How Amphipols Embed Membrane Proteins: Global Solvent Accessibility and Interaction with a Flexible Protein Terminus

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Abstract Amphipathic polymers called amphipols provide a valuable alternative to detergents for keeping integral membrane proteins soluble in aqueous buffers. Here, we characterize spatial contacts of amphipol A8-35 with membrane proteins from two architectural classes: The 8-stranded β-barrel outer membrane protein OmpX and the α-helical protein bacteriorhodopsin. OmpX is well structured in A8-35, with its barrel adopting a fold closely similar to that in dihexanoylphosphocholine micelles. The accessibility of A8-35-trapped OmpX by a water-soluble paramagnetic molecule is highly similar to that in detergent micelles and resembles the accessibility in the natural membrane. For the α-helical protein bacteriorhodopsin, previously shown to keep its fold and function in amphipols, NMR data show that the imidazole protons of a polyhistidine tag at the N-terminus of the protein are exchange protected in the presence of detergent and lipid bilayer nanodiscs, but not in amphipols, indicating the absence of an interaction in the latter case. Overall, A8-35 exhibits protein interaction properties somewhat different from detergents and lipid bilayer nanodiscs, while maintaining the structure of solubilized integral membrane proteins.

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Introduction

Biophysical and biochemical studies of integral membrane proteins are of key interest for characterizing their structure and function. Because membrane proteins are adapted to a hydrophobic phase, keeping them soluble in aqueous solutions is an intrinsic challenge, requiring the use of surfactants that can substitute the hydrophobic environment of a natural membrane (Raschle et al. 2010). Whereas detergent micelles represent the classical case, they can be destabilizing to some membrane proteins or to protein–

protein interactions. A number of alternative, milder systems have therefore been developed, such as lipid bilayer nanodiscs or amphipols (APols) (Bayburt et al. 2002; Bayburt and Sligar 2010; Popot et al. 2011; Tribet et al. 1996; Zoonens and Popot 2014). Results from multiple techniques, including NMR spectroscopy (Catoire et al. 2010, 2009; Planchard et al. 2014; Zoonens et al. 2005), electron microscopy (Althoff et al. 2011; Huynh et al. 2014; Liao et al. 2013) and molecular dynamics simulation (Perlmutter et al. 2014) indicate that APols associate with membrane proteins and keep them water soluble by adsorbing specifically onto their hydrophobic transmembrane surface. Structure, dynamics and function of a membrane protein may, however, depend on the surfactant type (Champeil et al. 2000; Dahmane et al. 2013; Elter et al. 2014; Etzkorn et al. 2013; Martinez et al. 2002; Perlmutter et al. 2014; Popot et al. 2011).

Here, we use high-resolution NMR spectroscopy to characterize the interactions of A8-35, a polyacrylate-based APol (Tribet et al. 1996) with proteins from two architectural classes, the β-barrel *Escherichia coli* outer membrane protein X (OmpX) and the α-helical bacteriorhodopsin (BR) from *Halobacterium salinarum*. Whereas previous NMR characterizations of OmpX/APol contacts were based on amide proton protection factors (Catoire et al. 2010), we are using here paramagnetic surface mapping, which provides a complementary and more extensive coverage. In addition, we examine the interaction of a polyhistidine (poly-His) tag with three different surfactants, APols, lipid bilayer nanodiscs and detergent micelles. Poly-His tags are of widespread use for protein purification and thus are frequently genetically fused to proteins. Because they are flexible, unstructured and relatively polar, they are expected to remain water accessible. However, in the case of membrane proteins, the uncharged nature of poly-His tags at alkaline pH can give rise to an interaction with the surfactants associated with the protein. We investigate, here, whether APol A8-35 interacts with a poly-His tag fused to the N-terminus of BR.

Materials and Methods

$[U\text{-}^{15}N, \sim 75\ \%\text{-}^2H]$-labeled OmpX was produced by overexpression in *E. coli* BL21(DE3)pLysS cells transformed with the plasmid pET3b-OmpX (Vogt and Schulz 1999). Cells were grown in D_2O-based M9 minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. Cells were induced after reaching an OD_{600} of 0.8 and harvested after 4 h by centrifugation. OmpX was purified from inclusion bodies and refolded into DHPC (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine) micelles as described (Fernández et al. 2004). Partially, deuterated A8-35 (batch DAPol-4) was synthesized by F. Giusti (UMR 7099) following published protocols

(Gohon et al. 2006, 2004) and added to the protein solution. Subsequently, the detergent was removed using BioBeads, following standard protocols (Zoonens et al. 2005). The remaining amount of detergent in the sample was assessed by 1D ^1H NMR measurements. The final NMR sample contained 1 mM OmpX with deuterated A8-35 in a standard NMR buffer (20 mM sodium phosphate, 100 mM NaCl, 5/95 % D_2O/H_2O, pH 8.0). Titration of the OmpX solution was performed by adding the paramagnetic relaxation enhancement agent Gd(DOTA)$^-$ (DOTAREM; a gadolinium ion chelated with 1,4,7,10-tetraazocyclododecane-*N,N′,N″,N‴*-tetraacetic acid; Laboratoire Guerbet, France) from an aqueous stock solution. The 2D [$^{15}N,^1H$]-TROSY spectra for the Gd(DOTA)$^-$ titration were recorded with 16 transients per increment, $t_{1max}(^{15}N) = 44$ ms, $t_{2max}(^1H) = 96$ ms, at 30 °C on a Bruker DRX 750 MHz NMR spectrometer with a triple resonance TXI probe. Measurement of the rotational correlation time was taken with the TRACT method (Lee et al. 2006). BR was expressed using a cell-free expression system and was refolded into DDM detergent micelles, DMPC lipid bilayer nanodiscs or A8-35 following published protocols (Etzkorn et al. 2013). NMR spectra of BR in the different surfactant environments were recorded under identical buffer conditions (20 mM sodium phosphate pH 7.3, 50 mM NaCl, 8 % D_2O).

Results and Discussion

The 8-stranded β-barrel membrane protein OmpX was refolded into dihexanoylphosphocholine (DHPC) detergent micelles (Fernández et al. 2004; Pautsch et al. 1999) and subsequently transferred to A8-35 (Catoire et al. 2009). The correct fold of the protein in A8-35 was assessed by comparing the 2D [$^{15}N,^1H$]-TROSY spectra of OmpX/A8-35 and OmpX/DHPC (Fig. 1a, b). The glycine resonances, which are upfield in the nitrogen dimension, as well as the β-sheet peaks, which are shifted downfield in the proton dimension, were found to feature highly similar chemical shifts in both spectra and thus virtually identical peak patterns resulted for 71 amide moieties. The high similarity of resonance frequencies for large parts of the protein backbone allows unambiguous transfer of resonance assignments from OmpX in DHPC micelles to OmpX/A8-35 complexes and, at the same time, directly indicates identical secondary and tertiary structure for these residues under both conditions. Importantly, most of the identified residues are located in the β-barrel region, confirming that the protein adopts the same fold in both environments.

Measurements of the ^1H amide proton resonance line widths show that these are increased in the complex with A8-35 amphipols compared with DHPC by a factor of approximately 2.0 (Fig. 1). Broader resonance lines of

Fig. 1 Global fold characterization of OmpX in partially deuterated A8-35. 2D [^{15}N,^{1}H]-TROSY spectra of **a** 1 mM [U-^{15}N, 75 %-^{2}H]-OmpX in complex with deuterated A8-35 amphipol and **b** OmpX in DHPC solution, both recorded at 30 °C. **c** 1D cross sections of two glycine resonances marked with an *arrow* in panels **a** and **b**. **d** TRACT analysis. *Symbols* represent for experimental data (integrals of the region 8.5–10.5 ppm, *circles* = R_α, *squares* = R_β). *Black lines* represent exponential fits, resulting in $R_\alpha = 33$ Hz, $R_\beta = 129$ Hz and $\tau_c = 39$ ns
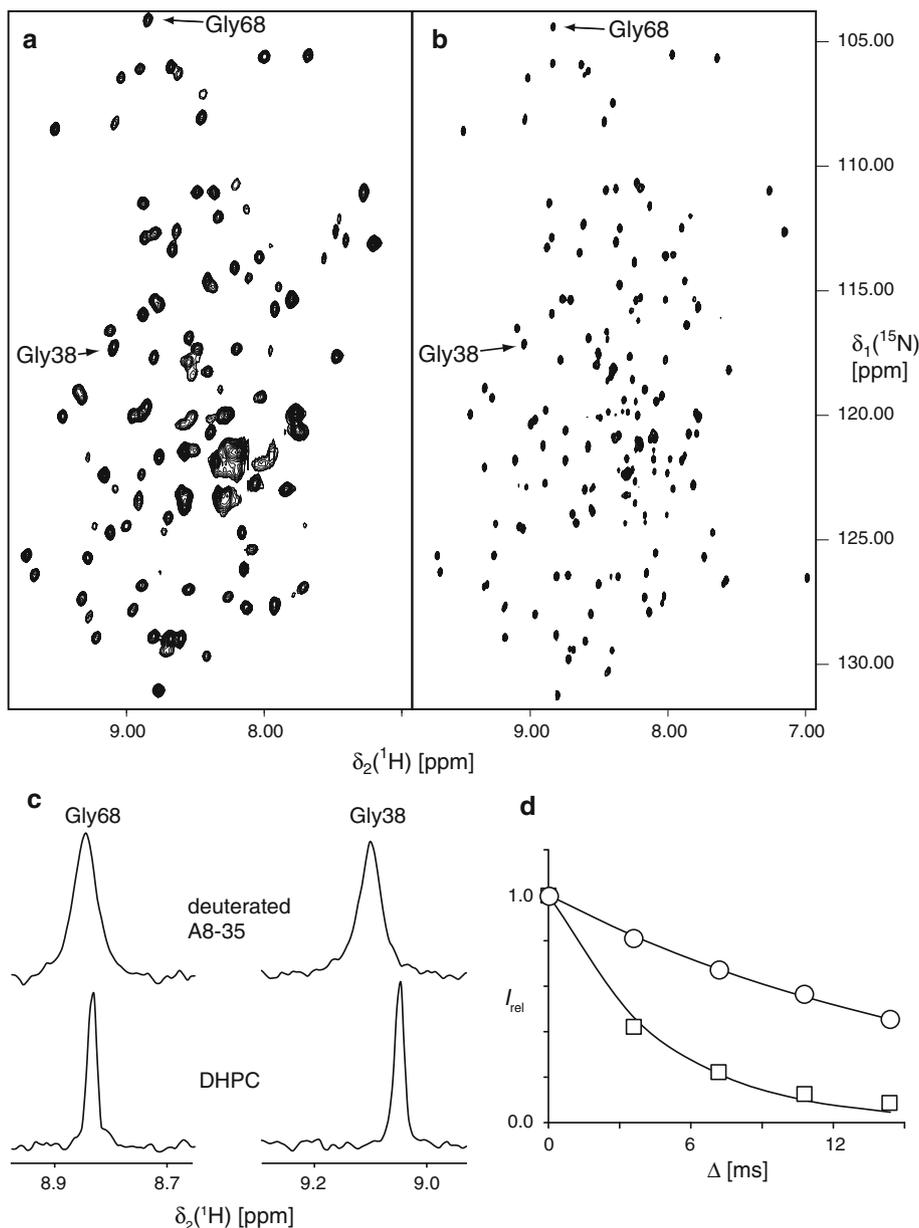
β-barrel membrane proteins in A8-35 as compared with detergent micelles have previously been observed for OmpX (Catoire et al. 2010) and for the transmembrane domain of OmpA (Zoonens et al. 2005). We address the contributions of decreased Brownian motion to the line width by measurements of the rotational correlation time τ_c with the TRACT method (Lee et al. 2006). Thereby, τ_c was found to be 39 ± 5 ns in our OmpX/A8-35 preparations, which are larger by a factor of 1.85 than the value of 21 ns reported in DHPC micelles (Lee et al. 2006). Since the resonance line width scales in first order linearly with τ_c, these measurements can largely rationalize the increased ^{1}H line width. Interestingly, previously reported preparations of OmpX/A8-35 complexes featured a τ_c value of 31 ns (Catoire et al. 2010). This difference may be attributed to the absence of EDTA in our sample buffer. EDTA has been shown to sequester residual Ca^{2+} ions, which can bridge A8-35 particles involved in complexes with membrane proteins, reducing molecular tumbling (Picard et al. 2006; Catoire et al. 2010). For solution NMR studies, it should thus be generally recommended to include EDTA in the preparations of MP/A8-35 samples.

The surface accessibility of amide moieties in OmpX/A8-35 complexes was mapped using the paramagnetic water-soluble relaxation agent Gd(DOTA)$^-$ (Fig. 2). In these experiments, the solvent accessibility is quantified by the intermolecular paramagnetic relaxation enhancement effect, as expressed by the relaxity constant ε (Caravan
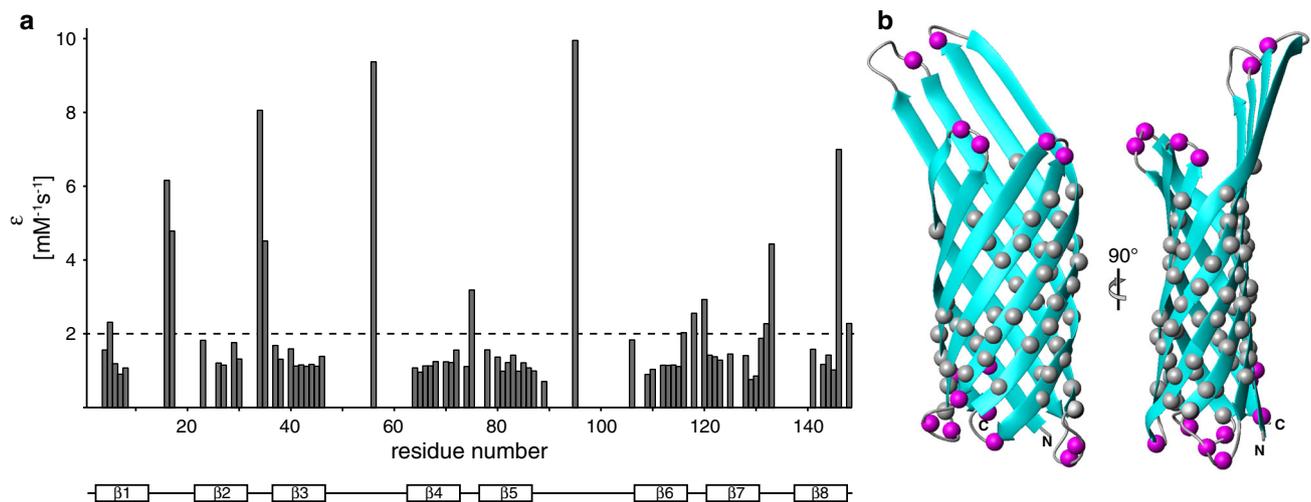
Fig. 2 Surface accessibility of OmpX/A8-35 complexes. **a** Paramagnetic relaxivity ε of backbone amide moieties by Gd(DOTA)$^-$, plotted versus the amino acid sequence of OmpX. The secondary structure elements of OmpX are indicated below. **b** Structure of OmpX [PDB 1QJ8; Vogt and Schulz (1999)], where all amide moieties of OmpX in A8-35 that could be unambiguously assigned are shown as *spheres*. *Gray* and *magenta colors* indicate protected and accessible amide moieties, respectively, as classified by the threshold level of $\varepsilon = 2$ s^{-1} mM^{-1} (*dashed line* in panel **a**)

et al. 1999; Hilty et al. 2004). Thereby, ε is an indicator of the minimal distance that the paramagnet can approach a given amide moiety. Large values of ε indicate close minimal distances and thus a high solvent accessibility. For amide groups in β-barrel membrane proteins, differences in ε correspond to shielding by adsorbed surfactant molecules, either detergent or APol. Using the value of $\varepsilon = 2$ s^{-1} mM^{-1} mM as threshold level for the classification of amide moieties into a "protected" and an "accessible" group, we find that of the 71 assigned resonances of OmpX in A8-35 amphipols, 57 are protected and 14 are accessible. The accessible amide moieties are located in the turns and loops of OmpX, and the protected amides are located in the barrel region (Fig. 2). In addition, 35 well-resolved, but unassigned resonances were detected in these titration experiments, out of which 30 are accessible and 5 are protected. The narrow chemical shift dispersion of these 35 resonances, suggests that most of them are located in loop and turn regions of OmpX. The relaxivities of OmpX in A8-35 correlate remarkably well with OmpX in the detergent DHPC (Fig. 3). Using the same classification of amide moieties, we find that of the 71 assigned resonances, 54 are protected and 10 are accessible in both conditions (Fig. 3). Only 7 resonances are in different classes in the two preparations. Overall, the solvent accessibility mapping shows that both surfactants adsorb specifically onto the hydrophobic surface of OmpX and in a similar topology (Hilty et al. 2004). These findings are consistent with previous NMR (Catoire et al. 2010) and recent MD (Perlmutter et al. 2014) data. A8-35 thus acts as a suitable membrane mimetic for OmpX.
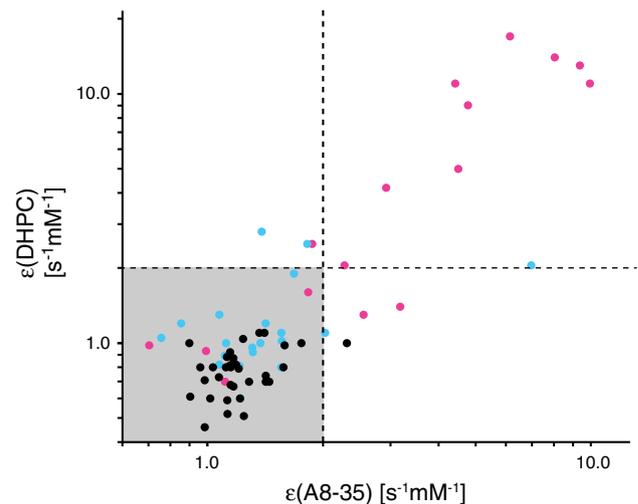

Fig. 3 Comparison of surface accessibilities of OmpX/A8-35 and OmpX/DHPC. Correlations of relaxivities ε of OmpX/A8-35 determined in the present work for 71 residues are correlated with the corresponding values in OmpX/DHPC (Hilty et al. 2004). Dashed lines denote the threshold level of $\varepsilon < 2$ s^{-1} mM^{-1} used for the classification of residues, and the *gray area* highlights the residues classified as protected in both conditions. Residues are colored according to their location in the OmpX structure (PDB 1QJ8): *black*, β-strand within >2 positions from end; *magenta*, loops and turns; *blue*, β-strand within ≤2 positions from end

In a second series of experiments, it was examined how a poly-His tag fused to the N-terminus of the archaebacterial α-helical membrane protein BR interacts with each of three different surfactant environments: The detergent DDM, DMPC-based lipid bilayer nanodiscs and A8-35. It has been shown previously that A8-35-trapped BR is both
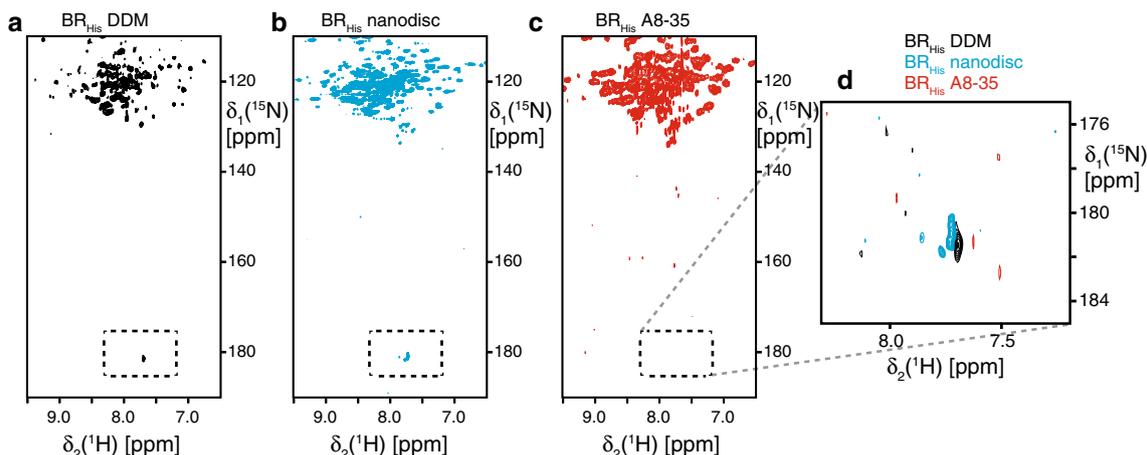
Fig. 4 Differential interaction between an N-terminal poly-His tag on bacteriorhodopsin and selected surfactants. **a–c** 2D [^{15}N,^{1}H]-TROSY NMR spectra of BR in **a** DDM micelles, **b** DMPC-based lipid bilayer nanodiscs, **c** amphipol A8-35. **d** Magnification of the highlighted region, characteristic for aromatic histidine resonance frequencies. Note that BR does not contain any histidine residue in its natural amino acid sequence and thus all of the imidazole NH-resonances arise from the poly-His tag

stable and functional (Dahmane et al. 2013; Gohon et al. 2008), and that its transmembrane, inner core region does not experience significant structural changes when embedded in either of these three different membrane-mimicking environments (Etzkorn et al. 2013). However, APols and nanodiscs do increase the thermal stability of BR as compared with detergent micelles (Etzkorn et al. 2013; Popot 2010). In addition, NMR data indicate that for several solvent-exposed loop residues, the APol environment shows closest similarity to solid-state NMR results obtained on BR in its native purple membrane (Etzkorn et al. 2013). Here, it was investigated whether a poly-His tag fused at the N-terminus of BR interacts with the surfactant belt. For this assessment, use was made of the differential proton exchange rates of unprotected and protected imidazole protons. Solvent-accessible imidazole protons of the histidine ring feature proton exchange rates with the surrounding water molecules in the fast chemical exchange regime and thus are not detectable in the NMR spectrum (Plesniak et al. 2011). 2D TROSY NMR spectra of BR in DDM solution and in lipid bilayer nanodiscs feature intense correlation crosspeaks in the imidazole spectral region (Fig. 4). These resonances could be unambiguously assigned to the poly-His tag, because BR does not contain any histidine residue in its natural amino acid sequence and because these resonances disappeared in equivalent preparations of the protein after selective cleavage of the poly-His tag with factor Xa (data not shown). The presence of histidine resonances indicates that the tag is at least partially protected from fast exchange with water in DDM and lipid bilayer nanodiscs. Interestingly, the resonances are not detected in BR/A8-35 complexes suggesting that the poly-His tag is directly accessible to the solvent and not embedded in the APol

belt. These observations show a remarkable difference between APols, detergent and nanodiscs, possibly due to the relatively small volume of polar moieties in A8-35 (carboxylate groups), as compared with glycerophosphatidylcholine in nanodiscs and maltoside in DDM.

Conclusion

As increasingly sophisticated surfactants are developed to handle membrane proteins in aqueous solutions, it becomes apparent that attention should be paid to their effects on membrane protein structure and dynamics, as compared with biological membranes (Zhou and Cross 2013). Recent NMR data, indeed, show that various environments may affect the extramembrane loops of BR (Etzkorn et al. 2013), whereas MD calculations suggest that the dynamics of both the β-barrel and loops of OmpX are damped in A8-35 as compared with DHPC (Perlmutter et al. 2014). The present experiments show, using these two membrane proteins as models, that amphipol A8-35 does not detectably interact with either the extramembrane regions of OmpX nor with a tag fused at the N-terminus of BR. Whereas the absence of such contacts cannot be generally inferred, the present data do confirm that APols represent a well-suited medium for biophysical and biochemical studies of integral membrane proteins in aqueous solutions.

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