Membrane Protein – Lipid Interactions in Mixed Micelles Studied by NMR Spectroscopy with the Use of Paramagnetic Reagents

Christian Hilty,[a] Gerhard Wider,*[a] César Fernández,[a, b] and Kurt Wüthrich[a]

For solution NMR studies of the structure and function of membrane proteins, these macromolecules have to be reconstituted and solubilized in detergent micelles. Detailed characterization of the mixed detergent/protein micelles is then of key importance to validate the results from such studies, and to evaluate how faithfully the natural environment of the protein in the biological membrane is mimicked by the micelle. In this paper, a selection of paramagnetic probes with different physicochemical properties are used to characterize the 60 kDa mixed micelles consisting of about 90 molecules of the detergent dihexanoylphosphatidylcholine (DHPC) and one molecule of the Escherichia coli *outer-membrane protein X (OmpX), which had previously been extensively studied by solution NMR techniques. The observation of highly selective relaxation effects on the NMR spectra of OmpX and DHPC from a water-soluble relaxation agent and from nitroxide spin labels attached to lipophilic molecules, confirmed data obtained previously with more complex NMR studies of the diamagnetic OmpX/DHPC system, and yielded additional novel insights into the protein – detergent interactions in the mixed micelles. The application of paramagnetic probes to the well-characterized OmpX/DHPC system indicates that such probes should be widely applicable as an efficient support of NMR studies of the topology of mixed membrane protein – detergent micelles.*

Introduction

By using modern NMR techniques, in particular transverse relaxation-optimized spectroscopy (TROSY)[1] in combination with appropriate isotope labeling,[2] mixed protein – detergent micelles can be structurally investigated in solution despite their large molecular size.[3–8] To evaluate the relevancy of results thus obtained to acquiring knowledge on natural systems, it is of interest to investigate how the interactions of detergents with the protein surface correspond to the situation in the natural lipid bilayer. In a previous publication, we used intermolecular nuclear Overhauser effects (NOE)[9] to identify the surface areas of the outer-membrane protein X from *Escherichia coli* (OmpX) that are in direct contact with dihexanoylphosphatidylcholine (DHPC) detergent molecules.[10] The work presented in this paper uses the effect of paramagnetic relaxation agents[11–13] on individual resonances in the NMR spectra of OmpX and of DHPC to further characterize OmpX/DHPC mixed micelles. Additionally, the incorporation of nitroxide spin labels[14] into the micelles was investigated with electron paramagnetic resonance (EPR) spectroscopy.[11]

Results

In this study on the surface solvation of the *E. coli* OmpX protein reconstituted in micelles of about 90 molecules of DHPC, the three paramagnetic compounds Gd(DOTA) (DOTAREM; Gd^{3+} chelated with 1,4,7,10-tetraazocyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid), 16-DSA (16-doxyl stearic acid), and 5-DSA (5-doxyl stearic acid) were added individually in stepwise fashion to solutions containing mixed micelles of [u-^{15}N,70%-^{2}H]-labeled OmpX and unlabeled DHPC. At each step of the titration, a 2D ^{15}N,^{1}H-TROSY and a 1D ^{1}H NMR spectrum were measured (Figure 1). Upon addition of Gd(DOTA), 16-DSA, or 5-DSA, the individual peaks in the 2D ^{15}N,^{1}H-TROSY spectrum of OmpX showed different relaxation enhancements for the three paramagnetic relaxation compounds; this was manifested by line broadening and intensity reduction. Gd(DOTA) caused the disappearance of resonances that were hardly affected by 5-DSA and 16-DSA, whereas other signals, which were not visibly affected by Gd(DOTA) were broadened beyond detection by 5-DSA and 16-DSA (Figure 1). Thus, the reference 2D ^{15}N,^{1}H-TROSY spectrum of OmpX recorded before addition of any relaxation agent (Figure 1 d) corresponds closely to the sum of the spectra in Figure 1 a and b, which were recorded in the presence of Gd(DOTA) and 16-DSA, respectively. Similarly, the resonances of the methyl end groups of DHPC are differently broadened by the three paramagnetic compounds, and the line shapes of the $-CH_3$ groups located at the tips of the hydrophobic tails of DHPC and $-N(CH_3)_3$ groups at its polar head show wide variations depending on the relaxation agent used (Figure 1, a – c). For example, Gd(DOTA) affects mostly the $-N(CH_3)_3$

[a] *Dipl. Phys. C. Hilty, Prof. Dr. G. Wider, Dr. C. Fernández, Prof. Dr. K. Wüthrich*
Institut für Molekularbiologie und Biophysik
Eidgenössische Technische Hochschule Zürich
8093 Zürich (Switzerland)
Fax: (+41) 1-633-1073
E-mail: gsw@mol.biol.ethz.ch

[b] *Dr. C. Fernández*
Current Address:
Novartis Pharma AG, P.O. Box, 4002 Basel (Switzerland)

 DOI: 10.1002/cbic.200300815

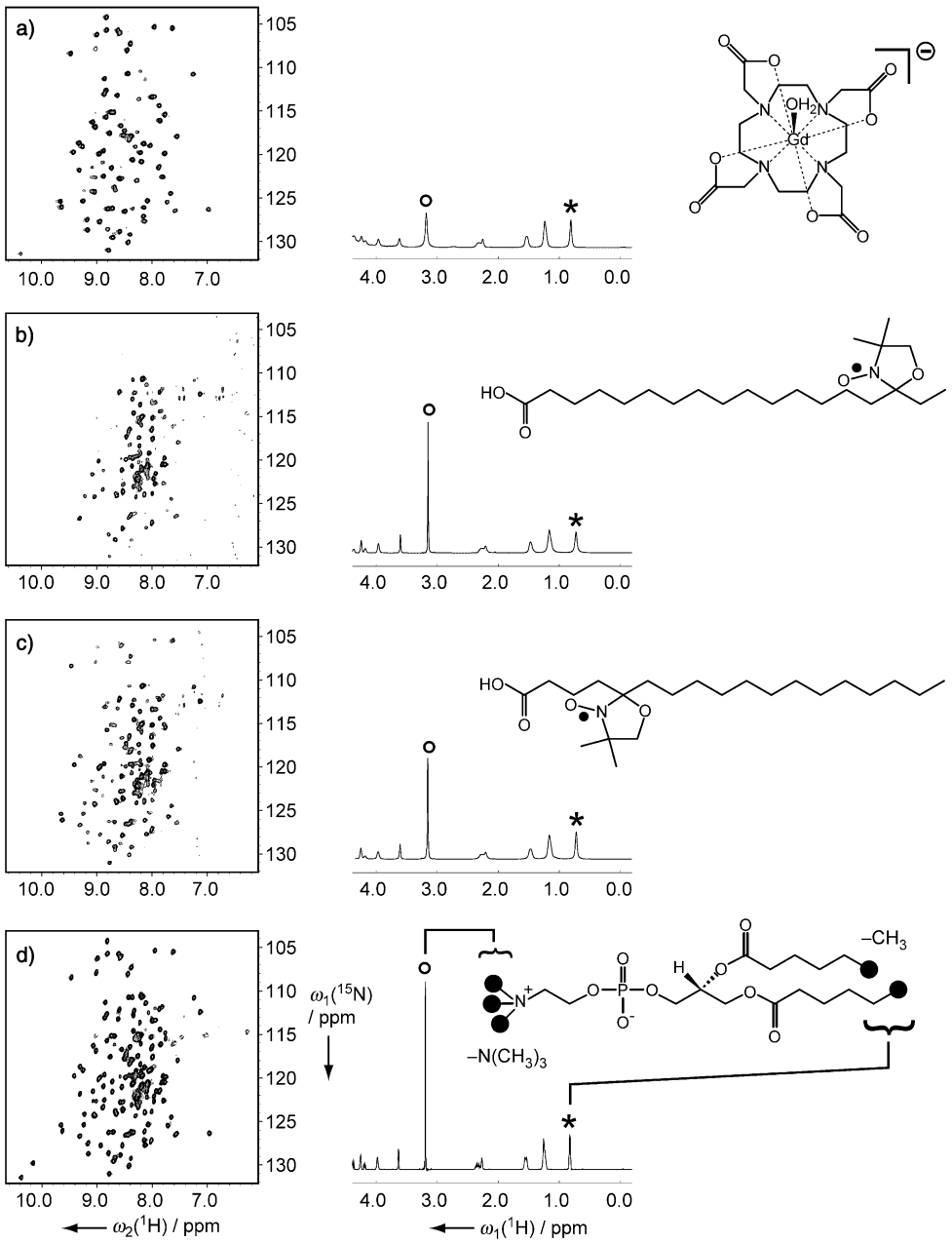

Figure 1. *Effects of the addition of three different paramagnetic relaxation probes to mixed OmpX/DHPC micelles in aqueous solution. For each paramagnetic probe, a 2D $^{15}N,^1H$-TROSY and a 1D ^1H NMR spectrum of OmpX/DHPC are shown, which present information on uniformly $^2H,^{15}N$-labeled OmpX and unlabeled DHPC, respectively. a) OmpX/DHPC in aqueous solution containing 28.4 mM Gd(DOTA). On the right, the molecular structure of Gd(DOTA) is shown, with a water molecule occupying the free metal coordination site.[24] The asterisk and the circle in the 1D ^1H NMR spectrum are explained in d) below. b) OmpX/DHPC with 4 mM 16-DSA. On the right, the structure of 16-DSA is shown, with the site of the unpaired electron marked by a filled circle. c) OmpX/DHPC with 4 mM 5-DSA. In the structure of 5-DSA, the site of the unpaired electron is indicated by a filled circle. d) OmpX/DHPC in the absence of a spin label. In the DHPC structure, the methyl end groups are drawn as filled circles. The resonances of these methyl groups in the 1D ^1H NMR spectra of DHPC are marked with an asterisk for the $-CH_3$ groups, and with a circle for the $-N(CH_3)_3$ group. All spectra were recorded at a ^1H resonance frequency of 750 MHz with NMR samples containing 0.5 mM OmpX and 100 mM DHPC at 30 °C (see text for details).*

resonance lines (Figure 1a), whereas 16-DSA affects mostly the $-CH_3$ resonances (Figure 1b).

In order to exclude possible effects on the relaxation data from mutual interactions between multiple molecules of the paramagnetic agents, we performed the titration within the concentration range in which relaxation enhancement is proportional to the bulk concentration of the reagents. The titration data for the methyl groups in DHPC are shown on the left-hand side of Figure 2. Transverse-relaxation enhancements ε were calculated from linear fits of the line widths in the 1D ^1H NMR spectra with Equation (1), below. A similar analysis for two residues of OmpX is shown on the right-hand side of Figure 2, here the line widths were measured in 2D $^{15}N,^1H$-TROSY spectra. The two residues T97 and G7 are located in loop L3 and in strand β1, respectively.[8] Since precise measurements of the width of the broad OmpX lines were rather difficult, we used the volumes

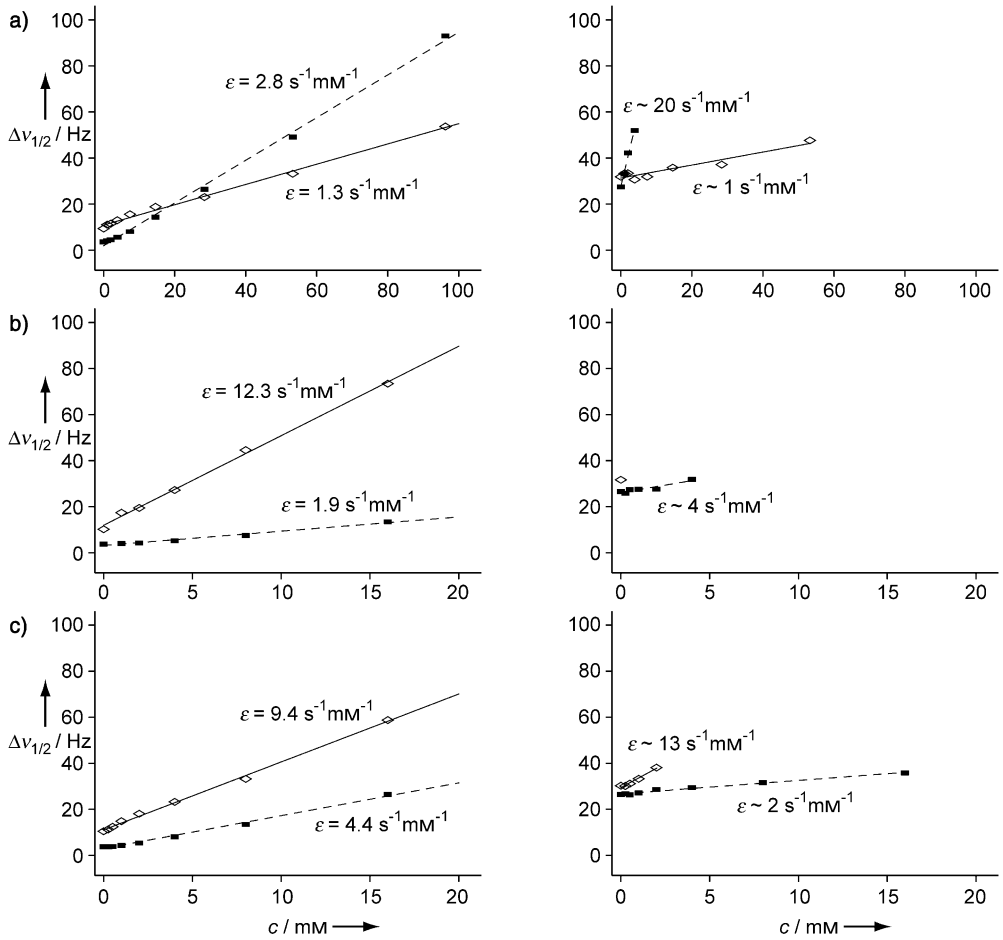

Figure 2. *Titration of OmpX/DHPC solutions with paramagnetic relaxation enhancement agents. a) Gd(DOTA). b) 16-DSA. c) 5-DSA. On the left, the line widths $\Delta\nu_{1/2}$ (full width at half height) of DHPC resonances in the 1D ^{1}H NMR spectra (Figure 1) are plotted against the concentration of the paramagnetic agents. The data for the $-CH_3$ resonances at 0.9 ppm (——◇——) and the $-N(CH_3)_3$ group at 3.1 ppm (– – –■– – –) are shown (Figure 1). The ε values obtained from linear fits (see text) are indicated. On the right, line widths of the OmpX resonances for the amide groups of G7 (——◇——) and T97 (– – –■– – –) are shown. The line widths were measured by fitting a Gaussian function to cross sections along ω_2(^{1}H) of the 2D ^{15}N,^{1}H-TROSY spectra used for the peak integration; the Gaussian function was chosen so as to account for the application of a cosine window to the FID before Fourier transformation. Only line widths for peaks with a signal-to-noise ratio $\geq$ 5 could be determined, and in all cases they are subject to considerable experimental error. Estimated relaxation enhancements ε based on the linear fits are indicated. All these values are within the range of values shown in Figure 4, which were obtained from analysis of peak integrals (Figure 3). In b), only one data point could be obtained for T97 of OmpX due to its fast relaxation in the presence of 16-DSA.*

of individual amide group signals in the 2D ^{15}N,^{1}H-TROSY spectra for quantitative analysis of the data. The decrease of the peak volumes during titration was analyzed by fitting a single exponential according to Equation (3), below (Figure 3). For both OmpX and DHPC, the data of Figures 2 and 3 nicely confirm the visual impression from Figure 1 that different molecular regions are affected by the different relaxation agents used.

The effective relaxation enhancements on the protein by the three paramagnetic agents are plotted versus the sequence positions of the corresponding amide groups in OmpX in Figure 4. As can be seen, the effect of Gd(DOTA) is largest for residues located in the solvent-accessible loops L1 – L4 of the protein (Figure 4 a). The shorter periplasmic turns are affected to a lesser extent, with the exception of T1, and the β-strands show only very small ε values. 16-DSA is highly specific and affects only the central parts of the β-strands (Figure 4 b). Also, 5-DSA mostly affects residues located in the β-strands, but sizeable ε values are seen for a larger range of amino acid residues (Figure 4 c). To visualize the different specificities of the three paramagnetic compounds used, the data in Figure 4 have been mapped onto the surface of the 3D structure of OmpX in DHPC micelles (Figure 5).

To check that the two nitroxide spin labels were indeed incorporated into the DHPC micelles, the EPR spectra of the spin labels free in solution were compared with those in the presence of DHPC and OmpX/DHPC micelles. Upon addition of DHPC or OmpX/DHPC, the lines in the EPR spectra of 16-DSA or 5-DSA broadened significantly, with the line broadening being larger for 5-DSA than for 16-DSA (Figure 6). The spectra in Figure 6 were used to calculate effective rotational correlation times, τ_r, for the two nitroxide spin labels in the different environments by using Equation (5), below. The resulting data (Table 1) confirm the visual impression from Figure 6 that the spin labels get incorporated into the micelles so that their effective correlation times are dominated by the overall tumbling motions of the mixed micelles.

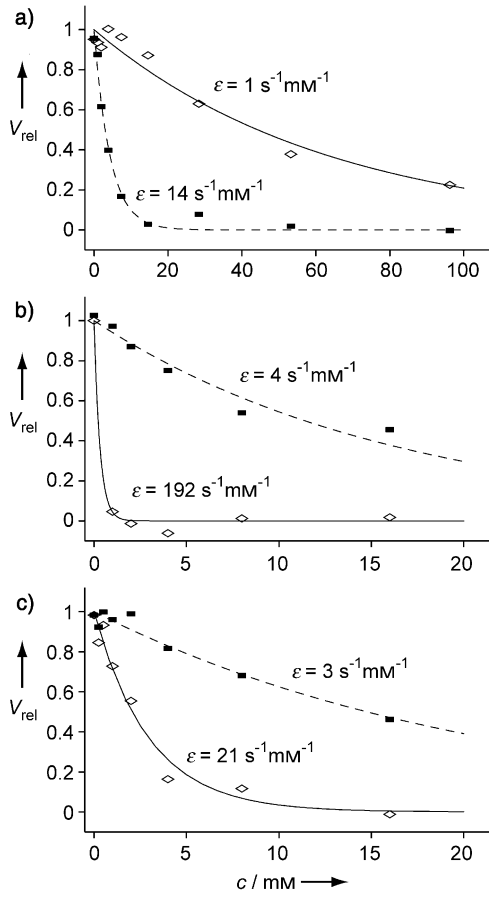

Figure 3. *Decay of relative peak volumes, V_{rel}, in 2D $^{15}N,^{1}H$-TROSY spectra of OmpX/DHPC upon titration with paramagnetic relaxation enhancement agents. a) Gd(DOTA). b) 16-DSA. c) 5-DSA. Normalized peak volumes for the amide groups of G7 (——◇——) and T97 (– – –■– – –) are plotted against the concentration of the paramagnetic agents. The positions of G7 in the lipid-covered β-barrel and T97 in a water-exposed loop are indicated in Figure 5. Single exponential fits were used to fit the experimental data (see text). The resulting ε values are indicated.*

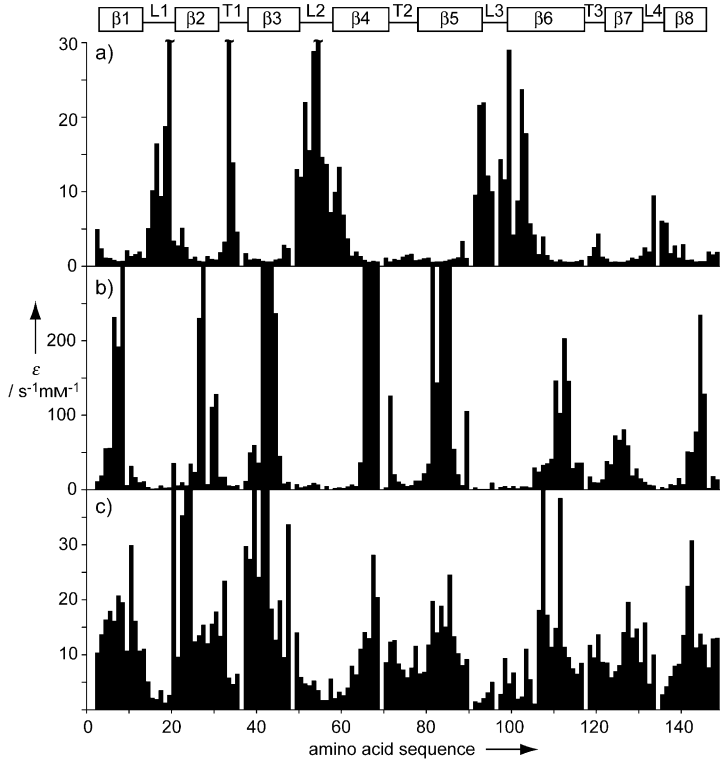

Figure 4. *Paramagnetic relaxation enhancement, ε, of the backbone $^{15}N-^{1}H$ groups of OmpX estimated from the decay of the resonances in 2D $^{15}N,^{1}H$-TROSY spectra upon titration with paramagnetic relaxation-enhancement agents (Figures 1 and 3): a) Gd(DOTA), b) 16-DSA, c) 5-DSA.*

Table 1. *Rotational correlation times and hyperfine splittings obtained from EPR measurements at 20 °C.*

	5-DSA	5-DSA DHPC	5-DSA DHPC OmpX	16-DSA	16-DSA DHPC	16-DSA DHPC OmpX
τ_r [ns][a]	0.24	2.06	2.27	0.14	0.42	0.45
$a_{^{14}N}$ [G][a]	15.8	14.9	14.8	15.8	14.6	14.6

[a] Rotational correlation times, τ_r, and ^{14}N isotropic hyperfine splittings, $a_{^{14}N}$, obtained from the line separations and line shapes in the EPR spectra of 5-DSA and 16-DSA at 20 °C in aqueous phosphate buffer at pH 6.5, in the same buffer after addition of 60 mM DHPC, and after further addition of 0.2 mM OmpX (computed with Equation (5) from the data in Figure 6).

Discussion

The OmpX/DHPC system has previously been extensively investigated by NMR, including the determination of the OmpX structure[8] based on complete sequence-specific resonance assignments,[3, 6, 15] and identification of protein surface – DHPC contacts from intermolecular $^{1}H,^{1}H$ NOEs.[10] The present paper, therefore, provides an opportunity to compare the information on protein solvation in DHPC micelles obtained from $^{1}H,^{1}H$ NOEs with the use of paramagnetic probes, and to compare practical aspects of the two approaches.

The ensemble of the paramagnetic relaxation enhancement data in Figures 1 – 5 and the previously identified surface areas of the protein showing NOEs between polypeptide hydrogen atoms and the $-CH_3$ resonance of the hydrophobic tails of DHPC, provide a qualitatively consistent view of OmpX solvation. The protein surface showing NOEs to the hydrophobic end of DHPC coincides quite closely with the regions affected by 5-DSA and 16-DSA. In contrast, as expected from its polar character (Figure 1), Gd(DOTA) is located in the aqueous phase and affects on the one hand mostly the $-N(CH_3)_3$ resonance of the polar headgroups of DHPC, which are located near the surface of the micelles (Figure 2), and on the other hand protein resonances in and near the loops and turns of the protein (Figures 4 and 5). These results thus confirm the prediction from the NOE studies that both ends of the OmpX β-barrel are accessible to aqueous solvent.[10] These data further substantiate the hypothesis that reconstitution in DHPC micelles should also be a suitable procedure for functional studies,[10] since the OmpX surface accessible for Gd(DOTA) (Figure 5 a) can be expected to be accessible for interactions with a wide variety of water-soluble compounds.

Based on relaxation enhancements with 16-DSA and 5-DSA (Figures 4 and 5), the present study adds more detailed

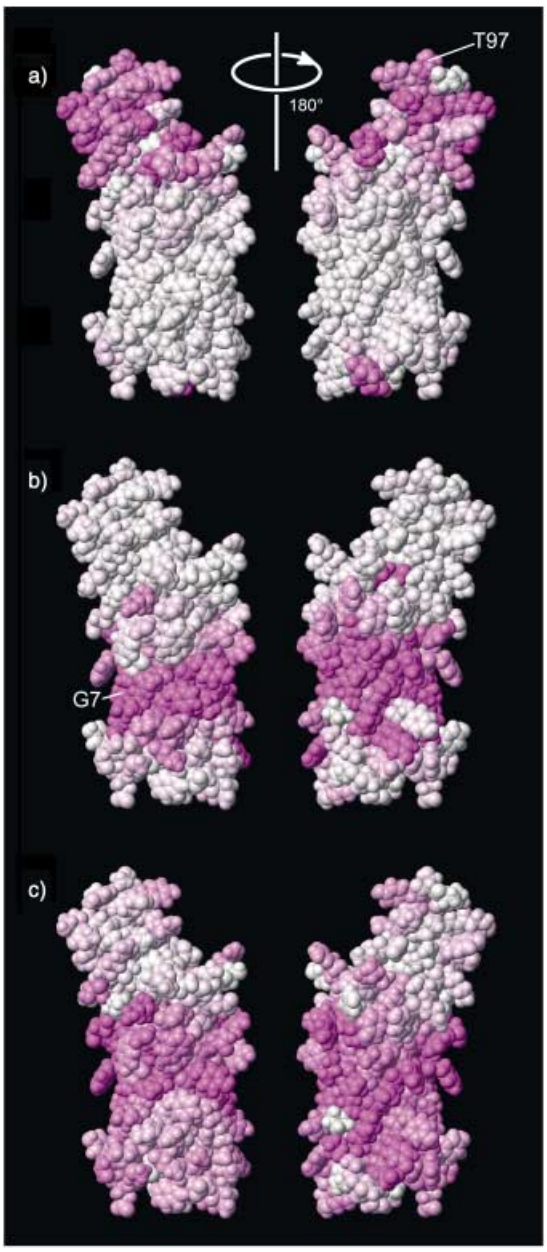

Figure 5. *Mapping of the paramagnetic relaxation-enhancement ε of the polypeptide backbone amide groups (Figure 4) onto the surface of OmpX[8] in mixed micelles with DHPC: a) Gd(DOTA), b) 16-DSA, c) 5-DSA. The protein is shown as an all-heavy atom space-filling model. The viewing angles of the drawings on the left and right differ by a rotation of 180° about a vertical axis. All atoms of each amino acid residue are colored according to the ε value measured for its backbone amide group. The ε values are linearly coded according to the color intensities, with white corresponding to $\varepsilon = 0$ and dark pink to $\varepsilon = \varepsilon_{max}$. The value for ε_{max} is independently chosen for each of the three paramagnetic compounds in order to give an optimal dispersion of colors (for quantitative data, see Figure 4). The residues G7 and T97, for which detailed data are given in Figures 2 and 3, are identified in A and B. The figure was prepared with the program MOLMOL.[25]*

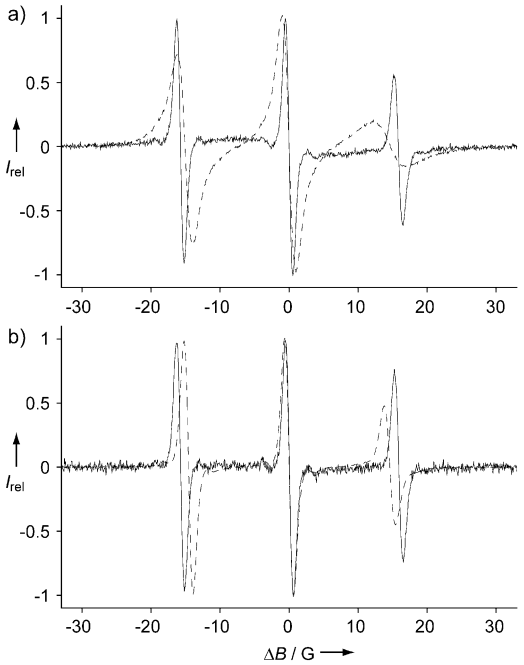

Figure 6. *X-band EPR spectra of the two nitroxide spin labels used in this study: a) 5-DSA, b) 16-DSA. The solid lines represent the spectra of the free spin labels in aqueous phosphate buffer solution. The broken lines show the spectra in the presence of DHPC micelles in samples containing 20 mM aqueous phosphate buffer at pH 6.5, 100 mM NaCl, 60 mM DHPC, 0.1 % NaN_3, and 0.1 mM spin label. The spectra were measured at 9.6 GHz on a Bruker ElexSys E500 spectrometer at 20 °C.*

information on the positioning of the DHPC headgroups in the mixed OmpX/DHPC micelles than was available from the NMR studies without paramagnetic probes (see below). Thereby, the interpretation of the paramagnetic relaxation effects on the protein is greatly aided by the concurrently collected additional information on the mixed micelles, that is, the relaxation enhancement on the resonances of DHPC (Figures 1 and 2), and the EPR data on the state of the two nitroxide spin labels (Figure 6). The EPR measurements present conclusive evidence that both spin labels are inserted into the micelles. That the spin label in 5-DSA is located more closely to the polar DHPC headgroups than the spin label in 16-DSA (Figure 1) is also clearly shown by the NMR relaxation data (Figure 2). Furthermore, it is seen that the spin label in 5-DSA is motionally more restricted, as evidenced by the fact that the correlation time for 16-DSA is significantly shorter than for 5-DSA (Table 1).[11, 16] For both spin labels, almost identical EPR spectra were obtained for DHPC micelles with and without OmpX (Table 1). This observation may suggest that the local environments experienced by the spin labels at the respective positions within the micelle[17] are similar, regardless of the presence of OmpX. However, we cannot yet exclude the possibility that the EPR spectra of the OmpX/DHPC sample are dominated by signals from empty DHPC micelles present in the sample.

It is readily apparent that a larger area of the protein surface is affected by 5-DSA than by 16-DSA (Figures 4 and 5). A more detailed comparison of the relaxation enhancements with 16-DSA, 5-DSA, and Gd(DOTA) (Figure 4) reveals two narrow bands around the β-barrel near the ends of each β-strand where only 5-DSA, but not 16-DSA or Gd(DOTA), causes significant relaxation. That this region of the OmpX surface is in close contact with DHPC headgroups was previously also suggested by intermolecular NOEs from the protein to $-N(CH_3)_3$ groups of DHPC.[10] It is

interesting that the association with the headgroups prevents significant access of the spin label in 16-DSA and Gd(DOTA) to the amide groups at both ends of the lipophilic central surface area of OmpX. This appears to be related to the immobilization of the molecular region near the nitroxide group in 5-DSA (Figure 1) in the DHPC micelles. Having established that the head groups of DHPC are in contact with the protein surface at the periphery of the dark pink areas in Figure 5c, it is then intuitively apparent from the schematic view in Figure 7 why 16-DSA induces relaxation enhancement primarily in the central part of the hydrophobic surface area of OmpX. The intermolecular ^{1}H,^{1}H NOEs cover a larger part of the hydrophobic surface, nearly identical to the area covered by 5-DSA (Figure 5c), since with the conditions used, there was spin diffusion along the entire hydrophobic tails of DHPC (Figure 1).[10]

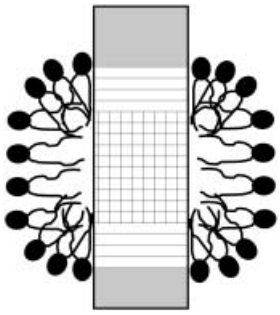

Figure 7. *Schematic representation of an OmpX/DHPC micelle, with OmpX drawn as a rectangle representing the same orientation of the protein as in Figure 5. Regions of the protein surface that are differently affected by the three paramagnetic agents are identified as follows: gray shading, area affected by Gd(DOTA); horizontally hatched, area affected by 5-DSA; cross-hatched, area affected by 16-DSA and 5-DSA.*

The experiments with paramagnetic reagents require the preparation of two or several ^{15}N,^{2}H-labeled protein samples, but only low protein concentrations are necessary for recording the highly sensitive 2D ^{15}N,^{1}H-TROSY and 1D ^{1}H NMR spectra. Because the relaxation-enhancement experiments are thus quite efficient, the use of a combination of two to four paramagnetic reagents with different solubilities in water and lipids may be a favorable alternative for the characterization of mixed membrane protein–detergent micelles. For example, use of paramagnetic agents may have added interest for work with detergents that have less favorable NMR spectral features than DHPC for the detection of intermolecular NOEs. Also, if the NOEs are more difficult to observe, for example, when the protein is not perdeuterated, the use of paramagnetic reagents is an alternative. Even for systems without protein-resonance assignments, NMR lines belonging to residues exposed to the aqueous phase and the lipid phase should be readily distinguished, so that the paramagnetic reagents could be used for an early global characterization of the system.

Experimental Section

Preparation of OmpX solutions for NMR experiments: Production of [u-^{15}N,70%-^{2}H]-labeled OmpX was accomplished by overexpression in *E. coli* BL21(DE3)pLysS cells harboring the plasmid pET3b-OmpX. Cells were grown in minimal medium (1 L) containing recycled heavy water with a deuteration level of 80%, glucose (4 g) as the sole carbon source, and $^{15}NH_4Cl$ (1 g, $>$ 98% ^{15}N) as the sole nitrogen source. Cells were harvested by centrifugation after reaching an optical density of 0.7 at 600 nm. OmpX protein was purified and refolded from inclusion bodies into DHPC (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine) micelles as described previously.[3] The preparation yielded material for five NMR samples with a volume of 0.5 mL (protein concentration 0.5 mM, solvent H_2O/D_2O (95:5%), 20 mM phosphate buffer pH 6.5, 100 mM NaCl, 100 mM DHPC).

Titration of the OmpX solutions with paramagnetic relaxation-enhancement agents: Titrations were performed by stepwise addition of a paramagnetic substance to a constant amount of protein. Compounds used were 16-DSA (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy, free radical; Aldrich) and 5-DSA (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy, free radical; Aldrich), which were used in the 0 to 16 mM concentration range, and Gd(DOTA) (DOTAREM; gadolinium chelated with 1,4,7,10-tetraazocyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; Laboratoire Guerbet, France), which was used over the 0–100 mM range.

Gd(DOTA) was added as an aqueous stock solution (0.1 M or 0.5 M). Stearic acid compounds were dissolved in methanol in order to provide aliquots of the necessary quantities. The solvent was subsequently evaporated in a SpeedVac. The OmpX/DHPC solution was then added to a given aliquot, which was dissolved by sonication for about 15 min. This procedure was necessary, since even the addition of a few microliters of methanol to the NMR sample induced chemical shift changes in the 2D ^{15}N,^{1}H-TROSY spectrum of OmpX; this indicates that methanol interacts with the OmpX/DHPC micelles. The pH was maintained at 6.5 by addition of a few microliters of NaOH (1 M) after each titration step. The 2D ^{15}N,^{1}H-TROSY spectra[18] for the titration were recorded with 16 transients per increment, $t_{1max}(^{15}N) = 37$ ms, $t_{2max}(^1H) = 48$ ms, time domain data size 85 $\times$ 512 complex points. The 1D ^{1}H NMR spectra were measured with water presaturation, 128 accumulated transients, time domain data size 2048 complex points. All spectra were recorded at 30 °C on a Bruker DRX 750 MHz NMR spectrometer equipped with a triple resonance TXI probe head with an actively shielded *z* gradient.

Estimation of relaxation enhancements ε: The enhancement of the nuclear spin relaxation caused by a paramagnetic substance can be described by Equation (1):

$$R = R^0 + \varepsilon c \qquad (1)$$

Here R and R^0 are the effective relaxation rates in the presence and absence of the paramagnetic compound, respectively.[19] The "relaxation enhancement factor" ε is specific for a given paramagnetic compound and is independent of its concentration in the bulk solution, c. Equation (1) can be written for both longitudinal and transverse relaxation. But here we focus on transverse relaxation, whereby the basis for the analysis are differences in the distribution of sizeable ε values in the structures of OmpX and DHPC when using water- or lipid-soluble paramagnetic substances, thus discriminating between the inside and outside of the mixed OmpX/DHPC micelles.

The relaxation effect of the paramagnetic compounds on individual amide groups of the protein was monitored by measuring the decay of the cross-peak volumes in a series of 2D ^{15}N,^{1}H-TROSY experiments, which were measured with different concentrations of a particular relaxation agent. The volume of a cross peak is proportional to the magnetization present immediately before the

detection of the signal, M_d, and is calculated for zero evolution time in the TROSY experiment.[20] M_d can be calculated from the steady-state magnetization M_0 with the relation:

$$M_d = M_0 \exp(-RT) \quad (2)$$

here T is the time during which the average effective transverse relaxation R is active. For zero chemical-shift-evolution time in the present experiments, T was 16.2 ms, which corresponds to the sum of the durations of the INEPT and ST2-PT elements. By substituting Equation (1) into Equation (2), the volume of a particular cross peak from residue i at concentration c of paramagnetic agent, $V_i(c)$, can be written as:

$$V_i(c) \propto \exp(-\varepsilon_i cT) \quad (3)$$

Here ε_i is the relaxation-enhancement factor for residue i. The ε_i values were estimated by fitting the experimental data with a single exponential according to Equation (3). For Gd(DOTA), which was added as an aqueous solution to the protein sample, the sample dilution was accounted for by multiplication with the corresponding dilution factors. Peak integrals were determined with the program XEASY.[21]

The effects of the paramagnetic compounds on the $-CH_3$ and $-N(CH_3)_3$ groups of DHPC were monitored by analyzing the full width at half height, $\Delta\nu_{1/2}$, of the resonance lines in the 1D ^{1}H NMR spectra. $\Delta\nu_{1/2}$ was determined by fitting a Lorentzian to the resonance lines in the spectra, which were processed without a window function. Subsequently, the relaxation enhancements ε were obtained from a linear fit of $\Delta\nu_{1/2}$ versus the overall concentration of paramagnetic agent by using Equation (4) for the transverse relaxation R in Equation (1):

$$R = 1/T_2 = \pi\Delta\nu_{1/2} \quad (4)$$

EPR measurements: X-band electron paramagnetic resonance (EPR) measurements were performed with the nitroxide spin labels 5-DSA and 16-DSA. Spectra of free spin labels (0.1 mM) were measured in aqueous solutions containing phosphate buffer (20 mM, pH 6.5), NaCl (100 mM), and NaN_3 (0.1 %). Spectra of the spin labels in the presence of DHPC micelles were measured with the same solution conditions in the presence of DHPC (60 mM). Spectra of the spin labels in the presence of mixed OmpX/DHPC micelles were obtained by further adding OmpX (0.2 mM). All EPR experiments were measured at 9.6 GHz, with a sweep range of 0.008 T on a Bruker ElexSys E500 spectrometer at 20 °C.

Rotational correlation times, τ_r, were calculated from the line shapes of the first-derivative EPR spectra with Equation (5):

$$\tau_r = 6.5 \times 10^{-10} \Delta H \left\{ \sqrt{\frac{I(0)}{I(+1)}} + \sqrt{\frac{I(0)}{I(-1)}} - 2 \right\} \quad (5)$$

Here ΔH is the peak-to-peak field difference of the zero transition, and $I(-1)$, $I(0)$ and $I(+1)$ the peak-to-peak amplitudes of the -1, 0 and $+1$ transitions, respectively.[22, 23]

Abbreviations

DHPC, dihexanoylphosphatidylcholine (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine); 5-DSA, 5-doxyl stearic acid (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy, free radical); 16-DSA, 16-doxyl stearic acid (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy, free radical); DOTA, 1,4,7,10-tetraazocyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; EPR, electron paramagnetic resonance; OmpX, outer-membrane protein X from *Escherichia coli*; TROSY, transverse relaxation-optimized spectroscopy.

Acknowledgements

Financial support was obtained from the National Center for Competence in Research (NCCR) Structural Biology. We thank Dr. Jeffrey Harmer in the group of Prof. Arthur Schweiger at the ETH Zürich for assistance with the EPR measurements and Sebastian Hiller for useful discussions.

Keywords: detergents · EPR spectroscopy · membrane proteins · micelles · NMR spectroscopy · spin labels

[1] K. Pervushin, R. Riek, G. Wider, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12366 – 12371.
[2] K. H. Gardner, L. E. Kay, *Annu. Rev. Biophys. Biomolec. Struct.* **1998**, *27*, 357 – 406.
[3] C. Fernández, K. Adeishvili, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 2358 – 2363.
[4] A. Arora, F. Abildgaard, J. H. Bushweller, L. K. Tamm, *Nat. Struct. Biol.* **2001**, *8*, 334 – 338.
[5] P. M. Hwang, W. Choy, E. I. Lo, L. Chen, J. D. Forman-Kay, C. R. H. Raetz, G. G. Privé, R. E. Bishop, L. E. Kay, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 13560 – 13565.
[6] C. Hilty, C. Fernández, G. Wider, K. Wüthrich, *J. Biomol. NMR* **2002**, *23*, 289 – 301.
[7] K. Oxenoid, F. D. Sönnichsen, C. R. Sanders, *Biochemistry* **2002**, *41*, 12876 – 12882.
[8] C. Fernández, C. Hilty, G. Wider, P. Güntert, K. Wüthrich, *J. Mol. Biol.* **2004**, in press.
[9] K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, **1986**.
[10] C. Fernández, C. Hilty, G. Wider, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 13533 – 13537.
[11] L. R. Brown, C. Bösch, K. Wüthrich, *Biochim. Biophys. Acta* **1981**, *642*, 296 – 312.
[12] J. Jarvet, J. Zdunek, P. Damberg, A. Gräslund, *Biochemistry* **1997**, *36*, 8153 – 8163.
[13] G. Pintacuda, G. Otting, *J. Am. Chem. Soc.* **2002**, *124*, 372 – 373.
[14] B. J. Gaffney, H. M. McConnell, *J. Magn. Reson.* **1974**, *16*, 1 – 28.
[15] C. Hilty, G. Wider, C. Fernández, K. Wüthrich, *J. Biomol. NMR* **2003**, *27*, 377 – 382.
[16] M. A. Brito, C. D. Brondino, J. J. G. Moura, D. Brites, *Arch. Biochem. Biophys.* **2001**, *387*, 57 – 65.
[17] H. Hauser, Biochim. *Biophys. Acta* **2000**, *1508*, 164 – 181.
[18] K. Pervushin, G. Wider, K. Wüthrich, *J. Biomol. NMR* **1998**, *12*, 345 – 348.
[19] H. Pfeifer, *Ann. Phys.* **1961**, *8*, 1 – 8.
[20] D. Braun, K. Wüthrich, G. Wider, *J. Magn. Reson.* **2003**, *165*, 89 – 94.
[21] C. Bartels, T. H. Xia, M. Billeter, P. Güntert, K. Wüthrich, *J. Biomol. NMR* **1995**, *6*, 1 – 10.
[22] T. J. Stone, T. Buckman, P. L. Nordio, H. M. McConnell, *Proc. Natl. Acad. Sci. USA* **1965**, *54*, 1010 – 1017.
[23] B. Cannon, C. F. Polnaszek, K. W. Butler, L. E. G. Eriksson, I. C. P. Smith, *Arch. Biochem. Biophys.* **1975**, *167*, 505 – 518.
[24] P. Caravan, J. J. Ellison, T. J. McMurry, R. B. Lauffer, *Chem. Rev.* **1999**, *99*, 2293 – 2352.
[25] R. Koradi, M. Billeter, K. Wüthrich, *J. Mol. Graph.* **1996**, *14*, 51 – 55.

Received: November 10, 2003 [F 815]

