Effects of pH on Bacterial Porin Function

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ABSTRACT: Porin is a trimeric channel-forming protein in the outer membrane of Gram-negative bacteria. Functions of the porins OmpF, OmpC, and PhoE from *Escherichia coli* K12 were analyzed at various pHs. Preliminary results from bilayer lipid membrane and liposome swelling assays indicated that in vitro porin has at least two open-channel configurations with a small and a large size. The small channels were stabilized at low pH while the larger channels were detected under basic conditions. The size switch occurred over a very narrow range near neutral pH, and the two major open-channel configurations responded differently to variations in voltage. The presence of two or more pH-dependent substates of porin could explain the variability in pore diameter measured by others and suggests a more dynamic role for porin in the cell.

Diffusion of small hydrophilic molecules (≤600 daltons) through the outer membrane of Gram-negative bacteria occurs via pore-forming proteins called porins. The two main nonspecific porins of *Escherichia coli* are OmpC and OmpF. OmpF has a larger channel than OmpC and is induced under conditions of low osmolarity. PhoE is an anion-selective porin whose production is induced by phosphate limitation. Recent studies have indicated that PhoE synthesis is also inhibited by high osmolarity (Meyer et al., 1990).

Because porins play such an important role in solute uptake, their structures have been examined extensively. The primary sequences of OmpC, OmpF, and PhoE from *E. coli* K12 have been determined and have been found to be 60% homologous (Inokuchi et al., 1986; Mizuno et al., 1983). Unlike other membrane proteins, porins have a high percentage of hydrophilic amino acids and lack long stretches of hydrophobic residues (Tommassen, 1988). Studies using various spectroscopic techniques have shown that porin's secondary structure is high in β-sheet (Kleffel et al., 1985; Markovic-Housley & Garavito, 1986; Nabedryk et al., 1988; Vogel & Jahnig, 1986). Such studies have led to two proposals regarding the tertiary structure of porin in the membrane: (1) The amphipathic character of amino acid sequences 9–10 residues long, though to be transmembrane β-strands, suggests an alignment of the hydrophobic sides toward the lipid bilayer with the polar sides facing the inside of the pore (Tommassen, 1988; Vogel & Jahnig, 1986). (2) The presence of polar and ionizable residues within the membrane may indicate networks of hydrogen and/or ionic bonds (Paul & Rosenbusch, 1985; Rosenbusch, 1990). Ionic bond formation could allow for pH-dependent structural changes. In terms of quaternary structure, porin is a trimer of ~36-kDa monomers. The three-dimensional structure of PhoE, OmpC, and OmpF has been examined by electron microscopic image reconstruction (Chang et al., 1985; Dorset et al., 1984; Jap et al., 1990, 1991). Using such a technique, Jap and co-workers analyzed PhoE and found that the channels in the trimer complex converge as they traverse the membrane but do not merge (Jap et al., 1990). Jap also detected a region of low density at the 3-fold axis of symmetry, which was suggested to be a site of bound lipopolysaccharide (LPS)[1] (Rocque et al., 1987; Jap, 1989). Previous studies have suggested that LPS plays a vital role in the structural integrity of porin (Hoenger et al., 1990; Rocque et al., 1987; Xu et al., 1986) and is critical for trimerization (Sen & Nikaido, 1991), insertion (Ried et al., 1990), and functioning (Schindler & Rosenbusch, 1981) of porin in the outer membrane. As a result of porin's structure as well as its strong interactions with LPS, the trimeric complex is stable to various denaturing conditions such as high temperatures, pH extremes, ionic detergents, and urea (Nakae et al., 1979; Rocque & McGroarty, 1990; Rosenbusch, 1974; Schindler & Rosenbusch, 1984).

Structural studies have been complemented by functional analysis of porin. Using the bilayer lipid membrane (BLM) assay, porin has been shown to exist in both an open- and a closed-channel conformation, but the significance of the closed conformation has been questioned (Benz, 1985; Benz et al., 1982; Hancock, 1987; Lakey et al., 1985; Sen et al., 1988). In a recent study, Lakey and Pattus (1989) detected voltage-induced channel closing (i.e., gating) using porin isolated by various techniques and modifying the BLM protocol; the results suggested that discrepancies among various investigators concerning voltage gating may be due to differences in BLM analysis techniques. The physiological significance of voltage gating has been questioned because (1) gating seems to occur at voltages (>100 mV) beyond that of the outer membrane's Donnan potential of 30 mV (Benz et al., 1982; Dargent et al., 1986; Schindler & Rosenbusch, 1978; Xu et al., 1986) and (2) porin channel permeability in intact cells is not affected by high Donnan potentials induced across the outer membrane (Sen et al., 1988). However, Stock et al. (1977) have suggested that the pH in the periplasm may be significantly below that in the media; such a lowered pH in vivo may decrease the threshold for voltage gating similar to the decrease detected in vitro at acidic pH (Xu et al., 1986). Also, the strength of the electric field in a narrow channel can be greater than that across the membrane (Itoh & Nishimura, 1986; Jap, 1989) and may allow for short-term potentials of up to 130 mV to occur at low salt concentration (Lakey, 1987).

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[1] Abbreviations: LPS, lipopolysaccharide; SDS, sodium dodecyl sulfate; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; BLM, bilayer lipid membrane assay; LSA, liposome swelling assay; MDO, membrane-derived oligosaccharide.

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In addition to affecting gating, pH has been found to influence the cooperativity (defined as the three monomers opening simultaneously in the trimer unit) of *E. coli* porin channels (Xu et al., 1986) and to affect channel size (Benz et al., 1979). Xu and co-workers (Xu et al., 1986) observed a decrease in *E. coli* porin channel cooperativity at low pH. Benz and co-workers (Benz et al., 1979) found an increase in channel size with pH. Similarly, Schindler and Rosenbusch (1978) observed "very high conductance levels" at pH values above pH 9.5. Also, Schindler and Rosenbusch (1982) observed increased lysine derivatization in porins modified with eosin isothiocyanate when carried out at higher pH. On the basis of their experiments, they suggested that this increased modification resulted from a pH-dependent conformational change in porin rather than solely from increased protonation of amino groups. Nonbacterial ion channels, including the mitochondrial voltage-dependent anion channel (VDAC) and sodium channels, have shown similar changes in channel function with pH. For example, at acidic pH, the conductance of VDAC has been shown to decrease sharply as membrane potential increases, while the decrease in conductance with voltage is not as great at basic pH (Ermishkin & Mirzabeko, 1990; Kinally, 1991). With batrachotoxin-modified sodium channels, single-channel conductance was found to decrease by 50% when the pH was changed from 7.4 to 4.9 (Daumas & Anderson, 1991). The functional properties of other transport systems have also been shown to be pH-dependent. The system A transport of alanine in hepatocytes is reported to be inactivated at pH 6.0 and to reach maximum activity at pH 8.0; it has been proposed that the titration of a histidine is involved with this pH-dependent change in function (Bertran et al., 1991).

In this study, we examined the effects of pH on *E. coli* K12 porin function. We have observed at least two main substates of the open channel: a small-size channel, stable at acidic pH, and larger-sized channels, stable at basic pH. We found that the pH-induced switch in channel size occurred in the neutral pH range and was affected by high voltage and, in some cases, by LPS depletion. The mechanism of the pH-induced changes and the possible physiological role of pH-induced regulation of porin channel activity in the cell are discussed.

MATERIALS AND METHODS

Cell Growth. OmpC was isolated from *E. coli* strain ECB 621 which lacks OmpF and LamB (gift of S. Benson). OmpF and PhoE were isolated from *E. coli* K12 strains PLB 3261 ($ompC^-$, $lamB^-$; Benson & Decloux, 1985) and JF 694 ($OmpF^-$, $OmpC^-$, gift of S. Benson), respectively. The strains were grown in 1% tryptone, 0.5% yeast extract, and 0.4% NaCl, pH 7.5, as described previously (Rocque et al., 1987; Rocque & McGroarty, 1989, 1990). Cells producing OmpC and PhoE were grown at 37 °C, and cells synthesizing OmpF were grown at 30 °C. Cells were harvested in late-logarithmic phase.

Porin Isolation. Porins were isolated by the method of Lakey et al. (1985) with some modifications (Rocque et al., 1987; Rocque & McGroarty, 1989, 1990). Cells were broken using a French press and treated with RNase and DNase. After the membranes were pelleted at 100000*g*, the inner membrane and some outer membrane proteins were dissolved with sodium dodecyl sulfate (SDS) and Tris-HCl. After centrifugation, the pellet contained outer membrane proteins, primarily porin, bound to the peptidoglycan. The porins were solubilized with high NaCl in the presence of mercaptoethanol, Tris-HCl, and SDS as described previously. The solubilized porin was dialyzed and precipitated with 90% acetone. Homogeneity of porin was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using the method of Laemmli (1970). The final preparations were suspended in 1% SDS, 10 mM Tris-HCl, pH 6.8, and 0.02% sodium azide (standard buffer). These "LPS-enriched" porin samples contained 6–9 molecules of LPS/trimer as determined by the thiobarbituric acid assay (Droge et al., 1970). To deplete porins of LPS, the samples were suspended in 30% SDS, applied to a Sephadex G-200 gel filtration column (2.5 × 100 cm), and eluted with 1% SDS, 10 mM Tris, 0.2 M NaCl, 1 mM EDTA, and 0.02% sodium azide, pH 9.0. This procedure removed all detectable LPS (by the thiobarbituric acid assay), but there probably was a small amount of "unremovable" LPS attached, as shown by Rocque et al. (1987). After LPS depletion, the porin remained in a trimeric configuration (Rocque et al., 1987). Porin was quantitated using the bicinchoninic acid protein assay (Pierce Chemical Co.), and porin trimeric structure and LPS association were monitored by SDS–PAGE (Rocque et al., 1987). The aggregation state of porin was monitored by measuring light scattering (at 320 nm for concentrations from 0.38 to 3.1 mg/mL) of porin solutions at pH 5.4 and 9.4.

Bilayer Lipid Membrane Assay (BLM). Analysis of electrical conductance across a bilayer lipid membrane was used to measure porin channel size, cooperativity, and voltage gating under various conditions. The electrical conductance was measured across a lipid bilayer comprised of 1% diphytanoylphosphatidylcholine (in *n*-decane). As described previously (Rocque & McGroarty, 1989, 1990; Xu et al., 1986), a small volume of porins suspended in standard buffer was added to the salt solution bathing the lipid membrane. This bathing solution contained 0.5 M NaCl and a 0.5 mM aliquot of an appropriate buffer [2-(*N*-cyclohexylamino)-ethanesulfonic acid (CHES) for pH 7.9–9.4; sodium phosphate for pH 6.25–7.5; or sodium succinate for pH 5.4–5.8]. It was difficult to stabilize the pH during the time period of each BLM study, but the pH was maintained to within 0.1 pH unit of the original pH. Silver–silver chloride electrodes were placed on either side of the membrane, and a constant voltage was applied using a 1.5-V battery. Changes in current were amplified using a Keithley Model 614 electrometer and recorded. The changes in current were reported as the size parameter λ/σ (channel conductance increment/bathing solution's specific conductance) versus the probability of occurrence of an event with a particular size. A statistically significant number of channels (≥200) were analyzed for each experimental condition.

Liposome Swelling Assay (LSA). LSA was performed exactly as described previously (Rocque & McGroarty, 1989, 1990) using the procedures of Nikaido and Rosenberg (1983). Solutions of 17% dextran and 120 mM dextrose, maltose, and maltotriose were prepared at pH 5.4 using 50 mM succinate and at pH 9.4 using 50 mM CHES. Liposomes were formed using ~6.2 μmol of phosphatidylcholine in the presence of 17% dextran and ~20 μg of LPS-enriched porin, either OmpC or OmpF. Control liposomes lacking porin were formed by adding only LPS in an amount equivalent to that present in the porin being tested. Liposomes were placed in the various test sugars at pH 5.4 or 9.4, and sugar influx was measured by the decrease in light scattering at 400 nm using a Gilford Response II spectrophotometer. The rate of swelling [$d(OD)/dt$] was monitored for the first 30 s following dilution and reported as the percent decrease in light scattering. The maximum measured $d(OD)/dt$ was −0.146 OD_{400} unit/min.

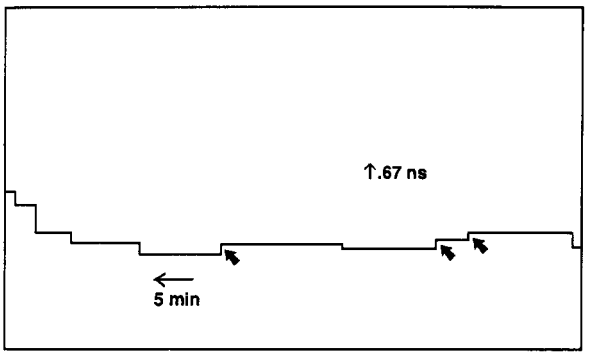

FIGURE 1: Stepwise current changes across a membrane comprised of phosphatidylcholine in the presence of LPS-depleted OmpF in a bathing solution of 0.5 M NaCl and 0.5 mM succinate (pH 5.4). The current decrements shown by arrows presumably represent channel-closing events. The voltage across the membrane was 75 mV. The tracing goes from right to left.

Nikaido and Rosenberg (1983) reported this method to be accurate at swelling rates [measured as d(OD)/dt] of between –0.005 and –0.200 OD_{400} unit/min.

RESULTS

pH Effects on Channel Size. In this study, we have identified pH-induced changes in channel size of porins from *E. coli* K12. Three porins of *E. coli* K12, OmpF, OmpC, and PhoE, were analyzed at different pHs using the BLM system. The resulting plots of stepwise current changes (or increments) versus time (Figure 1) were used to calculate the ratio λ/σ (channel conductance increment/specific conductance of the bulk aqueous phase) for the porins under various conditions (Figure 2). This ratio was used as a size parameter since (1) the ratio is proportional to the cross-sectional area of a pore (or set of pores) at its (their) narrowest point (assuming the pore is a cylinder filled with a solution of the same conductivity as the external solution; Benz & Bauer, 1988), (2) this ratio corrects for variations in the conductivity of the bulk aqueous phase (Benz & Bauer, 1988), and (3) no assumptions about the thickness of the membrane need to be made. Since the assumptions made in (1) may not be accurate and since the geometry of the channel is not well-defined (Nikaido, 1992), this ratio was not used to calculate channel diameters. *Comparisons* of channel size using this parameter are valid since current increments reflect channel cross-sectional areas. The results indicated that OmpF at pH 5.4 has a predominance of small channels (size parameter of ~1.6 Å) and very few large channels (size parameter of ~3.2–3.5 Å, see Figure 3). When measured at pH 6.55, this porin shows fewer small channels and an increase in the number of large channels. This trend continued as the pH increased to 7.5 and 8.15, until at pH 9.4 most channels were of the large-size type. This pattern is referred to as a pH-induced switch in channel size (or more correctly cross-sectional area, but "size" will be used for the purpose of discussion).

Histograms of OmpC channel sizes at different pHs showed a similar pattern, with a preponderance of small channels (size parameter of ~0.9 Å) occurring at pH 5.4 and a higher proportion of larger channels (size parameter of ~1.9–2.4 Å; see Figure 3) being detected as the pH was increased from 6.25 to 9.4. This pattern was also repeated with PhoE; at pH 7.9 and 9.2, a high proportion of large channels (size parameter of ~2.4 Å) was measured while the level of small channels (size parameter of ~1.3 Å) was increased at the lower pHs, e.g., pH 5.8. In all cases, the size of the large channel was approximately twice that of the small channel (for pHs <7.5; see below). No differences in light scattering between porin solutions at pH 5.4 and 9.4 were observed (for concentrations up to 3.1 mg/mL), indicating that the pH-induced switch in channel size, measured using BLM, was not a result of alteration in aggregation state. Also, we are assuming that the current increments of the small- and large-size channels are the result of conductance across the three channels in the

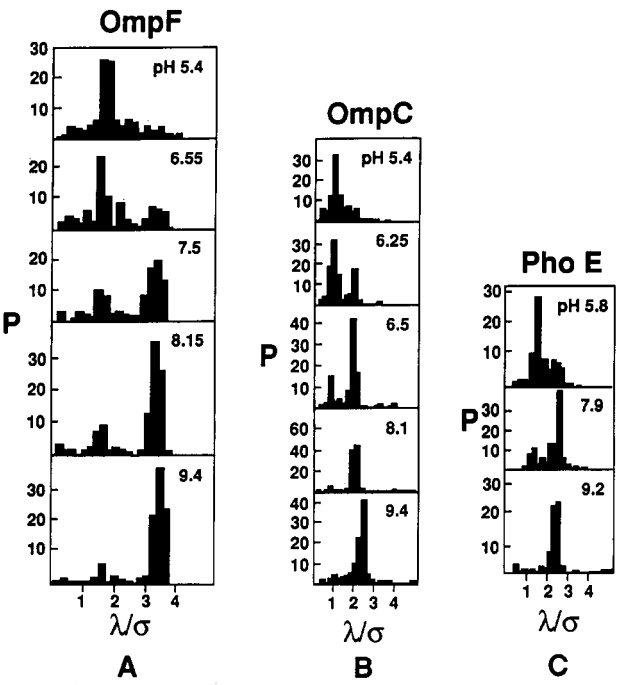

FIGURE 2: Probability distribution histograms of the size parameter λ/σ (Å) for LPS-enriched wild-type OmpF (A), OmpC (B), and PhoE (C) as measured in bilayer lipid membranes. The porins, solubilized in 1% SDS, 10 mM Tris, and 0.02% sodium azide, pH 6.8, were added to bathing solutions of 0.5 M NaCl containing 0.5 mM sodium succinate (pH 5.4 or 5.8), sodium phosphate (pH 6.25–7.7), or sodium 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES, pH 7.9–9.4). Electrical conductance was measured using a transmembrane potential of 25 mV. λ is the channel conductance increment, and σ is the specific conductance of the bathing solution. *P*, in arbitrary units, is the relative number of events with a given size parameter range. Both opening and closing events are included in the histogram of ≥200 events.

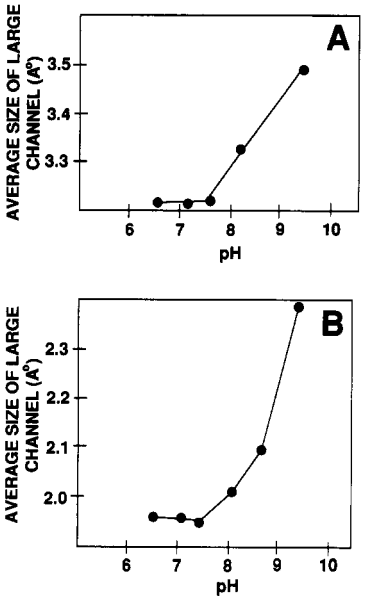

FIGURE 3: Average size (λ/σ) of the large channel at pHs above 6.5 for LPS-enriched OmpF (A) and OmpC (B). Each point is based on analysis of at least 200 channels at each pH.

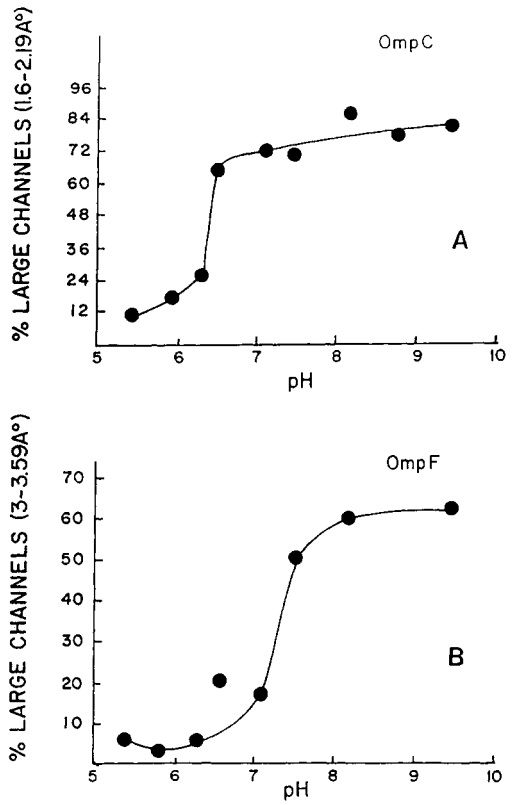

FIGURE 4: Titration curve for the pH-induced switch in channel size for OmpC (A) and OmpF (B), based on bilayer lipid membrane analysis performed as described in Figure 2. The porins, solubilized in 1% SDS, 10 mM Tris, and 0.02% sodium azide, pH 6.8, were added to bathing solutions of 0.5 M NaCl containing an appropriate buffer. Electrical conductance was measured using a transmembrane potential of 25 mV.

trimer complex as it inserts and opens cooperatively in the BLM (see Discussion).

Detailed analysis of the size of the large channel population for both OmpF and OmpC (Figure 3) measured at pHs above 6.5 indicated an increase in size beginning at pH 7.5 and continuing up to pH 9.4. This increase in the size of the large channel with increasing pH was greater for OmpC than for OmpF and suggests either multiple "large channel" substates or a gradual widening of the large channel as the pH was increased from 7.5 to 9.4.

To determine which amino acid(s) may be involved in the pH-induced switch in channel size, the pK_a of the switch was measured using BLM. Analysis of the change in proportion of large channels with pH for OmpC and OmpF (Figure 4) indicated that the switch from small to large channel size occurred over a fairly narrow pH range. This suggests that the change in channel size may be induced by the titration of a single group with an apparent pK_a of 7.2 for OmpF and 6.5 for OmpC. Also, the size switch was found to be reversible when the pH was adjusted between values above and below the pK_a of the size switch (pH 5.6 and 7.4; data not shown). In addition, porins treated to pH values as high as 8.5 were shown to completely switch to the smaller channel size when the pH was lowered (data not shown). We presume that the size switch is reversible over the pH range that the trimer is stable. The pH-induced change in channel sizes observed using BLM was confirmed using LSA analysis of OmpC and OmpF at different pHs (Table I). For OmpF at pH 9.4, the initial rate of influx of glucose and maltose was significantly greater than the rate at pH 5.4. A higher rate of influx at the higher pH was also observed with OmpC although the increase was not as great. With both porins, the difference in the influx rates of glucose measured at pH 9.4 and at 5.4 was statistically significant ($p < 0.05$, determined by the t test) despite the relatively high standard deviation. Control experiments using liposomes lacking porin indicated that there was no difference in leakage of solutes at the different pHs (Table I). In all cases, the liposomes were run on SDS–PAGE subsequent to the LSA to verify that porin denaturation had not occurred.

Effects of Voltage and LPS Depletion. LPS-depleted OmpF and OmpC samples were analyzed for single-channel conductance at different voltages between 10 and 140 mV, and the results were compared to those of the LPS-enriched samples (Figures 5 and 6). The larger channels of OmpF, detected at pH 9.4, were destabilized by the removal of LPS at the lower voltages such as 25 mV and by increasing the voltage for LPS-enriched OmpF to 75 mV and higher (see Figure 5). This can be seen by the significant increase in the number of the small channels detected under these destabilizing conditions. A similar destabilization of the larger channels of LPS-enriched OmpC at pH 9.4 was seen with increased membrane potential, although higher voltages were needed to induce a sizable number of small channels (see Figure 6). In the lower pH range, removal of LPS and elevation of the transmembrane potential resulted in very small conductance channels with OmpF which were approximately one-third and two-thirds the size of the small channel. Similar changes in OmpC's channel-forming activity at pH 5.4 were detected when the voltage was elevated; however, the shift to very small channels required higher voltages than needed with the OmpF sample. These very small channels detected at high voltage and low pH result either (1) from the loss of cooperativity of channel opening within the trimeric complex in the BLM [see Xu et al. (1986)] or (2) from the formation of additional porin conformations which have even smaller channel sizes.

Our previous work had shown that voltage-dependent gating of OmpF occurred at lower voltages when the samples were studied at low pH (Xu et al., 1986). Thus, we repeated these experiments using LPS-enriched and LPS-depleted samples (Tables II and III). The results indicate that for OmpF gating, or closing, of the channels was not significant below 100 mV unless the pH was below 6.5 (Table II). At pH 6.5, OmpF showed significant gating at 75 mV but only if the sample was LPS-depleted. Lowering the pH to 5.5 resulted in significant channel closing at 75 mV even for the LPS-enriched sample. For OmpF at pH 5.4, the voltage threshold for gating of LPS-enriched samples was 60 mV (Figure 7). Analysis of voltage gating of OmpC indicated that this protein is not as readily gated by high voltage even at low pH and with the removal of bound LPS. Significant levels of closed channels were detected at 75 mV only for LPS-depleted samples at acidic pH; LPS-enriched samples showed gating only at very high voltages (Table III, Figure 7). This is consistent with a recent report which showed that OmpC is largely insensitive to voltages below 200 mV in the near-neutral pH range (Lakey et al., 1991).

The effect of voltage on channel current for LPS-enriched OmpC indicated that the voltage-dependent change in current is ohmic up to 85–100 mV at both pH 9.2 and pH 5.6 (Figure 8B). At pH 5.6, the loss of linearity at high voltages is the result of an increase in closings (see Figure 7) and in the number of very small channels due perhaps to loss in cooperativity in the opening of the subunits within the trimers; the presence of small channels at 125 mV is indicated in Figure 6. At pH 9.2, the loss in linearity is the result of an increase

Table I: Liposome Swelling Assay: Percent Decrease in Light Scattering after 30 s in Test Sugar Solution

	OmpF[a]		OmpC[a]		no porin	
	pH 5.4	pH 9.4	pH 5.4	pH 9.4	pH 5.4	pH 9.4
glucose	3 ± 1	10 ± 2	4 ± 1	10 ± 1	1.3 ± 0.6	1.4 ± 0.5
maltose	2 ± 2	6 ± 1	2 ± 1	3 ± 1	0.6 ± 1.3	1.4 ± 0.5
maltotriose	0.4 ± 1	0.6 ± 0.5	0.2 ± 0.7	−1 ± 0.5	1.5 ± 0.5	1.2 ± 0.4
av	NA[b]	NA	NA	NA	1.1 ± 1.5	1.4 ± 0.8

[a] Corrected for control liposome swelling. [b] NA, not applicable.

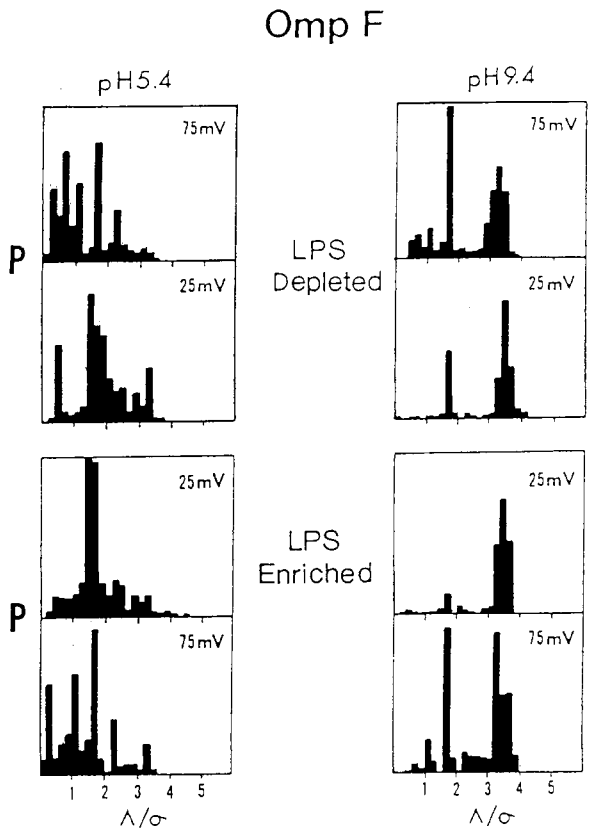

FIGURE 5: Probability distribution histograms of the size parameter λ/σ (Å) for LPS-enriched and LPS-depleted OmpF as measured in bilayer lipid membranes. The porins were prepared and added to bathing solutions at pH 5.4 or 9.4 as described for Figure 2. Electrical conductance was measured using transmembrane potentials of either 25 or 75 mV. Both opening and closing events are included in the histograms.

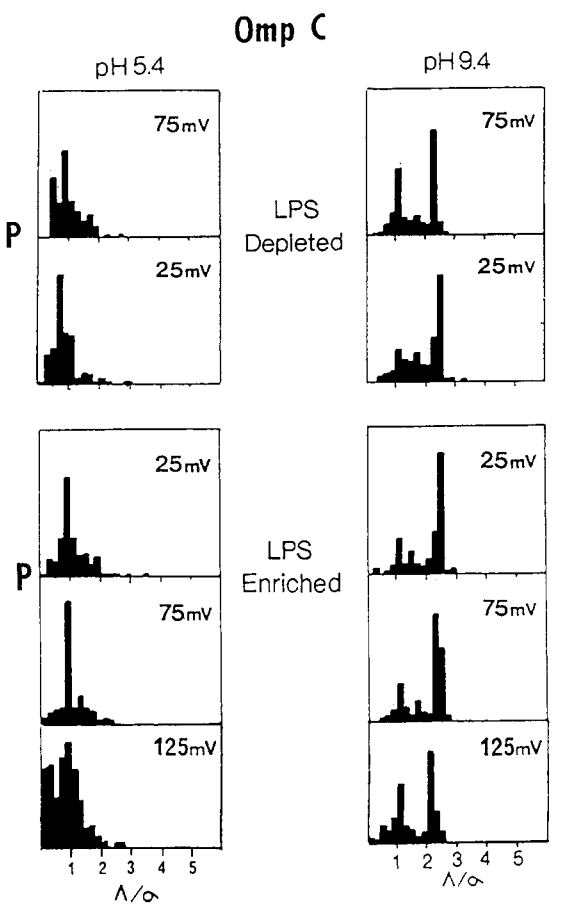

FIGURE 6: Probability distribution histograms of the size parameter λ/σ (Å) for LPS-enriched and LPS-depleted OmpC as measured in bilayer lipid membranes. The porins were prepared and added to bathing solutions at pH 5.4 or 9.4 as described for Figure 2. Electrical conductance was measured using transmembrane potentials of either 25, 75, or 125 mV. Both opening and closing events are included in the histograms.

in the percentage of small channels. Similar studies of the current–voltage relationship for LPS-enriched OmpF (Figure 8A) indicated that the current is ohmic up to 60 and 85 mV for pH 5.6 and 9.2, respectively. Like OmpC, the loss of linearity in the voltage–current relationship at high voltages for OmpF is the result of a shift to smaller channels when measured at pH 9.2, and the shift to very small channels (Figure 5) and an increase in channel closings (Figure 7) when measured at pH 5.6.

An estimate of the number of charges involved in gating was calculated by measuring the ratio of open channels to closed channels (N_o/N_c) at various membrane potentials (V_m) for each pH according to the equation (Morgan et al., 1990):

$$\ln (N_o/N_c) = qn(V_m - V_o)/kT$$

where n is the number of charges moving through the membrane potential, V_o is the potential for 50% of the channels closing, k is Boltzmann's constant, T is temperature, and q is the elementary charge (kT/q = 25 mV at room temperature). The calculated n for OmpC was 0.56 at both pH 9.2 and pH 5.6; for OmpF, the calculated n values were 0.61 and 0.75 at pH 9.2 and 5.6, respectively. The results suggest that ≤1 charge is involved in gating for both OmpC and OmpF. This compares with 2 charges calculated to be involved in gating of VDAC (Benz, 1990).

DISCUSSION

We propose that porins form substates of open channels with variable sizes which correspond to unique structural conformations. We have found that pH induces a change from one substate to another. Our BLM and LSA analyses of porin function show that increasing pH induces a switch from a small to a set of larger channel sizes.

Using BLM, the values we obtained for channel conductance increments were in a range similar to that obtained by others (Benz, 1985; Lakey, 1985; Nikaido & Rosenberg, 1983; Schindler & Rosenbusch, 1978, 1981; Xu et al., 1986) although this value varies, depending on the study. Our results may explain some of this variability as a result of differences in pH

Table II: Voltage Gating of OmpF: Relative Number of Closing Events Expressed as a Percentage of Total Events Detected in the BLM at Different pH Values and at Different Voltages[a]

voltage (mV)	pH 5.5		pH 6.5		pH 9.4	
	-LPS[b]	+LPS[c]	-LPS	+LPS	-LPS	+LPS
25	8	4	6	7	3	1
75	24	18	38	12	8	6
125		35				24

[a] ≥200 events measured for each data set. [b] LPS-depleted samples. [c] LPS-enriched samples.

Table III: Voltage Gating of OmpC: Relative Number of Closing Events Expressed as a Percentage of Total Events Detected in the BLM at Different pH Values and at Different Voltages[a]

voltage (mV)	pH 5.5		pH 6.5		pH 9.4	
	-LPS[b]	+LPS[c]	-LPS	+LPS	-LPS	+LPS
25	6	4	4		4	2
75	20	10	9		9	4
125		16				5

[a] ≥200 events measured for each data set. [b] LPS-depleted samples. [c] LPS-enriched samples.

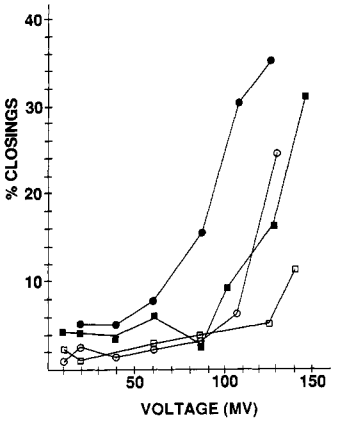

FIGURE 7: Analysis of voltage gating for LPS-enriched OmpF at pH 5.4 (●) and 9.2 (○), and for LPS-enriched OmpC at pH 5.6 (■) and 9.2 (□). Each point is based on the analysis of at least 200 channels.

especially since the channel-size switch occurs over a very narrow range and near neutral pH. Recent concerns about the use of these conductance measurements to calculate exact channel diameter may be valid but do not negate their use in qualitative comparisons to define porin substates under various experimental conditions. These substates might have physiological significance (see below) and certainly are relevant to structural studies. For example, the recent crystallization and X-ray analysis of *Rhodobacter capsulatus* porin (Nestel et al., 1989; Weiss et al., 1989, 1990) displayed a discrepancy between the effective pore diameter (1.6 nm) and that observed using 3-D analysis (1.0 nm). The presence of substates of porin structure and channel size as shown here may explain this discrepancy. Buehler and co-workers (Buehler et al., 1991) have also found variations in channel size with pH and proposed the presence of porin substates with unique configurations. However, they observed an *increase* in porin channel size at low pH (pH 4.5). This results contradicts our observation but could result from the initial steps in porin denaturation at pH 4.5 (Markovic-Housley & Garavito, 1986; Rocque & McGroarty, 1990); perhaps configurational changes occurring close to the denaturation point cause the channel to enlarge in diameter. Since the pK_a of their pH-induced change was not determined, our results are difficult to compare.

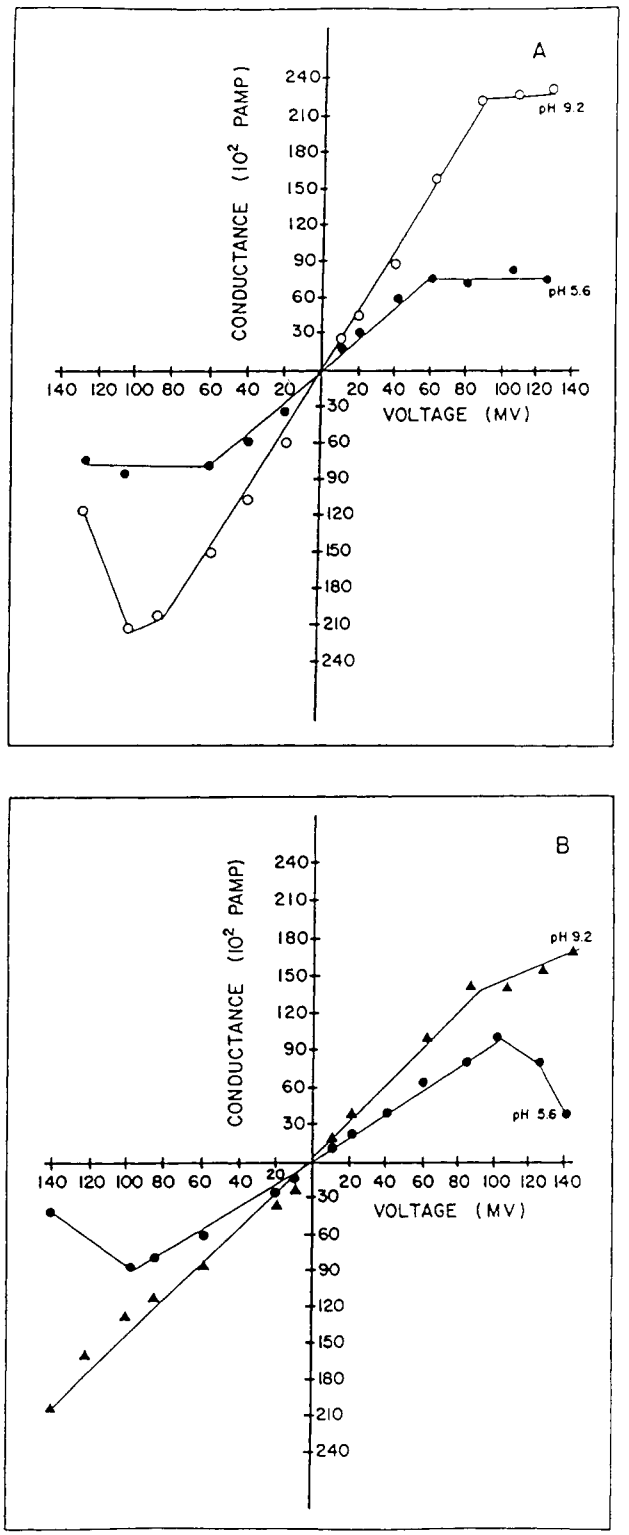

FIGURE 8: Effect of voltage on channel current for LPS-enriched OmpF (A) and OmpC (B) at pH 5.6 and 9.2. For each pH, the conductance at the different voltages was calculated by subtracting the conductance of the total number of closings from the conductance of the total number of openings for 200 events.

There is some question as to which of the observed channel conductance increments represents opening of the trimeric complex. We propose that both the small and the larger size channels, detected at low voltage, represent the cooperative and simultaneous opening of the three channels within the trimer but in a different configuration. This proposal is consistent with the following: (1) conductances approximately one-third and two-thirds of the conductance of the small

channel appear at low pH and high voltages which may result from monomers opening independently and noncooperatively [see Xu et al. (1986)]; (2) the LSA assay measures changes in monomer channel size, and this size did change with pH. The above discussion assumes that porin monomer channels do not merge as they cross the membrane; this assumption is supported by the recent 3-D analyses of PhoE (Jap et al., 1991). The further increase in the size of the large-size channel for both OmpC and OmpF at pHs greater than 7.5 and the presence of channel sizes intermediate between the small and large channel may suggest the presence of more substates than the two major ones we have defined.

The apparent pK_a's of the channel size switch (7.2, OmpF; 6.5, OmpC) were close to that of a histidine side chain (pK_a 6.0). Since PhoE, OmpF, and OmpC all have one histidine whose position is conserved among the three proteins, we suggest this histidine may be involved in the channel-size switch (see next paper).

The LSA data confirmed the BLM results showing an increase in monomer channel size with pH. Our liposome swelling rates were all within the range where the method is reported to be accurate (Nikaido & Rosenberg, 1983). Also, using essentially identical experimental conditions, we obtained swelling rates similar to those reported by Nikaido and Rosenberg (1983).

It may be possible that the larger channel sizes detected in the BLM analysis could be the result of trimer–trimer "dimerization" (i.e., aggregation of two porin trimers followed by simultaneous insertion into the bilayer) and not the result of a channel-size switch. However, five arguments can be given against such a "dimerization" process. First, the pI of porin is between 4 and 5; thus, at the higher pHs, a larger number of negative charges should result in charge repulsion rather than "dimerization". Second, the addition of Mg ions, which should stabilize cross-bridge formation between porin trimers by the association of the Mg^{2+} with LPS or detergent bound to porin, had no effect on channel size at either pH 5.4 or pH 9.4 (data not shown). Third, the LSA results indicated an increase in the initial rate of sugar flux through porin channels at high pH, suggesting increased channel diameter. Fourth, light-scattering measurements indicated no change in the aggregation state of porin at pH 9.4 compared to pH 5.4. Finally, if the histidine involved in the pH-induced switch in channel size [see Todt and McGroarty (1992)] is in the channel interior [as reported by Weiss et al. (1991) for *Rhodobacter capsulatus* porin], it could not be involved in a change in aggregation state.

The effect of voltage and LPS depletion on channel activity confirmed our earlier results which indicated that acidic pH stabilizes the closed state as does the removal of LPS (Xu et al., 1986). The results also suggest that the small channel configuration, stabilized at low pH, has a lower gating threshold than the large channel configuration stabilized at high pH. The structure of OmpC seems to be more stable and affected less by its environment than OmpF since it shows gating and a loss in cooperativity only at very high voltages. Since pH did not affect the number of charges (≤ 1) involved in gating, voltage gating is presumed to be a result of motion of a fixed charge rather than of a proton (Edmonds, 1990).

Two critical questions regarding this pH-dependent change in porin function include the following: (1) What is the mechanism by which pH induces a switch in channel size? (2) What is the physiological role (if any) of this phenomenom in the cell? Without additional data concerning the amino acid(s) involved in the channel size switch, it is difficult to define the exact mechanism. However, the conformational change could involve the protonation at low pH of an R group of an amino acid such as histidine which in turn induces a structural change that reduces the channel size [see Todt and McGroarty (1992)].

The physiological significance of the change in channel size with pH in the intact cell may be the more important question. In this regard, evidence supporting this phenomenon occurring in vivo was seen in several studies which showed that acidic conditions reduce the effectiveness of certain antibiotics as measured by MIC, bacteriocidal activity, and postantibiotic effect (Gudmundsson et al., 1991; Sabath et al., 1968; Laub et al., 1989). This may be of medical significance since the fluid from sites of bacterial infection in humans is acidic (Gudmundsson et al., 1991). It has been proposed that this reduced antibiotic effectiveness under acidic conditions may be due to permeability changes in the outer membrane of Gram-negative bacteria (Gudmundsson et al., 1991; Sabath et al., 1968; Laub et al., 1989). Our results suggest that an extracellularly induced switch in channel size could occur upon a pH change in the environment. Relevant to this proposal are the experiments of Heyde and Portalier (1987) which showed an increase in the synthesis of OmpC (i.e., smaller channels) and a decrease in OmpF (i.e., larger channels) with a drop in pH of the media. A pH-induced switch in channel size could complement permeability changes controlled at the level of porin synthesis. Alternatively, one could envision an intracellular mechanism whereby porin channel size is decreased during active aerobic metabolism as a result of a transient drop in pH in the periplasm resulting from proton pumping across the inner membrane. The resulting decrease in porin channel size and stability of the open state would increase the efficiency of ATPase by decreasing the rate of leakage of protons through the outer membrane. Also, since a variety of transport systems have reduced uptake at lower pH (Bertran et al., 1991; Ermishkin & Mirzabeko, 1990; Kinally, 1991; Daumas & Anderson, 1991; Benz et al., 1979; Schindler & Rosenbusch, 1978; Heyde & Portalier, 1987; Gudmundsson et al., 1991; Sabath et al., 1968), pH may have a common physiological role for controlling all of these systems.

A dynamic role for porin in cell function has been suggested by others (Delcour et al., 1989). Specifically, Delcour et al. (1992) found that membrane-derived oligosaccharides (MDO's) placed on the periplasmic side of the outer membrane decreased the single-channel conductance of mechanosensitive *E. coli* channels. They also found that MDO's increased the frequency of channel closing and affected the cooperativity of the gating in a concentration-dependent manner. Since MDO's are increased in the periplasm in low osmolarity solutions (Sen et al., 1988), it was suggested that MDO binds to the channel on the periplasmic side and prevents efflux of solutes down the concentration gradient. Whatever the mechanism or physiological role, the functional changes of porin with pH presented here indicate that the view of porin as a static filter may not be accurate.

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