Selectivity in Lipid Binding to the Bacterial Outer Membrane Protein OmpF

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ABSTRACT The outer membrane porin OmpF from *Escherichia coli* has been reconstituted into lipid bilayers of defined composition, and fluorescence spectroscopy is used to characterize its interaction with the surrounding lipid. OmpF is a trimer within the membrane. It contains two Trp residues per monomer, Trp^{214} at the lipid-protein interface and Trp^{61} at the trimer interface. The fluorescence of Trp-214 in the mutant W61F is quenched by dibromostearoylphosphatidylcholine (di(Br_2C18:0)PC), whereas the fluorescence of Trp^{61} in the mutant W214F is not quenched by di(Br_2C18:0)PC when fluorescence is excited directly through the Trp rather than through the Tyr residues. Measurements of relative fluorescence quenching for OmpF reconstituted into mixtures of lipid X and di(Br_2C18:0)PC have been analyzed to give the binding constant of lipid X for OmpF, relative to that for dioleoylphosphatidylcholine (di(C18:1)PC). The phosphatidylcholine showing the strongest binding to OmpF is dimyristoyloleoylphosphatidylcholine (di(C14:1)PC) with binding constants decreasing with either increasing or decreasing fatty acyl chain length. Comparison with various theories for hydrophobic matching between lipids and proteins suggests that in the chain length range from C14 to C20, hydrophobic matching is achieved largely by distortion of the lipid bilayer around the OmpF, whereas for chains longer than C20, distortion of both the lipid bilayer and of the protein is required to achieve hydrophobic matching. Phosphatidylcholine and phosphatidylethanolamine bind with equal affinity to OmpF, but the affinity for phosphatidylglycerol is about half that for phosphatidylcholine.

INTRODUCTION

The transmembrane regions of most intrinsic membrane proteins consist of one or more hydrophobic α-helices. Crystal structures of membrane proteins containing bundles of transmembrane α-helices show that many of these helices are markedly tilted with respect to the bilayer normal (see, for example, Doyle et al., 1998). The tilt angle adopted by any one transmembrane α-helix will depend on any packing constraints imposed by interhelical loops, on the interactions with any other transmembrane α-helices, and on interactions with the surrounding lipid bilayer. Optimal interaction between an α-helix and a lipid bilayer requires that the length of the hydrophobic helix matches the thickness of the hydrophobic core of the lipid bilayer because the cost of exposing either the fatty acyl chains or hydrophobic amino acids to water is high.

The importance of this concept of hydrophobic matching has been confirmed in a variety of theoretical models that consider how a lipid bilayer might become thinner or thicker around a rigid membrane protein with a hydrophobic length that does not match the hydrophobic thickness of the bulk lipid bilayer (Mouritsen and Bloom, 1984, 1993; Fattal and Ben-Shaul, 1993). The importance of hydrophobic matching has been confirmed in a number of experimental studies (Killian, 1998; Dumas et al., 1999). In particular, the presence of a membrane protein has been shown to increase the temperature of the gel-to-liquid crystalline phase transition for a phospholipid with short fatty acyl chains and to decrease the temperature of the phase transition for a phospholipid with long fatty acyl chains (Piknova et al., 1993; Dumas et al., 1999). This is consistent with the idea that short fatty acyl chains have to stretch to match the hydrophobic thickness of the membrane protein, whereas long fatty acyl chains have to compress (Piknova et al., 1993; Dumas et al., 1999). It has also been shown that bacteriorhodopsin preferentially partitions into gel-phase dilauroylphosphatidylcholine (di(C12:0)PC) when present in a bilayer containing a mixture of di(C12:0)PC and distearoylphosphatidylcholine (di(C18:0)PC) at low temperature in the gel-gel coexistence region (Dumas et al., 1997). In the two-phase region containing liquid crystalline and gel phases enriched in di(C12:0)PC and di(C18:0)PC, respectively, bacteriorhodopsin was found to partition preferentially into the liquid crystalline phase, although this could reflect a preference for the liquid crystalline phase over the gel phase rather than a preference for C12 chains over C18 chains. Unfortunately, at higher temperatures, when all of the lipid was in the liquid crystalline phase, it was not possible to tell whether bacteriorhodopsin showed any selectivity in its interaction with lipid (Dumas et al., 1997). However, in studies of the Ca^{2+}-ATPase of sarcoplasmic reticulum using either spin-labeled (London and Feigenson, 1981b) or brominated phospholipids (East and Lee, 1982), strengths of binding of liquid crystalline phase phospholipids to the ATPase were found to be independent of fatty acyl chain length. These results obtained with the Ca^{2+}-ATPase are not consistent with the expectations of hydrophobic

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matching theory and suggest that, in liquid crystalline bilayers, α-helical membrane proteins are not rigid but, in fact, can distort to match the thickness of the bilayer. Such a distortion could explain why bilayer thickness affects the activity of a membrane protein such as the Ca^{2+}-ATPase (Lee, 1998). The structural distortion could take the form of a change in the tilt of the transmembrane α-helices with respect to the bilayer normal or could be a change in the packing of the transmembrane α-helices. Studies of the incorporation of long hydrophobic α-helices into thin lipid bilayers suggest that, at least under some circumstances, single transmembrane α-helices can change their tilt angle to match the bilayer thickness (Webb et al., 1998).

In contrast to the α-helical membrane proteins, some membrane proteins adopt β-barrel structures that will be intrinsically more rigid than a bundle of α-helices. The β-barrel structure is adopted by the porins that are found in bacterial outer membranes (Cowan, 1993; Nikaido, 1994). It has also been suggested that the nicotinic acetylcholine receptor could adopt a mixed structure in which a central bundle of transmembrane α-helices is surrounded by a β-barrel structure (Unwin, 1993). Here we use a fluorescence quenching method to study the strength of binding of phospholipids to the *Escherichia coli* porin OmpF, making use of the two Trp residues per OmpF monomer (Fig. 1). The porin is reconstituted into bilayers containing the brominated phospholipid dibromostearoylphosphatidylcholine (di(Br_2C18:0)PC). Di(Br_2C18:0)PC behaves much like a conventional phospholipid with unsaturated fatty acyl chains, because the bulky bromine atoms have effects on lipid packing that are similar to those of a *cis* double bond, but contact between the bromine atoms and a Trp residue leads to quenching of the fluorescence of the Trp residue (East and Lee, 1982). In mixtures of brominated and nonbrominated phospholipids, the degree of quenching of the tryptophan fluorescence of the OmpF depends on the fraction of the surrounding phospholipids that are brominated and thus on the strength of binding of the nonbrominated phospholipid to the porin.

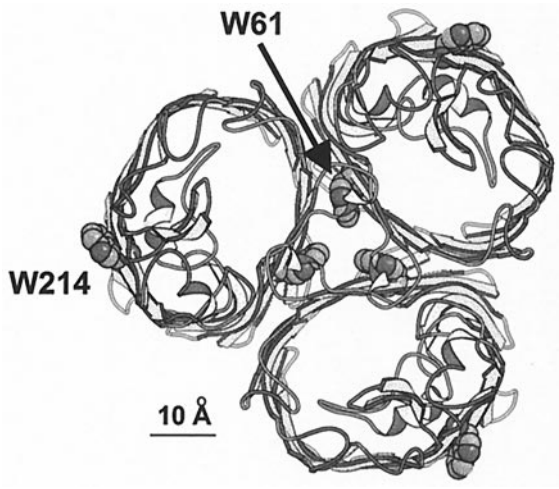

FIGURE 1 The structure of the OmpF trimer. The positions of the two Trp residues (W61 and W214) in each monomer are shown. The figure was prepared using Bobscript (Esnouf, 1999) and the coordinates in PDB 1OPF.

EXPERIMENTAL PROCEDURES

Octyl-polyoxyethylene (octyl-POE) was obtained from Bachem. Dilauroylphosphatidylcholine (di(C12:0)PC) was obtained from Sigma, and dimyristoleoylphosphatidylcholine (di(C14:1)PC), dipalmitoyloleoylphosphatidylcholine (di(C16:1)PC), dioleoylphosphatidylcholine (di(C18:1)PC), dieicosenoylphosphatidylcholine (di(C20:1)PC), dierucoylphosphatidylcholine (di(C22:1)PC), dinervonylphosphatidylcholine (di(C24:1)PC), dioleoylphosphatidylethanolamine (di(C18:1)PE), and dioleoylphosphatidylglycerol (di(C18:1)PG) were obtained from Avanti Polar Lipids. [^{3}H]Dipalmitoylphosphatidylcholine was obtained from New England Nuclear. Di(C18:1)PC and di(C14:1)PC were brominated to give di(Br_2C18:0)PC and dibromomyristoylphosphatidylcholine (di(Br_2C14:0)PC), respectively, as described by East and Lee (1982).

Purification of OmpF and mutagenesis

The general porin OmpF was purified from *E. coli* strain BZB1107 harboring the pGBF96 expression vector (Bainbridge et al., 1998). In this strain the native OmpF gene has been knocked out by Tn5 insertion mutagenesis, and expression of the specific porin LamB was inhibited by catabolite repression in a glucose-containing medium. *E. coli* was grown in LB broth medium containing ampicillin (60 μg/ml) and kanamycin (10 μg/ml) supplemented with 0.2% glucose at 37°C. Cells were induced at an optical density of ~0.6 with 0.5 mM isopropyl-β,D-thiogalactopyranoside. Cells were harvested in late logarithmic phase. Porins were isolated by the method of Rummel and Rosenbusch (personal communication). Cells were broken by homogenizing in a glass Teflon homogenizer at 60°C in buffer (2% sodium dodecyl sulfate (SDS), 20 mM Tris-HCl, pH 8.0) for 60 min. The envelope fraction was pelleted at 4°C at 100,000 $\times$ *g*. The pellet was washed with phosphate buffer (20 mM sodium phosphate, pH 7.3) to remove residual SDS and then extracted with 0.125% octyl-POE in phosphate buffer at 37°C for 60 min to remove the majority of the contaminants. The sample was pelleted at 4°C at 100,000 $\times$ *g*, and the pellet was then shaken at 37°C for 1 h with 3% octyl-POE in phosphate buffer and pelleted at 100,000 $\times$ *g* at 25°C. The supernatant containing the purified porin was dialyzed twice for 12 h at room temperature against phosphate buffer containing 1% octyl-POE and then aliquoted and stored at −20°C. Homogeneity of the porin was assessed by SDS-polyacrylamide gel electrophoresis, using the method of Laemmli (1970). Porin was estimated using the bicinchoninic acid protein assay (Pierce Chemical Co.) against bovine serum albumin as standard.

Site-directed mutagenesis was performed using the methods of Higuchi et al. (1988). Mutated porins were prepared with Trp residues substituted by Phe at position 61 (W61F), position 214 (W214F), or both positions (W61F, W214F). W61F was produced using oligonucleotide primers 5′-CCGGTTATGGTCAGTTTGAATATAACTTCCAGGG-3′, 5′-TTAATCTGTATCAGGCTG-3′ (primer 1), 5′-ATAACAATTTCACACAGG-3′ (primer 2), and 5′-CCCTGGAAGTTATATTCAAACTGACCATAACCGG-3′ (mutations are underlined). W214F was produced using primers 1 and 2 in combination with 5′-GACCAGTAGCAAACTGTTCAGC-3′ and 5′-GCTGAACAGTTTGCTACTGGTC-3′. The 1.08-kb fragments generated for each mutation were cloned back into the original vector, using *Eco*RI and HindIII restriction sites. The double mutant W61F, W214F was produced by cloning the 0.51-kb *Eco*RI, *Sal*I fragment from W61F into W214F.

Reconstitution of OmpF

Purified OmpF was reconstituted into lipid bilayers by mixing lipid and OmpF in octyl-POE, followed either by dilution or dialysis to remove the detergent. For fluorescence measurements, lipid (2.5 μmol) was dried from chloroform solution onto the walls of a thin glass vial. Buffer (200 μl; 10 mM HEPES, 15% (w/v) sucrose, pH 8.0) containing 0.6% octyl-POE by weight was added, and the sample was sonicated to clarity in a bath sonicator (Ultrawave). OmpF (0.15 mg) was then added, and the suspension was left at room temperature for 15 min, followed by incubation on ice for a further 60 min. The sample was then diluted 300-fold into buffer (20 mM Hepes, pH 7.2, 1 mM EGTA), and the Trp fluorescence spectra were recorded with excitation at 270 nm. Fluorescence spectra were recorded on an SLM 8000C fluorimeter at 25°C.

Gradient centrifugation was used to characterize the reconstituted preparation. Di(C18:1)PC (10 μmol) was mixed with [^{3}H]dipalmitoylphosphatidylcholine (0.1 nmol) in chloroform and dried onto the walls of a glass vial. The mixture was resuspended in buffer (0.8 ml; 10 mM Hepes, pH 8.0) containing 0.6% octyl-POE by weight and 15% (w/v) sucrose. The sample was sonicated to clarity in a sonication bath (Ultrawave). OmpF (0.6 mg) was added, and the mixture was left at room temperature for 15 min followed by 45 min on ice. The sample was then dialyzed at room temperature against two lots of buffer (500 ml; 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid, pH 7.1, 100 mM K_2SO_4) for a total of 5 h. Samples of dialysate (0.75 ml) were then loaded onto sucrose gradients containing the following solutions of sucrose (w/w) in 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (pH 7.1), 100 mM K_2SO_4: 2.5, 5.0, 10.0, 15.0, 20.0, and 30.0%; the 30% sucrose solution also contained 0.05% (w/v) Triton X-100. Samples were spun at 80,000 $\times$ *g* for 18 h at 4°C, and then 1.5-ml fractions were collected from the gradients and analyzed for lipid and protein by, respectively, liquid scintillation counting and protein assay (modified Lowry assay from Sigma). Phospholipids were extracted from the samples with chloroform/methanol/water at a final ratio (v/v) of 2:2:1.8, using the method of Bligh and Dyer (1959), and lipids were separated by thin-layer chromatography, using chloroform/methanol/acetic acid/water at a ratio (v/v) of 25:45:4:2.

Analysis of fluorescence quenching

Tryptophan fluorescence is quenched by bromine-containing molecules by a process of heavy atom quenching that requires contact between tryptophan and bromine. The fluorescence lifetime for tryptophan is considerably less than the time for two lipids to exchange position in a bilayer, so that quenching is proportional to the fraction of sites around the tryptophan residue occupied by quenching phospholipids (East et al., 1985). In the case of the Ca^{2+}-ATPase, quenching of tryptophan fluorescence by spin-labeled fatty acids has been shown to be a static process with no significant effect on fluorescence lifetime (Simmonds et al., 1982). Quenching can therefore be analyzed by the use of a lattice model of quenching (London and Feigenson, 1981a; Webb et al., 1998). The degree of quenching in the lattice model is proportional to the probability that a brominated lipid occupies a lattice site close enough to the Trp residue in the porin to cause quenching. For a random distribution of lipids, the probability that any lattice site is not occupied by a brominated lipid is $1 - x_{Br}$, where x_{Br} is the mole fraction of brominated lipid in the bilayer. The probability that any particular Trp residue will give rise to fluorescence is proportional to the probability that none of the *n* lattice sites close enough to the residue to cause quenching are occupied by a brominated lipid. Thus

$$F = F_{min} + (F_o - F_{min})(1 - x_{Br})^n \quad (1)$$

where F_o and F_{min} are the fluorescence intensities for the porin in nonbrominated and in brominated lipid, respectively, and F is the fluorescence intensity in the phospholipid mixture when the mole fraction of brominated lipid is x_{Br}.

The lattice model can readily be extended to describe quenching of the porin in a mixture of two lipids of different affinities for porin. At each site, an equilibrium will exist:

$$PL + Q \leftrightharpoons PQ + L$$

where PL and PQ are complexes of porin with nonbrominated lipid (L) and brominated lipid (Q), respectively. The equilibrium can be described by an equilibrium constant K, given by

$$K = [PQ][L]/[PL][Q] \quad (2)$$

where K is the binding constant of the brominated lipid relative to that of the nonbrominated lipid. Fluorescence quenching then fits the equation

$$F = F_{min} + (F_o - F_{min})(1 - f_{Br})^n \quad (3)$$

where the fraction of sites at the lipid-protein interface occupied by brominated lipid is f_{Br} (East and Lee, 1982). The fraction of sites occupied by brominated lipid is related to x_{Br} by

$$f_{Br} = Kx_{Br}/(Kx_{Br} + [1 - x_{Br}]) \quad (4)$$

Equation 3 was fitted to the experimental data using the nonlinear least-squares routine in the SigmaPlot package.

RESULTS

Reconstitution of OmpF

The first step in the reconstitution of OmpF into lipid bilayers is to dissolve purified OmpF in 1.0% octyl-POE; the minimum concentration of octyl-POE required for complete solubilization of OmpF was determined to be 0.3% from measurements of light scatter. The solution of OmpF in octyl-POE was mixed with phospholipid dissolved in 0.6% octyl-POE to give a molar ratio of phospholipid to OmpF of 600:1. The mixture was incubated for 15 min at room temperature, followed by an hour on ice, and was then diluted 300-fold into buffer, decreasing the concentration of octyl-POE to below its critical micelle concentration (0.23%; Moller et al., 1986) and thus reforming membranes.

It was confirmed in a number of ways that OmpF was fully reconstituted under these conditions. The Trp fluorescence for OmpF reconstituted with di(Br_2C18:0)PC was 50 $\pm$ 2% of that for OmpF reconstituted with di(C18:1)PC (Table 1). Increasing the concentration of octyl-POE in the original mixture or increasing the level of dilution was found not to affect the observed level of quenching (Table

TABLE 1 Effect of method of reconstitution on fluorescence quenching of OmpF by di(Br_2C18:0)PC

Method	F/F_o
0.6% octyl-POE/300-fold dilution	0.50 $\pm$ 0.02
0.6% octyl-POE/1500-fold dilution	0.54 $\pm$ 0.02
1.0% octyl-POE/300-fold dilution	0.51 $\pm$ 0.02
0.6% octyl-POE/dialysis	0.54 $\pm$ 0.02

F and F_o are fluorescence intensities for OmpF reconstituted in di(Br_2C18:0)PC and di(C18:1)PC, respectively. Fluorescence was excited at 270 nm, and emission was monitored at 318 nm.

1). Samples in which detergent was removed by dialysis for 3 h were found to give the same level of fluorescence quenching as observed with the dilution method (Table 1). The reconstituted OmpF was also characterized by centrifugation on a discontinuous sucrose gradient (Fig. 2). When OmpF and lipid were run separately on the gradient, all of the OmpF was found at the bottom of the gradient, and all of the lipid was found at the top. In contrast, when OmpF was mixed with di(C18:1)PC in 0.6% octyl-POE followed by dialysis to remove the detergent, OmpF was found at the 20% sucrose interface, with the lipid located partly with the OmpF and partly at the top of the gradient (Fig. 2). The molar ratio of lipid to protein for the OmpF-containing fraction was 460:1, compared to 600:1 in the original mixture. Thin-layer chromatography of the lipid extracted from the OmpF-containing fraction detected only phosphatidylcholine, whereas phosphatidylethanolamine was the main phospholipid component of the original purified OmpF (data not shown).

OmpF exists as a trimer in the *E. coli* outer membrane, the trimers being stable even at 75°C in sodium dodecyl sulfate (Jap and Walian, 1996). After reconstitution, OmpF still runs as a trimer when analyzed on sodium dodecyl sulfate-polyacrylamide gels (Fig. 3 *A*).

Fluorescence properties of OmpF

Each monomer of OmpF contains two Trp residues, Trp^{214} at the lipid-protein interface and Trp^{61} at the trimer interface (Fig. 1). The Trp mutants W61F and W214F were prepared. As shown in Fig. 3 *B*, these mutants, like the native protein, form trimers that are stable in SDS. The fluorescence emission spectra of native OmpF and the Trp mutants W61F and W214F are shown in Fig. 4 *A*. In all cases, the emission

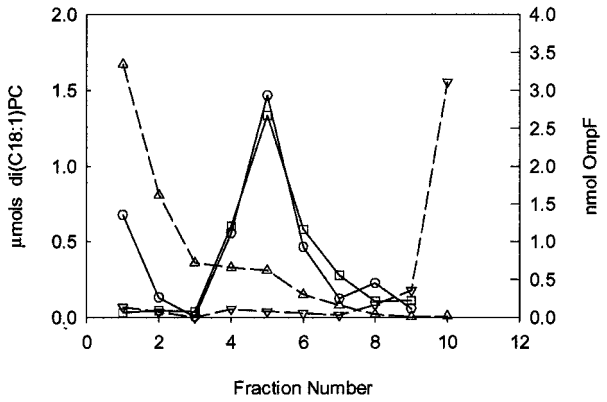

FIGURE 2 Sucrose gradient analysis of the reconstituted OmpF. A sample of OmpF reconstituted with di(C18:1)PC at a lipid to protein molar ratio of 600:1 was separated on a discontinuous sucrose gradient from 30 to 2.5% sucrose (○, □). Fractions (1.5 ml) were taken and analyzed for lipid (○) and OmpF (□). Lipid alone (△) or OmpF alone (▽) were also analyzed on the same gradient.

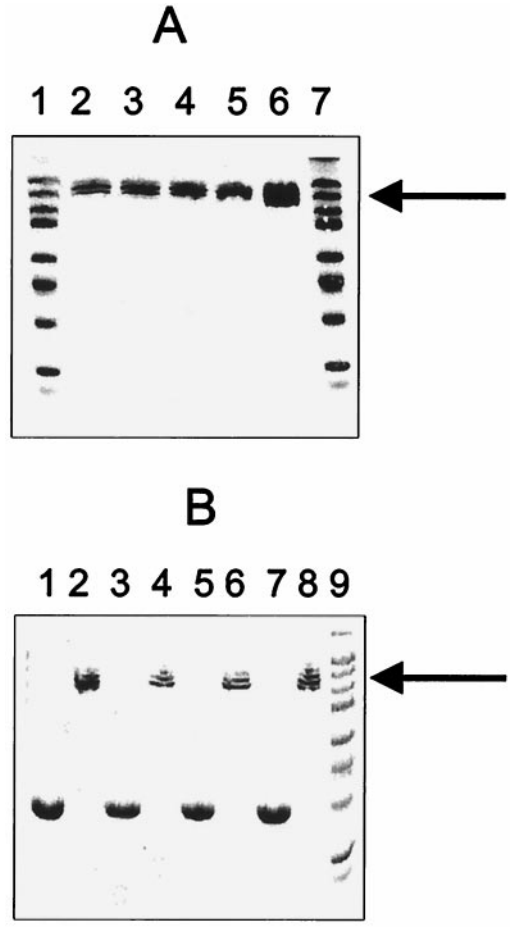

FIGURE 3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reconstituted native OmpF (*A*) and of mutated OmpF (*B*). (*A*) Lanes 2–5 contain OmpF reconstituted in di(C14:1)PC, di(C18:1)PC, and di(C24:1)PC, di(Br_2C18:0)PC, respectively. Lane 6 contains unreconstituted OmpF. Lanes 1 and 7 contain molecular weight markers; the arrow shows the expected position of the OmpF trimer. (*B*) Lanes 1, 3, 5, and 7 contain wild-type OmpF and mutants W61F, W214F and W61F, W214F, respectively, heat-denatured at 100°C for 5 min to produce monomer. Lanes 2, 4, 6, and 8 contain nondenatured native OmpF, W61F, W214F, and W61F, W214F, respectively. Lane 9 contains molecular weight markers. The position of the OmpF trimer is shown by the arrow; the apparent M_r of monomeric OmpF is 36,000.

spectra occur at relatively low wavelengths, indicating hydrophobic environments for both Trp residues (Lakowicz, 1983). The emission spectrum of Trp^{61} (mutant W214F) occurs at a slightly lower wavelength than that of Trp^{214} (mutant W61F). Because Trp emission wavelengths generally decrease with decreasing environmental polarity (Lakowicz, 1983), this would suggest that Trp^{61} at the trimer interface is in a very hydrophobic environment.

The mutant W61F, W214F lacking Trp residues shows a very high fluorescence emission centered at 305 nm due to fluorescence emission from the 29 Tyr residues in OmpF (Fig. 4 *A*). The lack of extensive Tyr fluorescence in the spectra of the wild-type or Trp-containing mutants of OmpF

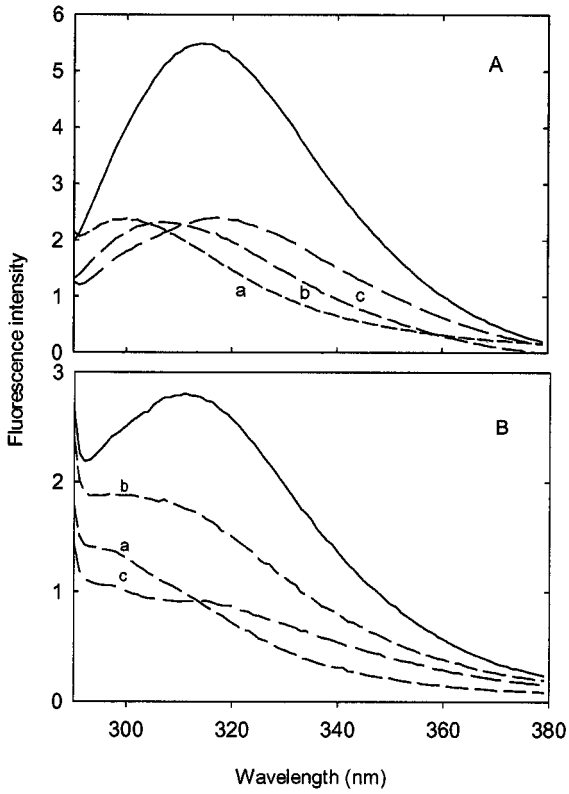

FIGURE 4 Fluorescence emission spectra of native and mutant OmpF. The solid line shows the fluorescence emission spectra of wild-type OmpF reconstituted in di(C18:1)PC (*A*) or di(Br2C18:0)PC (*B*). The spectra of mutants W61F, W214F (*a*), W214F (*b*), and W61F (c) are also shown as reconstituted in di(C18:1)PC (*A*) or di(Br_2C18:0)PC (*B*). Fluorescence was excited at 270 nm. For all spectra the concentration of OmpF was 37 nM and the molar ratio of lipid to protein was 1800:1. The buffer was 20 mM HEPES, 1 mM EGTA (pH 7.2).

suggest that Tyr fluorescence is quenched by energy transfer to the Trp, a process that has been shown to be highly efficient in other proteins (Lakowicz, 1983).

Reconstitution of OmpF into bilayers of di(Br_2C18:0)PC leads to quenching of 50 ± 2% of the Trp fluorescence of native OmpF, compared to 64% quenching of W61F but only 35% quenching of W214F, measured with an excitation wavelength of 280 nm (Fig. 4 *B*). The Tyr fluorescence of the double mutant W61F, W214F is quenched by 44% on reconstitution in di(Br_2C18:0)PC. Table 2 shows the level of quenching in wild-type and mutant OmpF as a function of excitation wavelength. Whereas the level of quenching observed for wild-type OmpF and for W61F is almost independent of excitation wavelength, the degree of quenching for W214F decreases with an increase in excitation wavelength from 270 to 290 nm. At 290 nm Trp fluorescence will be excited directly, whereas at 270 nm significant excitation by energy transfer from Tyr residues is possible. Thus these results suggest that the fluorescence of Trp^{61} in the mutant W214F is not significantly quenched

TABLE 2 Effect of excitation wavelength on the level of fluorescence quenching of OmpF in di(Br_2C18:0)PC

	F/F_o		
System	270 nm	280 nm	290 nm
Wild type	.48	.49	.54
W61F	.35	.37	.42
W214F	.70	.76	.96

F and F_o are fluorescence intensities for OmpF reconstituted in di(Br_2C18:0)PC and di(C18:1)PC, respectively. Fluorescence was excited at the given wavelengths, and emission was monitored at 320 nm.

by di(Br_2C18:0)PC and that the reduced fluorescence intensity shown in Fig. 4 *B* when fluorescence was excited at 280 nm was due to quenching of the Tyr residues. Thus di(Br_2C18:0)PC is unable to bind at the trimer interface.

Relative lipid binding constants

The fluorescence intensities of OmpF recorded in mixtures of di(C18:1)PC and di(Br_2C18:0)PC decrease with increasing content of di(Br_2C18:0)PC (Fig. 5 *A*); the data were fit to Eq. 1 with a value for n, the number of "sites" from which Trp fluorescence can be quenched, of 2.5 ± 0.25. As shown in Fig. 5 *A*, fluorescence quenching curves for OmpF in mixtures of di(Br_2C18:0)PC and di(C24:1)PC show more fluorescence quenching at intermediate mole fractions of di(Br_2C18:0)PC than is seen in mixtures with di(C18:1)PC. In contrast, less fluorescence quenching is seen at intermediate mole fractions in mixtures with di(C14:1)PC. These results show that the lipid-protein interaction is chain length dependent, with di(C14:1)PC binding to OmpF more strongly than di(C18:1)PC and di(C24:1)PC binding to OmpF less strongly than di(C18:1)PC. Analysis of the data in terms of Eq. 3 gives the binding constant for the lipid, relative to that for di(C18:1)PC; the results plotted in Fig. 6 show that the strongest binding is seen with a chain length of C14, with weaker binding for phosphatidylcholines containing shorter or longer fatty acyl chains.

Stronger binding of phosphatidylcholines with a C14 chain length than those with a C18 chain length was confirmed by studying quenching in mixtures of di(C18:1)PC and di(Br_2C14:0)PC (Fig. 5 *B*). The data fit Eq. 3 with a binding constant for di(C18:1)PC relative to di(Br_2C14:0)PC of 0.71 ± 0.21, equivalent to a relative binding constant for di(Br_2C14:0)PC relative to di(C18:1)PC of 1.40, compared with the value of 1.62 determined from quenching in mixtures of di(Br_2C18:0)PC and di(C14:1)PC (Figs. 5*A* and 6).

Quenching was also determined for W61F (Fig. 7). In mixtures of di(C18:1)PC and di(Br_2C18:0)PC the quenching data fit Eq. 1 with a value for n of 2.8 ± 0.22. The data for quenching in mixtures of di(Br_2C18:0)PC and di(C14:1)PC or di(C24:1)PC fit relative binding constants of 1.52 ± 0.3 and 0.66 ± 0.07, respectively, in good agree-

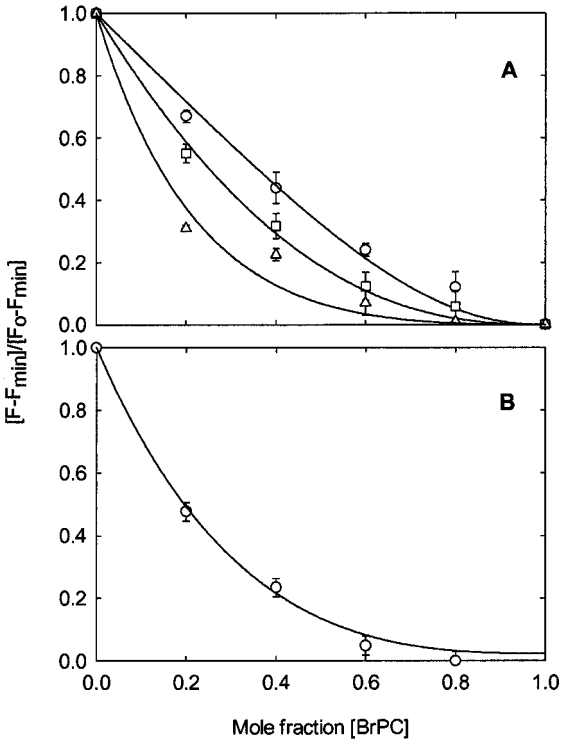

FIGURE 5 Fluorescence intensity for OmpF in mixtures containing di(Br_2C18:0)PC (*A*) or di(Br_2C14:0)PC (*B*). (*A*) OmpF was reconstituted in mixtures of di(Br_2C18:0)PC and di(C14:0)PC (□), di(C18:1)PC (□), or di(C24:1)PC (△). (*B*) OmpF reconstituted in mixtures of di(Br_2C14:0)PC and di(C18:1)PC. Fluorescence intensities are expressed as $(F - F_{min})/(F_o - F_{min})$, where F_o and F_{min} are the fluorescence intensities when the mole fraction of brominated lipid is 0 and 1, respectively, and F is the fluorescence intensity at intermediate mole fractions of brominated lipid. The solid lines show best fits to Eqs. 1–3. In *A*, the fit to the data for mixtures of di(Br_2C18:0)PC and di(C18:1)PC gives the value of n, and fits to the other sets of data with this value for n give the relative lipid binding constants plotted in Fig. 6.

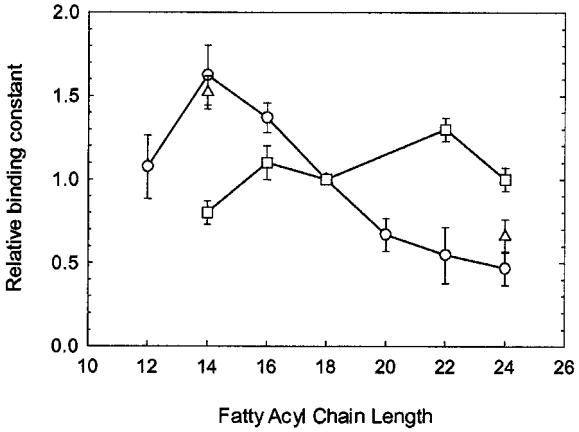

FIGURE 6 Relative lipid binding constants for OmpF. The binding constants of OmpF for phosphatidylcholines relative to that for di(C18:1)PC are plotted as a function of acyl chain length (□). Binding constants for W61F are also shown (△). The data are compared to relative lipid binding constants for the Ca^{2+}-ATPase of sarcoplasmic reticulum (□) taken from East and Lee (1982).

ment with the parameters obtained for wild-type OmpF (Fig. 6).

Effects of lipid headgroup on lipid affinity have also been determined. As shown in Fig. 8, fluorescence quenching in mixtures of di(C18:1)PE and di(Br_2C18:0)PC is comparable to that in mixtures of di(C18:1)PC and di(Br_2C18:0)PC, showing that di(C18:1)PE and di(C18:1)PC bind with similar affinity to OmpF (Table 3). In contrast, fluorescence quenching at intermediate mole fractions of di(Br_2C18:0)PC is higher in mixtures with di(C18:1)PG than in mixtures with di(C18:1)PC, showing that di(C18:1)PG binds with lower affinity to OmpF than does di(C18:1)PC (Table 3).

DISCUSSION

Hydrophobic mismatch

A particularly important feature of a membrane protein is the thickness of its hydrophobic, membrane-spanning region. The cost of exposing hydrophobic fatty acyl chains or peptide residues to water is such that the thickness of the hydrophobic region of the peptide should match the hydrophobic thickness of the bilayer. The question is then how the system responds when these do not match. Most models of hydrophobic mismatch assume that the lipid chains in the vicinity of the protein adjust their length to the hydrophobic thickness of the protein, with the protein acting as a rigid body. When the thickness of the bilayer is less than the hydrophobic length of the peptide, the lipid chains must be stretched. Conversely, when the thickness of the bilayer is greater than the hydrophobic length of the peptide, the lipid chains must be compressed. Stretching the fatty acyl chains

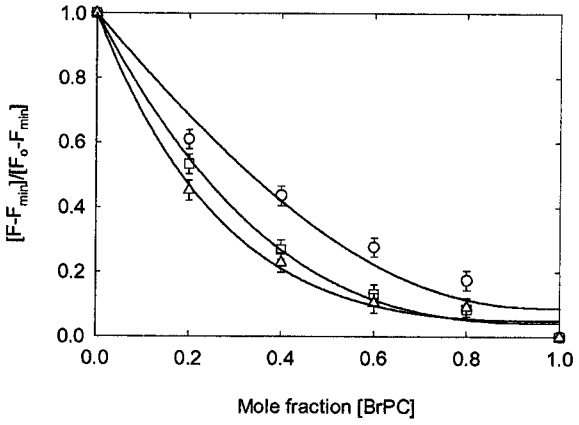

FIGURE 7 Fluorescence intensities for W61F in mixtures containing di(Br_2C18:0)PC. W61F was reconstituted in mixtures of di(Br_2C18:0)PC and di(C14:0)PC (□), di(C18:1)PC (□), or di(C24:1)PC (△).The solid lines show best fits to Eqs. 1–3. The relative lipid binding constants are plotted in Fig. 6.

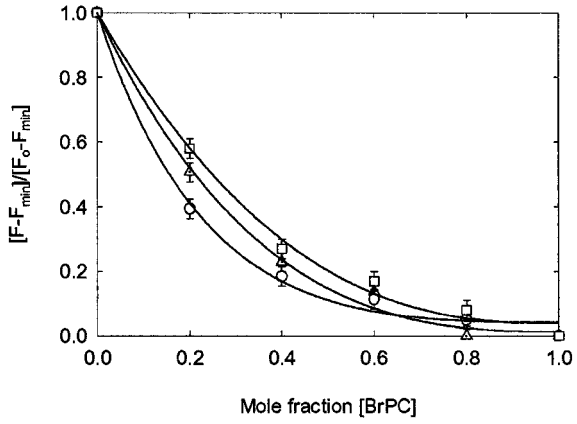

FIGURE 8 Fluorescence intensities for OmpF in mixtures of di(Br_2C18:0)PC and different headgroup phospholipids. OmpF was reconstituted in mixtures of di(Br_2C18:0)PC and di(C18:1)PC (□), di(C18:1)PE (△), or di(C18:1)PG (□). The solid lines show best fits to Eqs. 1–3 with the relative lipid binding constants listed in Table 3.

will effectively decrease the surface area they occupy on the membrane surface, and, conversely, compressing the chains will increase the effective area occupied on the surface.

A number of terms have been suggested to contribute to the total free energy cost of deforming a lipid bilayer around a protein molecule (Fattal and Ben-Shaul, 1993; Nielsen et al., 1998):

1. Loss of conformational entropy of the chains imposed by the presence of the rigid protein wall
2. Bilayer compression/expansion energy due to changes in the membrane thickness
3. Surface energy changes due to changes in area of the bilayer-water interface
4. Splay energy due to changes in the cross-sectional area available to the chains along their length, resulting from curvature of the monolayer surface near the protein

A number of models have been proposed to estimate these terms. Fattal and Ben-Shaul (1993) calculated the total lipid-protein interaction free energy as the sum of chain and headgroup terms. For the chains the loss of conformational entropy imposed by the rigid protein wall was positive (unfavorable), even for perfect hydrophobic matching. The other contribution to the chain term arose from the requirement for hydrophobic matching and the consequent stretching or compression of the chains. The term due to the head-group region was treated as an interfacial free energy, including an attractive term associated with exposure of the hydrocarbon core to the aqueous medium and a repulsive term due to electrostatic and excluded volume interactions between the headgroups. The resulting profile of energy of interaction as a function of hydrophobic mismatch was fairly symmetrical about the point of zero mismatch (Ben-Shaul, 1995). The calculated lipid perturbation energy F (in units of kT/Å of protein circumference) fits the equation

$$F = 0.37 + 0.005(d_P - d_L)^2$$

where d_P and d_L are the hydrophobic thicknesses of the protein and lipid bilayer, respectively, and the unperturbed bilayer thickness is 24.5 Å. The hydrophobic thickness of a bilayer of phosphatidylcholine in the liquid crystalline phase is given by

$$d_L = 1.75(N_c - 1)$$

where N_c is the number of carbon atoms in the fatty acyl chain (Lewis and Engelman, 1983b; Sperotto and Mouritsen, 1988). If all of the lipid perturbation energy were to be concentrated in the first shell of lipids around the protein and assuming that a lipid occupies 6 Å of the protein circumference, the lipid-protein interaction energy would change by 3.6 kJ mol^{-1} for a hydrophobic mismatch of 7 Å, corresponding to an increase in fatty acyl chain length of four carbons, and by 22.8 kJ mol^{-1} for a hydrophobic mismatch of 17.5 Å, corresponding to an increase in acyl chain length of 10 carbons. Changes in interaction energies of 3.6 and 22.8 kJ mol^{-1} correspond to decreases in lipid binding constants by factors of 4.3 and 10^4, respectively. If the change in lipid-protein interaction energy were to propagate out from the protein surface to affect more than the first shell of lipids, effects of hydrophobic mismatch would be reduced. For example, if effects were averaged over three shells of lipids, changes in fatty acyl chain lengths by 4 and 10 carbons from that giving optimal interaction would decrease lipid binding constants by factors of 1.6 and 21, respectively.

The approach of Nielsen et al. (1998) came to rather similar conclusions. The most important energy terms were found to be the splay energy and the compression-expansion term; the splay energy term is most important close to the protein, and the compression-expansion term is more important farther from the protein. Even though the bilayer deformation was calculated to extend some 30 Å from the protein, most of the deformation was found to be concentrated in the component immediately adjacent to the protein.

An alternative model for mismatch is the mattress model of Mouritsen and Bloom (1984, 1993). This again expresses mismatch as the sum of two terms. The first is an excess hydrophobic free energy associated with exposing either lipid chains or the protein surface to the aqueous medium. The second is proportional to the contact area between the lipid chains and the hydrophobic surface of the protein. The calculations showed that, for a protein of hydrophobic

TABLE 3 Relative lipid binding constants for OmpF

Lipid	Relative binding constant
Di(C18:1)PE	0.84 ± 0.12
Di(C18:1)PG	0.55 ± 0.07

Binding constants are calculated relative to the binding constant for di(C18:1)PC.

thickness 20 Å, which matches a bilayer of di(C14:0)PC in the liquid crystalline state, the binding constant for di(C14:0)PC is a factor of ~2.5 greater than for a bilayer of di(C18:0)PC, which will give a bilayer too thick by 7 Å (Sperotto and Mouritsen, 1993).

The results plotted in Fig. 6 show that the phosphatidylcholine with the highest relative lipid binding constant for OmpF is di(C14:1)PC; phosphatidylcholines with shorter or longer fatty acyl chains bind less strongly. The binding constant decreases by a factor of ~3 between di(C14:1)PC and di(C24:1)PC. This contrasts with results with the Ca^{2+}-ATPase, where the binding constants for phosphatidylcholines hardly change with changing chain length in the range C14 to C24 (Fig. 6). The changes in binding constant for OmpF from di(C14:1)PC to di(C18:1)PC are comparable to those calculated by the approaches of Fattal and Ben-Shaul (1993) and Mouritsen and Bloom (1984, 1993), which assume a rigid protein structure around which the lipid bilayer distorts to achieve hydrophobic matching. However, when the fatty acyl chain length is increased beyond C20, changes in the binding constant are relatively small (Fig. 6), whereas theory predicts a continuing decrease in interaction energy. These results suggest therefore that when the fatty acyl chain length is changed from C14 to about C20, the lipid bilayer thins around the β-barrel structure of OmpF, but that beyond a chain length of C20 the β-barrel also deforms to help maximize the interaction with the bilayer. In contrast, the transmembrane region of the Ca^{2+}-ATPase, composed of a bundle of 10 transmembrane α-helices, readily distorts to match changes in lipid bilayer thickness.

OmpF in the *E. coli* outer membrane

The predominant phospholipid in the outer membrane of *E. coli* is phosphatidylethanolamine, with some phosphatidylglycerol and cardiolipin (Harwood and Russell, 1984). The phospholipid component is concentrated in the inner leaflet, whereas the outer leaflet is mostly lipopolysaccharide (Harwood and Russell, 1984). The fatty acyl chain length of the phospholipid component is predominantly C16 or C18, but for the lipopolysaccharide component the major fatty acyl chain length is C14 with some C12 (Harwood and Russell, 1984). The hydrophobic thickness of OmpF, defined by the positions of the bands of aromatic residues at the two membrane-water interfaces, is ~25 Å (Martin, 1983), which matches the hydrophobic thickness of a bilayer of di(C14:1)PC (23 Å). This is consistent with the observation that the phosphatidylcholine showing the strongest binding to OmpF is di(C14:1)PC (Fig. 6). However, it should be pointed out, as discussed by Dumas et al. (1999), that local distortions of the lipid molecules surrounding a membrane protein mean that optimal hydrophobic matching may not necessarily occur when the hydrophobic thickness of the protein matches the hydrophobic thickness of an unperturbed lipid bilayer.

OmpF shows no significant selectivity for phosphatidylethanolamine over phosphatidylcholine (Table 3). However, OmpF has an affinity for the anionic phosphatidylglycerol that is about half that for phosphatidylcholine (Table 3). The structure of OmpF shows no clear regions of separated positive or negative charges in the regions around the hydrophobic transmembrane region that might have given rise to specificity in lipid headgroup binding based on charge.

One possible response of a membrane protein-to-hydrophobic mismatch is to change its state of aggregation, and, for example, bacteriorhodopsin has been shown to aggregate in bilayers containing short fatty acyl chains (Lewis and Engelman, 1983a). OmpF forms trimers in the native bacterial outer membrane that are stable in sodium dodecyl sulfate (Jap and Walian, 1996). After reconstitution into bilayers of phosphatidylcholines, OmpF remains trimeric in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3 *A*), suggesting that OmpF is probably trimeric in the reconstituted bilayer. This is consistent with the lower level of quenching by di(Br_2C18:0)PC observed for Trp^{61} than that observed for Trp^{214} (Fig. 4 and Table 2). Although reconstitution of W214F into bilayers of di(Br_2C18:0)PC leads to a reduced level of fluorescence when excited at 270 nm, the effect is much reduced when fluorescence is excited at 290 nm (Table 2), suggesting that the observed decrease in Trp fluorescence intensity follows from quenching of Tyr fluorescence, resulting in a reduction in Tyr-Trp energy transfer. These results suggest, therefore, that di(Br_2C18:0)PC is largely excluded from the trimer interface where Trp^{61} is located. The crystal structure of the porin of *Rhodopseudomonas blastica* shows three tightly bound detergent molecules located at the trimer threefold axis (Kreusch and Schulz, 1994), suggesting that hydrophobic molecules can bind in this region. Electron microscopic studies of porin reconstituted into bilayers of di(C14:0)PC suggest the presence of lipopolysaccharide at the trimer interface (Hoenger et al., 1990). It is possible, therefore, that di(Br_2C18:0)PC does not bind significantly at the trimer interface because it is unable to compete with tightly bound lipopolysaccharide.

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