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Structure and Assembly of β-Barrel Membrane Proteins*[S]

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Integral membrane proteins fall into two major structural classes; they consist of individual or bundled TM[1] α-helices, or they form monomeric, dimeric, or trimeric TM β-barrels. These folds are dictated by the necessity to form oriented hydrogen-bonded secondary structures in the highly ordered apolar environment of the lipid bilayer. No other structural motif has yet been confirmed for membrane proteins at high resolution, although the helical bundle structure of the nicotinic acetylcholine receptor may be surrounded by a concentric sheath of TM β-sheets (1). Apart from this possible exception, all membrane proteins of plasma and endoplasmic reticulum-derived membranes are α-helical, whereas the proteins of the outer membranes of Gram-negative bacteria and likely a fair number of proteins of the OMs of mitochondria and chloroplasts are of the β-barrel type. These two structural motifs are also recapitulated in various membrane-inserted toxins, which can be considered facultative integral membrane proteins. This review focuses on the structure and assembly of β-barrel membrane proteins. Because high resolution structures are known only for bacterial OM proteins and because our knowledge on the folding and assembly of these proteins is much more advanced than that of mitochondrial or chloroplast OM proteins, we will restrict our comments to the prokaryotic systems.

The Outer Membrane and Periplasmic Space of Gram-negative Bacteria

The lipid bilayer of the OM of Gram-negative bacteria is highly asymmetric. It consists of phospholipids (70–80% phosphatidylethanolamine, 20–30% phosphatidylglycerol, and cardiolipin) in the inner leaflet and LPS in the outer leaflet. LPS has multiple fatty acyl chains and complex polysaccharide structures linked to a glucosamine disaccharide backbone. LPS contains variable amounts of acidic saccharides and is therefore negatively charged. The fatty acyl chains of LPS are saturated and most are 14 carbon atoms long; some are hydroxylated at carbon 3. Thus, the outer leaflet may be more rigid than the inner leaflet, which is composed of phospholipids with many unsaturated chains. The periplasm between the inner membrane and OM contains a cross-linked peptidoglycan layer and a number of soluble proteins. The OM provides a more or less passive protective coat for this class of bacteria; most metabolic membrane functions are carried out by the inner membrane. Because the cells must exchange solutes with the environment, the OM must provide for the selective permeation of such solutes. Nutrients such as sugars, amino acids, vitamins, and metal cofactors need to be taken up. Specific and nonspecific pores, formed by the so-called porins, exist in the OM where they serve as molecular sieves. Scarce vitamins and metal complexes are actively taken up by specialized machines of the OM, which are connected to and powered by proteins of the inner membrane. Other OM proteins, such as TolC, are needed to export waste products and some proteins from Gram-negative bacteria. A brief description of the structures and functions of several outer membrane proteins is provided in the supplemental material to this minireview.

Assembly of β-Barrels in Outer Membrane

All OM proteins are synthesized with a signal sequence and are translocated through the inner membrane by the SecA/Y/E/G export machinery in an unfolded form and in a reaction that requires energy in the form of ATP. Once the protein has arrived in the periplasmic space, the signal sequence is removed by a signal peptidase. Although chaperone proteins may be present in the periplasm (see below), insertion and folding into the outer membrane is believed to be a spontaneous process. There is no specific signal in the sequence that targets this class of proteins to the outer membrane. It is rather the conformation of the mature protein that determines its insertion into the outer membrane (2). Pulse labeling and biochemical reconstitution experiments suggested that LPS might be important for the proper assembly of OmpA and PhoE into isolated outer membranes (3, 4). Unfortunately, these experiments are not entirely conclusive because LPS was offered in the form of micelles (with or without Triton X-100) and the proteins were prefolded into these micelles before they were incubated with outer membranes.[2] We have since learned that OmpA spontaneously refolds into micelles of a large variety of detergents and lipids (see below). Proteins that are prefolded in this manner may be easily inserted into OMs, especially when the membranes are offered in large excess, as was the case in these studies. Therefore, it is still unclear whether or not a prefolded intermediate in LPS really exists *in vivo* prior to insertion into the OM.

Various folding catalysts are known to exist in the periplasm of Gram-negative bacteria. Among them are protein disulfide isomerases, namely the *dsb* gene products, and peptidylprolyl isomerases. The Dsb proteins probably play no role in the folding and assembly of Omps because they generally contain no disulfide bonds. The peptidylprolyl isomerases found in the

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[S] The on-line version of this article (available at *http://www.jbc.org*) contains supplemental material and includes references, Fig. S1, and Table S1.

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[1] The abbreviations used are: TM, transmembrane; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; LPS, lipopolysaccharide; OM, outer membrane; TDFQ, time-resolved distance determination by fluorescence quenching.

[2] See also the third article in this series, "Detergents as Tools in Membrane Biochemistry" by R. M. Garavito and S. Ferguson-Miller (29).

periplasm include SurA and RotA. SurA has been implicated in the folding of OmpA, OmpF, and LamB (5). However, when a purified peptidylprolyl isomerase was included in a refolding reaction of OmpA *in vitro*, it had no effect on the refolding kinetics (6). In addition to its proline isomerization activity, SurA may also function as a molecular chaperone to prevent the misfolding and aggregation of proteins. Skp is another protein that has been postulated to function as a chaperone of Omps in the periplasm. It was the only major periplasmic protein that bound to OmpF on an affinity column (7). Purified Skp binds to OmpA, OmpC, OmpF, and LamB. It also binds to lipid monolayers through weak hydrophobic interactions (8). *In vivo*, Skp is associated with the plasma membrane and can be cross-linked to nascent translocated polypeptide chains of OmpA (9).

Folding of β-Barrels into Lipid Bilayers in Vitro

Even if chaperone proteins bind to unfolded Omps that are in transit from the inner to the OM to prevent their misfolding, all current evidence suggests that the membrane insertion and concomitant folding reaction itself is a spontaneous thermodynamically driven process, which does not require any accessory proteins. Surrey and Jähnig (10) were the first to show that OmpA can be completely refolded into artificial lipid bilayers *in vitro*. Because of the moderate hydrophobicity of the individual TM segments of β-barrel membrane proteins, these proteins can be extracted from membranes in an unfolded form with urea or guanidinium chloride *in the complete absence of detergent*. Several Omps have been shown by CD spectroscopy to be completely denatured in high concentrations of urea or guanidinium chloride. This obviates the need for chaperone molecules in *in vitro* refolding experiments. Rapid dilution of the denaturant in the presence of lipid bilayers leads to refolded, fully membrane-inserted, native conformations as shown for OmpA and OmpF. Refolding of OmpF is complicated by trimer formation after insertion into the membrane, which is an extremely slow process (11). Therefore, most refolding studies in membranes have been carried out with the smaller monomeric OmpA. Although OmpA (12–15), OmpX (16), and OmpF (17) have been refolded in detergents using various protocols,[3] we focus here on the mechanism of folding and insertion into lipid bilayers.

Detailed kinetic experiments on the refolding and insertion of OmpA into lipid bilayers led to the discovery of several kinetically distinguishable folding intermediates and consequently to a quite detailed folding mechanism. Most experiments have been carried out with small unilamellar vesicles of DOPC as model membranes. These membranes are highly curved and have a very fluid chain composition (one *cis* double bond in each chain; chain melting phase transition temperature at −22 °C). Both of these factors facilitate membrane insertion of OmpA in this *in vitro* system. Several different kinetic phases can be distinguished in fluorescence and gel shift experiments, indicating the existence of several intermediates on the folding pathway of OmpA (18). Compared with protein folding in solution, protein folding and insertion into membranes is very slow, taking 20–30 minutes to go to completion at 37 °C. This is not surprising, considering the highly ordered structure and the at least 100-fold higher viscosity of the hydrocarbon chains in even the most fluid lipid bilayers.

[3] The study by Kleinschmidt *et al.* (15) revealed that the most critical parameter for refolding is the presence of an aggregated substrate, *i.e.* the exposure of the protein to a micelle or bilayer surface. Monomeric detergent solutions do not support refolding of OmpA. At detergent concentrations greater than the critical micelle concentration details of the headgroup chemistry, chain composition and size of the micelle play only secondary roles, if any.

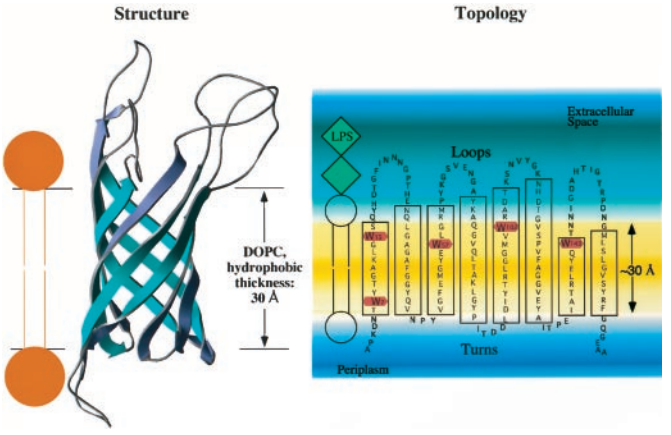

FIG. 1. **Structure and topological model of the transmembrane domain of OmpA.** The NMR structure of the refolded OmpA transmembrane domain (Protein Data Bank entry 1G90) was determined in dodecylphosphocholine micelles in solution (14). The eight transmembrane strands are connected with tight turns on the periplasmic side and long flexible loops on the outer surface. The five tryptophans of OmpA are *highlighted* in the topological model.

The faster phases of folding and membrane insertion are conveniently monitored by changes in tryptophan fluorescence. The fluorescence emission maximum shifts to lower wavelengths and the quantum yield increases when the Trps are transferred from water into the more apolar environment of the lipid bilayer. OmpA has five Trps in the TM domain and no Trp in the periplasmic domain (Fig. 1). The crystal and NMR structures of the TM domain of OmpA (13, 14) predict that all five Trps are located at the polar headgroup/hydrocarbon interface of the membrane.[4] Four Trps are expected to be translocated to the outer surface of the membrane, whereas one Trp (Trp-7) is expected to remain in the inner leaflet. Trp fluorescence experiments detected two kinetic phases. The faster phase was characterized by a rate constant of 0.16 min^{-1} and was quite independent of the measuring temperature. The slower phase was highly temperature-dependent, increasing from 0.35 to 3.3 h^{-1} in the 2–40 °C range. The activation energy of this process was 11.3 kcal/mol. The faster phase most likely corresponds to the binding of unfolded OmpA to the membrane surface, whereas the slower step with the high activation energy corresponds to at least two stages of membrane insertion, as will be discussed below.

Another convenient method of following the folding of OmpA is to utilize the different electrophoretic mobilities of folded and unfolded β-barrel proteins (19). If the samples are not boiled prior to loading on standard SDS-polyacrylamide gels, folded OmpA migrates with an apparent molecular mass of 30 kDa whereas unfolded (or incompletely folded) OmpA migrates at 35 kDa as expected from its sequence. Therefore, this assay can be used to monitor the formation of the β-barrel. Kinetic gel shift experiments show that completion of the β-barrel is slower (~0.6 h^{-1} at 37 °C) than the two fluorescence-detected processes described above (18). This strongly indicates that a closed β-barrel is only formed *after* the Trps are inserted into the lipid bilayer. At temperatures below ~25 °C, this slowest folding step becomes too slow to be detected, and kinetically trapped folding intermediates can be studied.

To better distinguish between different membrane-bound folding intermediates and to follow the path of Trps across the lipid bilayer at higher resolution, we developed a technique

[4] See also the first article in this series, "How Membranes Shape Protein Structure" by S. H. White, A. S. Ladokhin, S. Jayasinghe, and K. Hristova (30).

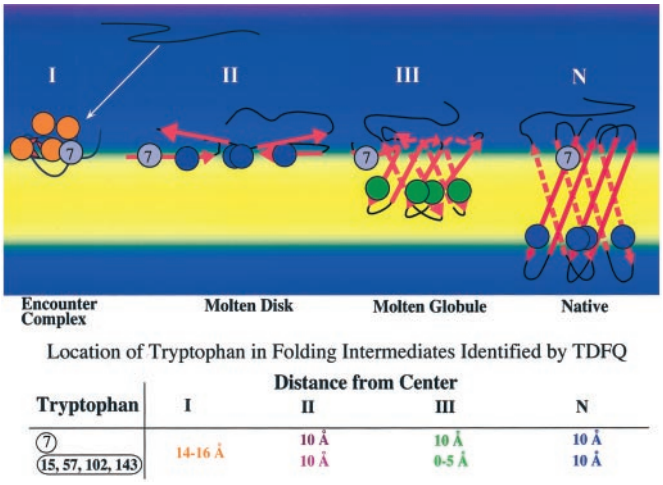

FIG. 2. **Model of folding and membrane insertion of OmpA.** Three membrane-bound intermediate states and the native state are shown. See text for a more detailed description of the folding intermediates. The distances of the Trps from the bilayer center as measured by fluorescence quenching are shown in the *lower part* of the figure.

that we call time-resolved distance determination by fluorescence quenching (TDFQ) (20). In these experiments, refolding is monitored by Trp fluorescence changes as before but in the presence of a series of membrane-bound quenchers of Trp fluorescence. Lipids that are selectively brominated in various positions along the *sn-2* fatty acyl chain are very effective collisional quenchers of Trp fluorescence. Only those Trps that are in the immediate vicinity of a bromine atom are quenched. Because the average positions of the bromines in the lipid bilayer are known to a resolution of a few Å, they serve as effective spectroscopic rulers to measure the average position of Trps during refolding and membrane insertion. Experiments of this type revealed that OmpA refolding proceeds through three membrane-bound folding intermediates (Fig. 2). The first is the fast intermediate that was detected before by the unquenched Trp fluorescence experiments. In this intermediate, the Trps are on average 14–16 Å from the bilayer center, *i.e.* at about the level of the glycerol backbone in a DOPC bilayer. The slower ($\sim$3.3 h^{-1} at 37 °C) process can now be broken up into populating two structurally distinct intermediates, namely a second membrane-bound intermediate with an average location of the Trps $\sim$10 Å from the bilayer center and a third intermediate with delocalized Trps (their average is found in the center of the bilayer). The 10 Å from the bilayer center found in the second intermediate corresponds to the upper hydrocarbon region in DOPC. The third intermediate and the subsequent native state are kinetically only accessible at temperatures >25 °C. The average Trp position in the native state, 9–10 Å, is very close to what was later determined in the crystal and NMR structures of OmpA (13, 14).

Single Trp mutants of OmpA offer the opportunity to separately follow the fate of each Trp during the membrane insertion and folding reaction (21). Because in OmpA four of the five Trps are located on the outer end of four different β-hairpins (Fig. 1), we can explore whether these hairpins translocate sequentially or synchronously in a concerted fashion. This question can be answered quite elegantly by measuring the TDFQ of the respective four Trp mutants and comparing the individual translocation rates. Table I shows the results from such experiments conducted in DOPC bilayers. The dominant translocation rate constants are very similar for all four Trps. These experiments provide strong evidence for a concerted translocation mechanism, *i.e.* the barrel forms as it is inserted. This mechanism makes much sense because a sequential

TABLE I

Rates of Trp translocation across the lipid bilayer during folding of OmpA in DOPC at 40 °C and single channel conductances of refolded OmpA and single Trp mutants in planar lipid bilayers

Mutant	Trp translocation rate[a]	Single channel conductance[b]
	min^{-1}	*pS*
Wild type		66
Trp-7	0[c]	51
Trp-15	0.55 ± 0.09	54
Trp-57	0.46 ± 0.02	67
Trp-102	0.26 ± 0.08	59
Trp-143	0.43 ± 0.09	65

[a] From Ref. 21.
[b] Small channel conductance in 1 M KCl and at 0.1 V (from Ref. 22). The standard errors are approximately 15 picosiemens (pS).
[c] Does not cross lipid bilayer.

mechanism would require the translocation of many open hydrogen bonds, which energetically would be extremely expensive. Table I also shows that the single Trp mutants that were used in these studies formed, after refolding, small channels of similar single channel conductance as the wild-type protein (22).

The slow folding rates of OmpA and the kinetic block of complete folding at low temperatures offers opportunities to study the structural properties of the various folding intermediates. In early experiments, the translocation of the polypeptide chain was arrested by choosing lipids in the gel state. Circular dichroism experiments showed that OmpA formed almost as much β-structure in DMPC in the gel phase (15 °C) as in DMPC in the fluid phase (30 °C) (10). However, the β-strands were not closed into a complete barrel at 15 °C; 30 °C was required for closure of the β-barrel. This result was confirmed by polarized Fourier transform infrared spectroscopy (23). Experiments conducted at room temperature indicated open, surface-oriented β-strands in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (phase transition temperature (T_m) = 41 °C), open but slightly more tilted strands in DMPC (T_m = 24 °C), and a closed β-barrel with inclined strands in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (T_m = −5 °C). More recent unpublished studies from our laboratory suggest that in fluid lipid bilayers the β-sheet content continues to increase as the protein inserts deeper into the membrane. In other words, some secondary structure forms only upon closure of the β-barrel. Despite this late growth of secondary structure, it is clear that some β-strands form early in the folding process, *i.e.* when the protein interacts with the lipid bilayer. Both the nucleation and the growth phase of β-structure formation of OmpA are reminiscent of the partition-folding coupling observed with small helical membrane proteins (24, 25).

Combining the structural and kinetic results, we conclude that the membrane-bound intermediates depicted in Fig. 2 are (**I**) a largely unstructured encounter complex, (**II**) a molten disc, and (**III**) an inside-out molten globule intermediate. Each of these intermediates must not be considered as a defined structure but as a rather large ensemble of structures characterized by common properties. The native state has a well defined structure in the membrane-inserted β-barrel portion, but as shown by NMR, the structures of the loops that emerge from the outer surface are still ill defined and highly dynamic (14). It is possible that interactions with LPS are required to fold the outer loops into a fixed conformation. This view is supported by the fact that OmpA forms a receptor for phage K3 only in the presence of LPS (19). We imagine the molten disc intermediate **II** to consist of individual or perhaps paired amphipathic β-strands that are located in the bilayer interface. Hydrogen bonds between neighboring strands, lipid head-

groups, and water are continuously formed and broken, giving it a relatively compact two-dimensional, yet nonspecific "molten disc" structure. In the inside-out molten globule intermediate **III**, the Trps are clearly more deeply inserted into the membrane. In fact, the largest fluctuations of the Trps relative to their average position in the bilayer are found in this state (20, 21). These fluctuations could arise from highly disordered protein or lipid states or a combination of the two. A complete barrel is still not formed in this state although the β-sheet content is very high. We imagine that in this state the polar residues form a central core that is shielded from the apolar environment of the lipid bilayer. This structural arrangement already reflects the general inside-out architecture of β-barrel membrane proteins. The structure is globally correct but locally incorrect. Hence, the protein forms an inside-out analogue of the "molten globule" state that has been well characterized for soluble proteins (26). We believe that error repair involving extensive remodeling of side chain contacts and main chain interstrand hydrogen bonding (27) eventually leads to the native structure. It is not surprising that this conformational search is many orders of magnitude slower in lipid bilayers than in water.

Conclusions and Outlook

Although we have made in the last few years considerable progress in understanding how β-barrels fold and assemble in lipid bilayer membranes, much still needs to be learned. Obviously, the rates of folding that were measured with pure lipid bilayers of DOPC are too slow to be physiological. However, we believe that this does not detract from the basic mechanisms and individual steps of folding that were discovered in this reconstituted system. We contend that the described *mechanism is physiologically relevant*. We are now faced with the task of finding the components that accelerate this basic mechanism. Periplasmic protein chaperones may serve such a function. However, preliminary experiments from our laboratory show that Skp does not significantly accelerate the folding kinetics of OmpA. It is not an active folding catalyst but rather a passive chaperone that binds to the unfolded chain to prevent misfolding in the periplasm. At very high concentrations, Skp even inhibits folding. Because Skp is bound to the inner membrane (9), it could function to prevent the spontaneous insertion of Omps into the inner membrane. Lipids of the OM could themselves act as active folding catalysts (28). The lipid composition of the inner leaflet of the OM is diverse, and the reason for this complexity is not well understood. It is conceivable that specialized lipids or mixtures of lipids could accelerate the basic folding and assembly reactions of Omps severalfold. It thus appears that Omps have evolved to spontaneously insert into the OM in a thermodynamically driven process. Their design as β-barrels allows them to do so. The lack of a specialized energy-driven folding machinery in the periplasmic space is probably the fundamental reason why Omps are β-barrels and not α-helical bundles.

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REFERENCES

1. Miyazawa, A., Fujiyoshi, Y., Stowell, M., and Unwin, N. (1999) *J. Mol. Biol.* **288,** 765–786
2. Freudl, R., Schwarz, H., Klose, M., Mova, N. R., and Henning, U. (1985) *EMBO J.* **4,** 3593–3598
3. Freudl, R., Schwarz, H., Stierhof, Y.-D., Gamon, K., Hindennach, I., and Henning, U. (1986) *J. Biol. Chem.* **261,** 11355–11361
4. de Cock, H., and Tommassen, J. (1996) *EMBO J.* **15,** 5567–5573
5. Lazar, S., and Kolter, R. (1996) *J. Bacteriol.* **178,** 1170–1773
6. Surrey, T., and Jähnig, F. (1995) *J. Biol. Chem.* **270,** 28199–28203
7. Chen, R., and Henning, U. (1996) *Mol. Microbiol.* **19,** 1287–1294
8. de Cock, H., Schäfer, U., Potgeter, M., Demel, R., Müller, M., and Tommassen, J. (1999) *Eur. J. Biochem.* **259,** 96–103
9. Schäfer, U., Beck, K., and Müller, M. (1999) *J. Biol. Chem.* **274,** 24567–24574
10. Surrey, T., and Jähnig, F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7457–7461
11. Surrey, T., Schmid, A., and Jähnig, F. (1996) *Biochemistry* **35,** 2283–2288
12. Dornmair, K., Kiefer, H., and Jähnig, F. (1990) *J. Biol. Chem.* **265,** 18907–18911
13. Pautsch, A., and Schulz, G. E. (1998) *Nat. Struct. Biol.* **5,** 1013–1017
14. Arora, A., Abildgaard, F., Bushweller, J. H., and Tamm, L. K. (2001) *Nat. Struct. Biol.* **8,** 334–338
15. Kleinschmidt, J. H., Wiener, M., and Tamm, L. K. (1999) *Protein Sci.* **8,** 2065–2071
16. Vogt, J., and Schulz, G. E. (1999) *Structure* **7,** 1301–1309
17. Eisele, J.-L., and Rosenbusch, J. P. (1990) *J. Biol. Chem.* **265,** 10217–10220
18. Kleinschmidt, J. H., and Tamm, L. K. (1996) *Biochemistry* **35,** 12993–13000
19. Schweizer, M., Hindennach, I., Garten, W., and Henning, U. (1978) *Eur. J. Biochem.* **82,** 211–217
20. Kleinschmidt, J. H., and Tamm, L. K. (1999) *Biochemistry* **38,** 4996–5005
21. Kleinschmidt, J. H., den Blaauwen, T., Driessen, A. J. M., and Tamm, L. K. (1999) *Biochemistry* **38,** 5005–5016
22. Arora, A., Rinehart, D., Szabo, G., and Tamm, L. K. (2000) *J. Biol. Chem.* **275,** 1594–1600
23. Rodionova, N. A., Tatulian, S. A., Surrey, T., Jähnig, F., and Tamm, L. K. (1995) *Biochemistry* **34,** 1921–1929
24. Tamm, L. K., and Bartoldus, I. (1990) *FEBS Lett.* **272,** 29–33
25. Han, X., and Tamm, L. K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13097–13102
26. Ptitsyn, O. B. (1995) *Curr. Opin. Struct. Biol.* **5,** 74–78
27. Rumbley, J., Hoang, L., Mayne, L., and Englander, S. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 105–112
28. Bogdanov, M., and Dowhan, W. (1999) *J. Biol. Chem.* **274,** 36827–36830
29. Garavito, R. M., and Ferguson-Miller, S. (2001) *J. Biol. Chem.* **276,** 32403–32406
30. White, S. H., Ladokhin, A. S., Jayasinghe, S., and Hristova, K. (2001) *J. Biol. Chem.* **276,** 32395–32398