to the bilayer normal are accessible by using this method.[18–23] In contrast to diffraction or solution NMR methods, the structural analysis of biological macromolecules by solid-state NMR spectroscopy does not require the formation of crystals or the fast reorientation of molecules, and therefore allows for the investigation of polypeptides in their natural bilayer environment. Solid-state NMR spectroscopy is also sensitive to the aggregation, dynamics and orientational mosaic spread of the membrane-associated molecules.[15,24,25] However, the strong dependence of the spectral line shapes on dynamical interchange can be detrimental to high-resolution structural investigations when conformational equilibria occur on intermediate or slow timescales because the spectral lines are too broad. In the past, solid-state NMR spectroscopy has often only realized its full potential when isotopic labels were introduced at specific sites. The preparation of samples for this type of investigation has taken advantage of the versatility of solid-phase peptide synthesis and the availability of a large number of amino acid building blocks that are labeled with stable isotopes.

By using single-site-labeled peptides it has thus been possible to analyze their secondary structure when associated with uniaxially oriented membranes,[16,26] and to investigate the alignment of α-helical domains with respect to the membrane surface.[10,21,27] The ^{15}N chemical shift information from ^{15}N-labeled amide bonds has proven particularly useful because it provides a direct indicator of the approximate helix-tilt angles of peptides that are reconstituted into oriented membranes.[14] Whereas transmembrane helices resonate at about 200 ppm, those that are aligned parallel to the membrane surface are found at <100 ppm. Therefore, the method seems particularly well suited to investigate the helix topologies of membrane-bound colicins, and to distinguish between the umbrella and the pen-knife model.

To prepare proteins in the quantities that are necessary for structural studies, strategies of bacterial overexpression and biochemical purification have been developed. Early on, these were used to prepare and uniformly ^{15}N label colicin channel domains or proteins of the Bcl-2 family, which were thereafter reconstituted into oriented phospholipid bilayers and investigated by solid-state NMR spectroscopy. By using this approach the helices of membrane-associated Bcl-x_L and t-Bid, two proteins of the Bcl-2 family, were found to be oriented approximately parallel to the membrane surface.[28,29] In contrast, the ^{15}N solid-state NMR spectra of oriented colicin channel domains are characteristic for the presence of a wide range of different alignments.[30,31] Although these latter spectra have provided a first global view on the orientational distribution of the peptide bonds and concomitantly of the helices of membrane-associated colicins, specific assignments are needed to provide a clearer interpretation of the spectral intensities. Strategies are therefore required that allow one to label only one or a few residues of membrane-inserted proteins at a time, and thereby to investigate selectively their local structure and dynamics by NMR spectroscopy.

In order to monitor the alignments and topological changes of helical domains in membrane proteins of the size of colicin channel domains or Bcl-2 proteins, we optimized biochemical labeling schemes and produced protein samples in quantitative amounts. The colicin E1 and B channel domains carry only a reduced number of methionines that can be moved by site-directed mutagenesis without changing the functionality of the protein (Figure 1). It was essential to optimize the fermentation conditions so that we could label the remaining methionine/s in the sequence without extensive dilution or scrambling. Finally, proton-decoupled solid-state ^{15}N NMR spectros-

copy of the membrane-associated colicin E1 thermolytic fragment directly reveals the coexistence of several conformers and/or alignments; this is suggestive of conformational flexibility of the membrane-inserted protein.

Results

By using site-directed mutagenesis in combination with an optimized fermentation protocol, it was possible to obtain colicin E1 and colicin B thermolytic fragments that were labeled at a single or a few selected sites. The fermentation medium consisted of a minimal medium that was complemented by the 20 common L-amino acids: 19 of them nonlabeled, and the ^{15}N-labeled analogue of methionine. To reduce the dilution of the label, it was essential to inoculate the fermentor with a bacterial culture that had been already grown in this medium. Furthermore the scrambling of methionine by transaminase reactions could be avoided by using a methionine auxotroph *E. coli* strain for the expression of the proteins.

Selective labeling was verified by recording the 2D HSQC spectra of the purified products. Indeed, when the above-described considerations were observed, the spectra were characterized by a number of peaks that correspond exactly to the number of methionines in the sequence (Figure 2). One peak is observed for each of the colicin E1 mutants L452M, part of helix 6 (Figure 2A), L467M (helix 7, Figure 2B), or L483M (helix 8, Figure 2C), V500M (helix 9, Figure 2D) or 516M (helix 10, Figure 2E). Furthermore Figure 2G and H are characterized by four and five intensities as expected for the colicin B wild-type protein and its M454L mutant, respectively. The high degree of methionine labeling and the limited amount of scrambling is further supported by HPLC-ESI-MS when at the same time Cys, Ser, Asp and Asn exhibit molecular weights predominantly at *m/z* ratios of the naturally abundant compound (not shown).

After the labeling protocol had been established, Val500 was converted into a methionine. In the crystal structure of colicin E1, which is thought to represent the globular conformation in aqueous environments, this site is located within helix 9, that is, within the predominantly hydrophobic helix–loop–helix region (Figure 1). The alignment of this loop constitutes the main difference between the umbrella model, in which helices 8 and 9 are transmembrane, and the pen-knife model, in which all helical domains are aligned approximately parallel to the membrane surface. After site-directed mutagenesis, single-methionine labeling with ^{15}N during bacterial overexpression, preparation of the thermolytic fragment, and reconstitution into oriented phospholipid bilayers at a protein-to-lipid ratio of 0.4 mol% (19.4 mg of protein), the sample was inserted into the magnetic field of a 9.4 Tesla solid-state NMR spectrometer with the membrane normal parallel to the magnetic field direction. Notably, the label concentration was quite low because the protein was "diluted" by the presence of lipids, water and glass plates. As a consequence, several days were required to record the spectra, that is, a time period similar to that necessary for acquisition of multidimensional solid-state NMR spectra.[30–32] However when the sample was frozen, a predominant peak at 85 ppm was obtained; this indicates

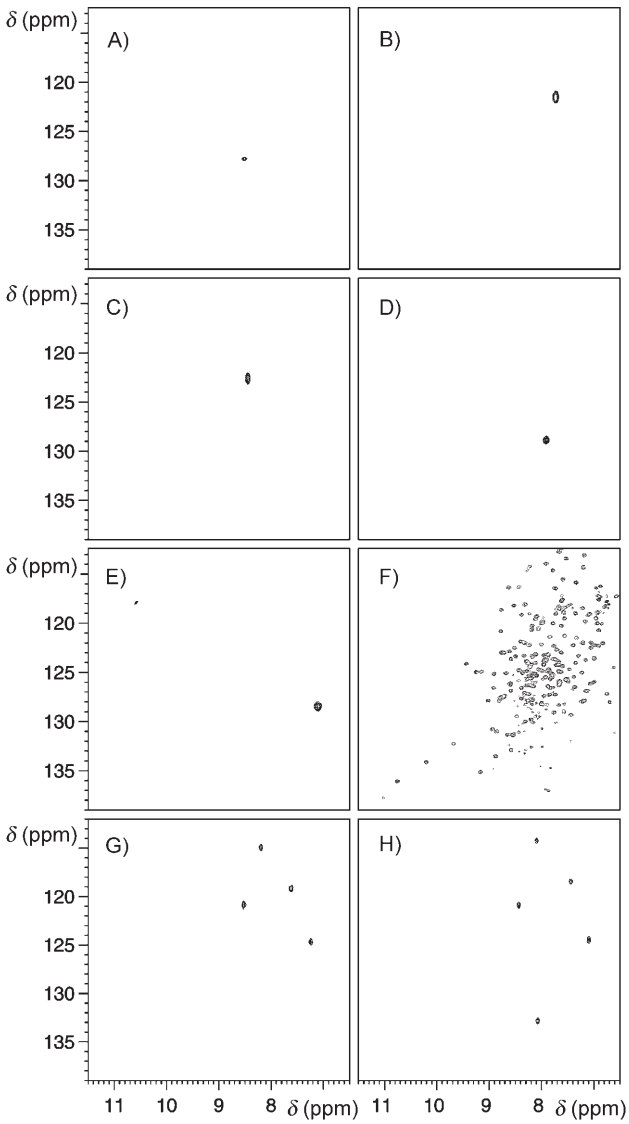

Figure 2. HSQC spectra of thermolytic fragments of colicin E1 (A–F) or colicin B (G, H) that had been labeled with ^{15}N at methionine positions (A–E, G, H) or uniformly (F). A) colicin E1 L452M, B) L467M, C) L483M, D) V500M, E) 516M, F) uniformly labeled thermolytic fragment of colicin E1; G) thermolytic fragment of colicin B and H) M454L. A comparison of G and H provides the assignment of L454.

that the protein is macroscopically well aligned, and that in the gel state the ^{15}N–^{1}H vector (helix 9) is oriented approximately parallel to the membrane surface (Figure 3B). In contrast, signal intensities between 70 and 220 ppm are observed at room temperature that therefore cover most of the chemical shift range. The chemical shift anisotropy indicates that the protein is immobilized by the membrane, and reveals the existence of a distribution of conformations and/or helix alignments (Figure 3A). The oriented spectrum of the cold sample that is shown in Figure 3A was obtained after the room temperature measurement (Figure 3B), and it excludes the possibility that the membrane alignment had degraded much during the acquisition period at ambient temperatures. Furthermore, the proton-decoupled ^{31}P NMR spectra from before and after acquisition of the ^{15}N spectra that are shown in

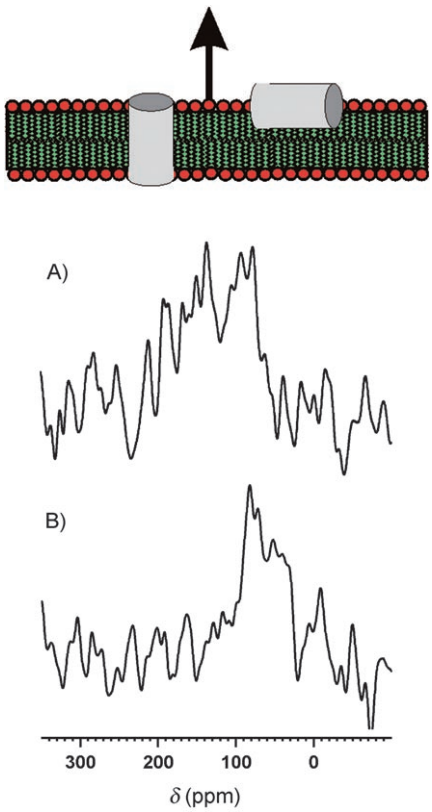

Figure 3. Proton-decoupled solid-state ^{15}N NMR spectra of 0.4 mol% colicin E1 thermolytic fragment labeled at a single ^{15}N methionine position (V500M) after reconstitution into oriented POPC/POPS (3:2) membranes. The membrane normal is oriented parallel to the magnetic field direction, A) at 25 °C and B) at −7 °C. The number of acquisitions were A) 110 000 and B) 60 000. The spectral intensities at 40–50 ppm arise from the phosphatidylcholine headgroups. The correlation between helix alignment and ^{15}N chemical shift is sketched on top of panel A; the arrow indicates the direction of the membrane normal/magnetic field direction.

Figure 3 are indicative of a constant quality of sample alignment and the absence of extensive lipid degradation during this time period (Figure S1). When other single-site labeled proteins were investigated, the signal intensities were too low to be analyzed in further detail (not shown).

Discussion

The preparation of oriented membrane samples for solid-state ^{15}N NMR spectroscopy required the production of several milligrams of pure thermolytic colicin fragments that were labeled at a single site. In our hands, it has proven important to define conditions that avoid scrambling or dilution of the isotopic label by transaminase reactions. The experimental requirements are more stringent than for example, those for solution NMR experiments as, due to the exchange broadening and the correspondingly lower resolution in the solid-state, it is more difficult to separate background signals from the methionine resonances. Furthermore, the low protein concentration results in low signal intensities that require several days of data acquisition; this defines the limits of what can reasonably be achieved with a 9.4 Tesla solid-state NMR spectrometer. The recording of the solid-state NMR spectra that is shown was, therefore, only possible after the fermentation conditions had been optimized.

The oriented membrane samples encompass approximately 0.5–1 μmol (10–20 mg) of protein. Although it has been possible to record solution NMR spectra of considerably less protein, or MAS solid-state NMR spectra of as little as 20 nmol of receptor-associated ligand,[33,34] the signal intensities of the membrane proteins that are investigated here are diminished due to the inherent mobility and the orientational heterogeneity. Whereas an isotropic line width in the range of a few ppm or less is typically observed under MAS conditions, the anisotropy of the ^{15}N chemical shift interaction is about 170 ppm and depends on the detailed properties of the labeled site. Therefore the line width of these oriented membrane samples can be rather broad (cf. Figure 3 A). However, because this reflects the dynamics, mosaic spread and topological heterogeneity of the protein in the membrane, these spectra provide important information about the proteins' structure and functioning.[35] Indeed, at room temperature the hydrophobic domain of colicin E1 that encompasses helix 9 exhibits a broad range of orientations, topologies and/or conformations (Figure 3 A), but a more homogenous in-plane alignment upon freezing the sample is observed (Figure 3 B). These temperature-related differences could be due to a more superficial association of the protein with the bilayer at low temperature. Furthermore, the signal intensities of more hydrophilic helices, which are probably only loosely or not at all membrane associated, remained too weak to be analyzed. Previously we and others have observed a large decrease in the ^{15}N solid-state NMR signal intensities of ^{15}N-labeled amides that are located within mobile loop regions,[35] or when part of helices that exhibit motions on an intermediate timescale.[24,36–38] It has been suggested that this is due to motional averaging of the ^{1}H–^{15}N dipolar couplings, which interferes with efficient cross-polarization.[39]

In previous investigations, 1D and 2D proton-decoupled solid-state ^{15}N NMR spectra of membrane-associated colicin E1 and B channel domains, in which all nitrogen atoms have been labeled uniformly with ^{15}N, indicated that approximately one fourth of the ^{15}N amides resonate at ^{15}N chemical shift frequencies $\geq$180 ppm, which is a chemical shift that is indicative of NH vectors that are aligned approximately parallel to the membrane normal.[30,31] Although this intensity distribution is suggestive of the presence of a transmembrane helical hairpin, and therefore the umbrella model, this interpretation remains speculative in the absence of more detailed spectral assignments. In particular, it is difficult to unambiguously discern on these previous spectra[30,31] two "helical wheels"[19,20] that encompass about 30–40 residues; this would provide a more reliable indication of a stable transmembrane helical loop. Signal intensities at about 200 ppm could also arise from residues that are in nonhelical conformations,[40] or helices other than helices 8 and 9 (Figure 1). Furthermore, signals from side-chain nitrogen atoms might be present in the spectra, but because their size and position strongly depend on their motions and alignments, they remain difficult to assess in a quantitative

manner. Most importantly, however, the data that are shown in Figure 3A indicate that at room temperature the conformation of the colicin E1 pore-forming domain is characterized by the coexistence of several orientational, topological and/or conformational states at the site of residue 500. The orientational distribution relative to the glass plates/B_0 field can be explained by the presence of a variety of conformations, and the spectrum is consistent with, for example, the coexistence of both the umbrella and the pen-knife model. In addition, the ^{31}P NMR spectra of the same sample (Figure S1) are indicative of protein-induced distortions in lipid alignment; this thereby suggests that changes in membrane curvature in the proximity of the colicin E1 channel domain have occurred that are concomitant with possible modulations of the protein orientation relative to the magnetic field direction. These observations have consequences also for the interpretation of the spectra from the uniformly labeled samples because the simultaneous presence of different topological/conformational states results in the ^{15}N signals of many, if not all, of the labeled sites being spread over a wide chemical shift region.

Here we have shown that the V500M site (helix 9 in the X-ray structure, Figure 4) exhibits a distribution of orientational and/or conformational states in a temperature-dependent manner. It remains possible that other environmental factors such as the lipid composition, interactions with other proteins such as TolA, or transmembrane electric fields have an effect on the membrane helical topologies of the colicin E1 pore domain. Our data are in agreement with fluorescence spectroscopy investigations on single-tryptophan mutants that are indicative of equilibria between at least two helix topologies of this protein.[41–43] In these experiments, the colicin E1 channel domain was inserted at 0.6 mol% into POPC/POPG (3:2) large unilamellar vesicles in aqueous buffer, at pH 4 and 100 mM NaCl.[42] Whereas the solid-state NMR experiments that are presented in Figure 3 were performed at neutral pH and in the presence of supported bilayers, the acidic conditions that were used in the fluorescence experiments also ensure colicin E1–membrane association in more dilute suspensions. By studying a number of tryptophan mutants, the combination of acrylamide quenching, fluorescence quantum yield, fluorescence decay times and red-edge excitation shift lead to a model where the helix 8–loop–helix 9 loop exchanges between different conformations.[41–43] The data suggest that an in-planar alignment is in equilibrium with a more membrane-inserted configuration, the latter being characterized by helices that are tilted with respect to the membrane normal. In particular, the fluorescence spectroscopy experiments position the loop region (residue 492) in the hydrophobic bilayer center; this is a finding that excludes a stable transmembrane alignment of helices 8 and 9 under the investigated conditions.[41]

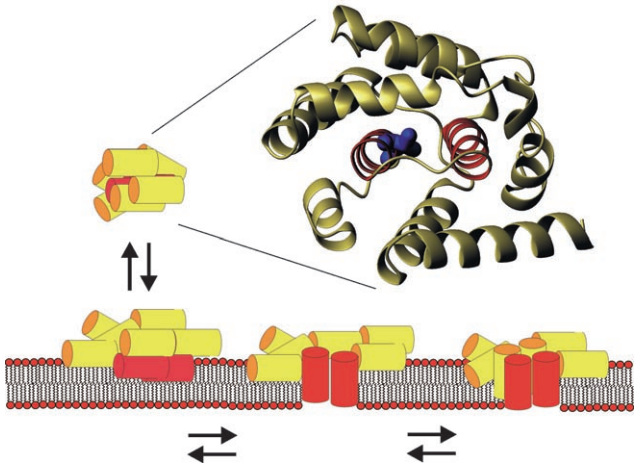

Figure 4. Model of the membrane equilibria of colicin and colicin-like proteins according to refs. [49] and [50]. The proteins adopt a globular structure in solution, which partially or fully unfolds upon membrane insertion. Depending on the environmental conditions and interactions with other proteins or with other colicin domains,[44] the hydrophobic helices 8 and 9 (in red) are oriented parallel or perpendicular to the membrane surface. It remains possible that other helices adopt transmembrane alignments for example, due to transmembrane electric potentials. The enlargement shows the PDB 2I88 structure[69] with the valine 500 site in blue.

In another series of experiments, the unfolding events of the colicin E1 channel domain have been characterized by a stopped-flow approach in which the protein and 100 nm vesicles were mixed under acidic conditions.[8] FRET between a fluorescence dye that was attached to Cys509 and single tryptophans was used to monitor the time-dependent changes in intramolecular distances during membrane insertion. These experiments indicate that unfolding, helix extension and membrane insertion occur within about 500 ms, and result in a highly mobile surface-associated state.[8] The solid-state experiments that are presented here (Figure 3A) are indicative that, once equilibrated, several topological states coexists that are in slow exchange on the 10^{-4} s timescale. At least one reversible folding intermediate has been discernible also for colicin B by using DSC, fluorescence and CD spectroscopies.[44,45]

The data that are presented in Figure 3 agree well with an extended 2D helical array of colicin E1 in the membrane,[46] where the interactions of individual helices are important determinants for their membrane alignment.[47] Notably, the hydrophobic region of colicin E1 is too short to span the POPC bilayer twice in a stable manner,[41,48] and the loop region that connects helices 8 and 9 lacks residues that could stabilize a transmembrane helical loop configuration (Figure 1). The resulting conformational flexibility might help to readily respond to external stimuli such as transmembrane electric fields, lipid composition or interactions with other proteins. The functional and structural data thereby reveal a flexible structure of the colicin E1 channel domain in membrane environments.[49]

In previous studies, Kienker et al.[50] have been able to trap individual states of the colicin Ia channel domain at both pH 6.2 and 8, and have shown that the topology of the helical hairpin loop 8–9 is in dynamic equilibrium, where its accessibility from the *trans* side of the membrane is a function of the experimental conditions (Figure 4). A number of other observations that are correlated with membrane insertion were observed by solid-state NMR spectroscopy; these include a reduced number of interhelical contacts,[10] increased motional amplitudes of the protein backbone and side chains, and aug-

mented average correlation times; these dynamic features correlate with optimal channel activity.[51,52] The timescales that were tested by these experiments include microsecond motions such as small-amplitude reorientation of entire helices or exchange between conformational substates[51] as well as cooperative segmental motions (tens of ms).[52]

Whereas the authors of these former studies have elegantly provided indirect evidence of the dynamic properties of colicin channel domains, for example by exploring the channel properties of free and trapped intermediates of colicin Ia,[50] in the present study a topological equilibrium of the colicin E1 helix 9 is directly visualized by the ^{15}N solid-state NMR spectra. Although variable alignments have also been observed when investigating membrane-associated peptides[29,53] this concept is less common for larger membrane proteins. However, it remains possible that such loosely folded membrane structures occur more often but escape our attention as such conformational flexibility is difficult to visualize by crystallographic techniques. Detergents, on the other hand, are known to promote the unfolding of tertiary structures, which leaves considerable doubts about the validity of solution NMR structures of membrane proteins that are obtained in micellar environments.[29,54] Therefore, the strategy presented here, which uses solid-state NMR spectroscopy of proteins that have been reconstituted into lipid bilayers, can provide a valuable supplementary view on the conformational heterogeneity and structural transitions of these proteins.

Experimental Section

Genes and plasmids: The autotrophic strains *E. coli* JM101 that bear the plasmid pColE1::Tn3M420/516[55] or pES3 that codes for colicin B,[56] respectively, were used to overexpress the colicin proteins. The pColE1 plasmid was a gift of Cheryl M. Hubbel, UCLA and was originally developed in the Levinthal laboratory.[55] The sequence carried two additional methionines at positions 420 and 516, which were changed back to encode the wild-type lysine (position 420) and isoleucine (position 516), respectively (Figure 1). Site-directed mutagenesis was used to create sequences that carry single methionines in the pore-forming domain at either L452M, L467M, L483M, V501M or 516M. These modifications were undertaken by using the site-directed mutagenesis kit from Stratagene. The bacteria and plasmids were handled according to standard protocols as described in ref. [57]. Oligonucleotide design and cycling conditions followed the recommendation of the manufacturer. The quality and function of the mutation was evaluated by restriction analysis, double-strand sequencing and spot tests.[58] The mutated clones showed the same lytic activities as the wild-type.

Preparation of the proteins: Overexpression and purification of thermolytic fragments of colicin E1 (colE1) and colicin B (colB) were based on published protocols.[7,56] Single colonies of the plasmid-bearing cells were picked and transferred to 2 mL of LB media. Further incubation was performed at 37 °C and by shaking at 250 rpm for 10 h. To dilute ^{14}N-methionine that was omnipresent in the LB culture medium, this overnight culture (2 mL) was used to first inoculate medium (100 mL) that had been selectively labeled with ^{15}N-methionine.[59,60] The composition of the medium followed the protocol of ref. [60] and contained sufficient concentrations of all of the 20 most common L-amino acids at their natural isotopic abundance, with the exception of methionine, which was replaced by 50 mg L^{-1} ^{15}N-methionine (Promochem, Wesel, Germany). All media were supplemented with 50 μg mL^{-1} ampicillin. The cells were grown in Erlenmeyer flasks for 16 h under shaking at 250 rpm. Only then was the overnight culture used to inoculate a BioFlo 3000 5-L fermentor (New Brunswick Scientific, New Jersey, USA) with the same medium that had been selectively enriched with ^{15}N-methionine. The cells were grown at 37 °C, pH 7.4 and an airflow of 3–4 L min^{-1} until an optical density at 600 nm of 1 was reached. At that time, protein expression was induced by adding mytomicin C (Sigma, Germany) to a final concentration of 0.26 mg L^{-1}. The fermentation was stopped after 2.5 h or when the optical density at 600 nm reached a plateau. The contents of the fermentor were harvested and cooled on ice. Afterwards the cells were concentrated and washed with low-salt TrisCl buffer (500 mL) by centrifugation (30 min, 6000 *g*). Cell lysis was performed by using a French pressure cell at 6000 psi (Thermo, Electron Corporation, USA). Cell debris was removed by centrifugation at 10000 *g* for 1 h. The supernatants that contained the colE1 or colB proteins were applied a CM Sephadex C-50 or SH Sephacryl R100 column (Amersham Pharmacia Biotech), respectively. Proteins were eluted by augmenting the salt concentration to 300 mM. Thereafter the eluate was dialysed once against a 100-fold volume of 20 mM TrisCl, 2 mM NaOAc, 6 mM β-mercaptoethanol at pH 7.8 by using a SpectraPor MWCO 10 kDa membrane. The C-terminal domains of colE1 or colB were obtained after incubation with 1% (*w*/*w*) thermolysin (Boehringer–Mannheim) for 2–4 h at 37 °C. The reaction was stopped by the addition of 10 mM EDTA. After dialysis against the same buffer, the proteins were applied on a Sephacryl-100 HR gel filtration column (Amersham Pharmacia Biotech) by following the protocol that was described by the manufacturer ("Gel Filtration, Principles and Methods", Amersham Bioscience) and finally concentrated with a Centricon3 system (Amicon–Millipore, Schwalbach, Germany). Most of the colicin proteins showed concentrations in the range of 0.8–1 mM, which was well suited for the preparation of NMR spectroscopy samples.

Mass spectrometric analysis of the hydrochloric acid hydrolysate: For mass spectrometric analysis 0.5–1 mg of pure protein was decomposed into its amino acids by acidic hydrolysis.[61] The proteins were dialyzed and lyophilized against double-distilled water before adding the hydrolysis mixture that consisted of 6 M HCl (500 μL), liquid phenol (5 μL) and dodecanethiol (5 μL). This mixture was frozen, and the glass container was sealed under vacuum prior to incubation at 110 °C for 24 h. Thereafter, the solution was lyophilized, dissolved in a redry solution of ethanol/water/triethylamine (2:2:1, *v*/*v*/*v*) and lyophilized again. The resulting amino acid mixture was derivatized[62] for 20 min in ethanol/water/triethylamine/phenylisothiocyanate (500 μL, 7:1:1:1, *v*/*v*). Before measurements, the mixture was lyophilized and dissolved in 20% acetonitrile (150 μL).

To determine the degree of ^{15}N labeling, derivatized protein hydrolysates were subjected to LC–MS analysis. The experimental setup consisted of a micro-HPLC pump (ABI140C), auto-sampler (PE200) and UV detector (ABI785 A) in row with a single-quadrupole mass spectrometer (ABI165, all from Applied Biosystems, Weiterstadt, Germany). In each case, a sample (10 μL) was injected, and the amino acids were separated on a reversed-phase HPLC column (Nucleosil C8, 125×1 mm, 5u, 120 Å, Macherey & Nagel, Düren, Germany) with a flow of 40 μL min^{-1}. A linear gradient from 5% to 95% eluent B over 45 min was applied (eluent A: 0.1% TFA in H_2O; eluent B: 0.1% TFA in acetonitrile). For MS detection, an ESI source was used in the positive ion mode with an ionization voltage of

4700 V and an orifice voltage of 10 V. During the whole run, MS full-scan spectra were recorded between *m/z* 100 and 1000 (2.7 s per scan). For relative quantification of the corresponding $^{15}N/^{14}N$ amino acid derivatives, ion chromatograms were extracted, and the resulting peaks were integrated with the vendors "Biotoolbox" software. The mass windows that were used for extraction (± 0.5 Da) were for methionine (^{15}N: 286.1 Da, ^{14}N: 285.1 Da), cysteine (^{15}N: 258.0 Da, ^{14}N: 257.0 Da), serine (^{15}N: 242.1 Da, ^{14}N: 241.1 Da), aspartic acid (^{15}N: 270.1 Da, ^{14}N: 269.1 Da) and asparagine ($2\times^{15}N$: 405.1 Da, ^{15}N-^{14}N: 404.1 Da, $2\times^{14}N$: 403.1 Da).

Multidimensional solution NMR spectroscopy: For heteronuclear solution NMR spectroscopy, purified protein (2–4 mg) was dissolved in 10 mM citrate (400–550 μL) at pH 7.0 or 20 mM phosphate buffer at pH 7.5. HSQC spectra were recorded on an Avance 500 NMR spectrometer by using a commercial triple-resonance NMR probe (Bruker, Rheinstetten, Germany). The GARP decoupling sequence was used on the ^{15}N channel during acquisition to eliminate the ^{1}H–^{15}N couplings. The intense water signal was reduced by a WATERGATE sequence.[63] A matrix of 128 rows, each of 2048 data points was acquired by using 64 scans per t_1 increment and TPPI. The spectral widths in the t_1 and t_2 domains were typically 2500 Hz and 8800 Hz, respectively, and the processing matrix of 1024×256 points. Before Fourier transformation, QSIN apodization functions were applied.

Solid-state NMR spectroscopy: To reconstitute the colicin thermolytic fragments into membranes,[30] POPC or POPC/POPS small unilamellar vesicles (Avanti Polar Lipids, Alabaster, AL) were prepared by extrusion through 50 nm polycarbonate filters (Avestin, Ontario, Canada). The protein was added to the appropriate amount of small unilamellar vesicles (50 mg phospholipid per mL) and the resulting dispersion was incubated at room temperature for 1 h. Thereafter the samples were concentrated to a final volume of about 1 mL by using centrifugal concentrators with a vertical paddle (Macrosep, Pall Filtron, Northborough, MA). The lipid–protein dispersion was evenly spread onto the center of 25 cover glasses (9×22 mm), slowly dried and equilibrated in 93% relative humidity before the glass plates were stacked on top of each other.

For solid-state NMR measurements, the samples were tightly sealed, inserted into flat coil NMR probes,[64] and introduced into the spectrometer with the bilayer normal parallel to the magnetic field direction. Proton-decoupled ^{15}N NMR spectra were acquired by using a Bruker Advance 400 spectrometer that was operating at 9.4 Tesla. A cross-polarization pulse sequence[65] was applied by using experimental conditions that were identical to those that resulted from the previous optimization of the spectral parameters, to afford best signal intensity and low RF heating by using uniformly labeled colicin E1 and B samples.[30] Typically the following parameters were used: ^{1}H B_1 field of approximately 40 kHz, 1.3 ms contact time, 3 s recycle delay, spectral width: 40 kHz, 256 data points. During acquisition the sample was cooled with a stream of air at room temperature. An exponential apodization function that corresponded to a line-broadening of 300 Hz was applied before Fourier transformation. NH_4Cl (41.5 ppm) was used as a reference, and corresponded to 0 ppm for liquid NH_3. To check the quality of sample alignment and to ensure that the sample did not show lipid degradation, proton-decoupled ^{31}P NMR spectra[66,67] were acquired before and after the multi-day solid-state ^{15}N NMR acquisitions.

Abbreviations: DSC: differential scanning calorimetry, ESI: electro spray ionization, FRET: fluorescence resonance energy transfer, MS: mass spectrometry, NMR: nuclear magnetic resonance, PC: phosphatidylcholine, POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, POPG: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol, POPS: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine.

Acknowledgements

We are most grateful to Cheryl M. Hubbel, Francoise Levinthal and Volkmar Braun for sending us the colicin E1 and colicin B strains. The valuable help and advice by Silvia Körner and Frank Siedler during the mass spectrometric analysis of the colicin hydrolytic products and derivatives is gratefully acknowledged. Without access to the fermentors in Dieter Oesterhelt's and the French press in Wolfgang Baumeister's departments this work would not have been possible. We also thank the Deutsche Forschungsgemeinschaft (Be-1247), the Agence Nationale de la Recherche sur le SIDA (grant to C.A.) and the Association pour la recherche sur le cancer (no. 3100) for financial support.

Keywords: Bcl-2 proteins · isotopic labeling · membrane proteins · NMR spectroscopy · transmembrane helical loop

[1] R. M. Stroud, K. Reiling, M. Wiener, D. Freymann, *Curr. Opin. Struct. Biol.* **1998**, *8*, 525–533.
[2] J. H. Lakey, S. L. Slatin, *Curr. Top. Microbiol. Immunol.* **2001**, *257*, 131–161.
[3] S. D. Zakharov, W. A. Cramer, *Biochim. Biophys. Acta Biomembr.* **2002**, *1565*, 333–346.
[4] J. O. Bullock, S. K. Armstrong, J. L. Shear, D. P. Lies, M. A. McIntosh, *J. Membr. Biol.* **1990**, *114*, 79–95.
[5] F. Levinthal, A. P. Todd, W. L. Hubbell, C. Levinthal, *Proteins* **1991**, *11*, 254–262.
[6] J. L. Hilsenbeck, H. Park, G. Chen, B. Youn, K. Postle, C. Kang, *Mol. Microbiol.* **2004**, *51*, 711–720.
[7] M. vB. Cleveland, S. Slatin, A. Finkelstein, C. Levinthal, *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 3706–3710.
[8] S. D. Zakharov, W. A. Cramer, *Biochimie* **2002**, *84*, 465–475.
[9] J. H. Lakey, D. Duche, J. M. Gonzalez-Manas, D. Baty, F. Pattus, *J. Mol. Biol.* **1993**, *230*, 1055–1067.
[10] W. Luo, X. Yao, M. Hong, *J. Am. Chem. Soc.* **2005**, *127*, 6402–6408.
[11] B. Bechinger, *Biochim. Biophys. Acta Biomembr.* **1999**, *1462*, 157–183.
[12] P. K. Kienker, K. S. Jakes, R. O. Blaustein, C. Miller, A. Finkelstein, *J. Gen. Physiol.* **2003**, *122*, 161–176.
[13] J. H. Davis, M. Auger, *Prog. Nucl. Magn. Reson. Spectrosc.* **1999**, *35*, 1–84.
[14] B. Bechinger, C. Sizun, *Concepts Magn. Reson.* **2003**, *18A*, 130–145.
[15] M. Hong, *Acc. Chem. Res.* **2006**, *39*, 176–183.
[16] T. A. Cross, *Methods Enzymol.* **1997**, *289*, 672–696.
[17] S. Luca, J. F. White, A. K. Sohal, D. V. Filippov, J. H. van Boom, R. Grisshammer, M. Baldus, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 10706–10711.
[18] B. Bechinger, Y. Kim, L. E. Chirlian, J. Gesell, J.-M. Neumann, M. Montal, J. Tomich, M. Zasloff, S. J. Opella, *J. Biomol. NMR* **1991**, *1*, 167–173.
[19] J. Wang, J. Denny, C. Tian, S. Kim, Y. Mo, F. Kovacs, Z. Song, K. Nishimura, Z. Gan, R. Fu, J. R. Quine, T. A. Cross, *J. Magn. Reson.* **2000**, *144*, 162–167.
[20] F. M. Marassi, S. J. Opella, *J. Magn. Reson.* **2000**, *144*, 150–155.
[21] C. Aisenbrey, B. Bechinger, *Biochemistry* **2004**, *43*, 10502–10512.
[22] R. Mani, S. D. Cady, M. Tang, A. J. Waring, R. I. Lehrer, M. Hong, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 16242–16247.
[23] A. Ramamoorthy, S. K. Kandasamy, D. K. Lee, S. Kidambi, R. G. Larson, *Biochemistry* **2007**, *46*, 965–975.
[24] C. Aisenbrey, B. Bechinger, *J. Am. Chem. Soc.* **2004**, *126*, 16676–16683.
[25] C. Aisenbrey, P. Bertani, P. Henklein, B. Bechinger, *Eur. Biophys. J.* **2007**, *36*, 451–460.
[26] B. Bechinger, M. Zasloff, S. J. Opella, *Biophys. J.* **1992**, *62*, 12–14.

[27] R. Smith, F. Separovic, T. J. Milne, A. Whittaker, F. M. Bennett, B. A. Cornell, A. Makriyannis, *J. Mol. Biol.* **1994**, *241*, 456–466.
[28] X. M. Gong, J. Choi, C. M. Franzin, D. Zhai, J. C. Reed, F. M. Marassi, *J. Biol. Chem.* **2004**, *279*, 28954–28960.
[29] C. Aisenbrey, U. S. Sudheendra, H. Ridley, P. Bertani, S. Nedelkina, J. H. Lakey, B. Bechinger, *Eur. Biophys. J.* **2007**, *36*, 451–460.
[30] S. Lambotte, P. Jasperse, B. Bechinger, *Biochemistry* **1998**, *37*, 16–22.
[31] Y. Kim, K. Valentine, S. J. Opella, S. L. Schendel, W. A. Cramer, *Protein Sci.* **1998**, *7*, 1–7.
[32] T. C. B. Vogt, S. Schinzel, B. Bechinger, *J. Biomol. NMR* **2003**, *26*, 1–11.
[33] P. T. F. Williamson, G. Grobner, P. J. R. Spooner, K. W. Miller, Watts, *Biochemistry* **1998**, *37*, 10854–10859.
[34] P. J. Spooner, L. M. Veenhoff, A. Watts, B. Poolman, *Biochemistry* **1999**, *38*, 9634–9639.
[35] C. Aisenbrey, U. Harzer, G. Bauer-Manz, G. Bär, I. N. Husmal Chotimah, P. Bertani, C. Sizun, A. Kuhn, B. Bechinger, *FEBS J.* **2006**, *273*, 817–828.
[36] H. Saitô, S. Tuzi, S. Yamaguchi, M. Tanio, A. Naito, *Biochim. Biophys. Acta Bioenerg.* **2000**, *1460*, 39–48.
[37] K. J. Hallock, D. K. Lee, J. Omnaas, H. I. Mosberg, A. Ramamoorthy, *Biophys. J.* **2002**, *83*, 1004–1013.
[38] L. Prongidi-Fix, P. Bertani, B. Bechinger, *J. Am. Chem. Soc.* **2007**, *129*, 8430–8431.
[39] D. E. Warschawski, J. D. Gross, R. G. Griffin, *J. Chim. Phys. Phys.-Chim. Biol.* **1998**, *95*, 460–466.
[40] J. H. Lakey, D. Massotte, F. Heitz, J. L. Dasseux, J. F. Faucon, M. W. Parker, F. Pattus, *Eur. J. Biochem.* **1991**, *196*, 599–607.
[41] S. E. Malenbaum, A. R. Merrill, E. London, *J. Nat. Toxins* **1998**, *7*, 269–290.
[42] M. C. Tory, A. R. Merrill, *J. Biol. Chem.* **1999**, *274*, 24539–24549.
[43] M. C. Tory, A. R. Merrill, *Biochim. Biophys. Acta Biomembr.* **2002**, *1564*, 435–448.
[44] A. Ortega, S. Lambotte, B. Bechinger, *J. Biol. Chem.* **2001**, *276*, 13563–13572.
[45] H. A. Sathish, M. Cusan, C. Aisenbrey, B. Bechinger, *Biochemistry* **2002**, *41*, 5340–5347.
[46] S. D. Zakharov, E. A. Kotova, Y. N. Antonenko, W. A. Cramer, *Biochim. Biophys. Acta Biomembr.* **2004**, *1666*, 239–249.
[47] B. Bechinger, *FEBS Lett.* **2001**, *504*, 161–165.
[48] U. Harzer, B. Bechinger, *Biochemistry* **2000**, *39*, 13106–13114.
[49] B. Bechinger, *Phys. Chem. Chem. Phys.* **2000**, *2*, 4569–4573.
[50] P. K. Kienker, X. Qiu, S. L. Slatin, A. Finkelstein, K. S. Jakes, *J. Membr. Biol.* **1997**, *157*, 27–37.
[51] D. Huster, L. Xiao, M. Hong, *Biochemistry* **2001**, *40*, 7662–7674.
[52] X. L. Yao, M. Hong, *Biochemistry* **2006**, *45*, 289–295.
[53] U. S. Sudheendra, B. Bechinger, *Biochemistry* **2005**, *44*, 12120–12127.
[54] J. A. Losonczi, E. T. Olejniczak, S. F. Betz, J. E. Harlan, J. Mack, S. W. Fesik, *Biochemistry* **2000**, *39*, 11024–11033.
[55] Y. K. Shin, C. Levinthal, F. Levinthal, W. L. Hubbell, *Science* **1993**, *259*, 960–963.
[56] U. Pressler, V. Braun, B. Wittmann-Liebold, R. Benz, *J. Biol. Chem.* **1986**, *261*, 2654–2659.
[57] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, **1989**.
[58] Q. R. Liu, V. Crozel, F. Levinthal, S. Slatin, A. Finkelstein, C. Levinthal, *Proteins* **1986**, *1*, 218–229.
[59] H. Senn, A. Eugster, G. Otting, F. Suter, K. Wuthrich, *Eur. Biophys. J.* **1987**, *14*, 301–306.
[60] D. C. Muchmore, L. P. McIntosh, C. B. Russell, D. E. Anderson, F. W. Dahlquist, *Methods Enzymol.* **1989**, 44–73.
[61] I. Davidson, *Methods Mol. Biol.* **1997**, *64*, 119–129.
[62] G. B. Irvine, *Methods Mol. Biol.* **1997**, *64*, 131–138.
[63] M. Piotto, V. Saudek, V. Sklenar, *J. Biomol. NMR* **1992**, *2*, 661–665.
[64] B. Bechinger, S. J. Opella, *J. Magn. Reson.* **1991**, *95*, 585–588.
[65] A. Pines, M. G. Gibby, J. S. Waugh, *J. Chem. Phys.* **1973**, *59*, 569–590.
[66] M. Rance, R. A. Byrd, *J. Magn. Reson.* **1983**, *52*, 221–240.
[67] B. Bechinger, R. Kinder, M. Helmle, T. B. Vogt, U. Harzer, S. Schinzel, *Biopolymers* **1999**, *51*, 174–190.
[68] M. W. Parker, J. P. Postma, F. Pattus, A. D. Tucker, D. Tsernoglou, *J. Mol. Biol.* **1992**, *224*, 639–657.
[69] P. Elkins, A. Bunker, W. A. Cramer, C. V. Stauffacher, *Structure* **1997**, *5*, 443–458.
[70] K. J. Hallock, D. K. Lee, A. Ramamoorthy, *Biophys. J.* **2003**, *84*, 3052–3060.
[71] R. Mani, A. J. Waring, R. I. Lehrer, M. Hong, *Biochim. Biophys. Acta Biomembr.* **2005**, *1716*, 11–18.

Received: August 30, 2007
Published online on March 12, 2008

