

Association of Spin-Labeled Lipids with β -Barrel Proteins from the Outer Membrane of *Escherichia coli*[†]

Muthu Ramakrishnan,[‡] Cosmin L. Pocanschi,[§] Jörg H. Kleinschmidt,^{*,§} and Derek Marsh^{*,‡}

*Abteilung Spektroskopie, Max-Planck-Institut für biophysikalische Chemie, 37077 Göttingen, Germany, and
Fachbereich Biologie, Universität Konstanz, 78547 Konstanz, Germany*

ABSTRACT: The interaction of spin-labeled lipids with β -barrel transmembrane proteins has been studied by the electron spin resonance (ESR) methods developed for α -helical integral proteins. The outer membrane protein OmpA and the ferrichrome–iron receptor FhuA from the outer membrane of *Escherichia coli* were reconstituted in bilayers of dimyristoylphosphatidylglycerol. The ESR spectra from phosphatidylglycerol spin labeled on the 14-C atom of the *sn*-2 chain contain a second component from motionally restricted lipids contacting the intramembranous surface of the β -barrel, in addition to that from the fluid bilayer lipids. The stoichiometry of motionally restricted lipids, 11 and 32 lipids/monomer for OmpA and FhuA, respectively, is constant irrespective of the total lipid/protein ratio. It is proportional to the number of transmembrane β -strands, eight for OmpA and 22 for FhuA, and correlates reasonably well with the intramembranous perimeter of the protein. Spin-labeled lipids with different polar headgroups display a differential selectivity of interaction with the two proteins. The more pronounced pattern of lipid selectivity for FhuA than for OmpA correlates with the preponderance of positively charged residues facing the lipids in the extensions of the β -sheet and shorter interconnecting loops on the extracellular side of FhuA.

Because of the favorable time scale of electron spin resonance (ESR)¹ spectroscopy, ESR spectra of spin-labeled lipids have proved to be a particularly useful means for studying lipid interactions with transmembrane proteins (see, e.g., ref 1). Spectra are resolved from the lipids interacting directly with the intramembranous surface of the protein. This allows determination of the stoichiometry and selectivity of the lipid–protein interaction and also the rate of exchange at the lipid–protein interface (2). Lipid–protein interactions with a large range of integral proteins have been studied in

this way, but so far these studies have been confined to α -helical proteins (3, 4). The sole exceptions are the β -sheet configurations of certain transmembrane peptides (5–7). So far, no such studies have been undertaken with the β -barrel proteins that are characteristic for the outer membranes of Gram-negative bacteria and of mitochondria. There are grounds to expect that lipid interactions with β -barrel proteins might differ from those with α -helical proteins. A β -barrel protein presents a rather different pattern of hydrophobic side chains to the lipids than do polytopic α -helical proteins. Also, the hydrophobic span of β -barrel outer membrane proteins is considerably shorter than those typical of α -helical proteins (8).

In the present paper, we describe spin-label ESR studies of lipid interactions with two β -barrel proteins from the outer membrane of *Escherichia coli* that are reconstituted in bilayer membranes of the anionic lipid dimyristoylphosphatidylglycerol. One of the proteins studied is the outer membrane protein OmpA, which is an eight-stranded β -barrel (9), and the other is the ferrichrome–iron receptor FhuA, which is a much larger 22-stranded β -barrel (10). Both proteins are monomeric, and therefore the whole of the external surface of the barrel is expected to contact lipid. Lipid–protein interactions with the β -barrel proteins are detected by suitably spin-labeled lipids and are found to have stoichiometries and selectivities that correlate well with the 3-D structures of the two proteins.

[†]M.R. is supported by a fellowship from the Alexander von Humboldt Foundation. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (KL1024/2-5) to J.H.K.

* Corresponding authors. J.H.K.: tel, +49-7531 88 3360; fax, +49-7531 88 3183; e-mail, joerg.helmut.kleinschmidt@uni-konstanz.de. D.M.: tel, +49-551-201 1285; fax, +49-551-201 1501; e-mail, dmarsh@gwdg.de.

[‡]Max-Planck-Institut für biophysikalische Chemie.

[§]Universität Konstanz.

¹ Abbreviations: OmpA, outer membrane protein A from *Escherichia coli*; FhuA, ferrichrome–iron receptor from *E. coli*; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 14-PGSL, 14-PCSL, 14-PESL, 14-PSSL, and 14-PASL, 1-acyl-2-[14-(4,4-dimethylloxazolidinyl-*N*-oxy)stearoyl]-*sn*-glycero-3-phosphoglycerol, -phosphocholine, -phosphoethanolamine, -phosphoserine, and -phosphoric acid; 14-DGSL, 1-acyl-2-[14-(4,4-dimethylloxazolidinyl-*N*-oxy)stearoyl]-*sn*-glycero-3-phosphoglycerol; 14-SASL, 14-(4,4-dimethylloxazolidinyl-*N*-oxy)stearic acid; LPS, lipopolysaccharide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; LDAO, *N*-lauroyl-*N,N*-dimethylammonium *N*-oxide; SDS, sodium dodecyl sulfate; ESR, electron spin resonance.

MATERIALS AND METHODS

Materials. *E. coli* strain P.400, expressing wild-type OmpA, was a generous gift from Dr. Ulf Henning, Tübingen. Wild-type OmpA was isolated and purified from the *E. coli* outer membrane in the unfolded form in 8 M urea as described in ref 11. *E. coli* strain BL21(AW740) carrying plasmid pHx405 and expressing wild-type FhuA was a generous gift from Dr. Wolfram Welte, Konstanz. His6-tagged wild-type FhuA was extracted from *E. coli* outer membranes and isolated and purified in *N*-lauroyl-*N,N*-dimethylammonium *N*-oxide (LDAO) detergent micelles, without unfolding, as described in ref 12. Dimyristoylphosphatidylglycerol (DMPG) was obtained from Avanti Polar Lipids (Alabaster, AL). Spin-labeled stearic acid, 14-SASL, was synthesized according to ref 13. Spin-labeled phosphatidylcholine, 14-PCSL, was synthesized by acylation of lysophosphatidylcholine with 14-SASL, as described in ref 14. Other spin-labeled phospholipids, 14-PGSL, 14-PSSL, 14-PESL, and 14-PASL, were prepared from 14-PCSL by headgroup exchange mediated by phospholipase D (14). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Unfolded OmpA was refolded into detergent micelles as described in ref 15. Briefly, 500 μ L of a 42 mg/mL solution of unfolded OmpA in 10 mM borate and 2 mM EDTA, pH 10, buffer that contained 8 M urea was diluted 20-fold with the borate buffer and mixed with an 800-fold molar excess of LDAO detergent. The mixture was incubated overnight at 40 °C to ensure complete refolding of the protein. Refolding was carried out at pH 10 because a yield of close to 100% was achieved at this pH value (15). Sample purity and folding were monitored by SDS–polyacrylamide gel electrophoresis according to the method of ref 16.

Reconstitution into Membranes. Phospholipid solutions containing 1 mol % of the desired spin-labeled lipid were prepared in CHCl_3 and dried under a stream of dry nitrogen gas. The resulting lipid film was desiccated overnight under vacuum and then covered with argon. The dry lipid film was hydrated with 10 mM Hepes and 2 mM EDTA buffer, pH 7.0, and frozen (in liquid nitrogen) and thawed (in a water bath at $\sim 5^\circ$ above the transition temperature of the phospholipid) seven times to obtain uniform lipid vesicles. The reconstitution was carried out by mixing 1 mg of the above lipid vesicles with protein to the desired lipid–protein ratio, and then 10% sodium cholate solution was added to give a final concentration of 0.2% sodium cholate in a total volume of 500 μ L. The sample was mixed well and incubated at room temperature for 1 h, vortexing from time to time. After 1 h incubation, the protein was precipitated with 52.5% ammonium sulfate solution that was added to achieve 35% final concentration. The pellet containing the reconstituted protein was then centrifuged for 30 min at 35000 rpm in a Beckman 50TI rotor.

Initial lipid/protein ratios were determined after ammonium sulfate precipitation by using the methods of refs 17 and 18 for lipid and protein, respectively. After this, the samples were adjusted to the required lipid/protein ratio by adding appropriate quantities of solubilized lipid vesicles. The pellet was dissolved and vortexed in 500 μ L of 10 mM Hepes buffer, pH 7.0, containing 2 mM EDTA and 250 mM NaCl. Detergent removal was achieved by extensive dialysis at 8

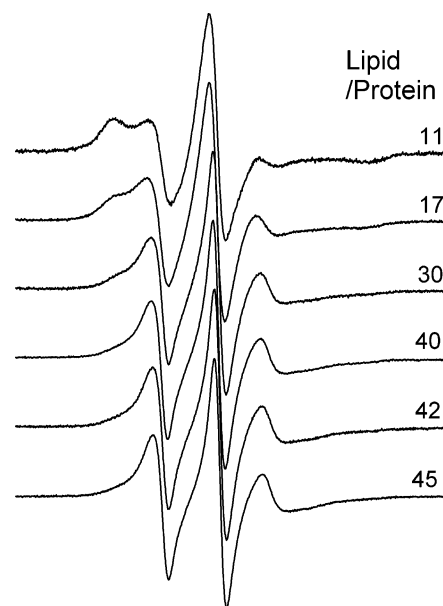


FIGURE 1: ESR spectra of phosphatidylglycerol spin labeled on the 14-C atom of the *sn*-2 chain (14-PGSL) in dimyristoylphosphatidylglycerol membranes containing OmpA at the lipid/protein mole ratios indicated in the figure. $T = 30^\circ\text{C}$; total scan width = 100 G.

$^\circ\text{C}$ against 10 mM Hepes buffer, pH 7.0, containing 2 mM EDTA and 250 mM NaCl, using 10 kDa cutoff dialysis membranes. Final lipid/protein ratios were determined after ESR spectroscopy.

Electron Spin Resonance Spectroscopy. ESR spectra were recorded on a 9 GHz Bruker EMX EPR spectrometer with a model ER 041 XK-D microwave bridge. Samples were placed in 50 μ L glass capillaries and flame sealed. The capillaries were placed in a standard 4 mm quartz sample tube containing light silicone oil for thermal stability. The temperature of the sample was maintained constant by blowing thermostated nitrogen gas through a quartz dewar. Spectra were recorded using the following instrumental settings: sweep width, 100 G; resolution, 1024 points; time constant, 20.48 ms; sweep time, 41.9 s; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; incident power, 5 mW. Spectral subtraction and integration were performed as described in ref 19. Spectral reference libraries for the fluid and motionally restricted components were obtained from 14-PCSL in egg phosphatidylcholine dispersions and in sonicated DMPC small unilamellar vesicles, respectively, at various temperatures.

RESULTS

OmpA Reconstituted in DMPG. Figure 1 shows the ESR spectra of spin-labeled phosphatidylglycerol, 14-PGSL, in reconstituted membranes of dimyristoylphosphatidylglycerol (DMPG) containing OmpA at different lipid/protein ratios. The spectra are recorded at 30 °C, which is above the chain-melting transition of DMPG bilayers. Figure 2 shows the transition curves for a sample with a lipid/protein ratio of 30:1 mol/mol, as registered by the central peak in the ESR spectra. This confirms that the OmpA/DMPG membranes are in the fluid phase at 30 °C.

The spin label is positioned at the 14-C atom of the *sn*-2 chain and gives rise to relatively sharp three-line spectra in

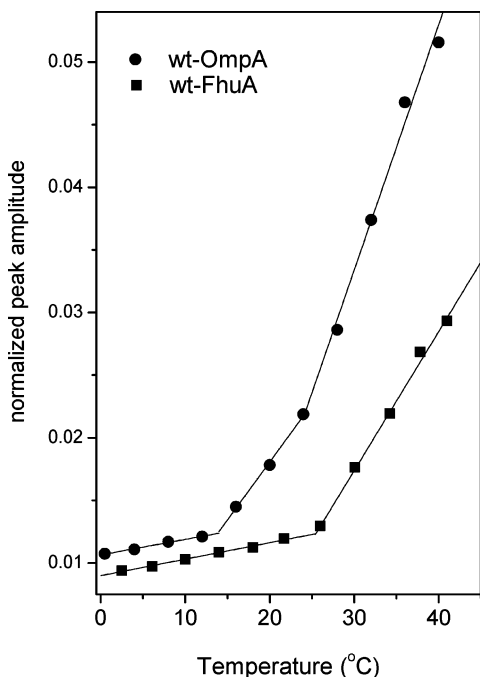


FIGURE 2: Temperature dependence of the central line height in the ESR spectra of the 14-PGSL spin label in dimyristoylphosphatidylglycerol membranes containing either OmpA (●) or FhuA (■) at lipid/protein ratios of 30 and 58 mol/mol, respectively. The spectral line height is normalized to the double integral of the first-derivative spectra in each case.

fluid bilayer membranes because of the rather large angular amplitude of segmental motion toward the terminal methyl end of the chain (see, e.g., ref 20). This is responsible for the line narrowing in the fluid phase reflected by the increase in peak amplitude that is found in Figure 2. It is seen from Figure 1 that the spin-label spectra from DMPG membranes that contain OmpA consist of two components. In addition to the three-line spectrum, a second component with larger hyperfine splitting is resolved in the outer wings of the spectrum. The proportion of this second component increases with the protein content of the membrane. A wide range of studies with α -helical transmembrane proteins (3) indicate that this motionally restricted spectral component represents the population of spin-labeled lipids whose chains contact the intramembranous surface of the protein directly.

Figure 3 illustrates the two-component nature of the ESR spectra from OmpA/DMPG complexes (Figure 3A) by means of difference spectroscopy. The fluid component in Figure 3A is matched by a spectrum from fluid egg phosphatidylcholine bilayers at 8 °C (dotted line in Figure 3C). Subtraction of $(1 - f) = 25\%$ of the normalized intensity of the latter from the spectrum in Figure 3A yields the solid-line difference spectrum given in Figure 3B. The latter motionally restricted component is reasonably well matched by the gel-phase spectrum from sonicated, small unilamellar DMPC vesicles at 9 °C (dotted line in Figure 3B). The complementary difference spectrum, which is given by the solid line in Figure 3C, is obtained by subtracting $f = 78\%$ of the normalized intensity of the gel-phase sonicated DMPC spectrum (i.e., the dotted line in Figure 3B) from Figure 3A and corresponds reasonably well with the fluid component used for subtraction (viz., the dotted line in Figure 3C).

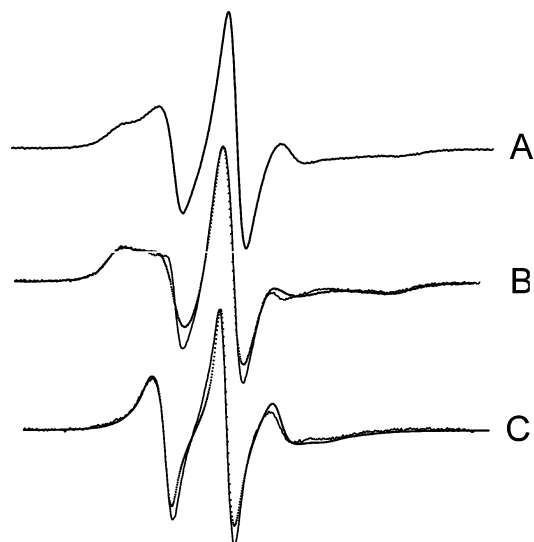


FIGURE 3: Spectral subtractions to quantitate the relative proportions of the fluid [fraction, $(1 - f)$] and motionally restricted (fraction, f) components from the ESR spectra of 14-PGSL in OmpA/DMPG membranes. (A) Experimental spectrum from OmpA/DMPG 1:17 mol/mol membranes at 30 °C. (B) Solid line: difference spectrum obtained by subtracting $1 - f = 25\%$ of the dotted-line spectrum in (C). Dotted line: motionally restricted comparison spectrum (sonicated DMPC vesicles at 9 °C). (C) Solid line: difference spectrum obtained by subtracting $f = 78\%$ of the dotted-line spectrum in (B). Dotted line: fluid comparison spectrum (egg phosphatidylcholine dispersion at 8 °C).

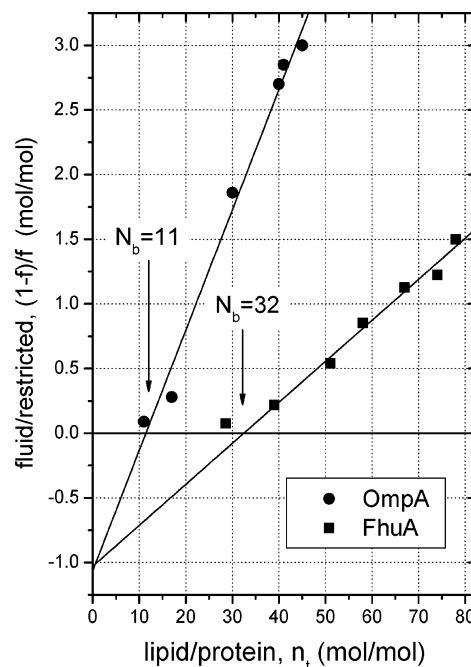


FIGURE 4: Dependence of the ratio of fluid to motionally restricted populations, $(1 - f)/f$, of 14-PGSL spin label on the lipid/protein ratio, n_t , in dimyristoylphosphatidylglycerol membranes containing either OmpA (●) or FhuA (■).

The relative proportions of fluid and motionally restricted spin-labeled lipid were determined by spectral subtraction and integration (19), as described in Figure 3. Figure 4 gives the ratio, $(1 - f)/f$, of the fluid to motionally restricted populations of 14-PGSL as a function of the total lipid/protein ratio, n_t , in the sample. The figure is plotted according to the equation for equilibrium lipid-protein exchange that

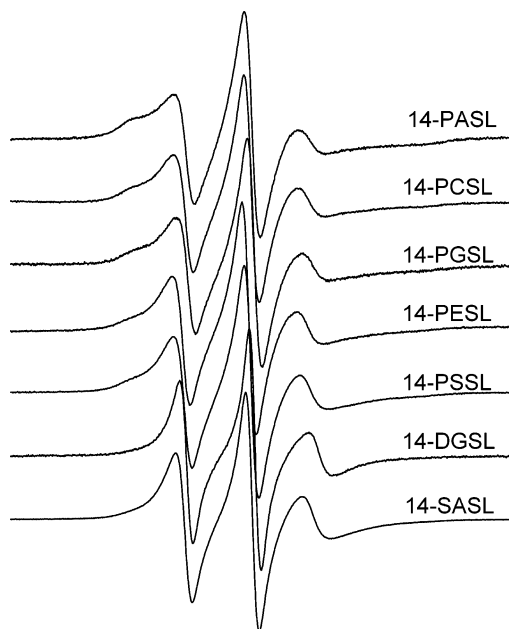


FIGURE 5: ESR spectra of spin-labeled phospholipids (14-PXSL) with different polar headgroups and of the corresponding spin-labeled diacylglycerol (14-DGSL) and stearic acid (14-SASL) in dimyristoylphosphatidylglycerol membranes containing OmpA at a fixed lipid/protein ratio of 24 mol/mol. $T = 30\text{ }^{\circ}\text{C}$; total scan width = 100 G.

has proved appropriate for analysis of the association of spin-labeled lipids with α -helical transmembrane proteins (21, 22):

$$(1 - f)/f = (n_t/N_b - 1)/K_r \quad (1)$$

where N_b is the number of lipid association sites at the perimeter of the β -barrel and K_r is the association constant of the spin-labeled lipid relative to that of the unlabeled host lipid (i.e., DMPG). The linearity of the plot according to eq 1 indicates that the number of association sites, $N_b \approx 11$ lipids/protein, remains constant, irrespective of the total lipid/protein ratio, just as for α -helical transmembrane proteins (3). Also, the relative association constant, $K_r \approx 1$, obtained from the y-intercept in Figure 4, indicates that there is relatively little selectivity between the spin-labeled and unlabeled phosphatidylglycerol for the interaction with OmpA.

Figure 5 shows the ESR spectra of different lipids spin labeled on the 14-C atom of the lipid chain in reconstituted OmpA/DMPG membranes of fixed lipid/protein ratio. A selectivity between the different lipids for interaction with OmpA is evident from the different proportions of the two components attributable to fluid and motionally restricted lipids in Figure 5. From eq 1, the ratio of the relative association constant, K_r , of a given lipid to that, K_r^{PG} , of phosphatidylglycerol is given by

$$K_r/K_r^{\text{PG}} = (1/f_{\text{PG}} - 1)/(1/f - 1) \quad (2)$$

where f_{PG} is the fraction of motionally restricted spin-labeled phosphatidylglycerol and f that of the spin-labeled lipid in question. Values for the selectivity, K_r/K_r^{PG} , deduced in this way by using difference spectra derived from Figure 5 are given in Table 1. Only phosphatidic acid displays an enhanced selectivity, relative to phosphatidylglycerol, for interaction with OmpA. All other negatively charged lipids

Table 1: Relative Association Constants, K_r , of 14-PXSL, 14-DGSL, and 14-SASL Spin-Labeled Lipids for Interaction with OmpA or FhuA in Dimyristoylphosphatidylglycerol Membranes^a

lipid	K_r/K_r^{PG}	$\Delta G - \Delta G^{\text{PG}}$ (kJ/mol) ^b	lipid	K_r/K_r^{PG}	$\Delta G - \Delta G^{\text{PG}}$ (kJ/mol) ^b
OmpA			FhuA		
14-PASL	2.5	-2.3	14-PASL	1.5	-1.0
14-PCSL	0.6	+1.1	14-PCSL	1.1	-0.3
14-PESL	0.5	+1.7	14-PESL	0.6	+1.2
14-PSSL	0.55	+1.6	14-PSSL	1.4	-0.8
14-DGSL	~ 0.1	$\sim +5.8$	14-DGSL	0.4	+2.5
14-SASL	~ 0.2	$\sim +4.1$	14-SASL	3.0	-2.8

^a Relative association constants, K_r , are normalized to the value for 14-PGSL either in OmpA/DMPG membranes of lipid/protein ratio 24 mol/mol or in FhuA/DMPG membranes of lipid/protein ratio 53 mol/mol. Measurements for OmpA are at 30 $^{\circ}\text{C}$ and for FhuA at 34 $^{\circ}\text{C}$. ^b Free energy of association, relative to 14-PGSL: $\Delta G - \Delta G^{\text{PG}} = -RT \ln(K_r/K_r^{\text{PG}})$.

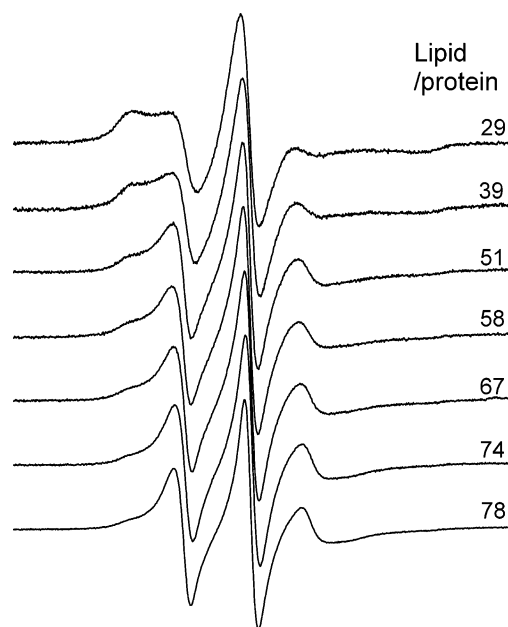


FIGURE 6: ESR spectra of phosphatidylglycerol spin labeled on the 14-C atom of the *sn*-2 chain (14-PGSL) in dimyristoylphosphatidylglycerol membranes containing FhuA at the lipid/protein mole ratios indicated in the figure. $T = 34\text{ }^{\circ}\text{C}$; total scan width = 100 G.

and zwitterionic lipids have a lower affinity for association with OmpA than does phosphatidylglycerol.

FhuA Reconstituted in DMPG. Figure 6 shows the ESR spectra of 14-PGSL in reconstituted membranes of DMPG that contain FhuA at different protein/lipid ratios. Spectra are recorded in the fluid phase of the lipid-protein membranes (cf. transition curve in Figure 2). As for membranes containing OmpA, the spectra consist of two components, and the proportion of the motionally restricted component increases with increasing protein content in the membrane. Figure 4 gives the dependence on lipid/protein ratio of the ratio of fluid to motionally restricted spin-labeled lipid associated with FhuA. The stoichiometry, N_b , of the lipid-protein interaction, $N_b \approx 32$ mol/mol, is considerably larger than that for the smaller β -barrel protein OmpA. Again, the association constant for spin-labeled phosphatidylglycerol is $K_r \approx 1$, relative to that for the unlabeled DMPG.

Figure 7 shows the ESR spectra of the different spin-labeled lipids in reconstituted FhuA/DMPG membranes of

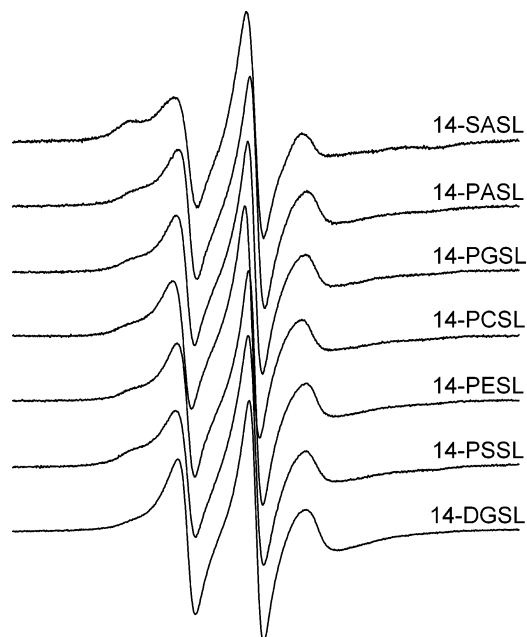


FIGURE 7: ESR spectra of spin-labeled phospholipids (14-PXSL) with different polar headgroups and of the corresponding spin-labeled diacylglycerol (14-DGSL) and stearic acid (14-SASL) in dimyristoylphosphatidylglycerol membranes containing FhuA at a fixed lipid/protein ratio of 53 mol/mol. $T = 34^\circ\text{C}$; total scan width = 100 G.

fixed lipid/protein ratio. Again, as for OmpA, a selectivity of interaction with FhuA is evident from the different proportions of the fluid and motionally restricted components in the spectra of the various spin-labeled lipids. Table 1 lists the selectivity of FhuA for the different spin-labeled lipids, in terms of the association constants, K_r/K_r^{PG} , relative to that for spin-labeled phosphatidylglycerol. The pattern of selectivity differs from that for OmpA. In particular with FhuA, a higher selectivity (relative to phosphatidylglycerol) is found for phosphatidylserine, and the association constant for phosphatidylcholine is comparable to that for phosphatidylglycerol. Additionally, the selectivity for stearic acid differs greatly between FhuA and OmpA.

DISCUSSION

Stoichiometry of the Lipid Interaction. The number of diacyl lipids that can be accommodated at one intramembranous surface of a β -sheet with n_β strands is (23)

$$N_b = n_\beta D_\beta / (d_{\text{ch}} \cos \theta_\beta) \quad (3)$$

where θ_β is the strand tilt within the sheet, $D_\beta = 0.47$ nm (24) is the interstrand separation, and $d_{\text{ch}} = 0.48$ nm is the width of a lipid chain. For OmpA ($n_\beta = 8$), the mean strand tilt is $\theta_\beta = 43.1^\circ$ deduced from the crystal structure (25), and $\theta_\beta = 46 \pm 1^\circ$ deduced from infrared dichroism in lipid bilayers (26). Both predict a lipid stoichiometry for OmpA of $N_b = 11$ lipids per monomer. For FhuA ($n_\beta = 22$), the strand tilt is $\theta_\beta = 38.3^\circ$ from X-ray (25) and $\theta_\beta = 44.5 \pm 1^\circ$ from infrared dichroism (26). This gives lipid stoichiometries for FhuA of $N_b = 27$ mol/mol from the crystal structure and $N_b = 30$ mol/mol in bilayers. Estimates of the stoichiometries for both proteins therefore correspond rather closely with the experimental determinations of the number of motionally restricted lipids interacting with the two β -sheet proteins.

Equation 3 applies to a continuous planar sheet and could be modified by the curvature of a β -barrel. For a barrel of approximately elliptical cross section, the number of lipids that can be accommodated at the perimeter is

$$N_b \approx \pi \left[\frac{3}{2}(a + b) - \sqrt{ab} \right] / d_{\text{ch}} \quad (4)$$

where a and b are the semiaxes of the ellipse. For OmpA, the cross section is only slightly elliptical: $a \approx b \approx 1.2$ nm (9) and thus $N_b \approx 16$ mol/mol. This value is significantly larger than the estimate for a planar sheet and larger than the population of motionally restricted lipid that is found experimentally. Evaluating the perimeter at the midpoint of the encircling lipid chains increases the estimate for OmpA even further to $N_b \approx 19$ lipids per protein. Because of the highly curved β -barrel, possibly not all lipids at the intramembranous perimeter of OmpA can interact optimally with the protein surface and experience direct motional restriction. For FhuA, $a \approx 2.3$ nm and $b \approx 1.9$ nm (10), and eq 4 yields a value of $N_b \approx 28$ mol/mol. This is close to the estimate from eq 3, because the FhuA barrel is relatively large in size, and curvature of the β -sheet is therefore less than for OmpA. Evaluating the perimeter at the chain midpoint increases the estimate for OmpA slightly to $N_b \approx 31$ mol/mol, which is close to the higher estimate from eq 3. Both types of estimate correspond reasonably well with the number of motionally restricted lipids that are associated with FhuA, as determined by ESR spectroscopy.

Selectivity of the Lipid-Protein Interaction. On the periplasmic side, the eight transmembrane β -strands of OmpA are connected by short turns, the charged residues of which are preponderantly acidic (27). Because there is only one basic residue (as opposed to four acidic residues), there is little basis for a selectivity for negatively charged lipids in the periplasmic leaflet. On the extracellular side, the loops connecting the β -strands of OmpA are larger, and the strands themselves extend beyond the intramembranous segments of the protein. There are no charged residues immediately adjacent to the transmembrane segments, but two basic side chains are located four residues away in extensions of the β -strand (27). These two positively charged residues are therefore situated ca. 1 nm above the hydrophobic core of the membrane, facing the lipid headgroups. Thus they could account for the selectivity of the negatively charged lipids, phosphatidic acid and phosphatidylglycerol, over the zwitterionic lipids, phosphatidylcholine and phosphatidylethanolamine, but not for the low selectivity for phosphatidylserine. The relative paucity of charged residues close to the lipid headgroups in OmpA is illustrated by the electrostatic surface of the protein that is shown in the upper panel of Figure 8. Under these circumstances, uncharged residues in the aromatic girdles, which are located at the polar-apolar interfaces (9, 27), may modulate the selectivity of lipid-protein interaction. A limited lipid selectivity of this type has been found recently for the interfacial tryptophan residues of gramicidin A channels reconstituted in DMPC (30). At pH 7.0, the fatty acid spin label (14-SASL) is expected to be protonated in negatively charged DMPG bilayers (31). The low selectivity for both diacylglycerol and protonated fatty acid is accounted for by the low polarity of these latter two lipids, which causes them to locate more deeply in the bilayer than do the phospholipids (32, 33).

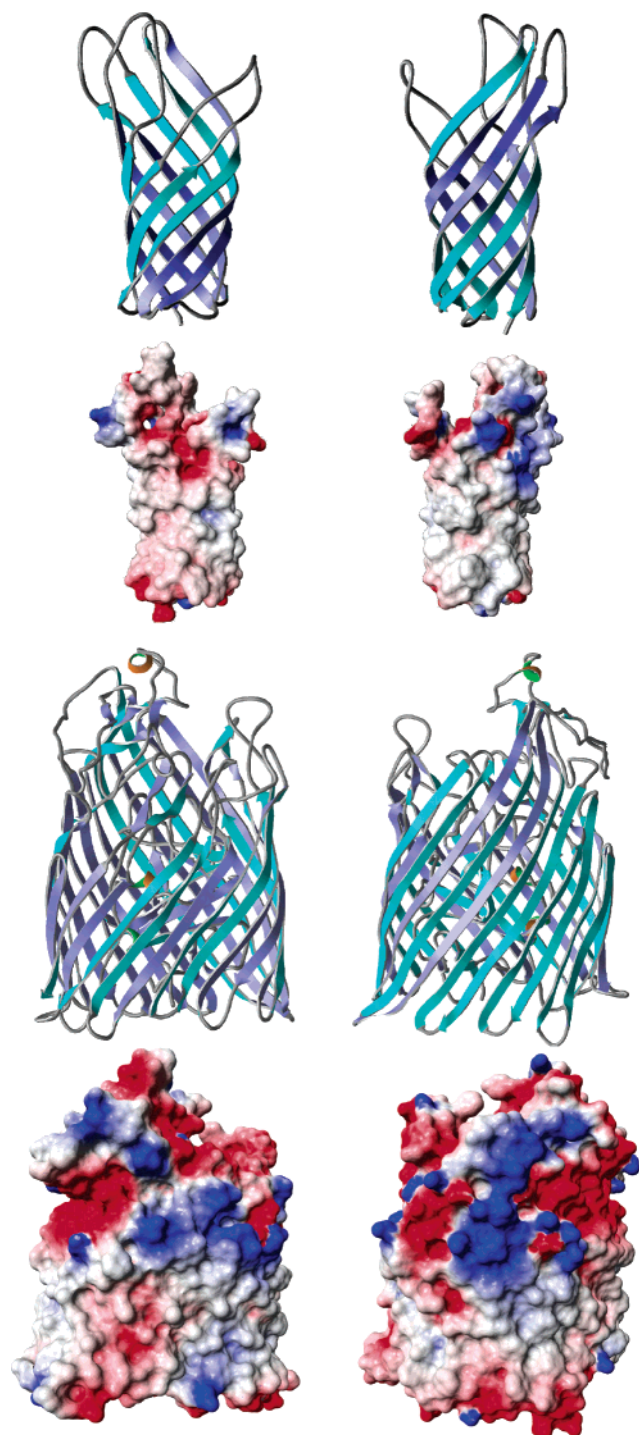


FIGURE 8: Ribbon diagrams and electrostatic surfaces of OmpA barrel residues 1–171 (upper panel; PDB code 1QJP; ref 9) and of FhuA (lower panel; PDB code 2FCP; ref 10). Ribbon diagrams were prepared with DeepView (28) and electrostatic surfaces with MolMol (29). In the electrostatic coloring, blue is positive, red is negative, and white is apolar. For each protein, a back and a front view is shown.

FhuA has 22 transmembrane strands and considerably more charged residues that are located close to the region of the phospholipid headgroups than does OmpA. This is clear from comparison of the electrostatic surfaces of the two proteins that are given in Figure 8. The periplasmic turns of FhuA contain 13 acidic residues and 4 basic residues (34). Although still bearing a net negative charge, the ratio of basic to acidic residues is somewhat higher than in the periplasmic

turns of OmpA. In the extracellular extensions of the FhuA β -strands, 6 basic residues and 2 acidic residues are located 2 residues away from the hydrophobic–polar interface; i.e., they face the lipid headgroups. A further 6 basic residues and 1 acidic residue are located 4 residues away from this surface. Additionally, 2 basic and 2 acidic residues are located in cytoplasmic loops, close to the hydrophobic transmembrane sector. There is thus a strong preponderance of positively charged residues at the extracellular surface that accounts for the marked selectivity of FhuA, relative to OmpA, for anionic lipids (see lower panel of Figure 8). The pattern of lipid selectivity with FhuA conforms more closely with that found for α -helical integral proteins. In the latter case, there is little selectivity between phosphatidylglycerol and phosphatidylcholine but a preferential selectivity for other negatively charged lipids (3).

In further connection with the selectivity of FhuA for certain negatively charged phospholipids, it is interesting to note the cluster of basic residues that is concentrated at the binding site of the single lipopolysaccharide (LPS) molecule which is resolved in the crystal structure of OmpA (PDB code 2FCP; ref 10). Arginines 382 and 384 and lysines 351, 439, and 441 are all located in the region of the phosphorylated glucosamines or of the acyl chain carboxyl groups of LPS. This part of the LPS molecule corresponds to the expected location of the phospholipid headgroups in a lipid bilayer.

As for OmpA, the selectivity of FhuA for apolar diacylglycerol is low. The selectivity of FhuA for stearic acid (14-SASL), however, is much higher than that of OmpA. The association constant of 14-SASL with FhuA is in the range that might be expected for the negatively charged, ionized form of the fatty acid (see, e.g., ref 3). Presumably, the negative surface potential of DMPG bilayers is neutralized locally in the regions of high positive charge on FhuA (see Figure 8) and results in ionization of the fatty acid at these association sites on the protein.

Currently, relatively little is known about the effects of specific lipids on the function of outer membrane proteins. Negatively charged lipopolysaccharide is, however, known to be involved in activation of the *E. coli* outer membrane protease OmpT (35). Furthermore, negatively charged lipids are found to be important for the folding and membrane insertion of outer membrane proteins, such as the *E. coli* phosphate transporter PhoE (36, 37) and including also OmpA (38).

ACKNOWLEDGMENT

We thank Frau B. Angerstein for synthesis of spin-labeled lipids. D.M. and J.H.K. are members of the COST D22 Action of the European Union.

REFERENCES

1. Marsh, D. (1989) Experimental methods in spin-label spectral analysis, in *Biological Magnetic Resonance* (Berliner, L. J., and Reuben, J., Eds.) Vol. 8, pp 255–303, Plenum Publishing Corp., New York.
2. Marsh, D., and Horváth, L. I. (1989) Spin-label studies of the structure and dynamics of lipids and proteins in membranes, in *Advanced EPR. Applications in Biology and Biochemistry* (Hoff, A. J., Ed.) pp 707–752, Elsevier, Amsterdam.
3. Marsh, D., and Horváth, L. I. (1998) Structure, dynamics and composition of the lipid–protein interface. Perspectives from spin-labelling, *Biochim. Biophys. Acta* 1376, 267–296.

4. Marsh, D., and Páli, T. (2004) The protein–lipid interface: perspectives from magnetic resonance and crystal structures, *Biochim. Biophys. Acta* (accepted for publication).
5. Horváth, L. I., Heimburg, T., Kovachev, P., Findlay, J. B. C., Hideg, K., and Marsh, D. (1995) Integration of a K⁺ channel-associated peptide in a lipid bilayer: conformation, lipid–protein interactions, and rotational diffusion, *Biochemistry* 34, 3893–3898.
6. Horváth, L. I., Knowles, P. F., Kovachev, P., Findlay, J. B. C., and Marsh, D. (1997) A single-residue deletion alters the lipid selectivity of a K⁺ channel-associated peptide in the β -conformation: spin label electron spin resonance studies, *Biophys. J.* 73, 2588–2594.
7. Wolfs, C. J. A. M., Horváth, L. I., Marsh, D., Watts, A., and Hemminga, M. A. (1989) Spin-label ESR of bacteriophage M13 coat protein in mixed lipid bilayers. Characterization of molecular selectivity of charged phospholipids for the bacteriophage coat protein in lipid bilayers, *Biochemistry* 28, 9995–10001.
8. Lee, A. G. (2003) Lipid–protein interactions in biological membranes: a structural perspective, *Biochim. Biophys. Acta* 1612, 1–40.
9. Pautsch, A., and Schulz, G. E. (2000) High-resolution structure of the OmpA membrane domain, *J. Mol. Biol.* 298, 273–282.
10. Ferguson, A. D., Hofmann, E., Coulton, J. W., Diederichs, K., and Welte, W. (1998) Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide, *Science* 282, 2215–2220.
11. Surrey, T., and Jähnig, F. (1992) Refolding and oriented insertion of a membrane protein into a lipid bilayer, *Proc. Natl. Acad. Sci. U.S.A.* 89, 7457–7461.
12. Ferguson, A. D., Breed, J., Diederichs, K., Welte, W., and Coulton, J. W. (1998) An internal affinity-tag for purification and crystallization of the siderophore receptor FhuA, integral outer membrane protein from *Escherichia coli* K-12, *Protein Sci.* 7, 1636–1638.
13. Hubbell, W. L., and McConnell, H. M. (1971) Molecular motion in spin-labeled phospholipids and membranes, *J. Am. Chem. Soc.* 93, 314–326.
14. Marsh, D., and Watts, A. (1982) Spin-labeling and lipid–protein interactions in membranes, in *Lipid–Protein Interactions* (Jost, P. C., and Griffith, O. H., Eds.) Vol. 2, pp 53–126, Wiley-Interscience, New York.
15. Kleinschmidt, J. H., Wiener, M. C., and Tamm, L. K. (1999) Outer membrane protein A of *E. coli* folds into detergent micelles, but not in the presence of monomeric detergent, *Protein Sci.* 8, 2065–2071.
16. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature (London)* 227, 680–685.
17. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, *Lipids* 5, 494–496.
18. Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193, 265–275.
19. Marsh, D. (1982) Electron spin resonance: spin label probes, in *Techniques in Lipid and Membrane Biochemistry* (Metcalf, J. C., and Hesketh, T. R., Eds.) Vol. B4/II, pp B426-1–B426/44, Elsevier, Amsterdam.
20. Marsh, D. (1981) Electron spin resonance: spin labels, in *Membrane Spectroscopy. Molecular Biology, Biochemistry and Biophysics* (Grell, E., Ed.) Vol. 31, pp 51–142, Springer-Verlag, Berlin, Heidelberg, and New York.
21. Brotherus, J. R., Griffith, O. H., Brotherus, M. O., Jost, P. C., Silvius, J. R., and Hokin, L. E. (1981) Lipid–protein multiple binding equilibria in membranes, *Biochemistry* 20, 5261–5267.
22. Marsh, D. (1985) ESR spin label studies of lipid–protein interactions, in *Progress in Protein–Lipid Interactions* (Watts, A., and de Pont, J. J. H. H. M., Eds.) Vol. 1, pp 143–172, Elsevier, Amsterdam.
23. Marsh, D. (1997) Stoichiometry of lipid–protein interaction and integral membrane protein structure, *Eur. Biophys. J.* 26, 203–208.
24. Fraser, R. D. B., and MacRae, T. P. (1973) *Conformation in Fibrous Proteins and Related Synthetic Peptides*, Academic Press, New York.
25. Páli, T., and Marsh, D. (2001) Tilt, twist and coiling in β -barrel membrane proteins: relation to infrared dichroism, *Biophys. J.* 80, 2789–2797.
26. Ramakrishnan, M., Pocanschi, C. L., Kleinschmidt, J. H., and Marsh, D. (2004) Orientation of β -barrel proteins OmpA and FhuA in lipid membranes. Chain length dependence from infrared dichroism (to be submitted for publication).
27. Pautsch, A., and Schulz, G. E. (1998) Structure of the outer membrane protein A transmembrane domain, *Nat. Struct. Biol.* 5, 1013–1017.
28. Guex, N., and Peitsch, M. C. (1997) Swiss model and the Swiss-Pdb viewer: An environment for comparative protein modeling, *Electrophoresis* 18, 2714–2723.
29. Koradi, R., Billeter, M., and Wüthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures, *J. Mol. Graphics* 14, 51–55.
30. Kóta, Z., Páli, T., and Marsh, D. (2004) Orientation and lipid-peptide interactions of gramicidin A in lipid membranes: polarized ATR infrared spectroscopy and spin-label electron spin resonance, *Biophys. J.* 86, 1521–1531.
31. Sankaram, M. B., Brophy, P. J., Jordi, W., and Marsh, D. (1990) Fatty acid pH titration and the selectivity of interaction with extrinsic proteins in dimyristoylphosphatidylglycerol dispersions. Spin label ESR studies, *Biochim. Biophys. Acta* 1021, 63–69.
32. Rama Krishna, Y. V. S., and Marsh, D. (1990) Spin label ESR and ³¹P-NMR studies of the cubic and inverted hexagonal phases of dimyristoylphosphatidylcholine/myristic acid (1:2, mol/mol) mixtures, *Biochim. Biophys. Acta* 1024, 89–94.
33. Schorn, K., and Marsh, D. (1996) Lipid chain dynamics and molecular location of diacylglycerol in hydrated binary mixtures with phosphatidylcholine: spin label ESR studies, *Biochemistry* 35, 3831–3836.
34. Locher, K. P., Rees, B., Koebnik, R., Mitschler, A., Moulinier, L., Rosenbusch, J. P., and Moras, D. (1998) Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes, *Cell* 95, 771–778.
35. Kramer, R. A., Brandenburg, K., Vandeputte-Rutten, L., Werkhoven, M., Gros, P., Dekker, N., and Egmond, M. R. (2002) Lipopolysaccharide regions involved in the activation of *Escherichia coli* outer membrane protease OmpT, *Eur. J. Biochem.* 269, 1746–1752.
36. de Cock, H., Brandenburg, K., Wiese, A., Holst, O., and Seydel, U. (1999) Non-lamellar structure and negative charges of lipopolysaccharides required for efficient folding of outer membrane protein PhoE of *Escherichia coli*, *J. Biol. Chem.* 274, 5114–5119.
37. de Cock, H., Pasveer, M., Tommassen, J., and Bouveret, E. (2001) Identification of phospholipids as new components that assist in the *in vitro* trimerization of a bacterial pore protein, *Eur. J. Biochem.* 268, 865–875.
38. Bulteris, P. V., Behrens, S., Holst, O., and Kleinschmidt, J. H. (2003) Folding and insertion of the outer membrane protein OmpA is assisted by the chaperone Skp and by lipopolysaccharide, *J. Biol. Chem.* 278, 9092–9099.

BI048858E