

Solution NMR Studies of Membrane-Protein–Chaperone Complexes

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Abstract: The biosynthesis of the bacterial outer membrane depends on molecular chaperones that protect hydrophobic membrane proteins against aggregation while transporting them across the periplasm. In our ongoing research, we use high-resolution NMR spectroscopy in aqueous solution as the main technique to characterize the structures and biological functions of these membrane-protein–chaperone complexes. Here, we describe NMR studies addressing three functional aspects of periplasmic membrane-protein–chaperone complexes. Firstly, the *Escherichia coli* outer membrane protein OmpX binds to each of the two chaperones, Skp and SurA, in structurally at least partially similar states despite fundamental differences between the three-dimensional structures of the chaperones. Secondly, we show that the Skp-bound state of OmpX is equivalent to a chemically denatured state in terms of its refolding competence into detergent micelles *in vitro*. Thirdly, we use amino acid mutation analysis to show that the interaction of OmpX to Skp is not dominated by the two most hydrophobic segments of OmpX.

Keywords: β -Barrel membrane proteins · Membrane biogenesis · Molecular chaperones · NMR spectroscopy · Protein complexes · Protein folding

Introduction

The biogenesis of bacterial outer membrane proteins involves a series of molecular chaperones and protein complexes for the transport, the stabilization and the membrane insertion (Fig. 1A).^[1] At the beginning of their biological lifetime, integral outer membrane proteins (Omps) are synthesized by the ribosome in the cytosol. The Omps are very hydrophobic polypeptides and would aggregate in the aqueous solution of the cytosol, if they were not stabilized by the chaperones trigger factor and SecB until they reach the Sec translocase.^[2] The Sec translocase is a protein-conducting channel, which guides the Omps across the inner membrane into the periplasmic space.^[3] In the periplasm, the insoluble proteins are then bound by further chaperones and transported to the outer membrane, where their simultaneous folding and membrane insertion is catalyzed by the Bam complex.^[1a,4] The Bam complex consists of five subunits out of which two are essential, the cen-

tral, pore-forming subunit BamA, and the indispensable lipoprotein BamD.^[1c,5] Our current research focus lies on the periplasmic chaperones that operate between the

Sec translocase and the Bam complex (Fig. 1A). In *E. coli*, these are mainly the three proteins SurA, Skp and DegP.^[1c] The relative functions of these three chaperones are

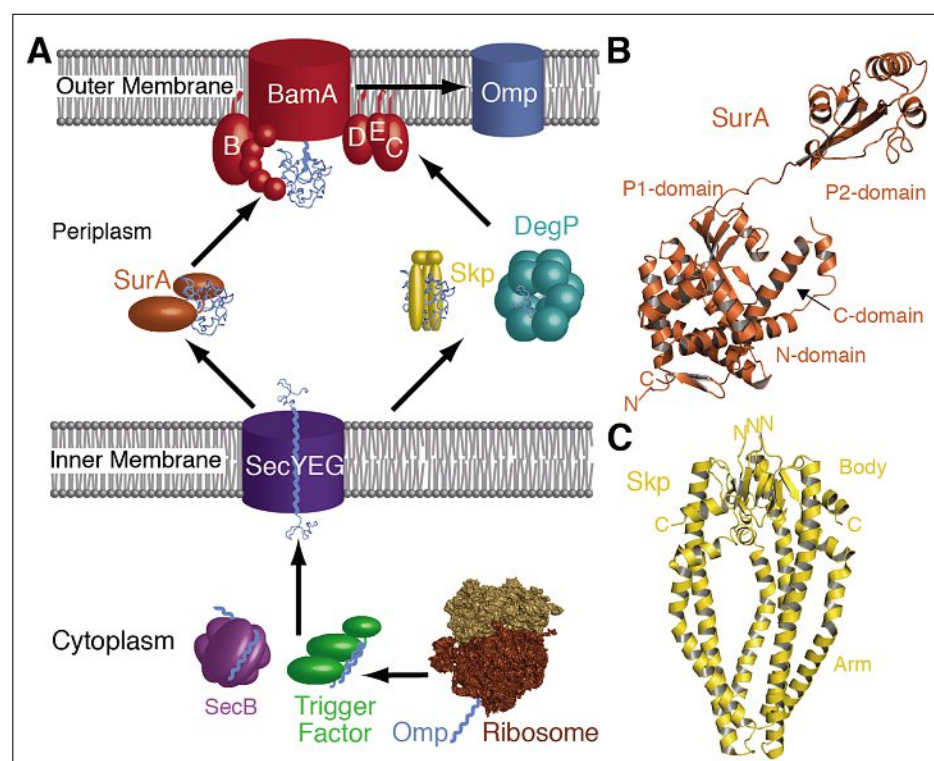


Fig. 1. A) Schematic overview of the chaperone assembly line for outer membrane biogenesis in *E. coli*. The individual biomolecules are symbolized by colored shapes. Outer membrane proteins (Omps, blue) are synthesized at the ribosome (brown) and follow the assembly pathway (black arrows) to their final destination in the outer membrane. See main text for details. The chaperones of key interest in this work are SurA (orange) and Skp (yellow). B) Crystal structure of SurA in ribbon representation^[7] (PDB 1M5Y). C) Crystal structure of the homotrimeric Skp in ribbon representation^[14a] (PDB 1SG2). In panels B and C, the N- and C-termini and the individual domains are indicated.

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not yet fully understood, however, the co-deletion of either Skp and SurA or SurA and DegP is lethal, indicating pairwise replaceable functions on the overall essential transport pathway.^[6]

SurA consists of an N-terminal domain (N-domain), two peptidyl-prolyl domains (P1- and P2-domain), and a C-terminal domain (C-domain; Fig. 1B).^[7] The N-domain, the P1-domain, and the C-domain form the structural core that harbors the chaperone function. The P2-domain, which is connected to this core *via* two flexible polypeptide linkers of 30 Å length, exhibits peptidyl-prolylisomerase activity *in vitro*.^[7,8] It is also of interest to note that SurA is structurally highly similar to the cytoplasmic chaperone trigger factor.^[7,9] It could be shown that the cellular abundances of eight β -barrel proteins, including the 8-stranded OmpX, were significantly reduced in SurA knock-out cells, demonstrating the involvement of SurA in their biogenesis pathway.^[10] SurA has been shown to bind short polypeptides as either monomeric or dimeric species^[11] with typical affinities in the lower micromolar range, thereby preferring substrates rich in aromatic residues arranged in alternating sequence.^[12] Such Ar-X-Ar motifs are occurring statistically more frequent in outer membrane proteins than in soluble or inner membrane proteins, and could thus provide the interaction points between the membrane protein substrates and SurA.^[12a,c] Still, the molecular mechanisms of the interaction between SurA with a full-length unfolded Omp are currently not understood in detail.

The second chaperone of interest, homotrimeric Skp (Fig. 1C) belongs to the class of cavity chaperones.^[13] Its crystal structure features a 'body'-domain unit that contains the trimerization interface and that consists of α -helical as well as of β -sheet secondary structure.^[14] Three 'arm'-domains of α -helical secondary structure protrude from the body-domain and these arms encompass a large central hydrophobic cavity that is the binding site for unfolded membrane proteins in aqueous solution.^[14a,15] Skp binds unfolded Omp substrates with nanomolar binding affinity.^[15b] Its substrate range comprises more than 30 different proteins from the outer membrane and the periplasm, several of them also being SurA substrates, including the outer membrane protein OmpX.^[15c] A key requirement for the functioning of both chaperones Skp and SurA comes from the fact that the periplasmic space is devoid of a cellular energy source, such as ATP. Therefore, binding, stabilization, as well as release of the protein substrates all have to be exergonic processes. This functional aspect, as well as several additional ones depend crucially on the molecular

details of the Omp-Skp and Omp-SurA complexes and their characterization is thus of key interest.

Our ongoing research activities aim at investigating the structures and biological functions of these protein assemblies, using high-resolution NMR spectroscopy in aqueous solution as the main technique. Here, we address three functional aspects of periplasmic membrane-protein-chaperone complexes: Firstly, we compare the NMR spectroscopic signatures of the substrate protein OmpX, when in complex with either Skp or SurA, to obtain information on its conformational state. Secondly, we investigate whether OmpX can be refolded directly from the Skp-bound state into detergent micelles, and show that refolding OmpX from either the Skp-bound or from a chemically denatured state results in the same three-dimensional protein structure. Thirdly, by a combination of NMR spectroscopy and mutational analysis, we characterize the influence of the two most hydrophobic OmpX segments on the OmpX-Skp interaction.

Results

Conformational Similarities between Two Different Chaperone-bound States

Solution NMR experiments of two different sample preparations, OmpX bound to Skp and OmpX bound to SurA, result in

meaningful 2D [¹⁵N,¹H]-correlation spectra with individually resolved resonance lines, despite the high molecular weight of the complexes of more than 60 kDa (Fig. 2A and 2B). These experiments depend crucially on the possibility to assemble the complexes *in vitro* from individually expressed and purified components. On the one hand, the high purity allows homogeneous samples in the concentration range of 100–500 μ M. On the other hand, this preparation scheme permits the use of protein-specific isotope labeling patterns for each of the two complex components separately. For example, the combination of uniformly ²H- and ¹⁵N-labelling for the substrate and the simultaneous unlabeled of the chaperone enables NMR spectra with a reduced number of resonances, facilitating the analysis (Figs. 2A and 2B).

Intriguingly, the 2D [¹⁵N,¹H]-TROSY spectra of OmpX bound to either SurA or Skp resolve a highly similar pattern of resonances (Fig. 2A and 2B). These two spectra feature the same subset of high-intensity peaks, which is particularly apparent in the glycine region, located upfield in the $\delta_1(^{15}\text{N})$ dimension. Additional temperature-dependent experiments, as well as sequence-specific resonance assignments^[16] confirm that indeed the same set of backbone amide moieties is appearing in both experiments, allowing a quantitative comparison of the resonance intensities (Fig. 2C). In both chaperone environments, peaks from the N-terminal region of

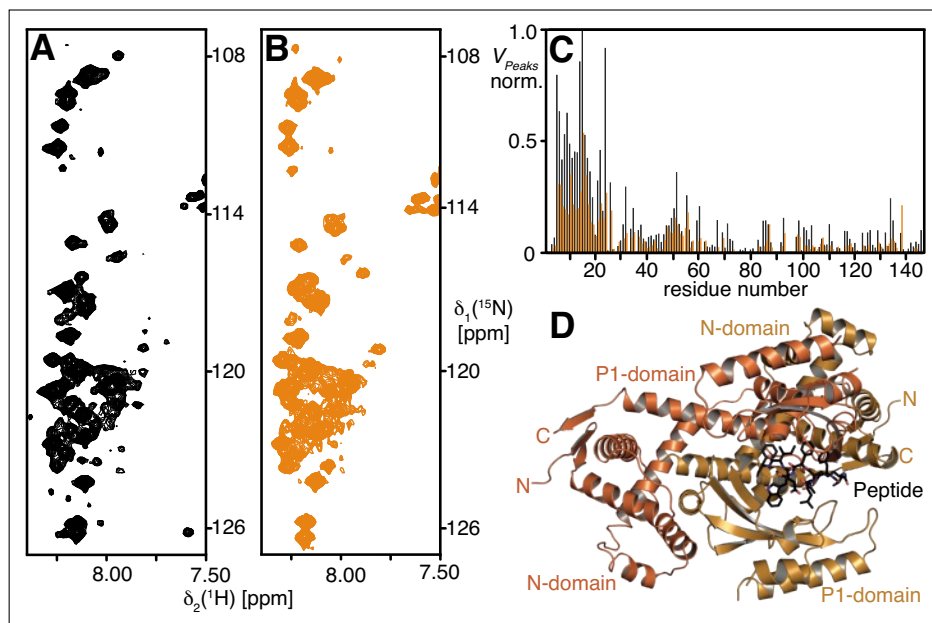


Fig. 2. NMR spectra of OmpX bound to different chaperones. A) 2D [¹⁵N,¹H]-TROSY spectrum of 215 μ M [^U-²H,¹⁵N]-OmpX bound to unlabeled Skp in NMR buffer at 31 °C. B) 2D [¹⁵N,¹H]-TROSY spectrum of 210 μ M [^U-²H,¹⁵N]-OmpX bound to unlabeled SurA in NMR buffer with 1% glycerol at 37 °C. C) Sequence-specific normalized peak volumes of backbone amide chemical shift correlations in the 2D [¹⁵N,¹H]-TROSY spectrum of Skp-OmpX (black) and SurA-OmpX (orange). Volumes were normalized for each spectrum to the peak with the highest volume (Q15). D) Crystal structure of SurA, lacking its P2-domain, in ribbon representation (orange) in complex with the NFLTKFWDIFRK peptide (black), which binds as a dimer^[11] (PDB 2PV3).

the OmpX substrate (residues 1–60) have the strongest intensities and highly similar resonance frequencies. Due to the strong dependence of the chemical shift on conformational and population changes, the high degree of correlation between resonance frequencies and amplitudes in the 2D [^{15}N , ^1H]-TROSY correlation spectra for Skp- and SurA-bound OmpX indicate that these parts of the OmpX polypeptide are in similar conformational ensemble states on both chaperones. This conclusion is striking, because of the substantially different three-dimensional structures of the two chaperones, based on which one would not necessarily expect similar substrate conformations (Fig. 1B and 1C). Whereas Skp binds the substrates in its central hydrophobic cavity, monomeric SurA does not contain such a structural element,^[7] but presumably binds Ar–X–Ar motifs within the Omp polypeptide with its binding cleft in either a monomeric or dimeric form.^[12] The binding site of the peptide is situated in the P1-domain of SurA (Fig. 2D) and appears to protect segments of the substrate in an extended form against aggregation, which would thus lead to a totally different substrate conformation than presumed for Skp and would thus be in contrast to our observations of highly similar 2D [^{15}N , ^1H]-TROSY spectra. Since Skp does not possess a specific peptide-binding cleft, these observations may indicate that multiple SurA molecule form a cavity around the unfolded Omp, resulting in a similar cavity as Skp. In one such scenario the resulting SurA cavity could structurally resemble the complex of trigger factor with ribosomal protein S7, which forms a 2:2 complex with one copy of partly folded S7 in the binding pocket of each trigger factor molecule.^[2c] At the moment, we are undertaking additional structural investigations into how the SurA–OmpX complex assembles and how the interaction take place.

Direct Refolding of OmpX from the Skp-bound State

Biochemical preparation schemes of β -barrel outer membrane proteins frequently exploit that these proteins can be refolded into detergent micelles from a chemically denatured state.^[17] Among many applications, the refolding approach has enabled the structure determination of several bacterial and a mitochondrial β -barrel outer membrane protein by solution NMR spectroscopy.^[18] Importantly, it is not guaranteed *a priori* that refolding a protein *in vitro* will end up in the three-dimensional structure that is adopted by the same polypeptide *in vivo*. Still, for several cases of refolded β -barrel outer membrane proteins such an identity has been verified.^[19] It is thus of high interest to assess an *in vitro* procedure for outer membrane protein re-

folding that uses the Skp-bound state as the biochemical starting point, instead of a chemically denatured state. It has been shown before, that folding of Omprs from Skp into lipid bilayers is possible,^[14b,20] but the resulting products were not analyzed structurally.

To this end, the detergent LDAO (lauryldimethylamine-oxide) was added to aqueous solution of Skp–OmpX complexes. The protein spontaneously refolded into the detergent micelles, as evidenced by the observation of a large amide proton chemical shift dispersion of about 4 ppm in the 2D [^1H , ^{15}N]-TROSY spectrum (Fig. 3A). A comparison of the spectra of OmpX refolded from the Skp-bound state with OmpX refolded from its denatured state in guanidine hydrochloride solution shows essentially identical resonance positions for all backbone amide moieties (Fig. 3B–D). Since the chemical shift is highly sensitive to changes in global or local structure, this identity directly shows that the folded three-dimensional structures of OmpX are in both cases identical. An additional verification of formation

of the correct three-dimensional structure was obtained by a pH titration. In a spectrum recorded at the elevated pH of 7.6, several signals appear with decreased intensity due to fast amide proton exchange with the solvent, resulting from low exchange protection factors. These signals could be assigned to the N-terminus, the extracellular loops L1, L2 and L3, as well as to the periplasmic loops T1 and T2 of folded OmpX^[21] (Figs. 3E and 3F). The location of these residues is highly correlated with those that were previously been identified to be locally flexible relative to the β -barrel^[18c] (Fig. 3E), confirming the correct structure and backbone dynamics of the OmpX sample refolded from the Skp-bound state. The Skp-refolded, as well as the guanidine hydrochloride-refolded sample are thus shown to result in the same thermodynamic state, irrespective of the different starting state and the thereof resulting different folding kinetics. At the same time these data also rule out the possibility that Skp might still be interacting with parts of the refolded OmpX. Another important conclusion is made by the ob-

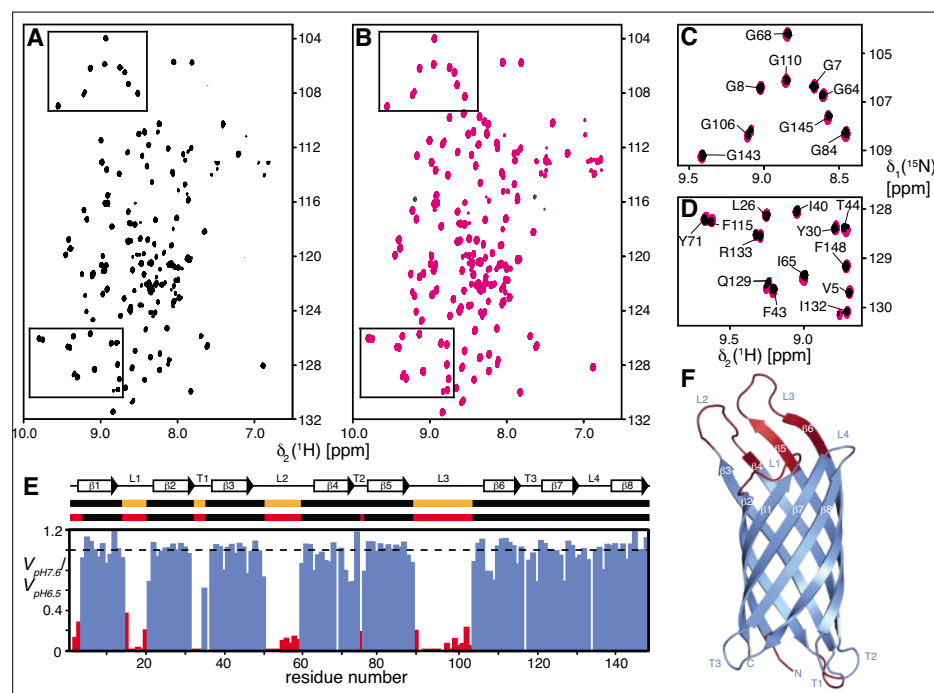


Fig. 3. OmpX refolded from two different denatured states. A) 2D [^{15}N , ^1H]-TROSY spectrum of 110 μM [U - ^2H , ^{15}N]-OmpX in NMR buffer refolded from the Skp-bound state into LDAO micelles. B) 2D [^{15}N , ^1H]-TROSY spectrum of 255 μM [U - ^2H , ^{15}N]-OmpX in NMR buffer refolded from 6 M guanidine hydrochloride solution into LDAO micelles. C), D) Enlarged view of two spectral regions of spectra in A (black) and B (magenta). The sequence-specific resonance assignment of the resonances is indicated. E) Bottom: Sequence-specific backbone amide peak volumes from 2D [^{15}N , ^1H]-TROSY spectra of OmpX refolded from the Skp-bound state at pH 7.6 relative to pH 6.5 (blue and red bars). Residues with a volume ratio <0.5 are highlighted in red. These residues are also displayed as red bars in the row above directly above. Residues of OmpX in DHPG micelles with a backbone amide ^{15}N [^1H]-NOE <0.5 , indicative for high flexibility, are shown as orange bars (values determined by Fernández *et al.*^[18c]). In the top row, the positions of regular secondary structure elements in folded OmpX are indicated. F) Solution NMR structure of OmpX refolded in DHPG micelles in ribbon representation^[18c] (PDB 1Q9F). Residues with strong amide exchange as identified in panel E are highlighted red. Termini, loops, turns and secondary structure elements are indicated.

servation that folding process from Skp is overall exergonic. The energy release occurring upon folding into the detergent micelle thus overcompensates for the tight binding affinity of Skp to the Omp, which is in the nanomolar range.^[15b]

Role of Local Hydrophobic Clusters for the Omp–Skp Interaction

OmpX unfolded in 8 M urea globally adopts a random coil state, but it also contains two polypeptide segments, which undergo a local hydrophobic collapse.^[22] These two hydrophobic clusters are located around the two only tryptophan residues in OmpX, W76 and W140.^[22] Local non-random clusters have frequently been found in structural characterizations of unfolded proteins, mainly by solution NMR techniques and their occurrence in unfolded OmpX is thus not unusual.^[23] However, whereas, for example, for the soluble protein lysozyme multiple hydrophobic clusters were found to be involved in long-range tertiary interactions, the two clusters around W76 and W140 of urea-unfolded OmpX do not interact.^[22b,23f] The two clusters in OmpX were found to comprise ten (Ile73 – Val82) and nine residues (Val137 – Gly145), respectively, as identified by non-random medium-range NOEs between these residues. Upon mutation of tryptophan to alanine, each of the two clusters are displaced from their wild type chemical shifts in a 2D [¹H, ¹⁵N]-correlation spectrum.^[22b] Importantly, the two clusters were found to bind to detergent micelles in 8 M urea solution. The two polypeptide segments that form the clusters in 8 M urea might thus also be key contact points for the hydrophobic interaction with Skp in aqueous solution.

To address this possibility, we characterized OmpX[W140A], OmpX[W76A], and the double mutant OmpX[W76A, W140A] in complex with Skp (Fig. 4). All three mutant proteins formed stable complexes with Skp, similar to the wild-type OmpX. Furthermore, they featured similar 2D [¹H, ¹⁵N]-TROSY spectra as wild-type OmpX. Only residues in the vicinity of the respective mutation showed significant chemical shift differences (Fig. 4). Interestingly, in the Skp bound state the chemical shift perturbation effects of the mutation on neighboring residues is more pronounced than in the urea-denatured state as for OmpX[W76A] and OmpX[W140A] sixteen (Ala70 – Val85) and fourteen residues (Val135 – Phe148), respectively, experienced a chemical shift change. For other residues mostly small changes were observed, but a few signals show chemical shift changes close to the threshold level for significance. This could possibly indicate weak long-range contacts of the hydrophobic clusters in the Skp-bound state,

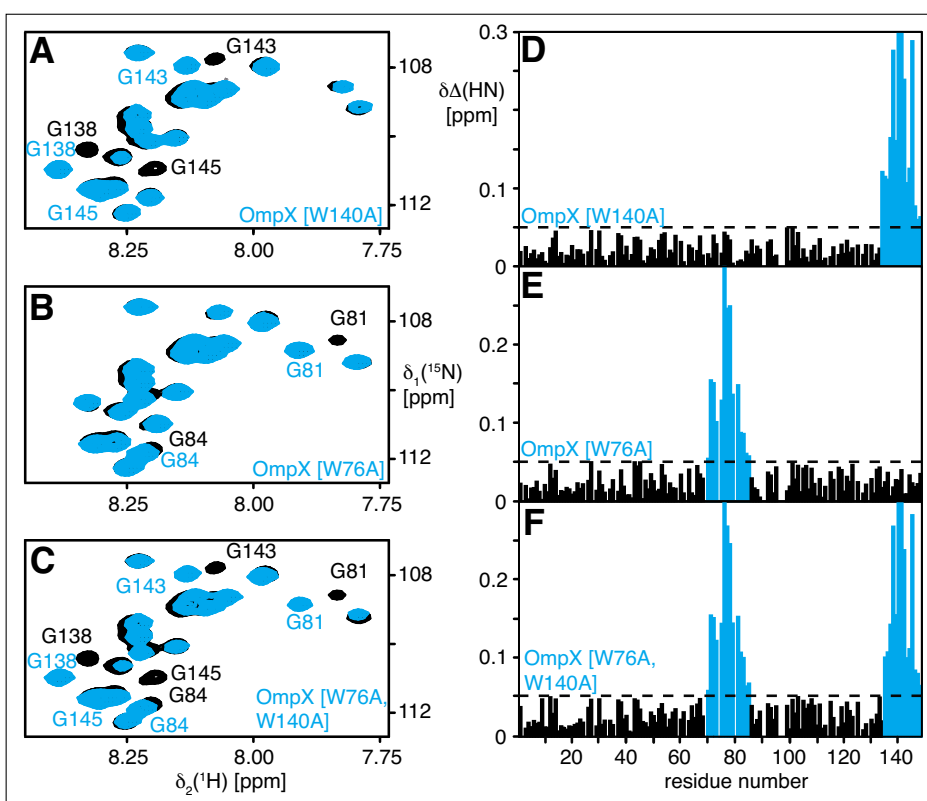


Fig. 4. Impact of the two most hydrophobic OmpX segments on the OmpX–Skp interaction. A) Overlay of the glycine region of two 2D [¹⁵N, ¹H]-TROSY spectra of [^{U-²H, ¹⁵N}]-wt-OmpX (black) and [^{U-²H, ¹⁵N}]-OmpX[W140A] (blue) bound to unlabelled Skp in NMR buffer. (B) Overlay of [^{U-²H, ¹⁵N}]-wt-OmpX (black) and [^{U-²H, ¹⁵N}]-OmpX[W76A] (blue). (C) Overlay of [^{U-²H, ¹⁵N}]-wt-OmpX (black) and [^{U-²H, ¹⁵N}]-OmpX[W76A, W140A] (blue). Selected glycine residues are indicated with their sequence-specific resonance assignments. D) Combined chemical shift differences of the amide moiety in 2D [¹⁵N, ¹H]-TROSY spectra for Skp-bound OmpX[W140A] relative to wt OmpX. (E) Same for OmpX[W76A]. (F) Same for OmpX[W76A, W140A]. In panels D–E, residues experiencing combined chemical shift changes larger than the significance level of 0.05 ppm (dashed line) are colored blue.

which were completely absent in the urea-denatured state. Importantly, the chemical shift changes in the double-mutant correspond essentially to a superposition of the effects of the two single mutations, showing also that no substantial long-range effects occur between the two cluster regions when OmpX is bound to Skp. Overall, the characterization of the tryptophan to alanine single- and double-mutants thus rule out that the two most hydrophobic polypeptide segments, which form hydrophobic clusters in urea solution, play a dominant role in the OmpX–Skp interaction.

Conclusion

The experiments described here have provided mechanistic insight into three central functional aspects of membrane-protein–chaperone interactions and at the same time illustrate the experimental approaches we apply to study and characterize these complexes by solution NMR spectroscopy. We have shown that the Skp- and SurA-bound states of OmpX have highly similar spectroscopic signatures, indicating also conformational similarities

of the bound substrates. Further, we demonstrated that Skp-bound OmpX is refold-competent *in vitro*, and that refolding from either the Skp-bound or the guanidine hydrochloride-denatured state leads to identical three-dimensional structures. Finally, we connected our work with previous findings on hydrophobic clusters, showing that the interactions between the chaperone Skp and the substrate OmpX are not dominated by the two most hydrophobic segments. Importantly, the functional studies shown here prove that the investigation of these high molecular-weight chaperone-bound states for the periplasmic chaperones is feasible with contemporary solution NMR methods. Besides their importance of the chaperone–substrate interactions in the bacterial outer membrane biogenesis, Skp and SurA are biotechnologically relevant targets for the enhancement of the solubility of proteins of interest.^[24] Our ongoing investigations aim at providing a detailed description of conformation and dynamics of these states, as well as an elucidation of the substrate handling between them. The biophysical principles found for these interactions may also well be transferable to other chaperone–substrate systems.

Experimental

Protein Expression and Purification

Skp and SurA were expressed recombinantly in *E. coli* BL21(λ DE3) cells in soluble form containing an N-terminal His₆-tag but without their N-terminal signal sequence. After cell lysis, protein purification was done with subsequent Ni²⁺-affinity and ion exchange chromatography as described.^[15a,25] OmpX lacking its signal sequence was expressed in inclusion bodies, subsequently washed and purified in the denatured state with ion exchange chromatography as described.^[22a] OmpX tryptophan mutants were expressed and purified accordingly.^[22b]

Refolding of OmpX into LDAO Micelles

One volume of denatured OmpX in 6 M guanidinium hydrochloride solution was dissolved in dropwise fashion into ten volumes of buffer containing 25 mM MES, 150 mM NaCl, 600 mM arginine, 1% LDAO, pH 6.5 under continuous stirring at 4 °C. The sample was washed with 25 mM MES, 150 mM NaCl, pH 6.5 in concentrators with a molecular weight cut-off of 30.000 Da. The final detergent concentration was ~250 mM, as determined by 1D ¹H-NMR.

NMR Spectroscopy

All NMR experiments for Skp–OmpX complexes were performed in NMR buffer (25 mM MES, 150 mM NaCl, pH 6.5). SurA–OmpX samples contained additionally 1% glycerol. All 2D [¹⁵N,¹H]-TROSY-HSQC experiments^[26] were recorded at 37 °C unless stated otherwise on Bruker 700 or 800 MHz spectrometers each equipped with a cryogenically cooled triple-resonance probe. Sequence-specific resonance assignments of OmpX bound to Skp are reported elsewhere.^[16] These assignments were transferred to SurA-bound OmpX by similarity, based on the high degree of identity between the two spectra. The combined chemical shift differences of the amide moieties were calculated as $\Delta\delta(\text{HN}) = (\Delta\delta(^1\text{H})^2 + 0.04 \cdot \Delta\delta(^{15}\text{N})^2)^{0.5}$.

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