Protein Folding

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Interactions with Hydrophobic Clusters in the Urea-Unfolded Membrane Protein OmpX**

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Proteins unfolded in denaturing solvents have in several instances been shown to contain nonrandomly structured polypeptide segments.[1–6] Biophysical characterization of these clusters is important for an understanding of their roles in early stages of global protein folding.[1,2,7,8] For example, long-range interactions between multiple hydrophobic clusters in urea-unfolded lysozyme were inferred from experimental studies[9] and advanced as an important new facet of protein folding.[10] Herein, we investigate inter- and intramolecular interactions of two hydrophobic clusters in positions 73–82 and 137–145 of the 148-residue *E. coli* outer membrane protein X (OmpX) unfolded in 8 M urea solution.[11] NMR spectroscopy experiments with designed variants of OmpX reveal that neither the thermodynamic stability nor the structure of the two clusters is affected by inter- and intramolecular long-range interactions between them. In contrast, we identify specific binding between the individual clusters and detergent micelles, which leads to rearrangement of the cluster architectures. These observations place previous studies with different proteins in a new frame of reference and present new perspectives for the role of initial hydrophobic collapse in protein folding and insertion of membrane proteins into ordered lipid structures.

Inter- and intramolecular interactions of well-structured hydrophobic clusters in urea-unfolded polypeptide chains are investigated herein with two different proteins. The main focus is on the outer membrane protein X (OmpX), which has two tryptophan residues, W76 and W140, among its 148 residues. In globally unfolded OmpX in 8 M aqueous urea solution, the polypeptide segments of residues 73–82 and 137–145 form two well-structured hydrophobic clusters, I and II.[11] In the soluble protein 434-repressor(1–63), the residues 41–60 have been shown to form a well-defined nonrandom structure in 8 M urea solution.[1,12]

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The characterization of interactions with the nonrandom local structures in OmpX and 434-repressor(1–63) is based on the observations that they are populated only to about 20–30% in the ensemble of protein molecules and that they are in rapid conformational exchange, on the NMR spectroscopy chemical shift time scale,[13] with the random-coil state.[1,11] On the other hand, the lifetime of each nonrandom local structure is in the slow-motion limit for the modulation of the dipole–dipole couplings that give rise to the ^{1}H–^{1}H nuclear Overhauser effect (NOE). Each individual nonrandom conformation thus has a lifetime between about 1 ms and 10 ns. In our approach we identify single amino acid variants of OmpX that lack one of the two clusters and then compare the population q' of the remaining cluster with its population q in the wild-type protein. Different population levels, $q' \neq q$, would then indicate stabilizing or destabilizing long-range interactions between the intact clusters in the wild-type protein.

To evaluate the populations of individual clusters, we use NMR spectroscopy chemical shift measurements. The chemical shift δ for a nucleus subjected to variable microsusceptibilities in an ensemble of rapidly interconverting states is given by Equation (1), where δ_i is the chemical shift of state i,

$$\delta = \sum_i p_i \delta_i \qquad (1)$$

p_i is the population of state i, and the summation is over all conformations present in 8 M urea, with $\Sigma p_i = 1$.[2,7,14] As the chemical shifts in nonrandom structures are different from the corresponding values in the random-coil state,[13] changes of the populations p_i will in general result in changes of the chemical shifts.

In the single amino acid variant OmpX[W76A], all the backbone amide resonances of the nine residues contained in cluster I are displaced from their positions in wild-type OmpX (Figure 1). Since short-range effects from amino acid exchanges in flexibly extended polypeptides extend only over two neighboring residues on each side,[9,15–17] these variations must arise from elimination of longer-range contacts between residues of cluster I. Since the chemical shifts in the variant protein are closer to the random-coil values than in wild-type OmpX (Figure 1), and there are no medium-range and long-range NOEs between hydrogen atoms of the cluster I residues, there is compelling evidence that the polypeptide segment 73–82 is in a flexibly extended conformation in OmpX[W76A]. Using the same approach, we find that the segment 137–145 is flexibly extended in the variant protein OmpX[W140A] (Figure 1). Furthermore, the combined ^{15}N and ^{1}H chemical shift differences, $\Delta\delta(^1\text{H},^{15}\text{N})$,

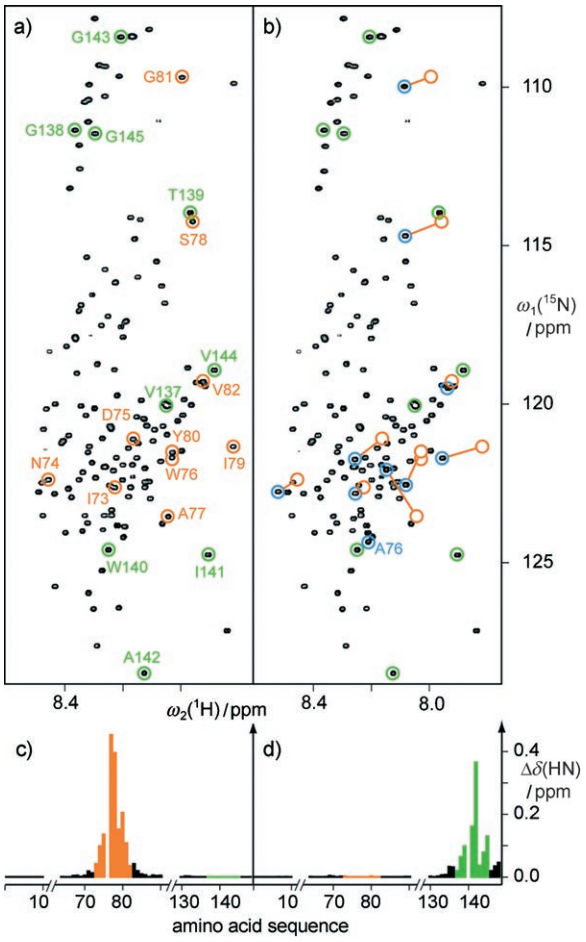

Figure 1. NMR spectroscopy data showing the impact of single amino acid replacements in the hydrophobic clusters identified in OmpX unfolded in 8 M urea solution. a), b) 2D [^{15}N,^{1}H]-HSQC NMR spectra of wild-type OmpX and OmpX[W76A], respectively; orange and green circles mark the positions of the backbone ^{15}N-^{1}H resonances of the hydrophobic clusters I (residues 73–82 in the 148-residue polypeptide) and II (residues 137–145), respectively, in the wild-type protein. In (b), additional blue circles mark the resonances of residues 73–82 in OmpX[W76A], and corresponding orange and blue circles are connected by orange lines. c) The chemical shift differences $\Delta\delta(^{15}N,^1H)$ between corresponding amide groups in OmpX[W76A] and OmpX are plotted versus the amino acid sequence, where the data for the clusters I and II are colored orange and green, respectively. No data is presented for residues 11 to 63 and 91 to 129, which show no chemical shift changes. d) Same as (c) for OmpX[W140A].

in the remaining cluster are not measurably affected (Figure 1c,d), which shows that the cluster architecture is maintained with the same population. With the precision of the chemical shift measurements achieved in these experiments, we estimate an upper limit for the free energy of interaction between the two clusters of $\Delta G = 0.13\ \mathrm{kJ\,mol^{-1}}$.

NMR spectroscopy experiments with the unlabeled polypeptide H-AYRINDWASIYGVVGVGY-OH, which corresponds to residues 70–87 of wild-type OmpX, were performed under solution conditions identical to those used for OmpX. Complete sequence-specific proton resonance assignments obtained with homonuclear 2D NMR spectroscopy experiments showed that the proton chemical shifts for the peptide are virtually identical to those of the corresponding residues in OmpX (Figure 2), whereas the deviations from the random coil values are as large as 0.4 ppm (Figure 2). The observation of identical proton chemical shifts indicates that

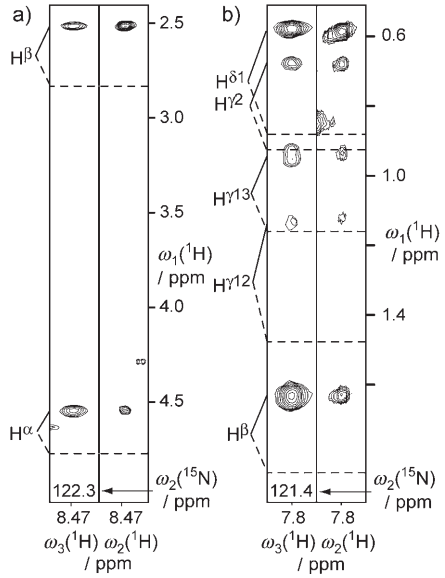

Figure 2. Comparison of corresponding side-chain ^{1}H chemical shifts in OmpX and the synthetic octadecapeptide H-OmpX(70–87)-OH in 8 M aqueous urea solutions. a) [$\omega_3(^1H)$,$\omega_1(^1H)$] strip at the amide proton chemical shift of residue N74 from a 3D ^{15}N-resolved [^{1}H,^{1}H]-TOCSY experiment with OmpX[11] (left panel) and [$\omega_2(^1H)$,$\omega_1(^1H)$] strip of the corresponding residue from a 2D [^{1}H,^{1}H]-TOCSY spectrum of OmpX(70–87). The random-coil chemical shifts are indicated by dashed lines. b) Same as (a) for residue I79.

the structure and population of cluster I in the peptide OmpX(70–87) are the same as in the full-length OmpX [Eq. (1)]. Data from two independent experimental approaches thus support that the formation of the hydrophobic clusters in urea-unfolded OmpX is encoded entirely in the local amino acid sequence and that there are no observable inter- or intramolecular long-range interactions between the two clusters in urea-unfolded OmpX.

As OmpX is an integral membrane protein, it was then of interest to investigate possible interactions of the clusters with ordered lipid structures. Stepwise addition of the detergent dihexanoylphosphatidylcholine (DHPC) to urea-unfolded OmpX caused chemical shift changes above a DHPC concentration of about 50 mM (Figure 3a), which closely matches the critical micelle concentration (cmc) of 45 mM for DHPC in 8 M urea (see the Supporting Information). Only the residues involved in the formation of the two hydrophobic clusters were strongly affected by DHPC, whereas other amide moieties showed only small chemical shift changes (Figure 3b). We thus have a specific interaction between the individual hydrophobic clusters and DHPC micelles, whereas there is no interaction with DHPC monomers. In the variant proteins OmpX[W76A] and OmpX[W140A], detectable interactions with DHPC micelles were present only for the remaining cluster (Figure 3c,d), and the chemical shift changes caused by micelle binding were identical in wild-

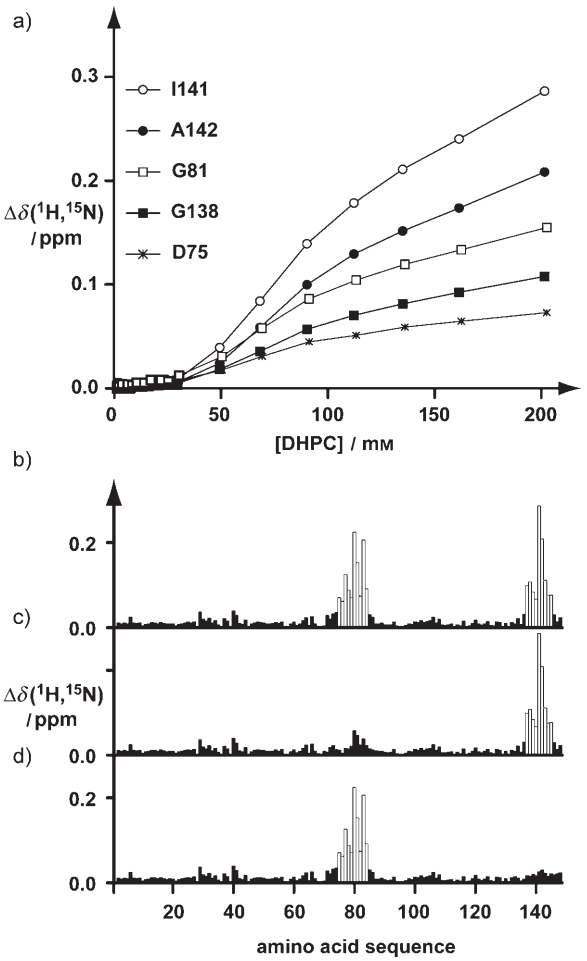

Figure 3. Detection of interactions of urea-unfolded OmpX with the detergent DHPC. a) Chemical shift differences $\Delta\delta(^1H,^{15}N)$ of the backbone ^{15}N-^1H moieties in unfolded OmpX in urea solution at variable DHPC concentrations. Data for five residues in the hydrophobic clusters I and II are shown. Complete data for all residues of the two clusters are given in Supporting Information, and the detergent-dependent shifts at 200 mM DHPC are shown in Figure 3b. b)–d) Plots versus the amino acid sequence of the chemical shift differences caused by interactions with DHPC micelles in aqueous 8 M urea solutions of the membrane protein OmpX and two variants thereof. $\Delta\delta(^{15}N,^1H)$ is the chemical shift difference of the backbone ^{15}N-^1H moiety with and without addition of 200 mM DHPC. b) Wild-type OmpX; c) OmpX[W76A]; d) OmpX[W140A].

type OmpX and OmpX[W140A] for cluster I and in wild-type OmpX and OmpX[W76A] for cluster II. In the reaction with DHPC micelles, we thus again have a complete absence of interactions between the two clusters in wild-type OmpX, with the implication that a DHPC micelle can only bind to one hydrophobic cluster.

Hydrophobic clusters have also been seen in urea-unfolded soluble proteins, including the DNA-binding domain of the 434-repressor 434(1–63), which contains the residues 41–53 and 54–60 in two hydrophobic clusters.[1,12] Adding 200 mM DHPC to 434(1–63) in 8 M aqueous urea solution produced selective chemical shift changes for the residues in the clusters (see the Supporting Information), showing that the affinity to micelles is not limited to hydrophobic clusters in urea-unfolded membrane proteins but seems to be a general property of such residual local structures in urea-unfolded proteins.

A structure determination of the OmpX hydrophobic clusters in the complex with DHPC micelles was based on complete assignment of NMR spectroscopy signals using standard triple-resonance experiments and collection of conformational constraints with 3D ^{15}N- and ^{13}C-resolved $[^1H,^1H]$-NOESY spectra. Only medium- and long-range ^1H-^1H NOEs were considered for the input for the structure calculation, and it was assumed that the random-coil conformers in the ensemble of molecules make at most negligibly small contributions to these NOEs.[7] For the clusters I and II, 37 and 26 such NOEs were observed, among which only five and three NOEs, respectively, coincide with NOEs observed in the absence of the micelles. The structure calculations for clusters I and II both converged with residual target function values of 0.0 Å^2. The backbone root mean square deviation (RMSD) for residues 73–82 was (0.67 ± 0.24) Å, and for the backbone atoms of residues 137–145 it was (0.42 ± 0.20) Å. The polypeptide backbone of cluster I adopts a helical conformation, with the hydrophobic side chains exposed to the outside (Figure 4a). A hydrogen bond between Val82-H^N and Ile79-CO is present in 14 of the 20 conformers. In cluster II, the backbone adopts a more extended helix-like conformation, with the hydrophobic side chains pointing to the exterior (Figure 4b). Two hydrogen bonds, Ala142-H^N to Thr139-CO and Gly145-H^N to Ala142-CO, both appear in 18 of the 20 conformers. For both clusters, comparison with the structures in the absence of micelles[11] reveals extensive differences for the backbone conformation as well as for the orientation of the hydrophobic side chains, which form a hydrophobic core in the interior of each of the clusters in the absence of the DHPC micelles.

In the natively folded state of OmpX, the central residues of the two polypeptide segments forming clusters in 8 M urea are separated by about 20 Å, and there are no direct contacts between any atom groups of the two clusters in the three-dimensional structure.[18,19] Thus, interactions between the two clusters in the urea-unfolded polypeptide would be non-native contacts. Non-native long-range interactions have previously been proposed to occur between different hydrophobic clusters in the denatured soluble protein lysozyme.[9,15] The different behavior of the two proteins might possibly be related to the different topologies and environments of the two proteins in their folded states, that is, OmpX as an integral membrane protein and lysozyme as a soluble protein in an aqueous milieu.

The observed intermolecular interactions between the hydrophobic clusters and detergent micelles can readily be rationalized with the free energy gain upon transfer of the hydrophobic clusters from the aqueous urea solution to the more hydrophobic environment of the ordered detergent structure. This binding reaction seems to be a general feature of hydrophobic clusters, as indicated by the experiment with the soluble 434(1–63) repressor protein, and it is tempting to speculate that it occurs in vivo as well as in vitro. Folding mechanisms of integral membrane proteins are biophysical

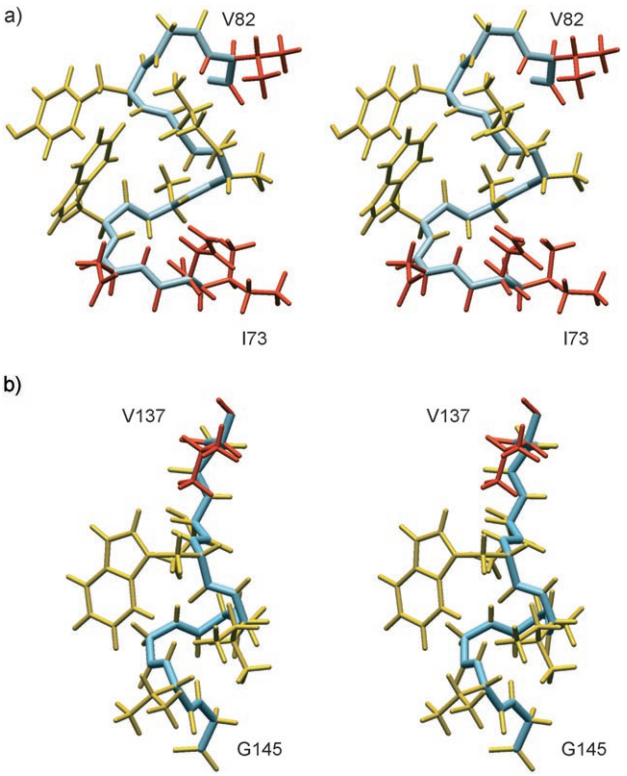

Figure 4. Stereo views of the two hydrophobic clusters of urea-unfolded OmpX in complex with DHPC micelles. a) Cluster I comprising residues 73–82. b) Cluster II comprising residues 137–145. From the bundle of 20 conformers used to represent the structure determined by NMR spectroscopy, the conformer closest to the mean coordinates is shown. Blue: backbone, yellow: side chains of residues with a mean global heavy-atom displacement below 1.0 Å in the 20 conformers aligned for their backbone atoms, red: other side chains.

processes of high complexity,[20–25] and the topology of the folded state of integral membrane proteins is adapted to an environment of alternating hydrophobic and hydrophilic phases, which also form the milieu for folding and insertion.[26] The affinity to the hydrophobic phase of detergent micelles presents a striking difference between the polypeptide segments that form hydrophobic clusters in 8 M aqueous urea and the other, randomly disordered parts of the polypeptide chain. In a common model for the folding mechanism of transmembrane β-barrel proteins, the unfolded polypeptide binds initially to the membrane surface, from where it is further inserted into the bilayer.[21,23] On the basis of the results presented herein, hydrophobic clusters would appear to be logical binding sites for the initial contacts with the membrane. The presence of similar clusters in unfolded soluble proteins then leads to the intriguing hypothesis that transient binding of hydrophobic clusters to ordered lipid surfaces might be an alternative chaperoning event in the folding pathway of some nonmembrane proteins.

Experimental Section

[*U*-^{13}C,^{15}N]-OmpX and [*U*-^{15}N]-OmpX were expressed and purified as reported.[11,27] The protein was transferred to OmpX NMR buffer (8 M urea, 20 mM phosphate, 0.1 mM NaN_3, 5% D_2O, 95% H_2O, pH 6.5). [*U*-^{15}N,^{13}C]-labeled 434-repressor(1–63) was expressed and purified as described.[28,29] The protein was dissolved in a buffer at pH 4.8 containing 7 M urea, 20 mM phosphate, 0.1 mM NaN_3, 5% D_2O, and 95% H_2O, which are the reference conditions from previous studies.[1] The samples had protein concentrations between 0.5 and 1 mM.

The single amino acid variants OmpX[W76A] and OmpX-[W140A] were obtained from a pET3b plasmid containing the OmpX wild-type gene using a QuickChange mutagenesis kit (Stratagene). Before further use, the correct DNA sequence of the variant genes was confirmed.

A peptide with the sequence H-AYRINDWASIYGVVGVGY-OH, corresponding to OmpX(70–87), was synthesized by Bio-Synthesis (Lewisville TX, USA) with a purity of greater than 95%, as confirmed by HPLC. Lyophilized peptide was dissolved in OmpX NMR buffer to a concentration of 1 mM and used without further purification.

NMR experiments were measured at 15 °C on a Bruker DRX 750 spectrometer. All experimental details are summarized in the Supporting Information.

For the calculation of three-dimensional structures, an upper limit distance constraint of 5.5 Å was applied for all observed medium-range and long-range NOEs.[1,13] Structure calculations were performed independently for the two polypeptide segments 73–82 and 137–145, using the program DYANA.[30] To limit chain-end effects, two extra residues were included at the N- and C-termini of these polypeptide segments.

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[1] D. Neri, M. Billeter, G. Wider, K. Wüthrich, *Science* **1992**, *257*, 1559–1563.
[2] L. J. Smith, K. M. Fiebig, H. Schwalbe, C. M. Dobson, *Folding Des.* **1996**, *1*, R95-R106.
[3] S. J. Demarest, Y. X. Hua, D. P. Raleigh, *Biochemistry* **1999**, *38*, 7380–7387.
[4] M. E. Hodsdon, C. Frieden, *Biochemistry* **2001**, *40*, 732–742.
[5] S. Schwarzinger, P. E. Wright, H. J. Dyson, *Biochemistry* **2002**, *41*, 12681–12686.
[6] K. A. Crowhurst, J. D. Forman-Kay, *Biochemistry* **2003**, *42*, 8687–8695.
[7] K. Wüthrich, *Curr. Opin. Struct. Biol.* **1994**, *4*, 93–99.
[8] E. R. McCarney, J. E. Kohn, K. W. Plaxco, *Crit. Rev. Biochem. Mol. Biol.* **2005**, *40*, 181–189.
[9] J. Klein-Seetharaman, M. Oikawa, S. B. Grimshaw, J. Wirmer, E. Duchardt, T. Ueda, T. Imoto, L. J. Smith, C. M. Dobson, H. Schwalbe, *Science* **2002**, *295*, 1719–1722.
[10] R. L. Baldwin, *Adv. Protein Chem.* **2002**, *62*, 361–367.
[11] H. Tafer, S. Hiller, C. Hilty, C. Fernández, K. Wüthrich, *Biochemistry* **2004**, *43*, 860–869.
[12] D. Neri, G. Wider, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 4397–4401.
[13] K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, **1986**.
[14] R. Mohana-Borges, N. K. Goto, G. J. A. Kroon, H. J. Dyson, P. E. Wright, *J. Mol. Biol.* **2004**, *340*, 1131–1142.
[15] J. Wirmer, C. Schlorb, J. Klein-Seetharaman, R. Hirano, T. Ueda, T. Imoto, H. Schwalbe, *Angew. Chem.* **2004**, *116*, 5904–5909; *Angew. Chem. Int. Ed.* **2004**, *43*, 5780–5785.

[16] S. Schwarzinger, G. J. A. Kroon, T. R. Foss, J. Chung, P. E. Wright, H. J. Dyson, *J. Am. Chem. Soc.* **2001**, *123*, 2970 – 2978.
[17] D. Braun, G. Wider, K. Wüthrich, *J. Am. Chem. Soc.* **1994**, *116*, 8466 – 8469.
[18] J. Vogt, G. E. Schulz, *Structure* **1999**, *7*, 1301 – 1309.
[19] C. Fernández, C. Hilty, G. Wider, P. Güntert, K. Wüthrich, *J. Mol. Biol.* **2004**, *336*, 1211 – 1221.
[20] P. J. Booth, R. H. Templer, W. Meijberg, S. J. Allen, A. R. Curran, M. Lorch, *Crit. Rev. Biochem. Mol. Biol.* **2001**, *36*, 501 – 603.
[21] J. H. Kleinschmidt, *Cell. Mol. Life Sci.* **2003**, *60*, 1547 – 1558.
[22] D. M. Engelman, Y. Chen, C. N. Chin, A. R. Curran, A. M. Dixon, A. D. Dupuy, A. S. Lee, U. Lehnert, E. E. Matthews, Y. K. Reshetnyak, A. Senes, J. L. Popot, *FEBS Lett.* **2003**, *555*, 122 – 125.
[23] L. K. Tamm, H. Hong, B. Y. Liang, *Biochim. Biophys. Acta Biomembr.* **2004**, *1666*, 250 – 263.
[24] S. H. White, G. von Heijne, *Curr. Opin. Struct. Biol.* **2004**, *14*, 397 – 404.
[25] W. Wickner, R. Schekman, *Science* **2005**, *310*, 1452 – 1456.
[26] S. H. White, W. C. Wimley, *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 319 – 365.
[27] C. Fernández, K. Adeishvili, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 2358 – 2363.
[28] D. Neri, T. Szyperski, G. Otting, H. Senn, K. Wüthrich, *Biochemistry* **1989**, *28*, 7510 – 7516.
[29] D. Neri, M. Billeter, K. Wüthrich, *J. Mol. Biol.* **1992**, *223*, 743 – 767.
[30] P. Güntert, C. Mumenthaler, K. Wüthrich, *J. Mol. Biol.* **1997**, *273*, 283 – 298.

