Journal of Bioenergetics and Biomembranes, Vol. 25, No. 6, 1993

Transport across the Bacterial Outer Membrane

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Received June 9, 1993; accepted July 15, 1993

Diffusion of small molecules across the outer membrane of gram-negative bacteria may occur through protein channels and through lipid bilayer domains. Among protein channels, many examples of trimeric porins, which produce water-filled diffusion channels, are known. Although the channels are nonspecific, the diffusion rates of solutes are often drastically affected by their gross physicochemical properties, such as size, charge, or lipophilicity, because the channel has a dimension not too different from that of the diffusing solutes. In the last few years, the structures of three such porins have been solved by X-ray crystallography. It is now known that a monomer unit traverses the membrane 16 times as β-strands, and one of the external loop folds back into the channel to produce a narrow constriction. Most of the static properties of the channel, such as the pore size and the position of the amino acids that produce the constriction, can now be explained by the three-dimensional structure. Controversy, however, still surrounds the issue of whether there are dynamic modulation of the channel properties in response to pH, ionic strength, or membrane potential, and of whether such responses are physiological. More recently, two examples of monomeric porins have been identified. These porins allow a very slow diffusion of solutes, but the reason for this low permeability is still unclear. Finally, channels with specific binding sites facilitate the diffusion of specific classes of nutrients, often those compounds that are too large to penetrate rapidly through the porin channels. Lipid bilayers in the outer membrane were shown to be perhaps 50- to 100-fold less permeable to uncharged, lipophilic molecules in comparison with the bilayers made of the usual glycerophospholipids. This is caused by the presence of a lipopolysaccharide leaflet in the bilayer, and more specifically, by the presence of a larger number of fatty acids in each lipid molecule, and by the absence of unsaturated fatty acids in the lipopolysaccharide structure.

KEY WORDS: Porin; channel; permeability; membrane protein; β-barrel; lipopolysaccharide; bilayer; fluidity

INTRODUCTION

Bacteria (or more correctly, eubacteria, which exclude archaebacteria) are divided into the gram-positive group and the gram-negative group. This distinction was originally made on the basis of a staining reaction, but it actually corresponds to a fundamental difference in the construction of the cell surface layers. Cells of gram-negative bacteria, which are far more numerous and diverse than the gram-positive group, are covered by the outer membrane, an additional membranous structure present outside the cytoplasmic membrane and the peptidoglycan layer (the "cell wall" in the narrow sense). The major function of the outer membrane is to act as a coarse sieve that excludes many of the noxious molecules that exist in the external medium (Nikaido and Vaara, 1985). This notion is corroborated by the observation that 95% of the newly discovered antibiotics act only on gram-positive bacteria, which are not covered by the outer membrane barrier (Vaara, 1993).

The gram-negative bacteria nevertheless must exchange some molecules with the environment: nutrients must come into the cytoplasm, and waste

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0145-479X/93/1200-0581$07.00/0

products must be excreted. Most of this flux through the outer membrane takes place through protein channels. There are several types of channels in this membrane.

(i) Proteins that we named porins contain large, open, water-filled channels that nonspecifically mediate the passive penetration of ions and small, hydrophilic nutrient molecules. We now know the existence of at least two types of porins. The "classical" trimeric porins that confer very high permeability, and the monomeric porins that confer much lower permeability.

(ii) Porin-like proteins containing specific binding sites carry out the facilitated diffusion of some nutrients that penetrate only slowly through the nonspecific porin channels. These proteins are called specific channels.

(iii) Large nutrient molecules that exist in very low concentrations outside (vitamin B_{12}, Fe^{3+}-chelator complexes) appear to be actively accumulated across the outer membrane by a system composed of a high-affinity outer membrane "receptor" and the TonB protein, which is anchored in the cytoplasmic membrane and is thought to couple solute translocation to energy input. Since the last group of proteins are discussed elsewhere in this issue, only the porins and specific channels are dealt with here. Because of the limitations in space, earlier data cited in the review of Nikaido and Vaara (1985) will often be presented without mention of the source. I also apologize for the inability to cite many important studies published in recent years.

CLASSICAL PORINS

Porins of enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, were identified by searching for outer membrane proteins that produced nonspecific diffusion channels in reconstituted proteoliposomes. The archetypal porins, such as OmpF (outer membrane protein F), OmpC, and PhoE of *E. coli* K12, are proteins of 36,000–38,000 Da, and in terms of mass they are usually the most abundant proteins in *E. coli*. They form tightly associated trimeric structures, which do not dissociate even in sodium dodecylsulfate, unless they are heated to about 70°C or above. Such trimeric porins are apparently widespread among various branches of gram-negative eubacteria (cited in Nikaido, 1992). Furthermore, a trimeric porin was discovered in *Thermotoga maritima* (Rachel *et al.*, 1990), an organism that belongs to the deepest and the most slowly evolving branch of eubacteria. Thus, the production of outer membrane containing trimeric porins appears to have been a very old property among eubacteria.

These porins allow the diffusional influx of small nutrient molecules, but exclude toxic compounds that are frequently large and lipophilic. The pore size was estimated in several ways. Initially, the efflux of radiolabeled, hydrophilic probes from proteoliposomes reconstituted with purified porins was measured. Experiments of this type with *E. coli* and *S. typhimurium* porins showed that although the efflux of disaccharides was nearly complete, tetrasaccharides remained completely within the vesicles after 10–20 min. This suggested that the "exclusion limit" of the channel was roughly 600 Da. In the second type of experiments, the rates of diffusion of sugars of various sizes were measured by following the osmotic swelling of porin-containing proteoliposomes. This approach is more informative because it yields the relative rates of diffusion of a series of sugars, rather than the absolute rates. This study led to an estimate for a pore diameter of about 11 Å for *E. coli* porins. In the third approach, several investigators attempted to determine the pore size from the conductivity of a "single channel," inserted into planar lipid bilayers. In this method, assumptions were made that the electrolyte solution inside the channel behaved as a continuous conductor, and showed the same electrical conductivity as the bulk solution. When we consider that the diameter of the hydrated ions is close to the diameter of the channel determined above, it becomes clear that this simplistic assumption cannot be used. Because what conducts electricity in solutions are the ions, the flux of ions through the channel must be treated as a movement of hydrated ions of discrete size, not as the flow of current through the hypothetical continuous matter. Nevertheless, it has been claimed that the method yielded similar estimates of about 10 Å diameter. It is clear now that this was due to the fortuitous circumstances that what was thought to be "a single channel" corresponded to the simultaneous insertion of three channels within a trimer, and the use of incorrect figures for the length of the channel (Nikaido, 1992).

In the earlier days of porin research, various claims were made that the channels are specific for ligands of certain types. However, to date there is no convincing evidence that real specificity, in the sense used in biochemistry, exists in the diffusion process through the porin channel. Since the channel is

narrow, the flux is obviously affected by the gross physicochemical properties of the pore. Thus, even among the solutes whose size is well within the "exclusion limit," the size exerts a very strong influence on diffusion rate through the channel. With *E. coli* OmpF porin for example, disaccharides of 342 Da diffuse at rates nearly one hundredfold less than the rate of diffusion of pentoses of 150 Da. In terms of the electrical charge, cations are slightly favored over anions by *E. coli* porins OmpF and OmpC, whereas the converse is true with PhoE porin. Among compounds of similar sizes, lipophilicity retards the penetration through the porin channel, possibly because of the presence of a water "cage" surrounding lipophilic solute molecules. Because of these restrictions, even the influx of small nutrient molecules frequently becomes retarded. It is presumably for this reason that an *E. coli* cell has nearly 100,000 copies of porins.

"Static" structures such as the permanently open, fixed-sized channels are rare in biology. Thus, there have been many attempts to show the existence of dynamic plasticity in porin function. Reconstitution of *E. coli* porins into planar lipid bilayers revealed, in several laboratories, that the channel could be closed by the application of voltage over a certain threshold value. This "voltage gating" phenomenon was thought by some to function in the regulation of the outer membrane permeability. The porins in the mitochondrial outer membrane are still referred to by some workers as VDAC (voltage-dependent anion channel). However, it is clearly impossible to have a sustained electrical potential difference across bacterial or mitochondrial outer membranes, which contain such high densities of nonspecific open channels. The only transmembrane potential that can exist across such membranes is the Donnan potential, which has, however, been shown to cause no closing or opening of the *E. coli* porin channel (Sen *et al.*, 1988). Thus, the voltage gating appears to be a laboratory artefact, although it is not without interest as a tool in studying the mechanisms of such processes.

Application of patch-clamping methods to spheroplasts of *E. coli* showed the existence of a pressure-sensitive channel, and the channel was thought to correspond to the porin channel in the outer membrane (Martinac *et al.*, 1987; Buechner *et al.*, 1990). This was intriguing as it was possible to hypothesize the opening and closing of porin channels in response to fine fluctuations in the consumption of nutrients and the excretion of waste products. However, similar pressure-sensitive channels were found in the cytoplasmic membrane of a gram-positive bacterium, *Streptococcis faecalis*, which lacks the outer membrane (Zoratti and Petronilli, 1988), and by fractionating *E. coli* membranes Berrier *et al.* (1989) showed conclusively that the pressure-sensitive channel comes only from the cytoplasmic membrane.

In one of the earliest studies of *E. coli* porins in black lipid films, Benz *et al.* (1979) observed that a decrease, from 9 to 3, in the pH of the bathing solution produced a significant decrease in the pore size. More recently, Todt *et al.* (1992) confirmed this finding and showed that the narrowing of the channel occurred already at pH 5.4. Since this is not far from the pH of some cultures of *E. coli*, this presumed conformational transition could indeed have some physiological significance. It will be important to see whether such a transition occurs in intact cells.

The porins are not hydrophobic proteins and do not contain any of the long stretches of hydrophobic amino acids typically found in integral proteins of the plasma membrane. These puzzling observations were explained by the solution of the three-dimensional structures of porins. Many of the features of the porin structure were initially elucidated by electron crystallography (Jap and Walian, 1990; Jap *et al.*, 1991). But the details became clear from the X-ray crystallographic structure of porin from *Rhodobacter capsulatus* at 1.8 Å resolution (Weiss *et al.*, 1991; Schiltz *et al.*, 1991; Weiss and Schulz, 1992). Like other classical porins, this porin exists as a stable trimer. The polypeptide chain of each subunit traverses the membrane 16 times as antiparallel β-strands, forming a β-barrel structure surrounding a large channel. Each subunit produces a channel, and the trimer therefore contains three channels. Because every other amino acid side-chain faces into the hydrophilic channel, a β-barrel spans the membrane without stretches of consecutive hydrophobic residues.

The solution of the crystal structure of *R. capsulatus* porin helped the study of *E. coli* OmpF porin, which is perhaps the first intrinsic membrane protein that yielded diffraction-grade crystals (Garavito and Rosenbusch, 1980). It became possible to determine the arrangement of OmpF trimers within a unit cell of the crystal, by using the molecular replacement method (Pauptit *et al.*, 1991). Selection of an appropriate crystal form then led to the solution of the crystal structure of *E. coli* OmpF and PhoE porins (Cowan *et al.*, 1992).

There is little sequence homology between the *E.*

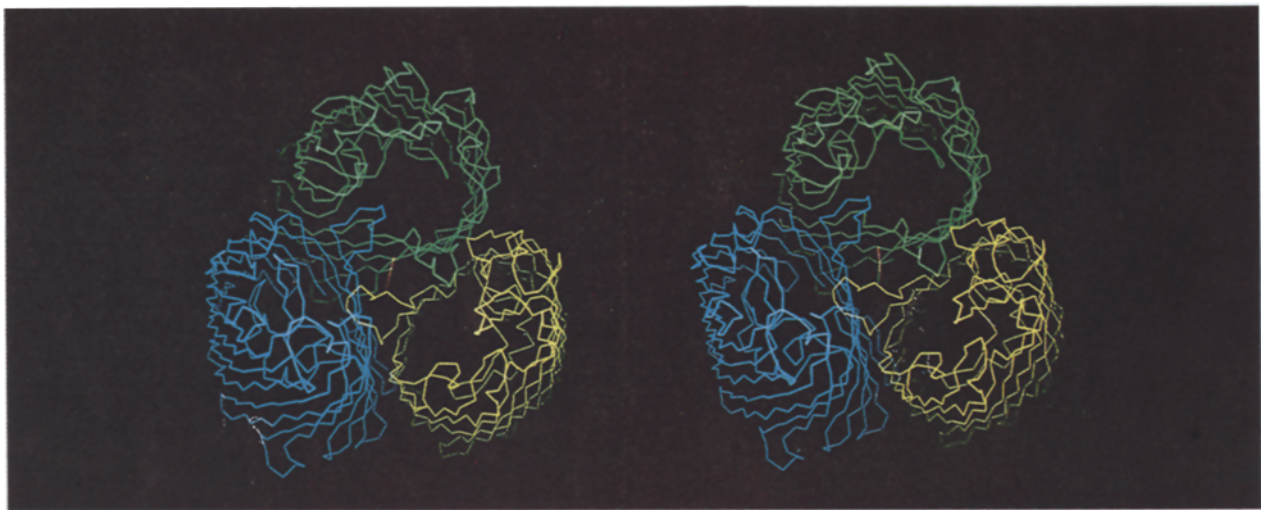

Fig. 1. Structure of porin OmpF trimer. The tracing of the α-carbons is shown, using a different colour for each subunit. Each subunit is seen to produce a central channel. Because the view is slightly askew from the three-fold axis (which is perpendicular to the membrane surface), one can see the construction of the β-barrels. From Cowan *et al.* (1992) with the permission of the authors and the publisher.

coli and *R. capsulatus* porins, yet these porins were shown to fold in a pattern remarkably similar to each other. Each subunit of these porins produces a transmembrane channel by forming a 16-strand β-barrel, as mentioned earlier (Fig. 1). However, the pores are narrowed at one point to a cross-section of 8×10 Å (*R. capsulatus*) or 7×11 Å (*E. coli* OmpF). The latter value is in remarkable agreement with the size determined from the relative diffusion rates of sugars, mentioned above. Because the distance between neighboring β-strands is 4.5 Å, 16 β-strands should produce a pore with a backbone-to-backbone diameter of $(4.5 \times 16)/\pi = 23$ Å. That the actual diameter is much less is due to the fact that one of the "extramembranous" loops folds back into the channel and produces a narrow region of limited depth. This arrangement produces a channel with a wide entrance, a wide exit, and a short central constriction. Such a channel serves well the physiological needs of the bacteria, because it is effective in excluding larger solutes while minimizing the frictional interactions between the solutes and the walls of the pore. Thus, small molecules can diffuse across the outer membrane quite rapidly.

The constriction, or the "eyelet," contains several negatively charged residues on one side, and several positively charged residues on the opposite side. Because of the electrostatic forces the side-chains of these charged residues are maximally extended, and thus this arrangement produces a structure of well-defined and rigid shape, a necessity for a pore that would exclude larger solutes efficiently.

Many porin mutants of larger channel size have been isolated in the laboratory of Benson and Misra by selecting for strains that are able to grow on maltodextrin oligosaccharides in the absence of the specific channel, LamB. Remarkably, most of the point mutants alter one of the charged residues that form the electrostatically rigidified eyelet mentioned above (Cowan *et al.*, 1992), replacing Arg residues at 42, 82, and 132 in OmpF (or corresponding Arg residues at positions 37, 74, and 105 in OmpC) with uncharged residues, or replacing Asp^{113} in OmpF (or corresponding Asp^{105} in OmpC) with an uncharged residue (Benson *et al.*, 1988; Misra and Benson, 1988). As expected, deletions within the eyelet-forming loop (L3) also produce widening of the channel (Cowan *et al.*, 1992).

PhoE porin, which is produced in *E. coli* under phosphate starvation conditions, has a preference for anions in contrast to the cation preference of OmpF or OmpC porins; this alteration in charge preference was traced to the Lys^{125} of PhoE, which replaces a glycine residue of OmpF (Bauer *et al.*, 1989). These residues are located in the eyelet-forming loop in terms of the primary structure. In terms of the tertiary structure, it can be seen that they are located at the narrowest portion of the channel, where a

strong influence on ion selectivity is expected (Cowan *et al.*, 1992; Struyve *et al.*, 1993).

In view of the assumptions made by some workers that porin channels are normally closed and become open only upon application of a stimulus, it is noteworthy that in all crystal structures the channel is always fully open. Furthermore, the β-barrel "backbone" of the pore appears quite rigid. However, since the solute-discriminating properties of the pore are determined not by the β-barrel itself but by one of the "external" loops, the possibility still remains that modulations of pore properties by external conditions, such as pH, may take place by the movement of one or more of the loops.

MONOMERIC PORINS

In addition to these classical trimeric porins, there are also monomeric proteins that exhibit pore-forming activity. The major porin OprF of *Pseudomonas aeruginosa* was a lone representative of this class for many years. This protein can be purified as an active monomer. The channels produced by this protein had puzzling properties. They allowed the diffusion of larger solutes in comparison with *E. coli* porins, and the channel diameter was estimated to be about 20 Å, about twice the width of *E. coli* porin channels. Yet the rate of diffusion of solutes through a unit amount of this porin was about two orders of magnitude slower both in a reconstituted system and in the intact outer membrane. Because of these unexpected properties, some workers proposed that OprF is not a porin. Such a claim appeared to have been supported when it was discovered that the amino acid sequence of OprF was homologous to that of OmpA of *E. coli*, a monomeric outer membrane protein that was not known to form pores. When the properties of OmpA were reexamined, however, it was found that OmpA was indeed a porin, although it allowed only slow diffusion of solutes (Sugawara and Nikaido, 1992). At the same time, the pore-forming functions of OprF was confirmed both in a reconstituted system (Nikaido *et al.*, 1991) and in intact cells (Bellido *et al.*, 1992).

The reason why these monomeric porins produce such low rates of penetration has not been elucidated. It seems possible that only a small fraction of the protein population folds in such a manner to produce functional channels. Alternatively, it is possible that the channels offer significant resistance to the passage of solutes, perhaps because amino acid side chains protrude into the channel.

SPECIFIC CHANNELS

The LamB protein (phage λ receptor) of *E. coli* is the best studied outer membrane transporter catalyzing a specific diffusion process. Since disaccharides such as maltose penetrate the porin channels rather slowly, these cells need a mechanism for facilitating the transport of maltose and larger maltodextrins across the outer membrane. The LamB protein is a porin-like trimeric protein most probably constructed also as a β-barrel (Ferenci *et al.*, 1988). This protein produces open channels that also allow the diffusion of small solutes structurally unrelated to maltose (Luckey and Nikaido, 1980; Benz *et al.*, 1987). The structural similarity between LamB and classical porins suggests that in both channels the solute diffusion occurs by an essentially similar mechanism, i.e., through water-filled channels. However, there is a specific binding site for maltose within each LamB channel (Benz *et al.*, 1987). Theory predicts that such a channel would produce Michaelis–Menten or saturation-type kinetics of diffusion (Benz *et al.*, 1987). Thus, the downhill diffusion of specific ligands through such a channel would become accelerated at low external concentrations. Indeed, this saturation phenomenon was experimentally demonstrated for LamB (Freundlieb *et al.*, 1988). Interestingly, a recent study suggests that under carbon starvation conditions the biosynthesis of LamB becomes derepressed, and that LamB plays a major role in the influx of small sugars and sugar alcohols such as glucose and glycerol, because the binding site of the LamB channel binds these compounds, albeit with low affinity (Death *et al.*, 1993).

Because the LamB channel is exposed on the cell surface, it binds even macromolecular ligands, such as starch. Researchers in Ferenci's group have devised ingenious methods to use starch binding for the isolation of *lamB* mutants altered in the ligand-binding activity, using either affinity columns (Ferenci and Lee, 1982) or retardation of chemotaxis in starch-containing plates (Heine *et al.*, 1988). Studies of these mutants showed that the maltose-binding activity was affected by mutations in residues 8–18, 74–82, and 118–121, the latter two groups occurring in the presumed third external loop (Heine *et al.*, 1988). Interestingly, it is this loop that in porins folds into the

β-barrel and produces the solute-discriminating constriction (Weiss *et al.*, 1991; Cowan *et al.*, 1992).

Another specific channel in *E. coli* is the Tsx (phage T6 receptor) protein. This protein was shown to facilitate the influx of nucleosides in intact cells, and the purified protein was shown to produce a channel with a specific binding site for nucleosides (Maier *et al.*, 1988). The sequence of the protein reveals no long hydrophobic stretches (Bremer *et al.*, 1990) and the protein therefore is likely to produce also a β-barrel. A plasmid-coded protein, ScrY, was shown to produce a channel with a somewhat LamB-like carbohydrate-binding site (Schulein *et al.*, 1991). This protein was crystallized in a form that diffracts up to 2.3 Å (Forst *et al.*, 1993).

P. aeruginosa outer membrane lacks a classical porin entirely, and the nonspecific diffusion across the outer membrane occurs very slowly in this organism. It is thus anticipated that specific channels will play a more important role in the uptake of nutrients in this organism. The function of one of them, protein D2 or OprD, was discovered because of its role in the influx of an antibiotic, imipenem. Imipenem is a carbapenem that shows excellent activity against *P. aeruginosa*, but its therapeutic use results in the rapid selection of resistant mutants. Quinn *et al.* (1986) showed that these mutants are deficient in one outer membrane protein, which was later identified as D2. It was also shown that this protein produces a diffusion channel, with a specific binding site for imipenem and basic amino acids (Trias and Nikaido, 1990). The diffusion of imipenem through this channel was shown to follow saturation kinetics (Trias *et al.*, 1989).

Another specific channel in the *P. aeruginosa* outer membrane is protein D1 or OprB, which is induced by growth in the presence of glucose (Hancock and Carey, 1980), and on reconstitution forms a channel that prefers D-glucose and D-xylose (Trias *et al.*, 1988). Protein P (OprP) is induced by growth under phosphate-starvation conditions (Hancock *et al.*, 1982). This protein, however, is quite different from *E. coli* PhoE protein that is induced under similar conditions. PhoE is a nonspecific porin, which has a slight general anion preference. It does not prefer phospate over other anions, and it has not been possible to show the existence of a specific phosphate-binding site in this protein (Benz *et al.*, 1984). (In this sense, it is misleading to call this protein "phosphoporin.") In contrast, OprP has a nearly absolute preference for anions, and one can show the existence of a phosphate-binding site with a K_D of 0.3 mM (Hancock and Benz, 1986). More recently, a pyrophosphate-specific channel, OprO, was discovered in *P. aeruginosa* (Hancock *et al.*, 1992).

β-BARRELS IN OUTER MEMBRANE PROTEINS

As in the porins and the LamB protein, the sequences of almost all of the outer membrane proteins appear to have β-sheet structures (see Nikaido, 1992). This forms a striking contrast to the better-known integral proteins of the plasma membranes, which seem to contain multiple transmembrane segments of hydrophobic α-helices. It seems likely that the β-barrel construction is related to the mechanism of export. Thus, the outer membrane proteins, which have a low overall hydrophobicity, may be exported easily into the periplasm (Sen and Nikaido, 1990). Upon insertion into the outer membrane, they refold into their stable β-barrel conformations through an interaction with lipopolysaccharide (LPS), a lipid unique to the outer membrane (Sen and Nikaido, 1991). If these proteins were to contain sequences that could form hydrophobic transmembrane α-helices, they probably would be retained by the plasma membrane and would not be exported into the periplasm. Interestingly, the β-barrel design appears to be present in at least one eukaryotic protein, forming large, open channels, the mitochondrial porin, found in the mitochondrial outer membrane (Blachly-Dyson *et al.*, 1990); possibly this is a legacy of the bacterial symbiont origin of this organelle.

DIFFUSION THROUGH THE LIPID BILAYER

The diffusion of hydrophilic solutes through the outer membrane is thus restricted by the properties of the individual pores and the number of channel-forming proteins. However, this constraint would become meaningless if noxious molecules could flow in by diffusing through the lipid domains of the outer membrane. Spontaneous diffusion across the lipid bilayer is often thought to be a process limited to very lipophilic solutes. However, this is not the case. For example, a fairly hydrophilic, zwitterionic cephalosporin of 415 Da (e.g., cephaloridine) is severely hindered in its diffusion through the monomeric porin channel of *P. aeruginosa*, OprF, showing a very low permeability coefficient of only 1×10^{-6} cm/s (Yoshimura and Nikaido, 1982). However,

similar-sized molecules with only a modest lipophilicity (oil/water partition coefficient of 0.1) traverse the usual glycerophospholipid bilayers 100 times faster, with a permeability coefficient of about 1×10^{-4} cm/s (Stein, 1967), and thus the restriction imposed by the property and the number of the pore would become meaningless, if this were to occur. These considerations suggest that the lipid domain of the outer membrane must have a much lower permeability in comparison with the usual glycerophospholipid bilayers.

Recently we determined the diffusion of highly lipophilic solutes across the lipid bilayer domains of the outer membrane (Plesiat and Nikaido, 1992). The approach was based on that of Zimmermann and Rosselet (1977), which had been used in measurement of the permeability through the porin pathway (Nikaido and Vaara, 1985). We incubated intact cells of gram-negative bacteria in solutions containing 3-oxosteroids. These steroid molecules diffuse spontaneously across the outer membrane bilayer, and then rapidly across the usual phospholipid bilayer of the cytoplasmic membrane, to be oxidized finally by the 3-oxosteroid dehydrogenase coded by a *P. testosteroni* gene cloned into a recombinant plasmid. Since the diffusion across the outer membrane is clearly the rate-limiting step, one can calculate the permeability of the outer membrane bilayer to various steroid molecules. We also confirmed that the diffusion was occurring through the lipid domain of the outer membrane, because the influx rate increased 25–100 times when the lipid domain was perturbed, either by adding 0.5 μg/ml of deacyl-polymyxin B, which binds to the LPS and disturbs their packing (Vaara, 1992), or by using mutant strains producing a very deficient, "deep rough"-type LPS (Nikaido and Vaara, 1985). Using this system, we found that the outer membrane bilayer is indeed less permeable, probably by a factor of 50–100, in comparison with the phospholipid bilayer of the typical cytoplasmic membrane. However, it still allows an influx of highly lipophilic solutes at a significant rate. For example, androstanediol diffuses with a permeability coefficient of 1×10^{-5} cm/s, a value comparable to the rate of diffusion through the OmpF porin channels of the *E. coli* outer membrane of a moderately lipophilic cephalosporin, cephalothin (Nikaido and Vaara, 1985).

The outer membrane bilayer thus is not a perfect barrier against the penetration of uncharged, hydrophobic agents, but it is a very effective barrier against amphiphilic compounds, containing charged groups. With the compounds of the latter class, the intrinsic low permeability of the bilayer is made far more effective because only a small fraction of such molecules would exist in the uncharged form at a physiological pH. This is probably a major reason that the *S. typhimurium* outer membrane was earlier found to be totally impermeable to nafcillin, a hydrophobic penicillin containing a carboxyl group. Similar reasoning shows that taurocholic acid, with its strongly acidic sulfate group, should be essentially impermeable through the outer membrane bilayer of enteric bacteria, in contrast to cholic acid, which is a weak acid. This makes ecological sense, because it is known that most of the bile acids exist as conjugated bile acids in the intestinal tract. However, these considerations show that the outer membrane cannot be an effective barrier against a lipophilic agent, a significant fraction of which exists in an uncharged form. Since lipophilic uncharged agents traverse the cytoplasmic membrane even more rapidly, the only defense gram-negative bacteria can use against these compounds would be either their inactivation, or active extrusion. This probably explains the existence of cytoplasmic enzymes that inactivate cholic acid in *E. coli* (Yoshimoto *et al.*, 1991), and the widespread occurrence of extrusion systems with different specificities (Levy, 1992).

What is the structural basis for this low permeability of the lipid domain of the outer membrane? Since LPS is a component that exists uniquely in the outer membrane, it is likely to be responsible for this property of the bilayer. Furthermore, the outer leaflet of this bilayer was found to contain only LPS molecules, and mutational alterations of LPS sometimes drastically increase the permeability of the outer membrane (Nikaido and Vaara, 1985). When lipophilic solutes traverse any lipid bilayer, they must first enter the hydrocarbon interior of the bilayer. This is thought to occur when transient lacunae are created in the bilayer, by the lateral mobility of the hydrocarbon chains. More lacunae will thus be created by an increase in both the intramolecular movement of hydrocarbon chains and the intermolecular movement of one lipid molecule against another. Increases in unsaturated fatty acid residues will obviously contribute to the former, but what determines the latter parameter is not immediately obvious. The answer is suggested by the finding that *Thermus aquaticus*, which can grow at temperatures of 70–80°C, renders the membrane sufficiently impermeable even at these temperatures by introducing into its membrane a glycolipid containing three, rather than

two, fatty acid chains (reviewed by Nikaido, 1990). Thus, increases in the number of hydrocarbon chains within a single lipid molecule increase the contact between the lipid molecules, and thus presumably prevent the formation of lacunae arising from the movement of one lipid molecule against another.

We can see that the LPS monolayer utilizes both of these principles to produce a structure of lower permeability. First, all of the fatty acid chains in the LPS are saturated fatty acids. Thus, the interior of the LPS leaflet has very low fluidity, as confirmed by experiments using spin labels (Nikaido *et al.*, 1977) and X-ray diffraction (Labischinski *et al.*, 1985, 1989). Second, each LPS molecule contains 6–7 fatty acid chains, in contrast to the glycerophospholipid molecule that contains only two such chains. That this results in a very large increase in the energy of interaction between neighboring LPS molecules can be seen from the fact that when an aggregate of LPS is diluted with glycerophospholipids, islands of LPS persists, presumably because of the strong interaction between LPS molecules, for several days or more in a sea of phospholipids, even at 37°C (Takeuchi and Nikaido, 1981).

An additional example of these strategies was recently discovered. Cell walls of mycobacteria were known to contain lipids of unusual structures, but the organization of these lipids remained unknown. Recent X-ray diffraction study (Nikaido *et al.*, 1993) showed that a major portion of the hydrocarbon chains in the cell wall are most probably arranged in a bilayer structure. The lipid domains of the "outer membrane" of this new type, however, are even less permeable than those of the gram-negative outer membrane (Nikaido *et al.*, 1993). Indeed, the major fatty acid species in the mycobacterial cell wall, mycolic acid, contains much longer hydrocarbon chains. (Mycolic acid has a branched structure, and thus one molecule contains a chain of more than 40 carbon atoms, and another with 22 carbon atoms.) There are very few double bonds in this structure. Finally, in contrast to LPS that contains 6–7 fatty acids per molecule, thousands of mycolic acid residues are joined to a single, common, macromolecular head group, arabinogalactan. Permeation of small, hydrophilic molecules was shown to occur through water-filled channels of mycobacterial porins (Trias *et al.*, 1992).

ACKNOWLEDGMENTS

Research in the author's laboratory was supported by grants from the National Institutes of Health (AI-09644) and from the Lucille P. Markey Charitable Trust.

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