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THE EFFECT OF ENVIRONMENTAL FACTORS ON THE FUNCTION AND STRUCTURE OF PORINS FROM *ESCHERICHIA COLI* K-12

By

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ABSTRACT

THE EFFECT OF ENVIRONMENTAL FACTORS ON THE FUNCTION AND STRUCTURE OF PORINS FROM *ESCHERICHIA COLI* K-12

By

Jill Cokeen Todt

In this thesis, we present evidence for an active role for porins in the regulation of the permeability of the outer membrane of *Escherichia coli* K-12. In particular, we have detected a pH-induced switch in channel size *in vitro* and *in vivo* with porins OmpF, OmpC, and PhoE. Small channels were detected at acidic pH with a transition to a set of larger-size channels (~double the cross-sectional area) at basic pH. This pH-induced switch between porin conformations occurred near physiological pH and appeared to be reversible. The pKa for this transition suggested that the only histidine, conserved in all three porins, may be involved in the pH-induced switch in channel size. Further evidence for the involvement of His21 came from studies showing that modification of His21 with diethyl pyrocarbonate eliminated the pH-induced switch in channel size (large-size channels were present at all pH's). Other factors besides pH were also found to affect channel size *in vitro*. These included the

amount of LPS bound to porin, voltage, the presence of MDO, and the tension of the membrane into which porin inserts. Some of these factors could be responsible for short-term regulation of channel size *in vivo*.

The functional changes in porin were correlated to structural alterations with pH measured using Fourier transformed infrared spectroscopy and intrinsic tryptophan fluorescence. This correlation was evidenced by: 1) the similarity of the pKa's of the structural change and of the channel size transitions, and 2) the induction of a change in structure by an alteration in pH similar to that induced by the modification of His21.

Based on this data, we propose that at acidic pH, His21 (which is across from the loop L3 between β strand 5 and 6 that defines the channel exclusion limit) becomes protonated causing L3 to come closer which narrows the channel. This mechanism, in combination with regulation of channel size by the synthesis of different levels of porins (OmpF is produced in high amounts at basic pH, while OmpC is produced at acidic pH), could protect the infectious cell from antibiotics or host defense mechanisms since fluid from sites of bacterial infection in humans has been found to be acidic.

This is dedicated

to my parents and my husband for

their unconditional love and support.

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LIST OF ABBREVIATIONS

BCA Bicinchoninic acid

BLM Bilayer lipid membrane

CHES 2-(N-cyclohexylamino)ethane sulfonic acid

DEPC Diethyl pyrocarbonate

DSC Differential scanning calorimetry

EDTA Ethylenediaminetetraacetate

FTIR Fourier transformed infrared spectroscopy

KDO 2-keto-3-deoxyoctulosonic acid

kDa Kilodaltons

LPS Lipopolysaccharide

LSA Liposome swelling assay

MIC Minimum inhibitory concentration

MDO Membrane-derived oligosaccharide

Omp Outer membrane protein

PAGE Polyacrylamide gel electrophoresis

SDS Sodium dodecyl sulfate

Tris Tris(hydroxymethyl)aminomethane

VDAC Voltage-dependent anion channel

CHAPTER 1

Literature Review

Gram Negative Bacterial Cell Envelope Structure

In gram-negative bacteria, the cell envelope acts as a selective barrier allowing in nutrients and precluding harmful agents such as antibiotics, proteases, or bile salts. The selectivity is determined by the structure and composition of the cell envelope (Figure 1) and has been discussed in many recent reviews (Nakae, 1986; Lugtenberg & Alphen, 1983; Nikaido & Vaara, 1985). The cell envelope is formed by an outer and an inner membrane. A periplasmic space located between the two membranes contains the peptidoglycan layer.

The periplasm also contains hydrolases, nutrient-binding proteins, and membrane-derived oligosaccharides (MDOs). MDOs are negatively charged polymers of glucose (8-10 residues) with sn-1-phosphoglycerol, phosphoethanolamine, or O-succinyl ester residues substituted at various positions (Kennedy, 1982). MDO production is increased in cultures under conditions of low osmolarity (Van Golde et. al, 1973; Kennedy, 1982; Sen et. al., 1988) presumably to keep the periplasm isoosmolar with the cytoplasm preventing the cytoplasmic membrane from swelling significantly under these conditions (Stock et. al., 1977). The presence of fixed anions in the periplasm creates a Donnan potential across the outer membrane of 30 mV (Sen et. al., 1988).

The outer membrane contains lipopolysaccharide (LPS) in the outer leaflet, phospholipid in the inner leaflet, and

Figure 1. Schematic representation of the cell envelope of *Escherichia coli*. OM represents the outer membrane, PG=peptidoglycan layer, CM=cytoplasmic membrane. Lipopolysaccharide is represented by *a*, the outer membrane structural protein OmpA is represented by *b*, the outer membrane porins=*c*, phospholipids=*d*, and lipoprotein=*e*.

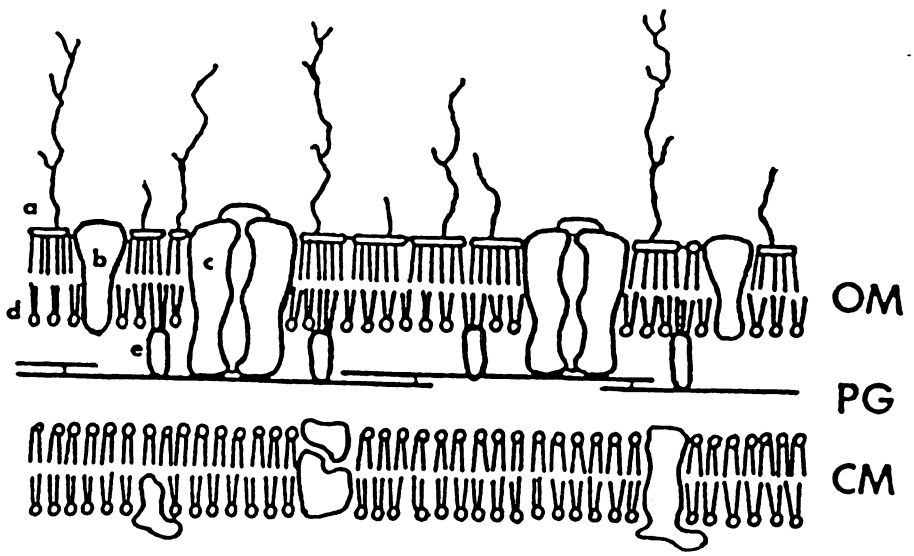

FIGURE 1

a number of major and minor proteins. LPS consists of lipid A, core polysaccharide and an O-antigen (Lugtenberg & Alphen, 1983). Lipid A consists of β 1,6 linked D-glucosamine disaccharides head groups covalently bound with fatty acyl chains, two of which are amide bound. Lipid A is essential for the endotoxin activity of LPS. The LPS core oligosaccharide contains 3-deoxy-D-manno-octulosonic acid (KDO) and a number of sugars such as glucose, galactose, and N-acetyl-D-glucosamine. The O-antigen, used in O-serotyping to identify substrains of one species, consists of 40 or more repeating units each containing 3-6 sugar residues. These sugars can be substituted with O-acetyl groups, phosphate, amino acids, or ethanolamine triphosphate; this can contribute significantly to the net surface charge of the bacterial cell. In some strains, such as *E. coli* K12, LPS is missing the long O-antigen (Figure 2).

Escherichia coli Porins and Regulation of Synthesis

Nutrients enter the cell through outer membrane proteins called porins. A number of reviews (Benz & Bauer, 1988; Benz, 1985; Nikaido, 1992; Rosenbusch, 1990; Nikaido & Vaara, 1985; Nakae, 1986; Tommassen, 1988) have described porin structure and function; these proteins from enteric species form nonspecific water-filled pores (in the outer membrane) with a diffusion limit of ~600 Daltons. The two main nonspecific porins of *Escherichia coli* are OmpC and OmpF. OmpC has a smaller channel than OmpF and is produced

Figure 2. Tentative structure of *Escherichia coli* K-12 LPS. Ions and other non-covalently attached constituents are not included. Amide and ester bonds are indicated by -N- and -O- links respectively. The following abbreviations are used: Glc=glucose, Gal=galactose, Hep=heptose, KDO=2-keto-3-deoxyoctulosonic acid, Rha=rhamnose, GlcN=N-acetylglucosamine, EA=ethanolamine, P=phosphate.

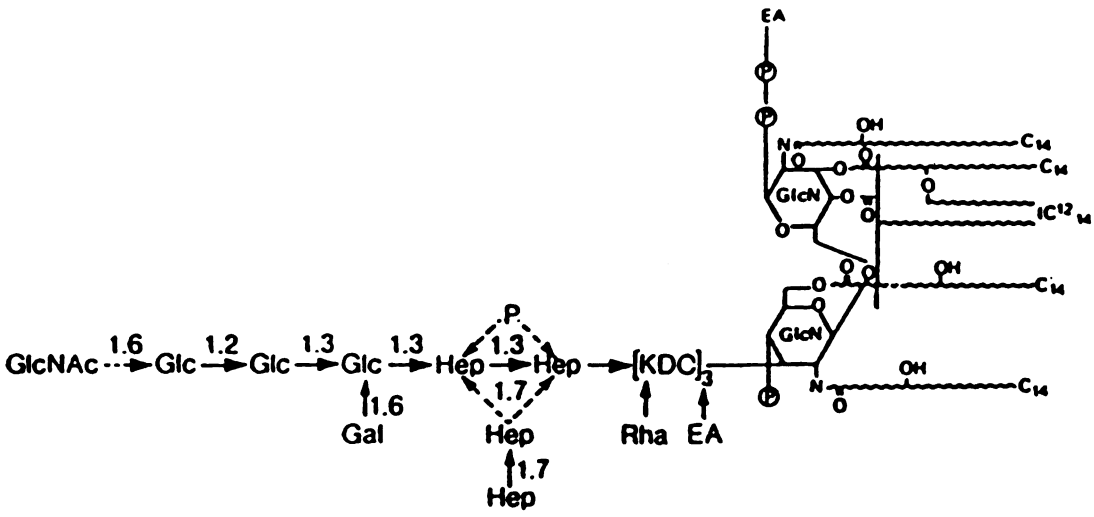
EA
P
P
OH
N
O
O
O
GlcN
O
O
O
C14
C14
O
IC12 14
OH
C14
O
O
GlcN
O
N
O
OH
C14
P
GlcNAc
1.6
Glc
1.2
Glc
1.3
Glc
1.3
Hep
1.3
Hep
[KDC]3
P
1.7
1.6
Gal
Hep
Rha
EA
1.7
Hep

Figure 2

under conditions of high osmolarity, low cAMP concentration, high temperature (Nakae, 1986), or low pH (Heyde & Portalier, 1987). The production of PhoE is also reduced under conditions of high osmolarity (Meyer et. al., 1990). PhoE, OmpF, and OmpC are defined as general diffusion pores since the movement of ions in their channels is similar to that in free solution; however, PhoE (induced by phosphate limitation) has binding sites for anionic solutes which makes it an anion-selective channel (Brunen et. al., 1992). OmpC and OmpF are slightly cation-selective (Benz et. al., 1979). LamB, whose production is activated by maltose or maltodextrin-containing media, is a specific porin since it shows saturation behavior with increasing substrate concentrations (Schulein & Benz, 1990).

Bacterial Porin: Structure

Because porins play such an important role in nutrient uptake, their structure has been examined extensively (Jap et. al., 1990; Chang et. al., 1985; Jap et. al., 1991; Dorset et. al., 1984; Cowan et. al., 1992; Weiss et. al., 1991; Fourel et. al., 1992; Jeanteur et. al., 1991). The primary sequences of OmpC, OmpF (Figure 3), and PhoE from *E. coli* K12 have been determined and found to be 60% homologous (Inokuchi et. al., 1982; Mizuno et. al., 1983). Unlike other membrane proteins, porins have a high percentage of hydrophilic amino acids and lack long stretches of

Figure 3. Topology of OmpF, with amino-acid sequence in one-letter code. The view is from outside the 16-stranded antiparallel β-barrel, which is unrolled. The last two β-stands are repeated on the right-hand side (hatched) to emphasize the barrel. Secondary structural elements are indicated (bold if their side chains are external) by diamonds for barrel β-strands, rectangles for α-helices and circles for turns and loops. The subunit contact regions are shaded.

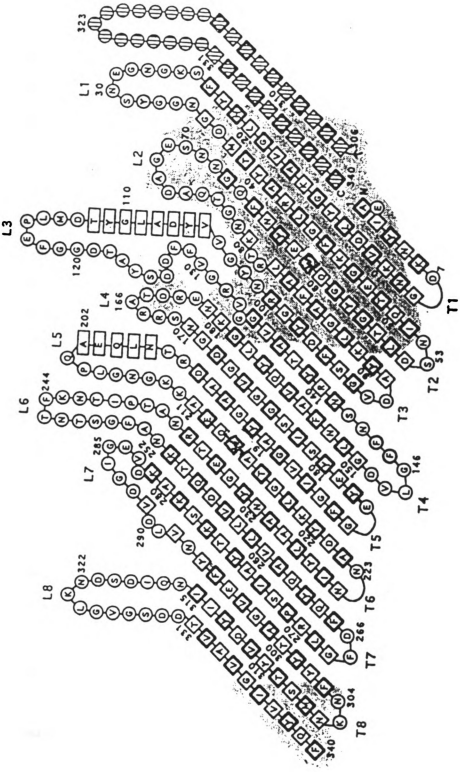

Figure 3

hydrophobic residues (Tommassen, 1988). Studies using circular dichroism spectroscopy (Markovic-Housley, 1986), infrared spectroscopy (Kleffel et. al., 1985; Nabedryk et. al., 1988), and Raman spectroscopy (Vogel & Jahnig, 1986) have shown that porin's secondary structure is high in beta sheet. These observations have lead to two proposals regarding the structure of porin in the membrane: 1) the observed amphipathic character of amino acid sequences 9-10 residues long, which are thought to be transmembrane β-strands suggests the alignment of the hydrophobic sides to face toward the lipid bilayer with the polar side facing the inside of the pore (Tommassen, 1988; Vogel & Jahnig, 1986), and 2) the presence of polar and ionizable residues within the membrane may indicate networks of hydrogen and/or ionic bonds (inside the channel lumen, between subunits, and between headgroups of the membrane lipid and the porin exterior.) (Vogel & Jahnig, 1986; Rosenbusch, 1990).

The recent structural analysis of crystallized OmpF to a resolution of 2.4 Å has corroborated this model and permitted a more detailed description of porin structure (Figure 4) (Cowan et. al., 1992). Porin is a trimer of ~36 kDa monomers which bind ionically to peptidoglycan as well as interacting strongly with LPS. OmpC, OmpF and PhoE monomers when synthesized simultaneously have been shown to randomly associate into heterotrimers (Gehring & Nikaido, 1989). Each monomer consisting of a 16-stranded antiparallel β-barrel forms a pore or channel (Cowan et.

Figure 4. RIBBON diagram of OmpF. Arrows represent β-strands and are labelled 1 to 16 starting from the strand after the first short turn. The short β-strand at the N-terminus continues the C-terminal strand β-16 and is therefore called β16′. The long loops are denoted L1 to L8, the short turns at the other end T1 to T8. Loop L2 protudes towards the viewer. Loop L3 (black) folds inside the barrel.

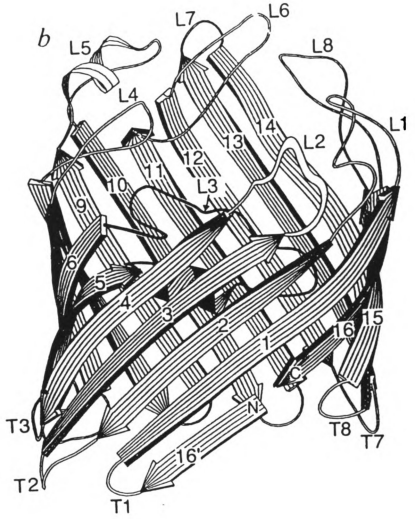
b
L7
L6
L5
L8
L4
L1
14
13
L2
12
11
10
L3
9
6
5
4
3
2
1
16
15
C
N
T3
T8
T7
16
T2
T1

Figure 4

al., 1992). The exterior surface of the trimer complex contains a hydrophobic band of R groups whose upper and lower limits consist of aromatic amino acids, with small size aliphatic residues in between. The interface between monomers is a combination of hydrophobic and hydrophilic interactions. Loops (connecting the β-strands) on the cell exterior, in general, are longer than those facing the periplasm; thus the exterior of the protein has a "rough" surface. On this surface are a number of carboxyl groups which could presumably bind to the anionic LPS via divalent cations. One large loop between β-strand 5 and 6 (L3) folds into the channel lumen approximately midway in the channel interior creating an eyelet and forming a constricted zone. This zone determines the exclusion limit of the pore and is segregated into negatively-charged amino acids on one side within the loop (Asp113 and Glu117) and positively-charged amino acids across from the loop (Arg42, Arg82, Arg132, Lys16). The amino acids in this constriction zone are highly conserved among OmpF, PhoE and OmpC.

In terms of interactions between porin and other membrane components, LPS seems to be important in the association of porin into trimers as well as its assembly into the outer membrane (Ried et. al., 1990; Sen & Nikaido, 1991). The isolated trimer is associated with variable amount of LPS as illustrated by the ladder of bands of trimers with different levels of associated LPS seen on a SDS-polyacrylamide gel (Rocque et. al., 1987). One result

of the unique structure of porin is its stability to various denaturing conditions such as high temperatures, pH extremes, ionic detergents, and urea (Schindler & Rosenbusch, 1984; Rocque & McGroarty, 1990; Rosenbusch, 1974; Nakae et. al., 1979).

Bacterial Porin: *In vitro* Functional Analyses

Various methods have been used to analyze porin function *in vitro* and *in vivo*. Two commonly used *in vitro* techniques are the bilayer lipid membrane (BLM) assay (Schindler & Rosenbusch, 1978; Benz & Bauer, 1988; Benz et. al., 1979) and the liposome swelling assay (LSA) (Nikaido & Rosenberg, 1983). Using the BLM, conductance is measured across a lipid membrane painted across a small hole in a teflon partition separating two chambers. These chambers contain a salt solution with a small amount of porin dissolved in a detergent. Insertion of porin into the membrane results in a measurable increase in membrane conductance. The conductance measured can indicate the insertion and opening of channels as well as their closing. Parameters that can be measured using the BLM assay include the cross-sectional area of the porin channel at its narrowest point, cooperativity of channel opening and closing and ion selectivity. Cross-sectional area is calculated by dividing the specific channel conductance increment, λ, by the specific conductance of the bathing solution, σ. Channel cooperativity is defined as the

opening or closing of the 3 porin monomers as a unit indicated by the conductance levels (trimer channel size). Noncooperativity of channel opening or closing is indicated by channel sizes 1/3 or 2/3 the size of the trimer (Xu et. al., 1986). Effects of various environmental factors that have been examined include pH, voltage, temperature, as well as the presence of LPS. It has been shown that ion flow through the porin channels is similar to movement in free solution (ie. the average λ/σ is fairly constant over a range of salt concentrations) (Benz & Bauer, 1988; Benz et. al., 1978; Brunen et. al., 1992) although small variations in conductance which have been measured may be due to interaction of carboxyl groups in the pore with cations since amidation of carboxyl groups significantly reduced the variability of λ/σ (Benz et. al., 1984). Limitations of this method are due to: 1) difficulties in ascertaining which conductance increment represents trimer opening, 2) the fact that the conductance increment is a concerted effect of surface potentials (LPS or phospholipid), charge of porin trimers, and potential difference across the membrane (Brunen et. al., 1992; Jap, 1989), and 3) the fact that the permeability only of ions or charged molecules can be measured and not uncharged solutes.

LSA is a technique whereby the channel exclusion limit for uncharged solutes can be determined (Nakae, 1986; Lugtenberg & Alphen, 1983). Liposomes are formed in the presence of porin in a solution containing an impermeant

solute. The rate of change in the light scattering of such liposomes which swell upon dilution into a solution containing a specific-sized test solute reflects the relative size of the channel. Limitations of this method include the following: 1) the Donnan potential of the intact cell is not reproduced in liposomes and 2) if charged molecules are present, a membrane potential can be created, inducing a complex movement of buffer and other ions (Bellido et. al., 1991).

Using BLM analysis, 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. Some investigators have observed an increase in the number of channel closings with increased transmembrane voltage, a phenomenon referred to as voltage gating (Schindler & Rosenbusch, 1978; Schindler & Rosenbusch, 1981; Dargent et. al., 1986; Xu et. al., 1986; Delcour et. al., 1989). Others have been unable to detect this gating phenomenon (Benz, 1985; Benz et. al., 1978; Lakey et. al., 1985). In a recent paper, Lakey and coworkers detected voltage gating using porin isolated and analyzed applying a variety of methods; they suggested that differences in measuring voltage gating may be due to variations in the BLM technique (Lakey & Pattus, 1989). The physiological significance of voltage gating has been questioned because: 1) gating reportedly occurs at voltages (>100 mV) (Schindler & Rosenbusch, 1978; Dargent et. al., 1986; Xu et. al., 1986) beyond that of the

outer membrane's Donnan potential of 30 mV (Stock et. al., 1977) and 2) Sen and coworkers have found that porin channel permeability in intact cells is not affected by high Donnan potentials induced across the outer membrane (Sen et. al., 1988). However acid pH, as is found in the periplasm (Stock et. al., 1977), may reduce the voltage threshold *in vivo* as it has been found to do *in vitro* (Xu et. al., 1986). Also a change in electrostatic field strength can be amplified across the narrow porin channels (Itoh & Nishimura, 1986; Jap, 1989) which may allow short term potentials of up to 130 mV to occur at low salt concentrations (Lakey, 1987). Alternatively, Lakey and Pattus (1989) have suggested that the removal of the porin-peptidoglycan linkage, occurring as a result of porin purification, may induce voltage dependence and this voltage control may be an expression of an *in vivo* closing regulated by another mechanism. Others have suggested that voltage gating is a protection to prevent porin, if mistakenly inserted into the cytoplasmic membrane, from collapsing the transmembrane potential (Nikaido, 1992).

In addition to affecting gating, pH has been found to influence porin channel size (Benz et. al., 1979; Buehler et. al., 1991) and cooperativity (Xu et. al., 1986). Buehler and coworkers (1991) have observed an increase in channel size at low pH (pH 4.5). On the other hand, Benz and coworkers (1979) detected <u>decreased</u> channel size (the most frequently observed conductance, λ, halved) with pH

decreases from 9 to 2. Also Schindler and Rosenbusch (1982) observed "very high conductance levels" at pH values above pH 9.5. Non-bacterial ion channels, including the mitochondrial voltage dependent anion channel (VDAC) and sodium channels, have shown changes in channel function with pH similar to those seen in Benz's studies of bacterial porins. VDAC switches to substates of lower permeability with increasing voltage (Benz, 1990; Mannella, 1990); at acidic pH, the conductance of VDAC has been shown to decrease more sharply (as compared to basic or neutral pH) as membrane potential increases (Ermishkin & Mirzabekov, 1990). With batrachotoxin-modified brain sodium channels, single channel conductance was found to decrease by 50% when the pH was changed from 7.4 to 4.9 (P. Daumas and O.S. Anderson, Abstr. Annu. Meet. Biophys. Soc. 1991, p. 259). Changes in function, which are pH-dependent, have been observed in other transport systems. For example, 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 activity. (Bertran et. al., 1991). Xu and coworkers detected decreased channel cooperativity at low pH (Xu et. al., 1986). Voltage also appears to control channel cooperativity. PhoE and OmpF show a loss of cooperativity at high potentials as indicated by predominance of monomer and dimer-sized conductances under these conditions

(Schindler & Rosenbusch, 1981; Schindler & Rosenbusch, 1978; Xu et. al., 1986).

Most investigators have found that bound LPS can modify bacterial porin function (Schindler & Rosenbusch, 1981; Schindler & Rosenbusch, 1978) while some have found that LPS removal has no effect (Benz et. al., 1978; Hancock, 1987). It has been shown that methods used to deplete porin of LPS do not totally remove LPS (Rocque et. al., 1987,) and this could explain why some investigators do not detect an LPS-dependent effect.

The anionic MDO has also been found to affect porin function. Declour and coworkers, using patch clamping techniques found that with polarities opposite to the Donnan potential, MDO increased the number of closings of porin from *E. coli* wild type AW740 (Delcour et. al., 1992). They found that phosphoethanolamine (a substituent of MDO) mimicked this effect and hypothesized that the depolarization of cells adapted to low osmolarity, which occurred when cells were suddenly placed in high-salt medium, caused the MDO-dependent closure of porin channels. Similarly polyanions have been found to affect VDAC function; Konig's polyanion has been found to induce VDAC to close at lower potentials (Mannella, 1992). Colombini and coworkers have isolated a modulator protein from mitochondria which has the same effect as polyanions (Liu & Colombini, 1992).

Another factor which has been found to affect function for some *E.coli* channels is the tension of the membrane lipid. Specifically, Martinac and coworkers have discovered channels in the outer membrane (and in the inner membrane) which can be activated by suction or pressure (Martinac et. al., 1990). These "mechanosensitive" channels were also activated by amphipaths (Markin & Martinac, 1991). This presumably occurred via a mechanism, first described by Sheetz and Singer (1974), whereby the insertion of an amphipath preferentially into one leaflet of an asymmetrical bilayer (either due to the asymmetric distribution of proteins or polar lipids), caused an alteration of the contour of the membrane, ie. a "cupping" effect. Activity of mechanosensitive channels was also increased in the presence of increased osmolarity. These results in combination prompted Martinac and coworkers (1990) to propose the presence of mechanosensitive biosensors in the living cell which monitor membrane expansion during cell growth and volume or osmolarity changes.

Bacterial Porin: *In vivo* Functional Analyses

Methods used to study porin function *in vivo* include the measurement of uptake of metabolizable solutes such as antibiotics or maltose. Since β-lactamases which degrade β-lactams are located primarily in the periplasm, Zimmermann and Rosselet (1977) have developed a method to measure outer membrane permeability by monitoring the decrease of β-lactam

(measured as a decrease in absorbance at 260 nm) using intact cells. Assuming that the β-lactams penetrate the outer membrane primarily through porins and that the K_m and V_{max} for β-lactamase is the same for cell-bound and periplasmic enzyme, they proposed that at a given β-lactam concentration, a steady state is rapidly established such that:

$$C(S_o - S_e) = S_e \times V_{max}/(S_e + K_m)$$

where C = permeability parameter, S_o = external antibiotic concentration, and S_e = periplasmic antibiotic concentration (Zimmermann & Rosselet, 1977). These assumptions were found to be accurate, at least in experiments with *E. coli* (Zimmermann & Rosselet, 1977; Sawai et. al., 1977; Nikaido et. al., 1983), allowing the prediction of minimum inhibitory concentrations (MICs) (Livermore & Davy, 1991). Using this method, Nikaido and coworkers (1983) showed that OmpC and OmpF excluded large hydrophobic and negatively-charged solutes. This may help explain how these bacteria protect themselves against hydrophobic and negatively-charged bile salts in their natural environment, the intestinal tract (Nikaido, et. al., 1983). They also found using hydrophilic compounds that OmpC-containing cells had consistantly lower permeability coefficients than OmpF-containing cells, reflecting OmpC's relatively smaller channels.

Using whole cells, investigators have observed that acidic pH reduces the effectiveness of certain hydrophilic

antibiotics as measured by the MIC, bacteriocidal and post-antibiotic effects (suppression of bacterial growth which persists after limited exposure of organism to antimicrobial agent) (Gudmundsson et. al., 1991; Laub et. al., 1989; Sabath et. al., 1968). It has been suggested that this was due not to the altered ionization state of the antibiotic but to a change in "receptor" (ie. porin) affinity on the bacterial cell (Sabath et. al., 1968).

Bacterial Porin: Structure-Function Studies

A variety of approaches have been used to define regions of porin structure that are vital for function. Chemical modification (Tokunaga et. al., 1981; Benz et. al., 1984; Hancock et. al., 1986; Schindler & Rosenbusch, 1982), mutation (Misra & Benson, 1988a; Misra & Benson, 1988b), and hybrid porin gene construction (Tommassen et. al., 1985; Benz et. al., 1989; van der Ley et. al., 1987; Nogami et. al., 1985) experiments have been utilized to determine which amino acids are important in defining channel function. Chemical modification of amino and carboxyl groups in porin has been found to slow the diffusion of negatively and positively charged molecules respectively; neither of these modifications has been shown to affect diffusion of uncharged solutes (Tokunaga, 1981). Chemical modification has also been used to analyze exposure of amino acids and to probe conformational changes (Schindler & Rosenbusch, 1984; Schindler & Rosenbusch, 1982). Chemical modification of

lysine with eosin isothiocyanate was shown to increase with increasing pH (Schindler & Rosenbusch, 1984); these workers suggested that the change in modification with pH resulted from a pH-dependent conformational change in porin rather than solely from increased protonation of amino groups at basic pH.

Benson and coworkers have isolated strains of *E. coli* producing mutant OmpC and OmpF porins which allow the cells to grow on maltodextrins in the absence of the maltodextrin-specific porin LamB (Dex^+ phenotype) (Misra & Benson, 1988a; Misra & Benson, 1988b). These porins apparently have enlarged channels and the change in channel structure in these Dex^+ strains caused an increased sensitivity to hydrophilic antibiotics as well as an increased rate of ^{14}C-maltose uptake in the intact cells. The mutant porins contained amino acid deletions, insertions and single residue substitutions. Benson observed that these mutations all occurred in the first third of the protein and involved a small number of charged amino acids (R42, R82, R132, D113 for OmpF) (Misra & Benson, 1988a, Misra & Benson, 1988b). According to the most recent structural analysis of OmpF, these amino acids contribute to the eyelet of the channel; changes in the length or charge of these amino acids can change the exclusion limit of the porin channel (Cowan et. al., 1992).

Bacterial Porin: Significance of Structure-Function Studies

There are three major reasons for looking at structure-function relationships in bacterial porin. First, such studies may help discover ways to increase the antibiotic suseptibility of pathogenic bacteria. For example, Sabath and coworkers indicated that alkalinization of the media had an enhancing effect on the action of erythromycin against gram-negative bacteria (Sabath et. al., 1985). The second reason for studying porin structure-function relationships is as a model for other channel-forming proteins. As described previously, mitochondrial porins are similar to bacterial porins in function as well as structure (Ermishkin & Mirzabekov, 1990; Blachly-Dyson et. al., 1990). However bacterial porins are more amenable to study due to the ease of genetic manipulation as well as to the level of protein that can be isolated. Also the primary sequence and three dimensional structure of bacterial porin is more defined. Finally, there has recently been a study showing that porins play a role as pathogenicity determinants in gram-negative bacterial infections (Galdiero et. al., 1990; Li et. al., 1991). Thus elucidation of porin structure-function relationships could be a step toward altering pathogenicity.

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CHAPTER 2

Effects of pH on Bacterial Porin Function

ABSTRACT

Porin is a trimeric channel-forming protein in the outer membrane of gram-negative bacteria. Function 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 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.

INTRODUCTION

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 non-specific 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, thought 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; Jap et. al., 1991). Using such a technique, Jap and coworkers 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). Cowan and coworkers' (1992) recent X-ray diffraction analysis of crystallized OmpF at a resolution of 2.4 Å has allowed more detailed analysis of porin. Each monomer consists of a 16-stranded antiparallel β-barrel containing a pore (Cowan et. al., 1992). The exterior surface contains a hydrophobic band whose upper and lower limits consist of aromatic amino acids, with small size aliphatic residues in between these limits. The interface between monomers contains a combination of hydrophobic and hydrophilic interactions. The loops (connecting the β-strands) facing the cell exterior in general are longer than those facing the periplasm and thus

create a "rough" outer surface. On this surface are carboxyl groups which could presumably bind to LPS via divalent cations. One large loop between β-strand 5 and 6 folds into the channel luman about half-way down creating an eyelet or a constricted zone. This zone determines the exclusion limit of the pore and is segregated into negatively-charged amino acids on one side (Asp113 and Glu117) and positively-charged amino acids on the other (Arg42, Arg82, Arg132, Lys16). The amino acids in this constriction site are conserved among OmpF, PhoE, and OmpC.

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 & Pattus (1989) detected voltage-induced channel closing (ie., 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).

In addition to affecting gating, pH has been found to influence the cooperativity (defined as the 3 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; Schindler & Rosenbusch, 1978). Xu and coworkers (1986) observed a decrease in *E. coli* porin channel cooperativity at low pH. Benz and coworkers (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 modification of lysine with eosin isothiocyanate when the derivitization of porin was carried out at higher pH. Based on their experiments, they suggested that this increased modification resulted from a pH-dependent conformational change in porin rather than solely from decreased protonation of amino groups. Non-bacterial 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 & Mirzabekov, 1990; K. W. Kinally, Abstr. Annu. Meet. Biophys. Soc. 1991, p. 177). With batrachotoxin-modified brain sodium channels, single channel conductance was found to decrease by 50% when the pH was changed from 7.4 to 4.9 (P. Daumas and O. S. Anderson, Abstr. Annu. Meet. Biophys. Soc. 1991, p. 259). 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 conformations 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; Rocque & McGroarty, 1990). Cells producing OmpC and PhoE were grown at 37°C, and cells synthesizing OmpF were grown at 30°C. Cells were harvested late in logarithmic growth phase.

Porin Isolation

Porins were isolated by the method of Lakey et. al. (1985) with some modifications (Rocque et. al., 1987; Rocque & McGroarty, 1989, Rocque & McGroarty, 1990). Cells were broken using a French press and treated with RNAse and DNAse. After pelleting the membranes at 100,000xg, 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, including most of the 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 x 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 was monitored by SDS-PAGE (Rocque et. al., 1987) and by the thiobarbituric acid assay. 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)

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% diphytanoyl phosphatidylcholine (in n-decane). As described previously (Rocque & McGroarty, 1989; Rocque & McGroarty, 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 0.5 mM of an appropriate buffer (2-(N-cyclohexylamino)ethanesulfonic acid, CHES, for pH 8.1-9.4; sodium phosphate for pH 6.25-7.9; 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 units of the original pH. Silver-silver chloride electrodes were placed on either side of the membrane and a constant voltage was applied using a 1.5V battery. Changes in current were amplified using a Keithley Model 614 electrometer and recorded. The changes in current were reported as a size parameter λ/σ (channel conductance increment/bathing solution's specific conductance) versus the probability of the occurrence of an event with a particular size. A statistically significant number of channels ($\geq$200) were analyzed for each experimental condition.

Liposome Swelling Assay (LSA)

LSA was performed exactly as described previously (Rocque & McGroarty, 1989; Rocque & McGroarty, 1990) using the procedures of Nikaido & 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 umol phosphatidylcholine in the presence of 17% dextran and ~20 ug of LPS-enriched porin, either OmpC or OmpF. Control liposomes lacking porin were formed adding only LPS in an amount equivalent to that present in the porin being tested. Liposomes were added to solutions of 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 seconds following dilution and reported as percent decrease in light scattering. The maximum measured d(OD)/dt was -0.146 OD_{400} units/min. 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} units/min.

RESULTS

pH Effects on Channel Size

In this study, we have identified pH-induced changes in channel size of porins from *E. coli* K-12. Three porins of *E. coli* K-12, OmpF, OmpC, and PhoE, were analyzed at different pHs using the BLM system. The resulting plots of stepwise current changes 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 the pore at its 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

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.

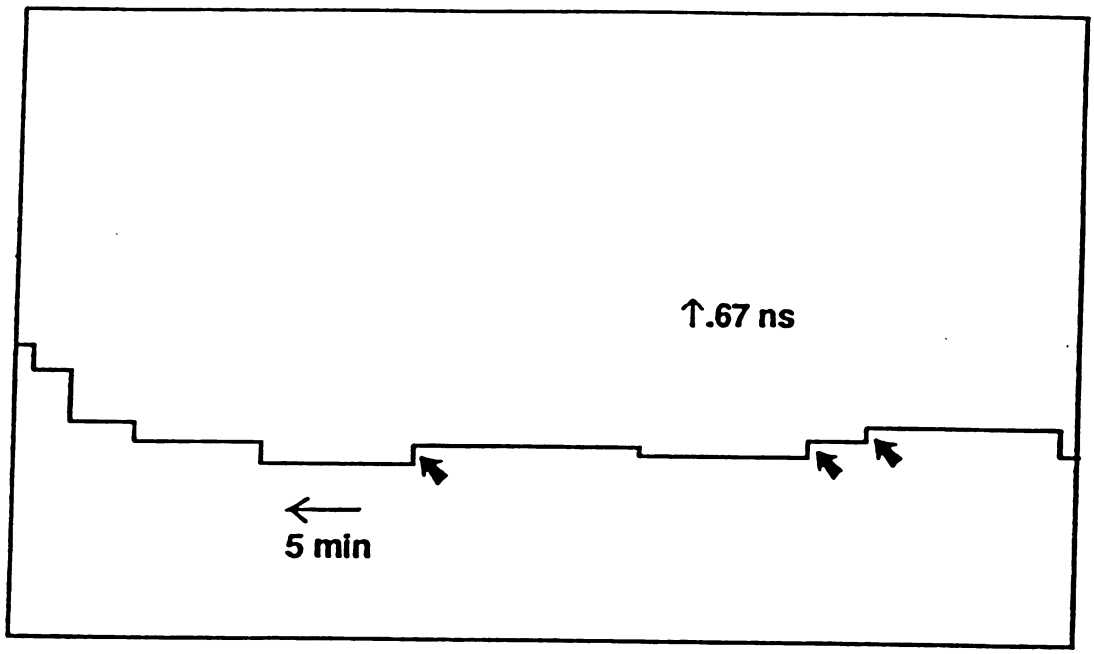

Figure 1

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, 0.02% sodium azide, pH 6.8, were added to bathing solutions of 0.5 M NaCl containing 0.5 mM of either sodium succinate (pH 5.4 or 5.8), sodium phosphate (pH 6.25-7.9), or sodium 2-(N-cyclohexylamino)ethanesulfonic acid (CHES, pH 8.1-9.4). 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 $\geq$200 events.

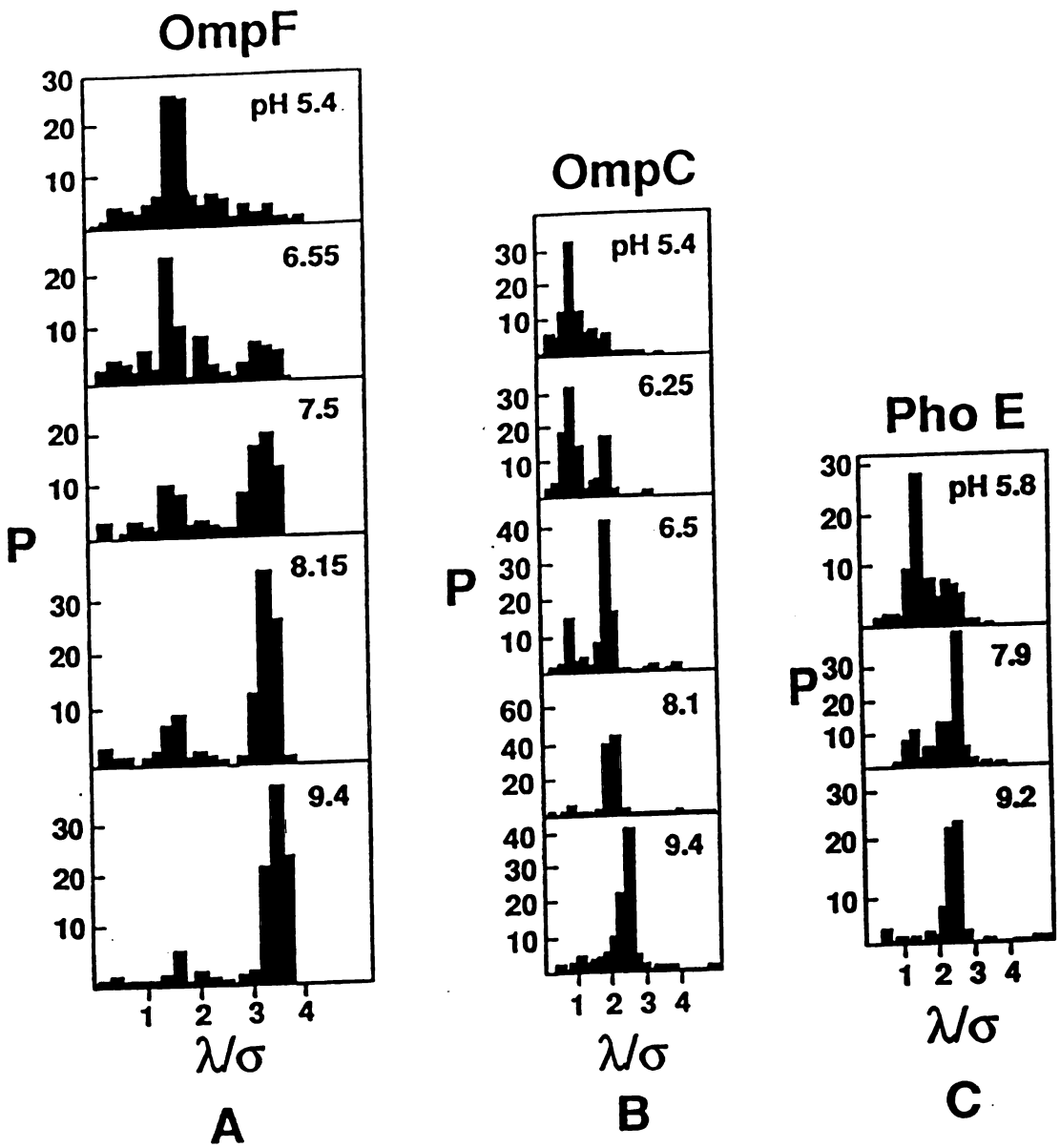
OmpF
pH 5.4
6.55
7.5
8.15
9.4
P
λ/σ
A
OmpC
pH 5.4
6.25
6.5
8.1
9.4
P
λ/σ
B
Pho E
pH 5.8
7.9
9.2
P
λ/σ
C

Figure 2

~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 was 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 pH's 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 6.5, 8.1, and 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 pH's, e.g. pH 5.8. In all cases, the size of the large channel was approximately twice that of the small channel (for pH's < 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

Figure 3. Average size (λ/σ) of the large channel at pH's 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.

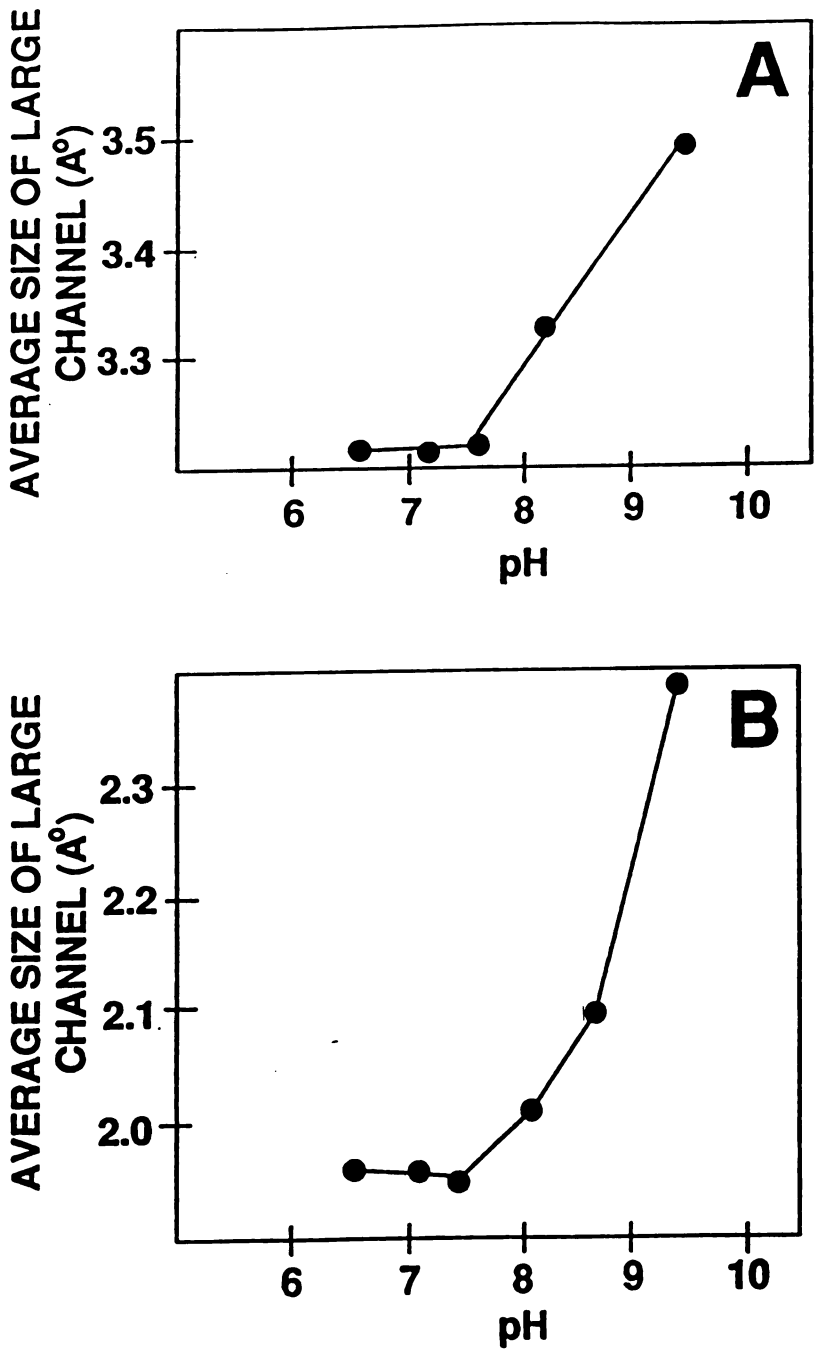
A
AVERAGE SIZE OF LARGE CHANNEL (A°)
3.5
3.4
3.3
6
7
8
9
10
pH
B
AVERAGE SIZE OF LARGE CHANNEL (A°)
2.3
2.2
2.1
2.0
6
7
8
9
10
pH

Figure 3

aggregation state. Also, we are assuming that the current increments of the small and large size channel are the result of conductance across the three channels in the 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 pH's 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 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 at 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

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, 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.

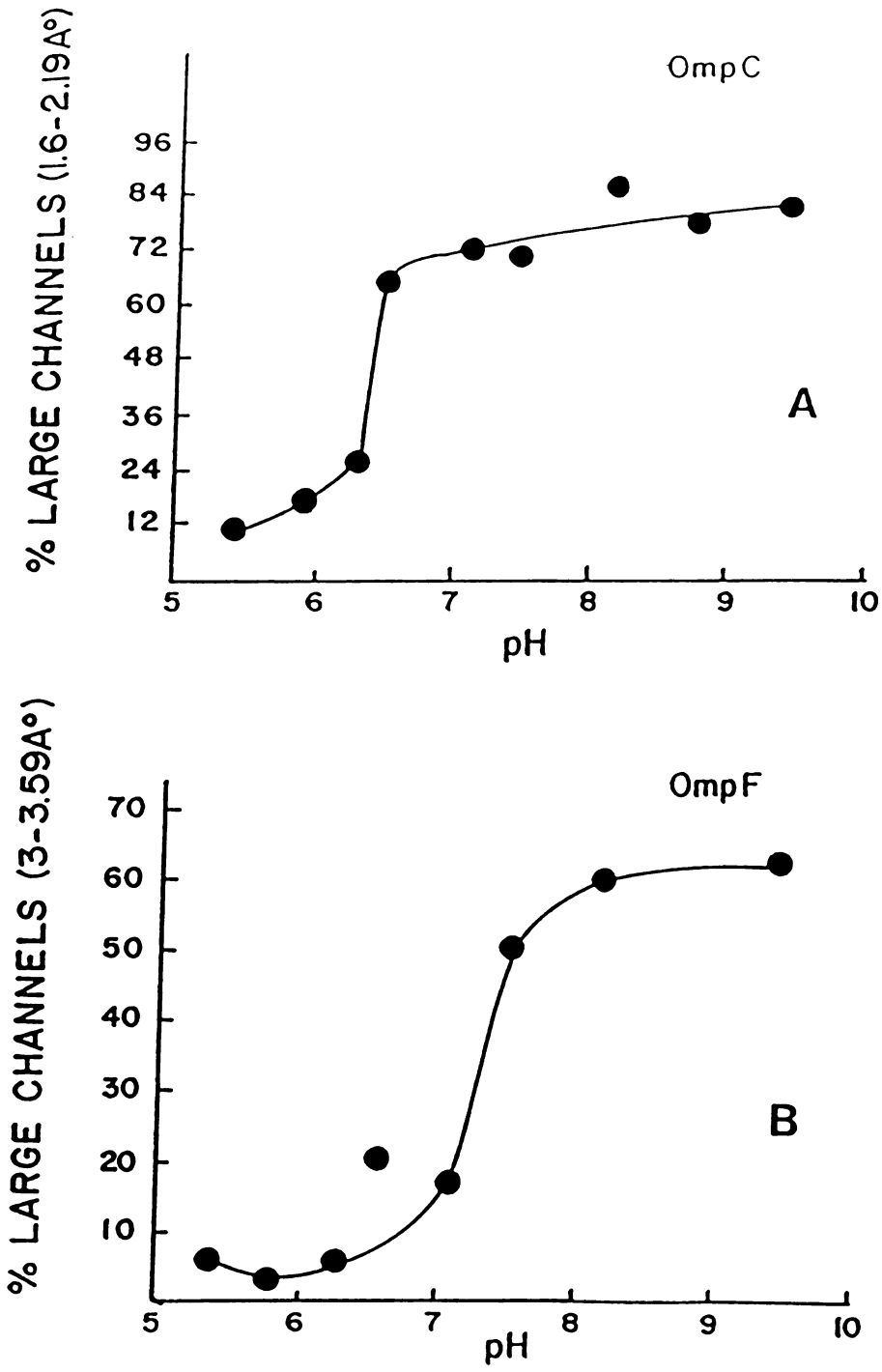
OmpC
% LARGE CHANNELS (1.6-2.19A°)
96
84
72
60
48
36
24
12
5
6
7
8
9
10
pH
A
OmpF
% LARGE CHANNELS (3-3.59A°)
70
60
50
40
30
20
10
5
6
7
8
9
10
pH
B

Figure 4

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 1). 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 pH 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 1). 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 that of the LPS-enriched samples (Figure 5 and Figure 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

TABLE 1:

Liposome Swelling Assay
Percent Decrease in Light Scattering
After 30 Seconds in Test Solute

Test Solute	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%
Average	N.A.[b]	N.A.	N.A.	N.A.	1.1% ± 1.5%	1.4% ± 0.8%

[a]Corrected for control liposome swelling

[b]N.A. 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.

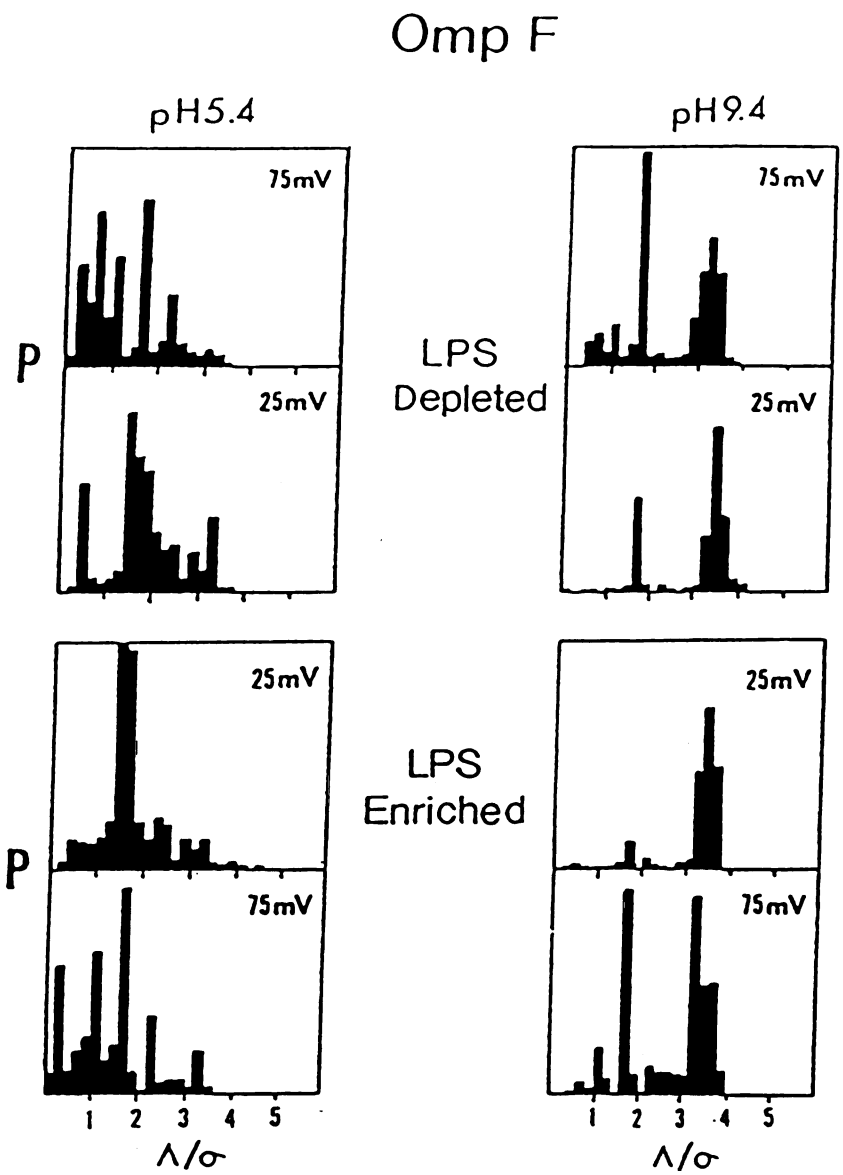
Omp F
pH5.4
pH9.4
75mV
25mV
P
LPS
Depleted
LPS
Enriched
1 2 3 4 5
Λ/σ

Figure 5

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.

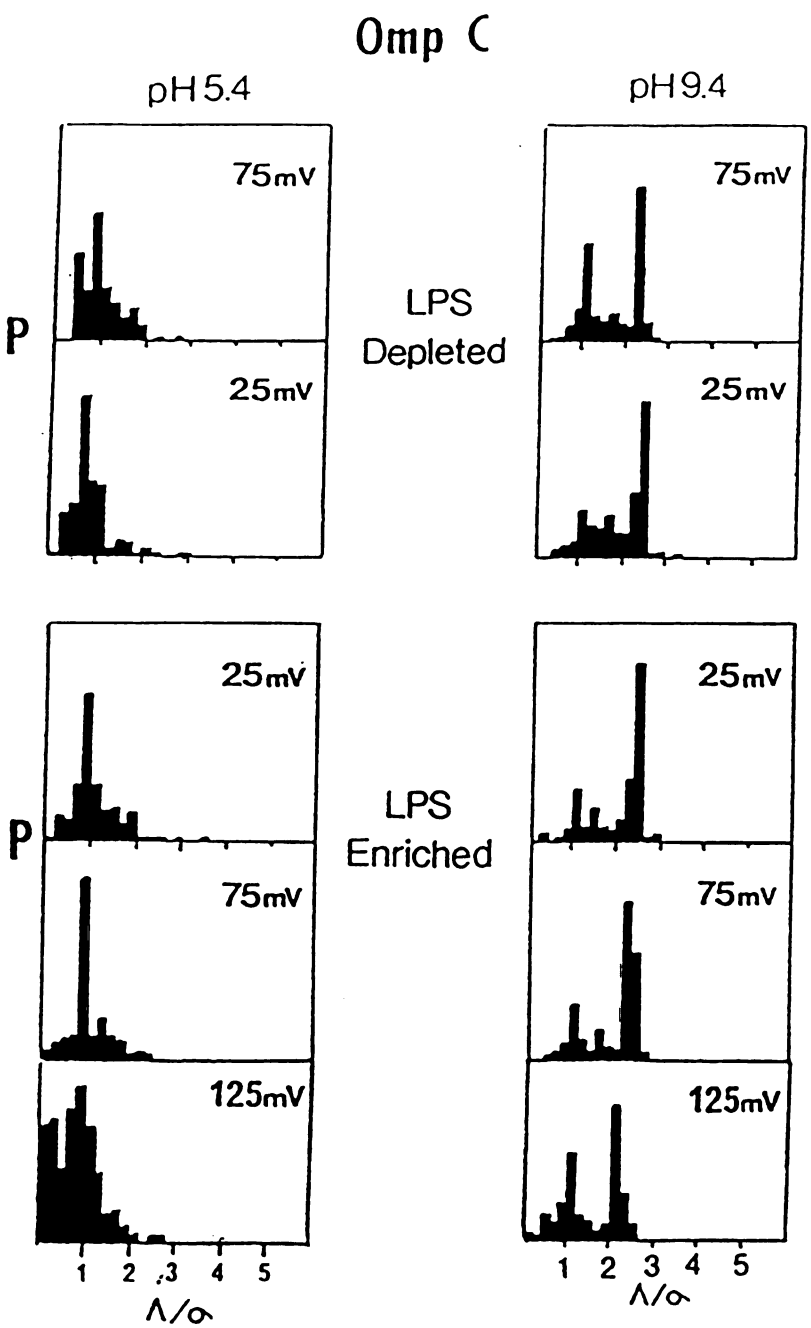
Omp C
pH 5.4
pH 9.4
75mV
25mV
P
LPS
Depleted
25mV
75mV
125mV
P
LPS
Enriched
1 2 3 4 5
Λ/σ

Figure 6

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 1/3 and 2/3 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 was needed with the OmpF sample. These very small channels detected at high voltage and low pH result from either 1) the loss of cooperativity of channel opening within the trimeric complex in the BLM (see Xu et. al., 1986) or 2) 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 2 and 3). For OmpF, the results indicate that gating, or closing, of the channels was not significant below 100 mV unless the pH was below 6.5 (Table 2). At pH 6.5, OmpF showed significant gating at 75 mV but

TABLE 2[a]

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.

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 3[a]

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.

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

only if the sample was LPS-depleted. Lowering the pH to 5.5 resulted in a significant increase in 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 3, 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 to 100 mV at both pH 9.2 and 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 in the percentage of small channels. Similar studies of the current-voltage relationship for LPS-enriched OmpF (Figure 8A) indicated

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.

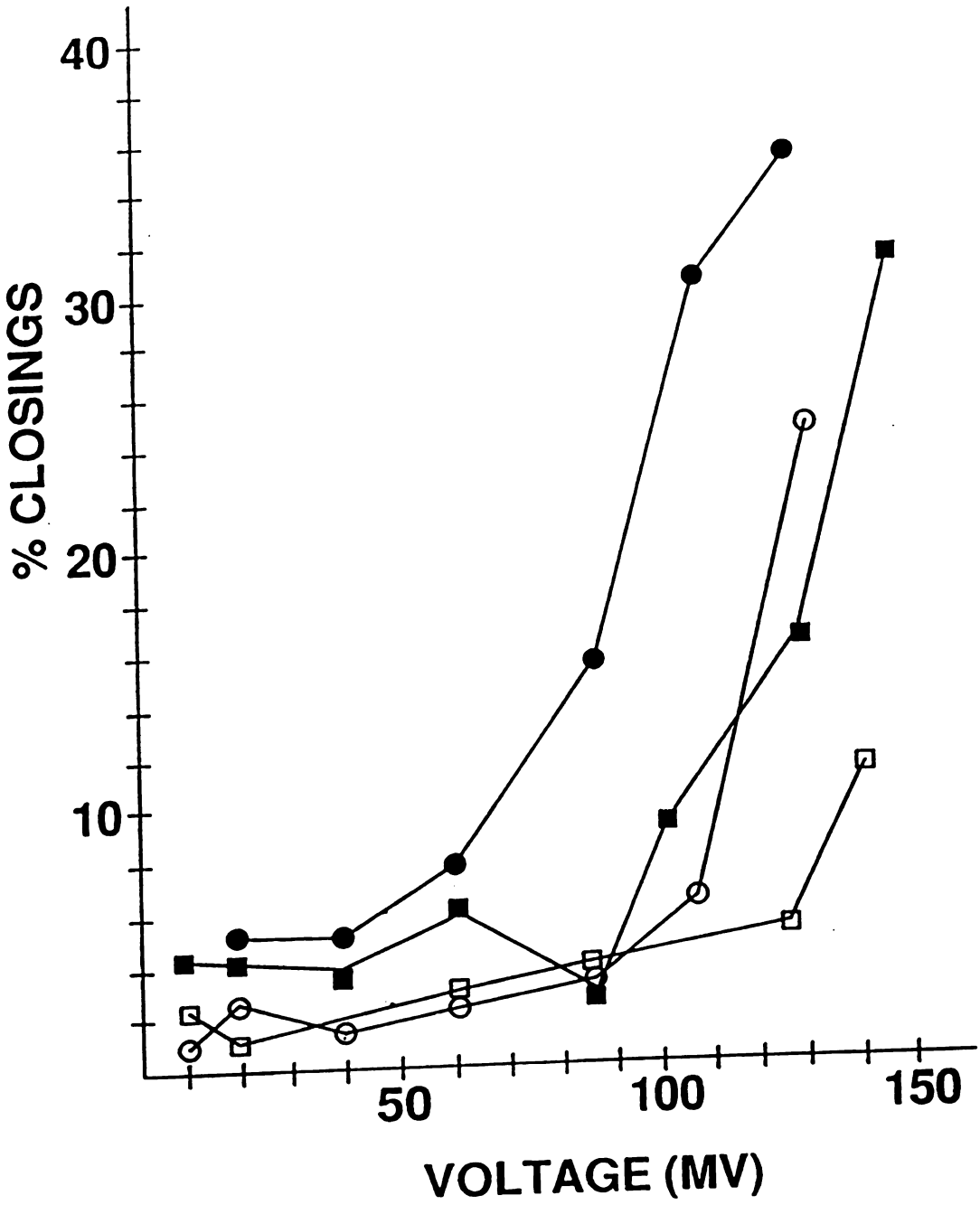

Figure 7

Figure 8. The effect of voltage on channel current for LPS-enriched OmpF (A) and OmpC (B) at pH 5.6 and pH 9.2. For each pH, 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.

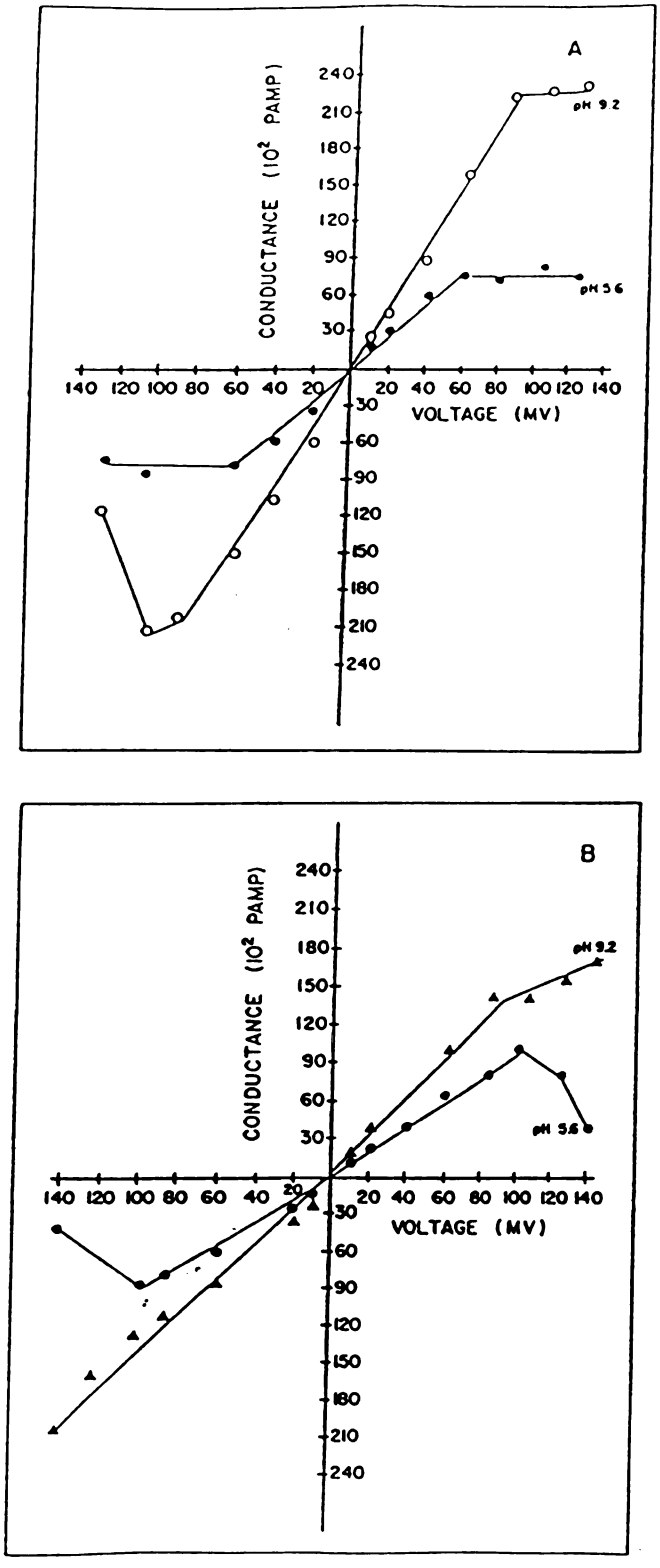
A
CONDUCTANCE (10^2 PAMP)
240
210
180
150
120
90
60
30
pH 9.2
pH 5.6
140 120 100 80 60 40 20
20 40 60 80 100 120 140
VOLTAGE (MV)
30
60
90
120
150
180
210
240
B
CONDUCTANCE (10^2 PAMP)
240
210
180
150
120
90
60
30
pH 9.2
pH 5.6
140 120 100 80 60 40 20
20 40 60 80 100 120 140
VOLTAGE (MV)
30
60
90
120
150
180
210
240

Figure 8

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 following 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 5.6; for OmpF, the calculated n was 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 open channels with variable sizes which correspond to unique structural conformations. We have found that pH induces a change from one conformation 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; Schindler & Rosenbusch, 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 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 does 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; Weiss et. al. 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 coworkers (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 (Rocque & McGroarty, 1990; Markovic-Housley & Garavito, 1986); 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.

There is some question as to which of the observed channel conductance increments represent opening of the trimeric complex. We propose that both the small and 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 ~1/3 and 2/3 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); and 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) and OmpF (Cowan et. al., 1992). The further increase in the size of the large-size channel for both OmpC and OmpF at pH's 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_as 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 3 proteins, we suggest this histidine may be involved in the channel-size switch (see next chapter).

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" (ie. aggregation of 2 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 pH's, a larger number of negative charges should result in charge repulsion rather than "dimerization". Secondly, 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 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 which may be involved in the pH-induced switch in channel size (see next chapter) 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 states 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:

(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's 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 next chapter).

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 phenomena 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 change in pH in the environment. Relevant to this proposal are the experiments of Heyde & Portalier (1987) which showed an increase in the synthesis of OmpC (ie. smaller channels) and a decrease in OmpF (ie. 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. Regardless, the fact that a variety of transport systems have reduced uptake at lower pH (Bertran et. al., 1991; Ermishkin & Mirzabekov, 1990; K. W. Kinally, Abstr. Annu. Meet. Biophys. Soc. 1991, p. 177; P. Daumas and O. S. Anderson, Abstr. Annu. Meet. Biophys. Soc. 1991, p. 259; Benz et. al., 1979; Schindler & Rosenbusch, 1978; Heyde & Portalier, 1987; Gudmundsson et. al., 1991; Sabath et. al., 1968) may indicate a common physiological role.

A dynamic role for porin in cell function has been suggested by others (A. H. Delcour, E. Martinac, E. P.

Kennedy, J. Adler and C. Kung, Abstr. Annu. Meet. Biophys. Soc. 1991, p. 459). Specifically, Delcour et. al. (1989) found that membrane derived oligosaccharides (MDO) placed on the periplasmic side of the outer membrane decreased the single channel conductance of mechanosensitive *E. coli* channels. They also found that MDO 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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CHAPTER 3

Involvement of His21 in the pH-Induced Switch In Porin Channel Size

ABSTRACT

Porin is a channel-forming protein in the outer membrane of gram-negative bacteria. In chapter 2, we showed that pH induced a switch in the channel size *in vitro* for porins OmpF, OmpC and PhoE. In the results presented here, His21 of OmpC and OmpF from *Escherichia coli* was chemically modified with diethyl pyrocarbonate. Functional analysis of these modified porins at different pH's suggested that this histidine is involved in the pH-induced switch in channel size. Secondary structure analysis of porins at various pH's using Fourier transform infrared spectroscopy indicated that there was no global change in structure accompanying the pH-induced switch in channel size.

INTRODUCTION

The outer membrane of gram-negative bacteria contains trimeric channel-forming proteins called porins through which influx of nutrients and antibiotics occurs. The structure and function of bacterial porins have been reviewed extensively (Benz, 1985; Nikaido & Vaara, 1985; Rosenbusch, 1990; Tommassen, 1988, Cowen et. al., 1992); however, the exact mechanism for the regulation of porin function has yet to be defined. In recent functional studies, we found that porins of *Escherichia coli* K-12 can be stabilized in at least two open channel configurations *in vitro*, a small channel detected at acidic pH and a larger channel stabilized under basic conditions (Todt et. al. 1992). The pKa of the channel-size switch suggested the involvement of the single histidine present in both OmpC and OmpF (His21). In this study, we present further evidence for the involvement of histidine in the pH-induced functional changes by chemically modifying the residue with diethylpyrocarbonate (DEPC). Furthermore, using Fourier transform infrared spectroscopy (FTIR), we show that this functional change does not involve a structural alteration of a global nature since there is no change in porin secondary structure with pH.

MATERIALS AND METHODS

Cell growth

LPS-enriched OmpC and OmpF were isolated from *E. coli* K-12 strains ECB 621 ($ompF^-$,$lamB^-$; gift of S. Benson) and PLB 3261 ($ompC^-$, $lamB^-$; Benson & Decloux, 1985) respectively. Cultures were grown in 1% tryptone, 0.5% yeast extract, and 0.4% NaCl, pH 7.5 as described previously (Rocque & McGroarty, 1989; Rocque & McGroarty, 1990; Xu et. al., 1986). Cells producing OmpF were grown at 37°C, while cells producing OmpC were grown at 30°C. Cells were harvested late in logarithmic growth phase.

Porin isolation

Porins were isolated by the method of Lakey et. al. (1985) with some modifications (Rocque & McGroarty, 1989; Xu et. al., 1986). Cells were broken using a French pressure cell and treated with RNAse and DNAse. After pelleting the membranes at 100,000xg, the inner and some outer membrane proteins were dissolved with sodium dodecyl sulfate (SDS) in Tris-HCl. After centrifugation, the pellet contained outer membrane proteins, including most of the porins, bound to the peptidoglycan. The porins were solubilized with high NaCl in the presence of mercaptoethanol, Tris-HCl, and SDS. 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 levels of LPS bound to porin isolates were quantitated by the thiobarbiturate assay for 2-keto-3-deoxyoctulosonic acid (Droge et. al., 1970). The final preparations were suspended in 1% SDS, 10 mM Tris-HCl, pH 6.8, and 0.02% sodium azide (standard buffer). To adjust the pH of the porin samples for FTIR studies, samples were dialyzed against 1.0% SDS containing either 10 mM phosphate (for pH 5.8) or 10 mM CHES (for pH 9.25). For bilayer lipid membrane (BLM) studies, the samples were dialyzed against 0.5% SDS and 20 mM phosphate pH 6.0 (phosphate-SDS buffer). Porin was quantitated using the bicinchoninic acid protein assay (Pierce Chemical Co.), and porin trimer structure was confirmed by SDS-PAGE (Rocque et. al., 1987).

Carbethoxylation and decarbethoxylation of porin

Carbethoxylation of porin with DEPC was performed in phosphate-SDS buffer using the method of Bindslev and Wright (Bindslev & Wright, 1984) with modifications. A DEPC stock solution was prepared immediately prior to use by diluting an aqueous solution of DEPC (Sigma Chemical Co.) with equal volumes of anhydrous ethanol and determining the DEPC concentration by quantitative dilution of a small aliquot (1-5ul) into 3 ml of 10 mM imidazole, pH 7.5. Absorbance of this solution at 230 nm was converted to DEPC concentration using an extinction coefficient of 3000 $cm^{-1}M^{-1}$. OmpC and OmpF (1 ml of a 0.5-2 mg/ml solution) were modified by the addition of a small volume (1-2ul) of DEPC stock solution to

a final concentration of between 0.18-4 mM (18.5-80 mole DEPC/mole protein). Carbethoxylation of porin's histidine was detected at 246 nm and converted to concentration using an extinction coefficient of 3200 $cm^{-1}M^{-1}$. Tyrosine modification was monitored at 278 nm. Analysis was performed on modified porin only after determining by absorbance at 246 nm that one residue was modified per monomer of porin. This modified residue was stable for at least 3 hours at pH 5.6 and 8.0. The carbethoxy group was removed from the modified OmpC and OmpF by the addition of hydroxylamine (in 20 mM phosphate, 0.5% SDS, pH 6.8) to a final concentration of 20 mM. The reaction was allowed to proceed until the number of residues modified (as detected at 246 nm) was close to 0 (2-10 min.). The reaction was stopped by dilution of porin with phosphate-SDS buffer to a concentration of 10 ug/ml.

Bilayer lipid membrane assay (BLM)

Analysis of electrical conductance across a bilayer lipid membrane was used to measure the channel size of modified and unmodified porins. The electrical conductance was measured across a lipid bilayer comprised of 1% diphytanoyl phosphatidylcholine (in n-decane). As described previously (Rocque & McGroarty, 1989; Rocque & McGroarty, 1990), a small volume of porin suspended in standard buffer was added to the salt solution bathing the lipid membrane. This bathing solution contained 0.5 M NaCl and 0.5 mM buffer

at an appropriate pH. It was difficult to maintain the pH precisely during the time period of the BLM analysis; however, the pH was controlled to within 0.1 pH units 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 the occurrence of an event with a particular size. A statistically significant number of channels ($\geq$200) were analyzed for each experimental condition.

FTIR

For FTIR measurements, porin samples were diluted to a concentration of 2 mg/ml and analyzed along with control samples lacking the protein but containing LPS at concentrations equivalent to the amount present in porin samples. The porin and control samples were dialyzed against either pH 5.8 or 9.25 buffer. The samples were then lyophilized and resuspended in D_2O. For FTIR analysis, samples were placed in a CaF_2 Harrick cell. FTIR spectra were recorded on a Nicolet 710 spectrophotometer and an atmospheric background was recorded before sample scanning. Eight hundred interferograms were averaged, apodized with a triangular squared function and Fourier transformed to a

resolution of 2 cm^{-1}. The spectrum of the control sample at the appropriate pH was subtracted from each sample spectrum. A BP Decon self-deconvolution program was used to deconvolute each background-subtracted spectrum and to obtain intensity values for each peak. Two constants required as computer input were σ, the estimated half-width at half-height of unresolved bands, and k, the resolution "efficiency" factor. Values entered for σ and k were 90 cm^{-1} and 2.3 respectively.

RESULTS

In previous work, we have found that a switch in porin channel size may be induced by the titration of a single group with an apparent pKa of 7.2 for OmpF and 6.5 for OmpC. (Todt et. al., 1992). Since these pKas are close to that of a histidine side chain (pKa 6.0), of which OmpF and OmpC contain only one, porin was modified with DEPC and the effects on function were examined. DEPC has been used to specifically modify histidine to study its function in proteins (Bindslev & Wright, 1984; Blanke & Hager, 1990; Miles, 1977; Sams & Matthews, 1988; Takeuchi et. al., 1986). Unmodified and DEPC-modified OmpF (Figure 1) and OmpC (Figure 2) were analyzed at different pHs in the BLM apparatus, measuring channel conductance. As seen in our previous results, there was an increase in the proportion of larger-size channels (λ/σ ~3.1-3.5 Å for OmpF, ~1.9-2.5 Å for OmpC) and a decrease in the relative number of small channels (λ/σ ~1.55 Å for OmpF, ~0.9 Å for OmpC) at basic pH. In contrast, histograms of DEPC-modified OmpC and OmpF showed a high proportion of larger-size channels at both acidic and basic pH suggesting that the modified residue was involved with controlling the pH-induced switch in channel size. There was a slight increase in the number of small-size channels with DEPC-modified OmpF at pH 8.0 compared to wild type at pH 8.1 which was probably due to that fact that the pH was slightly lower. Removal of the carbethoxy group

Figure 1. Probability distribution histograms of the size parameter λ/σ (Å), for OmpF (A), DEPC-modified OmpF (B) and DEPC-modified OmpF treated with 20 mM hydroxylamine (C) as measured in bilayer lipid membranes. The porins were added to bathing solutions of 0.5 M NaCl containing 0.5 mM of sodium phosphate. 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 histograms of $\geq$200 events.

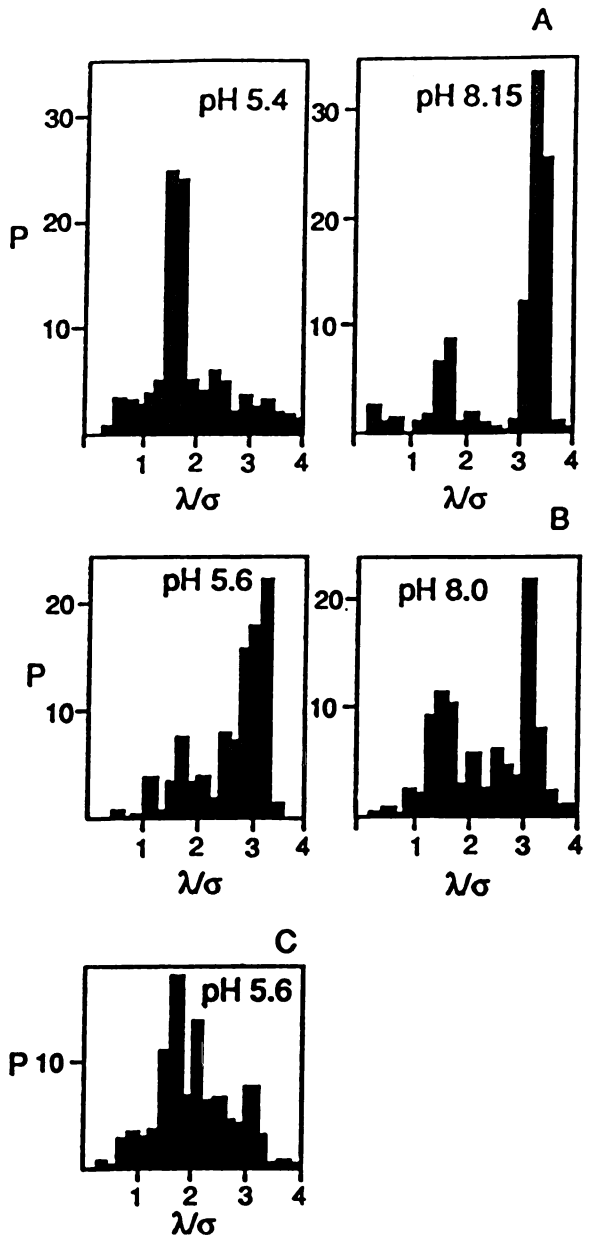
A
pH 5.4
pH 8.15
30
20
10
P
1 2 3 4
λ/σ
B
pH 5.6
pH 8.0
20
10
P
1 2 3 4
λ/σ
C
pH 5.6
P 10
1 2 3 4
λ/σ

Figure 1

Figure 2. Probability distribution histograms of the size parameter λ/σ (Å) for OmpC (A), DEPC-modified OmpC (B), and DEPC-modified OmpC treated with 20 mM hydroxylamine (C) as measured in bilayer lipid membranes. The porins were added to bathing solutions and electrical conductance was measured as described in Figure 1.

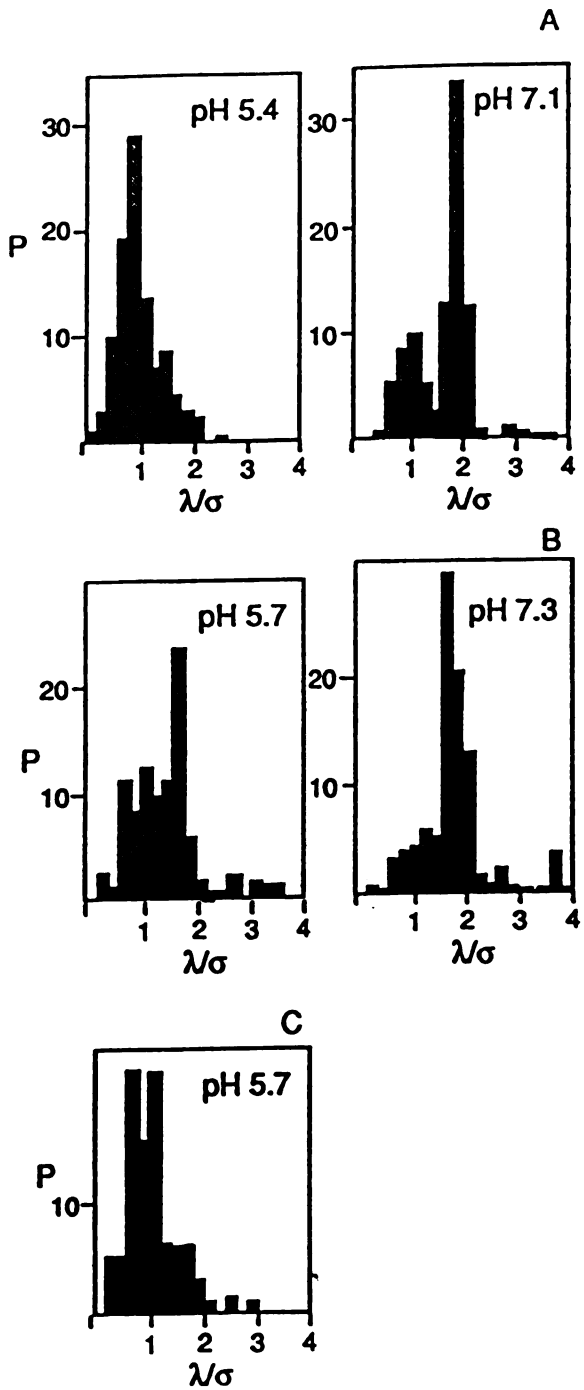

Figure 2

from histidine (with hydroxylamine) restored the small channel configuration at low pH (Figure 1C and 2C).

To determine whether the pH-induced switch in channel size involved global structural changes, FTIR analysis of porins at various pH's was performed. FTIR spectra have been used to measure structural changes in proteins (Alvarez et. al., 1987; Haris et. al., 1989; Susi et. al., 1967; Wantyghem et. al., 1990). The secondary structure of porin can be measured by examining the amide I region (1620-1690 cm^{-1}) of the absorption spectra. Amide I peaks of OmpC and OmpF were analyzed at pH 5.8 and pH 9.25 after suspending the samples in D_2O. When comparing porin samples at pH 5.8 to pH 9.25, the deconvoluted amide I peaks were essentially identical (Figure 3) indicating little change in secondary structure in this pH range.

Figure 3. Amide I region of the FTIR spectra of LPS-enriched OmpF (A) or OmpC (B) suspended in 1% SDS and either 10 mM succinate, pH 5.8 (---) or 10 mM CHES, pH 9.25 (——). The spectra were recorded on a Nicolet 710 FTIR spectrometer and deconvoluted using the BP Decon package. Spectra of control samples containing the same buffer, detergent and LPS as the porin samples were subtracted from each porin spectra. The spectra at pH 5.8 and 9.25 were offset.

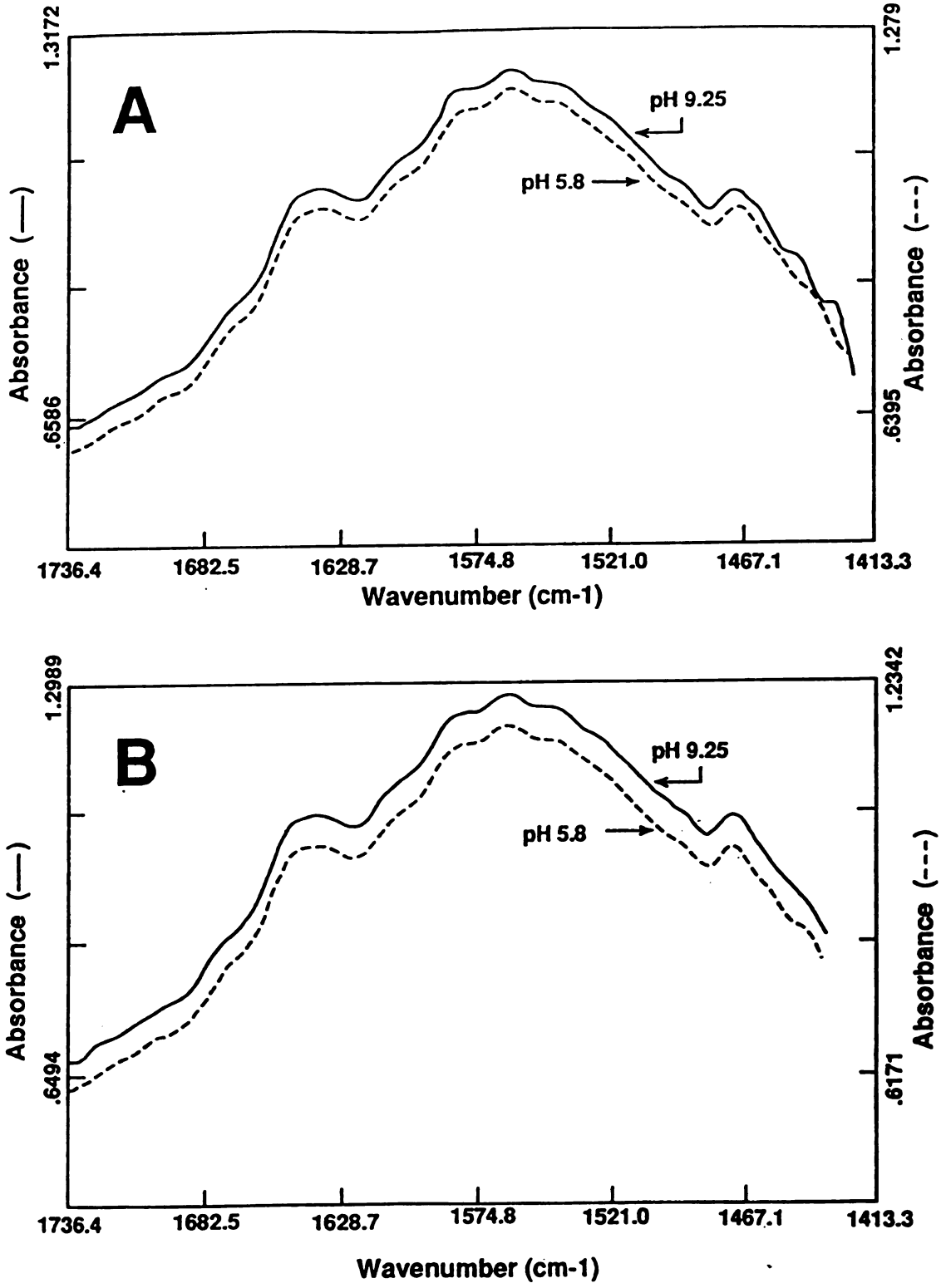
A
pH 9.25
pH 5.8
1.3172
.6586
1.279
.6395
Absorbance (—)
Absorbance (---)
1736.4
1682.5
1628.7
1574.8
1521.0
1467.1
1413.3
Wavenumber (cm-1)
B
pH 9.25
pH 5.8
1.2989
.6494
1.2342
.6171
Absorbance (—)
Absorbance (---)
1736.4
1682.5
1628.7
1574.8
1521.0
1467.1
1413.3
Wavenumber (cm-1)

Figure 3

DISCUSSION

These results corroborate our previous functional analysis of *E. coli* porins which showed that porins exist in at least two open channel configurations, a small-size channel stable at acidic pH and a larger-sized set of channels stable at basic pH. Since the pKa of the switch in channel size indicated the involvement of a histidine (Todt et. al., 1992) and since OmpC and OmpF (as well as PhoE) contain one histidine residue (His 21), an attempt was made to specifically modify this histidine using DEPC and study the effect on function using BLM analysis. The results indicated that the DEPC-modified residue was involved in the pH-induced switch in channel size. Evidence that this modified residue was histidine includes: 1) only one residue per monomer was modified for OmpC and OmpF, and both porins contain only one histidine per monomer, 2) hydroxylamine addition, which is known to remove the carbethoxy group from modified histidyl and tyrosine groups (but not from modified lysyl or sulfhydryl groups) (Miles, 1977), reversed the effect of DEPC on OmpF and OmpC (Figure 1C and 2C respectively), and 3) there was no indication of tyrosine modification by DEPC since there was no drop in absorbance at 278 nm (Burstein et. al., 1974) and the addition of 20 mM hydroxylamine (a concentration too low to reverse tyrosine modification; Burstein et. al., 1974) still reversed the effect of DEPC on porin. Thus our results

indicate that histidine is involved in the pH-induced switch in porin channel size.

Upon analyzing the amide I peak for changes in porin secondary structure, no differences were detected for either OmpF or OmpC between pH's 5.8 and 9.25. This indicated that any conformational alteration accompanying the pH-induced change in function does not involve a global alteration in structure.

In combination, these results indicate the involvement of His21 in a change in porin channel size with pH which results in a small conformational change but a fairly large alteration in function. Histidine has been found to be involved in the regulation of transport through a variety of other channels (Bertran et. al., 1991; Cain & Simoni, 1988; Padan et. al., 1985; Yamaguchi et. al., 1991; C. K. Abrams, K. S. Jakes, S. Finkelstein and S. L. Statins, Abstr. Annu. Meet. Biophys. Soc. 1991, p. 458a; C. Pederzolli, L. Cescatti, and G. Menestrina, Abstr. Annu. Meet. Biophys. Soc. 1991, p. 458a). Evidence for small changes in porin structure resulting in large changes in function has also been found by Benson and coworkers (1985 and 1988) who isolated mutants with single residue changes in OmpF and OmpC which allowed the uptake of significantly larger maltodextrins presumably due to enlarged porin channels. Also, the presence of channel substates has been proposed for VDAC (Mannella et. al., 1992) and similar substates in bacterial porin could explain some of the

discrepancies among channel sizes reported by various investigators (Benz et. al., 1984; Jap et. al., 1991; Weiss et. al., 1990; Xu et. al., 1986). The mechanism of the pH-induced switch in channel size may involve the movement of a protonated histidine toward a neighboring carboxyl at acidic pH which results in a reduction in channel size. Structural analysis of OmpF porin has shown that this histidine would be in a positively-charged section lining the channel, close to the trimer center (Cowan et. al., 1992). This would place histidine across from the large, negatively-charged loop between strands β5 and β6 which defines the exclusion limit for diffusing particles. Therefore, at acidic pH, the protonated histidine could cause this negatively-charged loop to move closer causing a change in channel size.

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CHAPTER 4

Effects of pH on Bacterial Porin Structure; The Involvement of His21 in a pH-Induced Conformational Change

ABSTRACT

Porin is a channel-forming protein in the outer membrane of gram-negative bacteria. In previous chapters, it was shown that pH induced a switch in the channel size *in vitro* for porins OmpF, OmpC and PhoE. In the results presented here, this pH-induced switch in channel size was correlated with a structural change detected using intrinsic tryptophan fluorescence and Fourier transformed infrared spectroscopy. The pH-induced alteration in conformation correlated with the pH-induced functional changes as indicated by: 1) the similarity of the pKa's of the structural change and of the channel size transitions and 2) the induction of a change in structure by an alteration in pH similar to that induced by the modification of His21; this amino acid has been identified as involved in this pH-induced change in function (Todt & McGroarty, 1992).

INTRODUCTION

The outer membrane of gram-negative bacteria contains trimeric channel-forming proteins called porins through which influx of nutrients and antibiotics occurs. The structure and function of bacterial porins have been described extensively (Benz, 1985; Nikaido & Vaara, 1985; Rosenbusch, 1990; Tommassen, 1988); however, the exact mechanism, if any, for the regulation of porin has yet to be defined. In recent functional studies, we found that porins of *Escherichia coli* K-12 can be stabilized in at least two open channel configurations *in vitro*, a small channel detected at acidic pH and a larger channel stabilized under basic conditions (see Todt et. al., 1992). In this study, the pH-induced switch in channel size was correlated with a structural alteration detected using Fourier transformed infrared spectroscopy (FTIR) and intrinsic tryptophan fluorescence. His21 is proposed to be involved in the pH-induced switch in channel size (Todt & McGroarty, 1992). Evidence for the involvement of this histidine in the switch in channel size was found by measuring changes in structure and function of porins modified with diethyl pyrocarbonate (DEPC).

MATERIALS AND METHODS

Cell growth

LPS-enriched OmpC and OmpF were isolated from *E. coli* K-12 strains ECB 621 (ompF$^-$, lamB$^-$; Misra & Benson, 1988) and PLB 3261 (ompC$^-$, lamB$^-$; Benson & Decloux, 1985; Benson et. al., 1988) respectively. PhoE was isolated from *E. coli* K12 strain JF 694 (ompF$^-$, ompC$^-$; Benson et. al., 1988). Cultures were grown in 1% tryptone, 0.5% yeast extract, and 0.4% NaCl, pH 7.5 as described previously (Rocque & McGroarty, 1989; Rocque & McGroarty, 1990; Xu et. al., 1986). Cells producing OmpF or PhoE were grown at 37°C, while cells producing OmpC were grown at 30°C. Cells were harvested late in logarithmic growth phase.

Porin Isolation

Porins were isolated by the method of Lakey et. al. (1985) with some modifications (Rocque & McGroarty, 1989; Xu et. al., 1986). Cells were broken using a French pressure cell and treated with RNAse and DNAse. After pelleting the membranes at 100,000xg, the inner and some outer membrane proteins were solubilized with sodium dodecyl sulfate (SDS) in Tris-HCl. After centrifugation, the pellet contained outer membrane proteins, including most of the porin, bound to the peptidoglycan. The bound porins were released with high NaCl concentration in the presence of mercaptoethanol, Tris-HCl, and SDS. 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 levels of LPS bound to porin isolates were quantitated by the thiobarbiturate assay for 2-keto-3-deoxyoctulosonic acid (Droge et. al., 1970). The final preparations were suspended in 1% SDS, 10 mM Tris-HCl, pH 6.8, and 0.02% sodium azide (standard buffer). To adjust the pH of the porin samples for fluorescence studies, the samples were dialyzed against 0.1% SDS containing 1.0 mM of either succinate (pH 5.6), sodium phosphate (pH 6.1-7.6) or sodium 2-(N-cyclohexylamino)ethanesulfonic acid (CHES, pH 7.9). To adjust the pH of the porin samples for FTIR studies, samples were dialyzed against 0.1% SDS containing 10 mM of either phosphate (pH 5.6) or CHES (pH 8.5). Porin was quantitated using the bicinchoninic acid protein assay (Pierce Chemical Co.), and trimeric structure was confirmed by SDS-PAGE (Rocque et. al., 1987).

Carbethoxylation and decarbethoxylation of porin.

Carbethoxylation of porin with DEPC was performed in phosphate-SDS buffer using the method of Bindslev and Wright (1984) with modifications. A DEPC stock solution was prepared immediately prior to use by diluting an aqueous solution of DEPC (Sigma) with equal volumes of anhydrous ethanol and determining the DEPC concentration by quantitative dilution of a small aliquot (1-5ul) into 3 ml

of 10 mM imidazole, pH 7.5. Absorbance of this solution at 230 nm was converted to DEPC concentration using an extinction coefficient of 3000 $cm^{-1}M^{-1}$. OmpC and OmpF (1 ml of a 0.5-2 mg/ml solution) were modified by the addition of a small volume (1-2ul) of DEPC stock solution to a final concentration of between 0.18-4 mM (18.5-80 mole DEPC/mole protein). Carbethoxylation of porin's histidine was detected at 246 nm and converted to concentration using an extinction coefficient of 3200 $cm^{-1}M^{-1}$. Tyrosine modification was monitored at 278 nm. Analysis was performed using modified porin only after determining from the absorbance at 246 nm that one residue per monomer was modified. This modified residue was stable for at least 3 hours at pH 5.6 and 8.0. The carbethoxy group was removed from the modified OmpC and OmpF by the addition of hydroxylamine (in 20 mM phosphate, 0.5% SDS, pH 6.8) to a final concentration of 20 mM. The reaction was allowed to proceed until the number of residues modified (as detected at 246 nm) was reduced to near 0 (2-10 min.). The reaction was stopped by diluting the porin to 10 ug/ml with phosphate-SDS buffer.

FTIR

For FTIR measurements, porin samples were diluted to 2 mg/ml and analyzed along with control samples lacking the protein but containing LPS at concentrations equivalent to the amount present in porin samples. The porin and control samples were dialyzed against either pH 5.6 or pH 8.5

buffer. The samples were then lyophilized and resuspended in D_2O. OmpF, OmpC and control samples were modified with DEPC while an equivalent amount of ethanol was added to unmodified porin and control samples. For FTIR analysis, samples were placed in a CaF_2 Harrick cell. FTIR spectra were recorded on a Nicolet 710 spectrophotometer and an atmospheric background was recorded before sample scanning. Unmodified OmpC, OmpF and control samples were analyzed at pH 5.6 and 8.5 while modified porin and control samples were analyzed at pH 5.6. Eight hundred interferograms were averaged, apodized with a triangular squared function and Fourier transformed to a resolution of 2 cm^{-1}. The spectrum of the control sample at the appropriate pH was subtracted from each sample spectrum. A BP Decon self-deconvolution program was used to deconvolute each background-subtracted spectrum and to obtain intensity values for each peak. Two constants required for computer analysis were σ, the estimated half-width at half-height of unresolved bands, and k, the resolution "efficiency" factor. Values entered for σ and k were 90 cm^{-1} and 2.3 respectively. The intensities of the amide II peaks were normalized to the corresponding amide I peak to correct for concentration effects (Wantyghem et. al., 1990).

Intrinsic Tryptophan Fluorescence

To measure intrinsic tryptophan fluorescence, porin samples were diluted to approximately 0.5 mg/ml. OmpC, was

dialyzed against pH 5.6 and 7.9 buffers; PhoE was dialyzed against pH 6.1 and 6.9 buffers. OmpF was dialyzed against pH 6.1, 6.6, 7.1, 7.3, and 7.6 buffers. Also, an amino acid solution was prepared at pH 5.6 (in 10 mM succinate) and at pH 7.9 (in 10 mM CHES), containing concentrations of tyrosine and tryptophan equivalent to that in the OmpC sample; i.e., 40 μM tryptophan and 290 μM tyrosine. Intrinsic tryptophan fluorescence measurements were made on a Perkin Elmer Model 512 double beam spectrofluorometer as modified by Adamsons et. al. (1982). A longer excitation wavelength of 295 nm was utilized to preferentially excite tryptophan residues. PhoE, OmpF and OmpC samples, and the amino acid solution were analyzed at various pH's. Also analyzed were control samples consisting of buffer at the appropriate pH. Emission spectra were plotted as quantum efficiency vs. emission wavelength.

RESULTS

To correlate the pH-induced switch in channel size observed in *in vitro* functional studies (Todt et. al., 1992) with structural alterations induced by pH, bacterial porins were analyzed at various pH's using FTIR and intrinsic tryptophan fluorescence. FTIR spectra can be used to measure structural changes in proteins (Alvarez et. al., 1987; Haris et. al., 1989; Susi et. al., 1967; Wantyghem et. al., 1990). The secondary structure of porin can be measured by examining the amide I region (1620-1690 cm^{-1}) of the absorption spectra. Alterations in the tertiary structure can be detected by measuring the change in the number of exchangeable hydrogens. This is shown by a shift in the midpoint of the amide II peak (1480-1575 cm^{-1}) from ~1550 cm^{-1} to ~1450 cm^{-1} when hydrogen is completely exchanged for deuterium (Alvarez et. al., 1987; Susi et. al., 1967; Rainteau et. al., 1989). Amide II peaks of OmpC and OmpF were analyzed at pH 5.6 and pH 8.5 after suspending the samples in D_2O. In previous studies, when comparing porin samples at pH 5.6 to those at pH 8.5, we found that the deconvoluted amide I peaks were essentially identical indicating little change in secondary structure in this pH range (Todt & McGroarty, 1992). In contrast, the amide II peak for both OmpC and OmpF showed a decrease in absorbance intensity (after normalization to the amide I peak intensity, $\Delta A=4.5 \pm 0.4\%$) at ~1560 cm^{-1} when comparing

samples at pH 8.5 to those at pH 5.6 (Figure 1); the ΔA indicated an increase in the level of hydrogen-deuterium exchange at the higher pH. This ΔA did not change significantly between 10 minutes and 25 hours following D_2O addition (0.85 ± 0.41% after 25 hrs) at both pH's for OmpF and OmpC, indicating that the major intensity difference with pH was not a kinetic effect. Overall, these results suggest that an alteration in tertiary structure occurred in OmpC and OmpF upon changing the pH between 8.5 and 5.6.

Since it has been shown that His21, the only histidine in these porins, is involved in the pH-induced switch in channel size (Todt & McGroarty, 1992), porin was modified with DEPC, which specifically alters histidine (Bindslev & Wright, 1984; Blanke et. al., 1990; Miles, 1977; Sams & Matthews, 1988; Takeuchi et. al., 1986), and the effects of the modification on structure were examined with FTIR spectroscopy. Previously we found that DEPC-modified porin, at all pH's, had predominantly large-size channels as did unmodified porin at pH 8.5 (measured by the bilayer lipid membrane assay; Todt & McGroarty, 1992). Therefore, to corroborate that the same structural change that occurred with a pH change was occurring with DEPC modification, FTIR analysis was performed on modified porin and compared to unmodified samples. Amide II peaks of DEPC-modified and unmodified OmpF and OmpC were analyzed at pH 5.6 (Figure 2). Comparing the amide II peaks of unmodified porins at pH 8.5 and at 5.6, there was a nearly identical drop in

Figure 1. Amide II regions of the FTIR spectra for OmpF (A) or OmpC (B) suspended in 0.1% SDS and either 10 mM CHES, pH 8.5 or 10 mM phosphate, pH 5.6. The spectra were recorded on a Nicolet 710 FTIR spectrometer and deconvoluted using a BP Decon package. Spectra of control samples containing the same buffer, detergent and LPS as the porin samples were subtracted from each porin sample spectra. The intensities of the amide II peaks were normalized to the intensity of the corresponding amide I peak to give relative absorbance intensity.

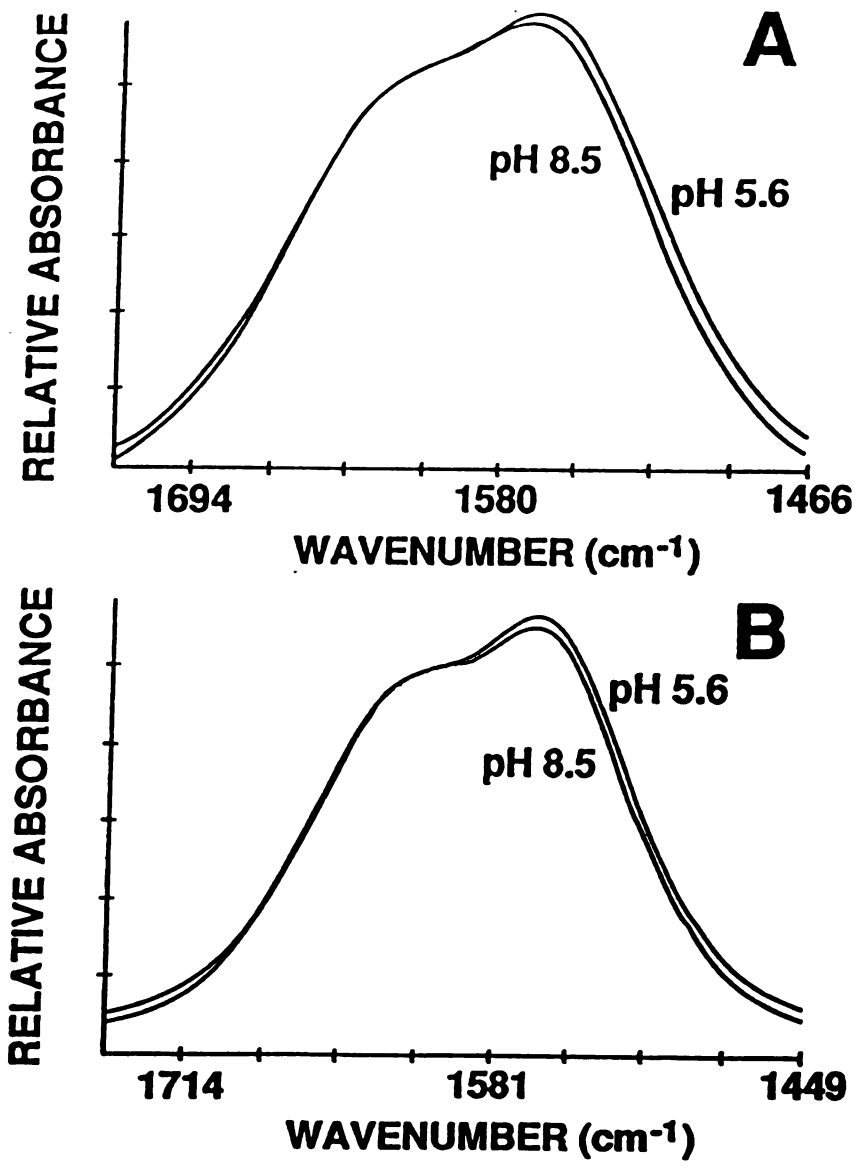
A
RELATIVE ABSORBANCE
pH 8.5
pH 5.6
1694
1580
1466
WAVENUMBER (cm-1)
B
RELATIVE ABSORBANCE
pH 5.6
pH 8.5
1714
1581
1449
WAVENUMBER (cm-1)

Figure 1

Figure 2. Amide II regions of the FTIR spectra for either OmpF (A) or OmpC (B) suspended in 0.1% SDS, 10 mM phosphate pH 5.6 and treated with DEPC (a) or ethanol (b). The spectra were recorded and processed as described for Figure 1.

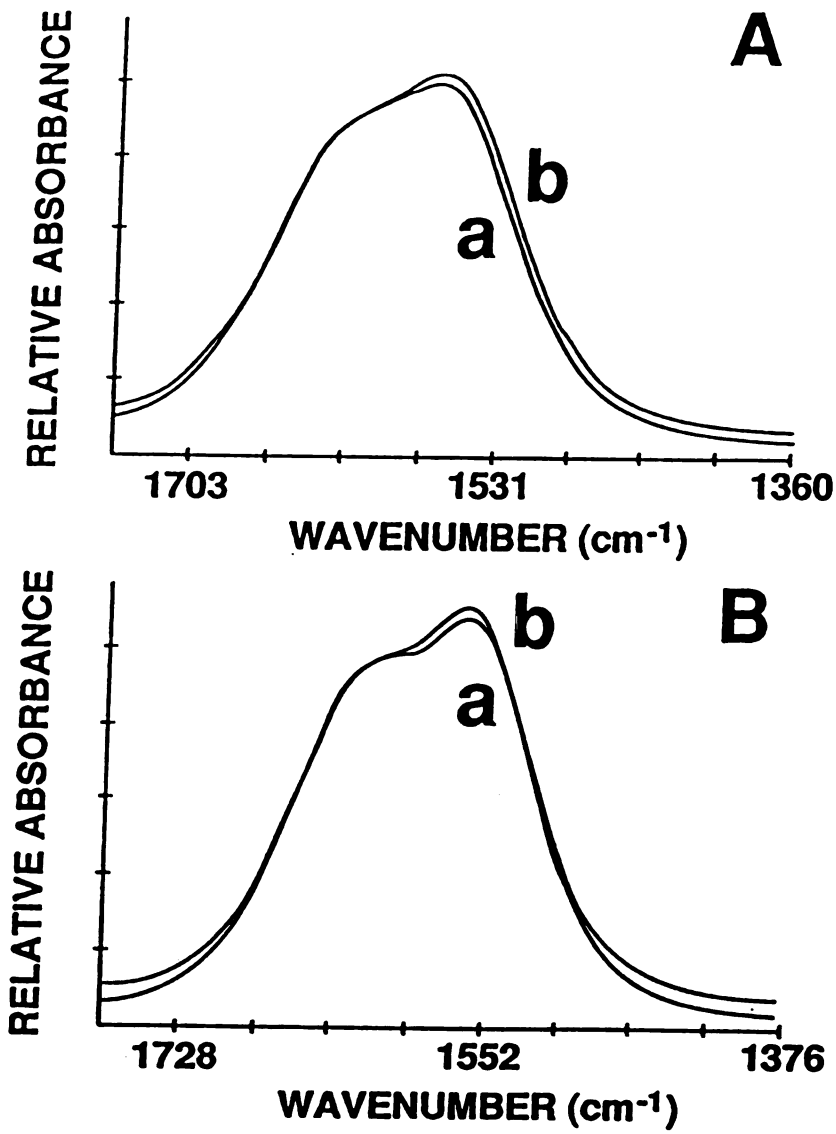
A
b
a
RELATIVE ABSORBANCE
1703
1531
1360
WAVENUMBER (cm-1)
B
b
a
RELATIVE ABSORBANCE
1728
1552
1376
WAVENUMBER (cm-1)

Figure 2

absorbance intensity (4.1 ± 0.4%) seen when comparing DEPC-modified porins to unmodified porin at pH 5.6. This indicated that a similar change in structure occurred with the elevation in pH as occurred with modification of His21.

Alterations in the tryptophan fluorescence of proteins have also been used to measure changes in structure. The porins OmpC, PhoE and OmpF have 4, 3, and 2 residues of tryptophan respectively. Changes in the tryptophan fluorescence with pH can indicate alterations in the environment of these residues. Tryptophan fluorescence of OmpC, OmpF, and PhoE was examined at various pH's and compared to a control solution containing tryptophan and tyrosine (Figure 3). The fluorescence of the tryptophan/tyrosine solution changed very little between pH 5.6 and 7.9. In contrast the fluorescence of both OmpF and PhoE, was greater at pH 6.6 (for OmpF) or pH 6.9 (for PhoE) compared to pH 6.1. For OmpC, however, fluorescence did not change between pH 5.6 and 7.9. The results indicate that for porin OmpF and PhoE, a pH-induced change in the localized region containing tryptophan residues occurred. The pKa for the change in OmpF tryptophan fluorescence was determined by plotting quantum efficiency versus pH (Figure 4). The pKa was between 6.1 and 6.6 which is close to the pKa determined for the pH-induced switch in channel size determined using *in vitro* functional assays (7.2) (Todt et. al., 1992).

Figure 3. Relative quantum efficiency of the fluorescence emission spectra (λ_{ex}=295 nm) for OmpF (A), PhoE (B), OmpC (C), and tryptophan and tyrosine at 40 uM and 290 uM respectively (D). OmpC and the amino acid solution were analyzed at pH 5.6 (1.0 mM succinate, 0.1% SDS), and 7.9 (1.0 mM CHES, 0.1% SDS) as described in the Materials and Methods. OmpF was analyzed at pH 6.1 and 6.6, while PhoE was analyzed at pH 6.1 and 6.9. Spectra were recorded on a modified Perkin- Elmer Model 512 Fluorescence spectrometer.

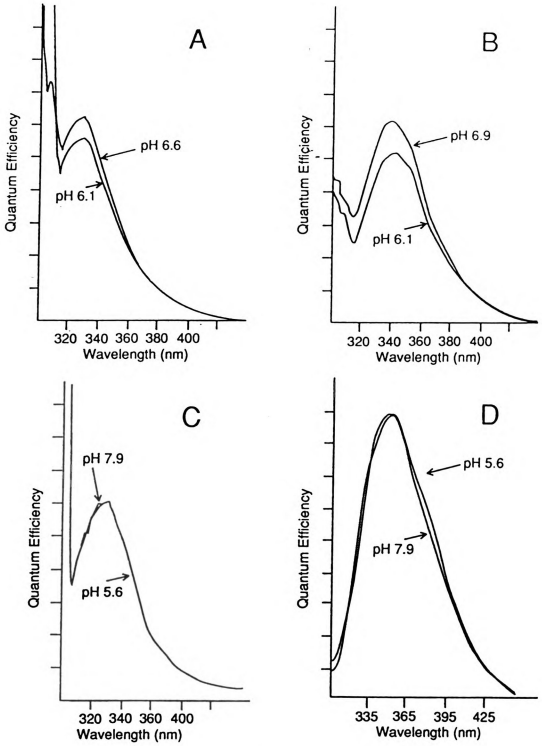
A
Quantum Efficiency
pH 6.6
pH 6.1
320 340 360 380 400
Wavelength (nm)
B
Quantum Efficiency
pH 6.9
pH 6.1
320 340 360 380 400
Wavelength (nm)
C
Quantum Efficiency
pH 7.9
pH 5.6
320 340 360 400
Wavelength (nm)
D
Quantum Efficiency
pH 5.6
pH 7.9
335 365 395 425
Wavelength (nm)

Figure 3

Figure 4. Relative quantum efficiency at peak maximum of the fluorescence emission spectra (λ_{ex}=295 nm) for OmpF plotted against the pH of the buffer. Buffers consisted of 0.1% SDS and 1.0 mM Na phosphate, pH 6.1, 6.6, 7.1, 7.3, or 7.6. Quantum efficiency was obtained using a modified Perkin Elmer Model 512 fluorescence spectrometer.

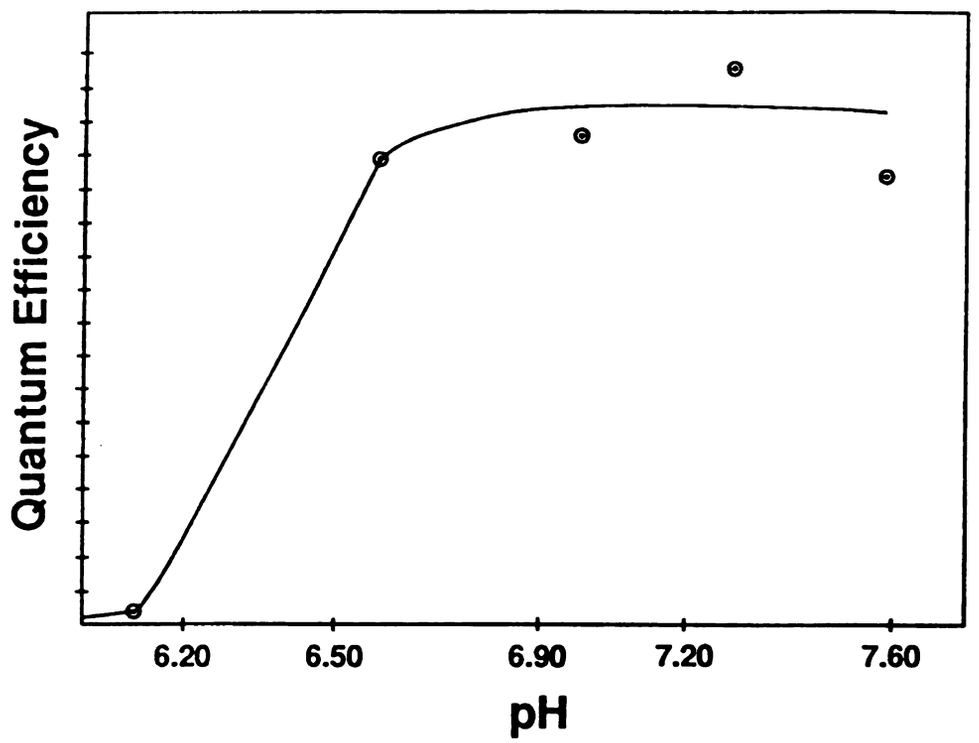

Figure 4

DISCUSSION

Our preliminary structural studies suggest a small localized conformational change in porin structure with pH. To characterize changes in protein conformation with pH across the neutral range, porins were analyzed using FTIR. Previously, upon analyzing the amide I FTIR peak for changes in porin secondary structure, no differences were detected for either OmpF or OmpC between pH's 5.6 and 8.5, indicating that any conformational alteration was subtle (Todt & McGroarty, 1992). However, changes in tertiary structure were detected with the decrease in the absorbance intensity of the amide II peak (1560 cm^{-1}) which indicated increased hydrogen-deuterium (H-D) exchange at the higher pH. A decrease in absorbance intensity (ΔA) at ~1560 cm^{-1} was detected at pH 8.5 compared to pH 5.6 for both OmpC and OmpF. H-D exchange is know to be catalyzed by base (Kim & Baldwin, 1982); therefore, to eliminate the possibility that the pH effect was due to a pH-dependent change in this exchange rate, a kinetic analysis of H-D exchange was followed for up to 25 hours. The fact that the drop in the intensity with time of the 1560 cm^{-1} peak for both OmpC and OmpF was significantly smaller compared to ΔA with pH suggests that, if the pH-induced ΔA is a kinetic effect resulting from differences in H-D exchange rates, it occurs in less than 10 minutes (the shortest sample analysis time). Also the fact the ΔA does not change significantly with time

for up to 25 hours suggests the drop in absorbance reflected a conformational change with pH. The value of ΔA, although small, is in a range found by others to be significant (Wu & Lentz, 1991; Wantyghem, 1990).

Our FTIR analysis of modified and unmodified porins at pH 5.6 indicated that DEPC modification causes an alteration in porin tertiary structure similar to that induced by basic pH. These results along with our previous kinetic study indicate that the drop in absorbance at ~1560 cm^{-1} with pH is not a kinetic effect of base-catalyzed hydrogen-deuterium exchange since the drop also occurred in the absence of a pH change.

When measuring intrinsic tryptophan fluorescence, alterations in the emission maximum or quantum yield can results from changes in the polarity of the environment of the amino acid residues; this change in polarity could be due to a conformation change. The tryptophan fluorescence of the solution of free amino acids (trp/tyr) did not change in the pH range of 5.6 to 7.9 while tryptophan fluorescence using either OmpF or PhoE showed a decrease in quantum efficiency when comparing samples at pH 6.1 to those at pH 6.6 (for OmpF) or 6.9 (for PhoE). OmpC on the other hand, did not show this change in quantum efficiency between pH 5.6 and 7.9. Since OmpF and PhoE contain a tryptophan which is lacking in the OmpC sequence (trp210 in PhoE, trp214 in OmpF), we propose that the environment of this common tryptophan is altered with pH. The fact that the pKa of

this change in tryptophan environment is close to the pKa for the pH-induced switch in channel size suggests a correlation between these structural and functional alterations with pH. The small difference in the pKas could result from the difference in ion concentration as well as detergent concentration used in the structural assays (1.0 mM salt, 0.1% detergent) as compared to that used in functional studies (0.55 M salt, 1% detergent). Variations in the environment of the amino acid side chain is known to alter or modify pKa values (Schulz & Schirmer, 1979; White et. al., 1968). In the latest model for OmpF by Cowan and coworkers (1992), trp214 is in an intramembranous β strand (β10) with its side chain facing the lipid environment. Trp214 is near a loop (L3) between β strands 5 and 6 which defines the channel exclusion limit. We have proposed that the protonation of His21 (which is across from L3) at acidic pH causes L3 to move closer to His21 resulting in a reduction in channel size (Todt & McGroarty, 1992). Thus, movement of this charged loop resulting in a change in polarity of the trp214 environment is consistent with our previous results and with the current model of porin structure.

In conclusion, structural analysis of porin using FTIR and tryptophan fluorescence indicated that there is a small, but reproducible pH-induced change in porin tertiary structure which correlates with a previously reported pH-induced switch in channel size (Todt et. al., 1992) since

the pKas are similar for the structural and functional transitions. We also have analyzed OmpF and OmpC at acidic and basic pH using differential scanning calorimetry and the results suggest that structural changes occur in this pH range (data not shown). Further evidence for correlation of the change in structure and function is provided in the histidine modification studies which indicated that this residue is involved in the structural as well as the functional alteration with pH; histidine modification caused a similar alteration in porin tertiary structure as a change to basic pH. This modification also induced the same changes in channel size as high pH. The conformation change induced by changes in pH is probably not a result of an alteration in aggregation state with pH because there was no change in light scattering of porin solutions within the pH range studied (Todt et. al., 1992). The alterations in structure and function of bacterial porin over a narrow pH range has implications for structural analysis of porin using, for example, x-ray crystallography since small changes in the pH used in different analyses could cause changes in the channel diameter. This could explain discrepancies such as that between the functionally effective diameter (1.6 nm) determined for *Rhodobacter capsulatus* porin and that observed using 3-D analysis (1.0 nm) (Nestel et. al., 1989; Weiss et. al., 1989; Weiss et. al., 1990).

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CHAPTER 5

Factors Affecting the pH-Induced Switch in Channel Size of OmpF and OmpC

ABSTRACT

Previous *in vitro* studies described a pH-induced switch in channel size for *Escherichia coli* porins OmpF, OmpC, and PhoE. As the pH was raised, there was a switch from a small channel to a set of larger-sized channels as detected in bilayer lipid membrane assays. In this study, we examined the effect of two factors on this pH-induced switch in channel size: 1) the addition of membrane-derived oligosaccharides, and 2) the addition of porin to only one side of the bilayer lipid membrane. We found that MDO had a biphasic effect on porin channel size even at very low concentrations and that reduced pressure on the membrane (exerted on the side of porin addition) seemed to increase the insertion and opening of porins with the large size channel configuration.

INTRODUCTION

In chapter 2, we reported a pH-induced switch in channel size for porins from *E. coli* K-12, OmpF, OmpC, and PhoE (Todt et. al., 1992). *In vitro* experiments showed that as the pH was increased, there was a switch in porin configuration from a small-size to a set of larger-size channels. It is possible that other factors affect this pH-induced switch *in vivo*. Delcour and workers (1992) have reported that membrane-derived oligosaccharides (MDO's) promote the closing of porins from *E. coli* AW740 at membrane potentials opposite to that of the Donnan potential (using patch clamp techniques). MDO production is increased in the periplasm of gram-negative bacteria under conditions of low osmolarity (Kennedy, 1982). The level of MDO is presumed to play a role in keeping the periplasm isoosmolar with the cytoplasm and to prevent the cytoplasm from swelling significantly under conditions at low osmolarity (Stock et. al., 1977). Delcour and coworkers have found that phosphoethanolamine (a substituent of MDO) mimicked the effect of MDO. Therefore, they hypothesized that the depolarization of cells adapted to low osmolarity, which occurred when cells were suddenly placed in high-salt medium, caused the MDO-dependent closure of porin channels.

Morgan and coworkers (1990) have reported an asymmetric voltage gating effect for *E. coli* 0111:B4 porin. They found that voltage gating occurred *in vitro* only when porin was

added to one side of the membrane in the bilayer lipid membrane (BLM) assay. This is in contast to the findings of Todt and coworkers (1992) who found that voltage gating occurred when *E. coli* K-12 porins were added to both sides of the BLM (using the same assay). Therefore, the effect of two different factors on the pH-induced switch in porin channel size was studied: 1) the addition of MDO to both sides of the BLM and 2) addition of porin to only one side of the BLM.

MATERIALS AND METHODS

Cell Growth

OmpC and OmpF were isolated from *E. coli* K12 strains ECB 621 (ompF$^-$lamB$^-$; gift of S. Benson) and PLB 3261 (ompC$^-$ lamB$^-$; Benson & Decloux, 1985) respectively. MDO was isolated from *E. coli* K12 strain D21. 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; Rocque & McGroarty, 1990). Cells were harvested in late logarithmic growth phase.

Porin Isolation

Porins were isolated by the method Lakey et. al. (1985) with some modifications (Rocque et. al., 1987; Rocque & McGroarty, 1989; Rocque & McGroarty, 1990). Cells were broken using a French press and treated with RNAse and DNAse. After pelleting the membranes at 100,000 x g, the inner membrane and some outer membrane proteins were solubilized with sodium dodecyl sulfate (SDS) and Tris HCl. After centrifugation, the pellet contained outer membrane proteins, primarily porins, bound to the peptidoglycan. The porins were solubilized with high NaCl concentrations 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 10 mM Tris HCl pH 6.8, 0.02% sodium azide containing either 1% Triton or 1% SDS. Porin was quantitated using the bicinchoninic acid protein assay (Pierce Chemical Co.).

MDO Isolation

MDO was isolated using the method of Rotering & Raetz (1983) with a few modifications. Briefly, *E. coli* K-12 D21 cells were extracted with 50% cold ethanol for 1 hour in an ice bath. Precipitate was removed by centrifugation at 15,000 x g (4°C). The supernatant solution, containing MDO, was lyophilized and resuspended in 10 mM Tris-HCl pH 7.3, 0.1 N LiCl, 7% propanol (TLP). This sample was applied to a Biogel P-10 column (2.5 x 80 cm) and eluted with TLP. Fractions containing hexoses (indicating MDO) as detected by the anthrone assay (Spiro, 1966) were pooled and lyophilized. The lyophilized sample was resuspended in distilled water and desalted on a Sephadex G-10 column (2.2 x 48 cm). Hexose-containing fractions were pooled, lyophilized and resuspended in distilled water.

Bilayer Lipid Membrane Assay (BLM)

Analysis of electrical conductance across a bilayer lipid membrane was used to measure porin channel size under various conditions. The electrical conductance was measured across a lipid bilayer comprised of 1% diphytanoyl

phosphatidylcholine (in n-decane). As described previously (Rocque & McGroarty, 1989; Rocque & McGroarty, 1990; Xu et. al., 1986), a small volume of porin was added to either side or both sides of a lipid membrane bathed in a salt solution. This bathing solution contained 0.5 M NaCl and 0.5 mM of an appropriate buffer, 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), for pH 7.0, 7.9 and 9.4 or sodium succinate for pH 5.1. For the MDO studies, the pH was adjusted after the addition of MDO to the bathing solution. Silver-silver chloride electrodes were placed on either side of the membrane and a constant voltage was applied using a 1.5V battery. Changes in current were amplified using a Keithley Model 614 electrometer and recorded. The changes in current were reported as a size parameter λ/σ (channel conductance increment/bathing solution's specific conductance) versus the probability of the occurance of an event with a particular size. A statistically significant number of channels ($\geq$200) were analyzed for each experimental condition.

RESULTS AND DISCUSSION

The effect of MDO on the pH-induced switch in channel size was examined for OmpF (Figure 1) and OmpC (Figure 2). For OmpF at pH 5.1 and 7.9, there was a biphasic MDO effect. At pH 5.1 using low concentrations of MDO, there was an increase in the percentage of large-size channels (compared to that present in the absence of MDO) followed by a drop as the concentration of MDO was increased further. At pH 7.9, the effect was reversed with the percent of large channels decreased at low MDO concentration followed by an increase as the MDO concentration was raised. This trend was repeated with OmpC although the biphasic effect was not as pronounced. Possibly there are two sites on porin to which MDO binds in a pH-dependent manner; ie. at low pH, MDO binds to one site, while at high pH, MDO binds to the other. At low MDO concentration under acidic conditions, MDO could bind to one site (such as the negatively-charged aspartic acid present in a periplasmic loop of porin; D266 in OmpF) causing a conformational change which increased channel size; at higher pH (either pH 7.0 or 7.9), a change in the protonation of the phosphates on the MDO could cause it to bind to another site (such as the positively-charged lysine present in a periplasmic loop of porin; K305 in OmpF) causing a decrease in channel size. Interpretation of the effect of MDO at higher concentrations is difficult due to various factors. Since MDO has an effect

Figure 1. Effect of MDO on the percentage of large-sized OmpF channels based on bilayer lipid membrane analysis performed as described in Figure 3. Porins were solubilized in 1% SDS, 10 mM Tris, 0.02% sodium azide pH 6.8. MDO was added to both sides of the membrane.

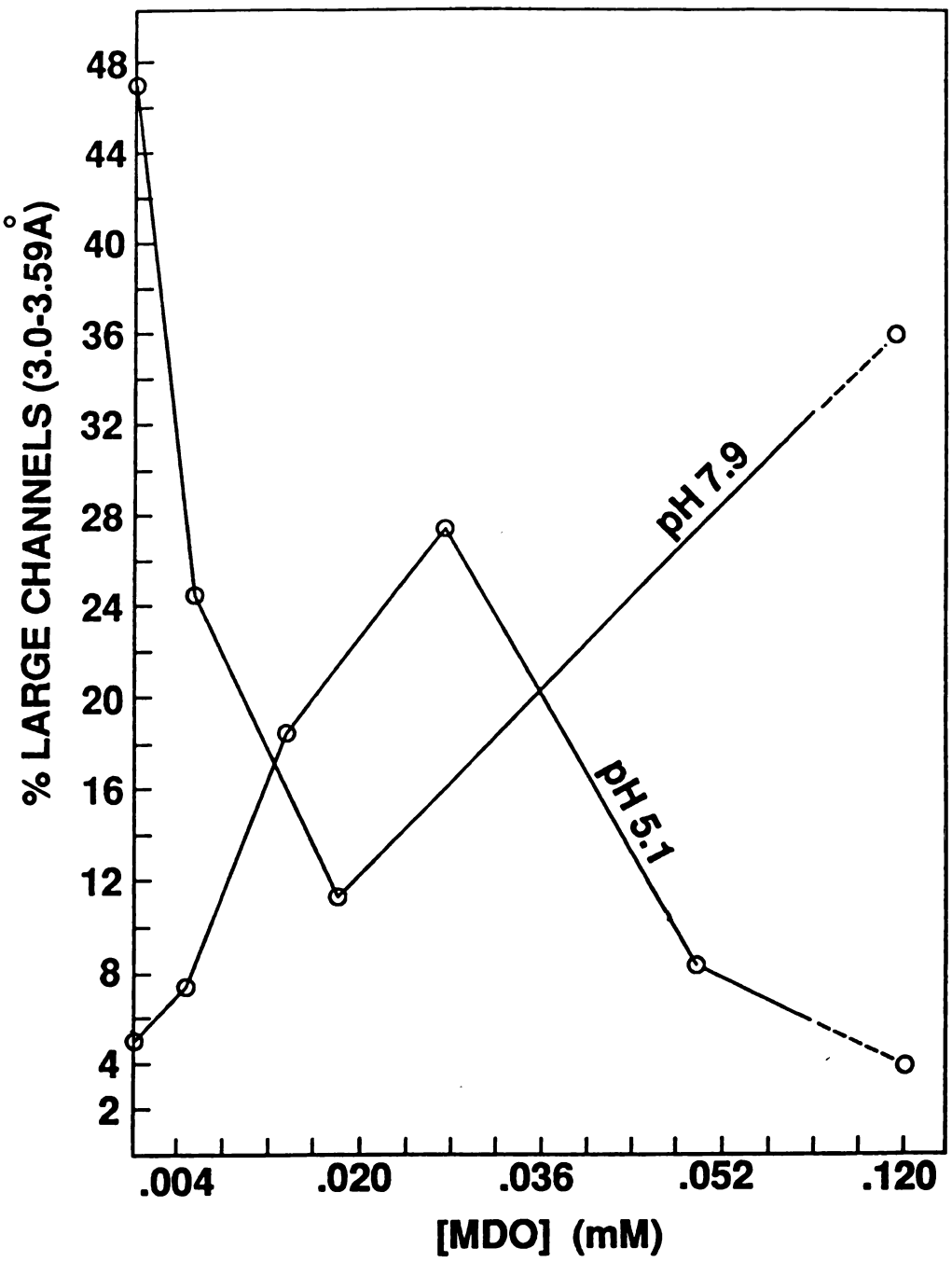
% LARGE CHANNELS (3.0-3.59Å)
48
44
40
36
32
28
24
20
16
12
8
4
2
.004
.020
.036
.052
.120
[MDO] (mM)
pH 7.9
pH 5.1

Figure 1

Figure 2. Effect of MDO on the percentage of large-sized OmpC channels based on bilayer lipid membrane analysis performed as described in Figure 3. Porins were solubilized in 1% SDS, 10 mM Tris, 0.05% sodium azide pH 6.8. MDO was added to both sides of the membrane.

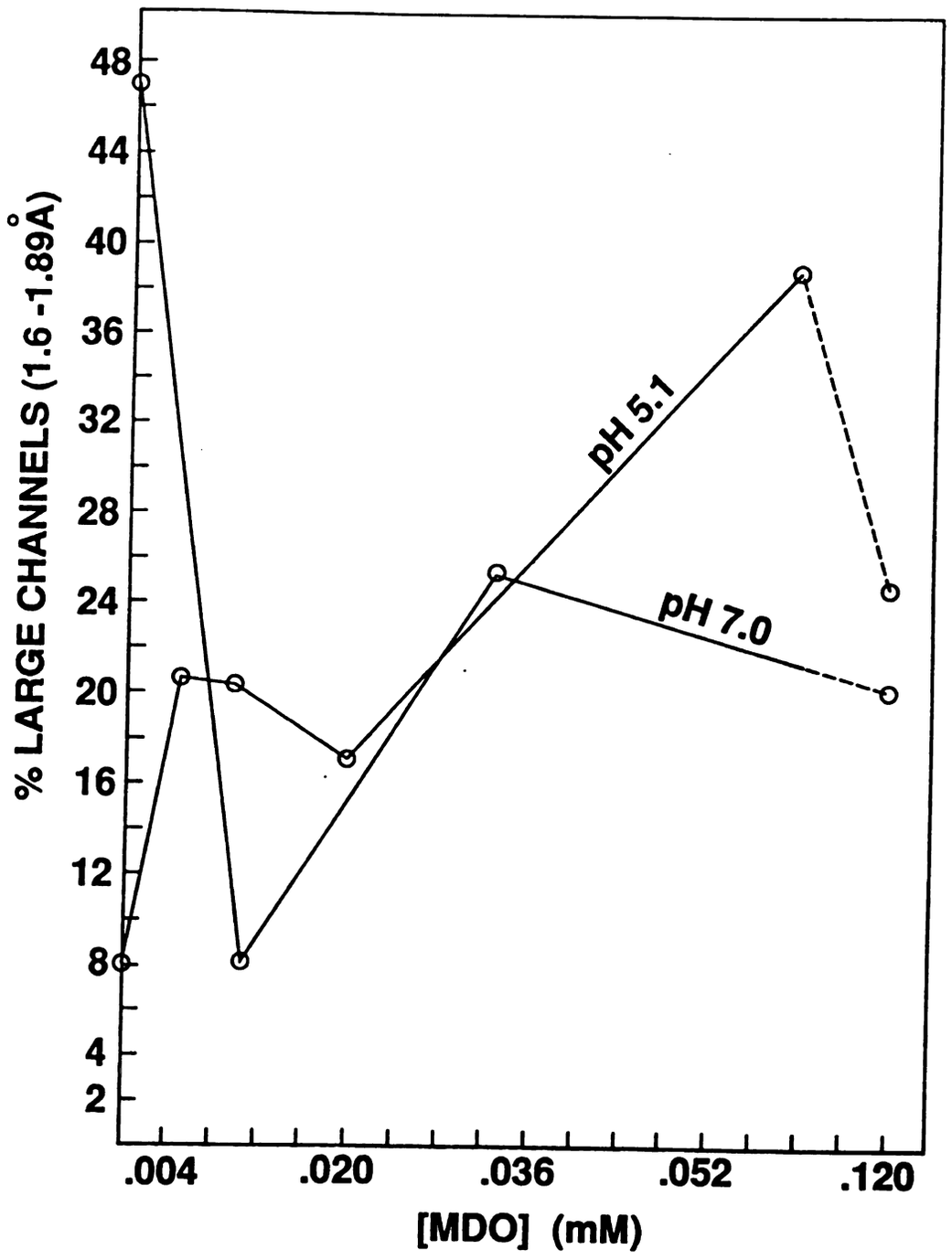
% LARGE CHANNELS (1.6 -1.89Å°)
48
44
40
36
32
28
24
20
16
12
8
4
2
pH 5.1
pH 7.0
.004
.020
.036
.052
.120
[MDO] (mM)

Figure 2

only when added to the positive side of the membrane with respect to porin addition, differences in MDO addition with respect to the side of the membrane may create localized changes in the potential across the porin channel. This along with small differences in MDO concentrations across the membrane may cause fluctuations in potential resulting in variability in conductance. Also changes in pressure, which may be caused by a small differential in MDO concentration across the membrane, have been found to prefentially allow the insertion of large-size channels (see following discussion). Porin response to MDO levels is complicated by the fact that these experiments were all carried out using MDO concentrations below those present in the periplasm; the concentration of MDO in that compartment can reach as high as 20 mM (Sen et. al., 1988). Regardless, it appears that even at low concentrations MDO has an effect on porin channel size *in vitro*.

In a second set of experiments, we examined the effect of adding porin to only one side of the membrane in the BLM apparatus (Figure 3). We found that the addition of detergent, LPS, or denatured porin to one side of the membrane was necessary for the insertion of porin on the opposite side of the membrane (regardless of the polarity of the applied voltage). Also, the addition of OmpF or OmpC to only one side of the membrane eliminated the pH-induced switch in channel size; primarily large-sized channels were present at both pH 5.1 and 9.4. We propose that the

Figure 3. Probability distribution histograms of the size parameter λ/σ (Å) for OmpF and OmpC as measured in bilayer lipid membranes. Porins, solubilized in 1% triton, 10 mM Tris HCl, 0.02% sodium azide pH 6.8 were added either to both sides (A) or to one side (B) of the lipid membrane which was bathed in a solution containing 0.5 M NaCl with 0.5 mM of either sodium succinate (pH 5.4) or sodium 2-(N-cyclohexylamino)ethanesulfonic acid (CHES, pH 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 $\geq$200 events.

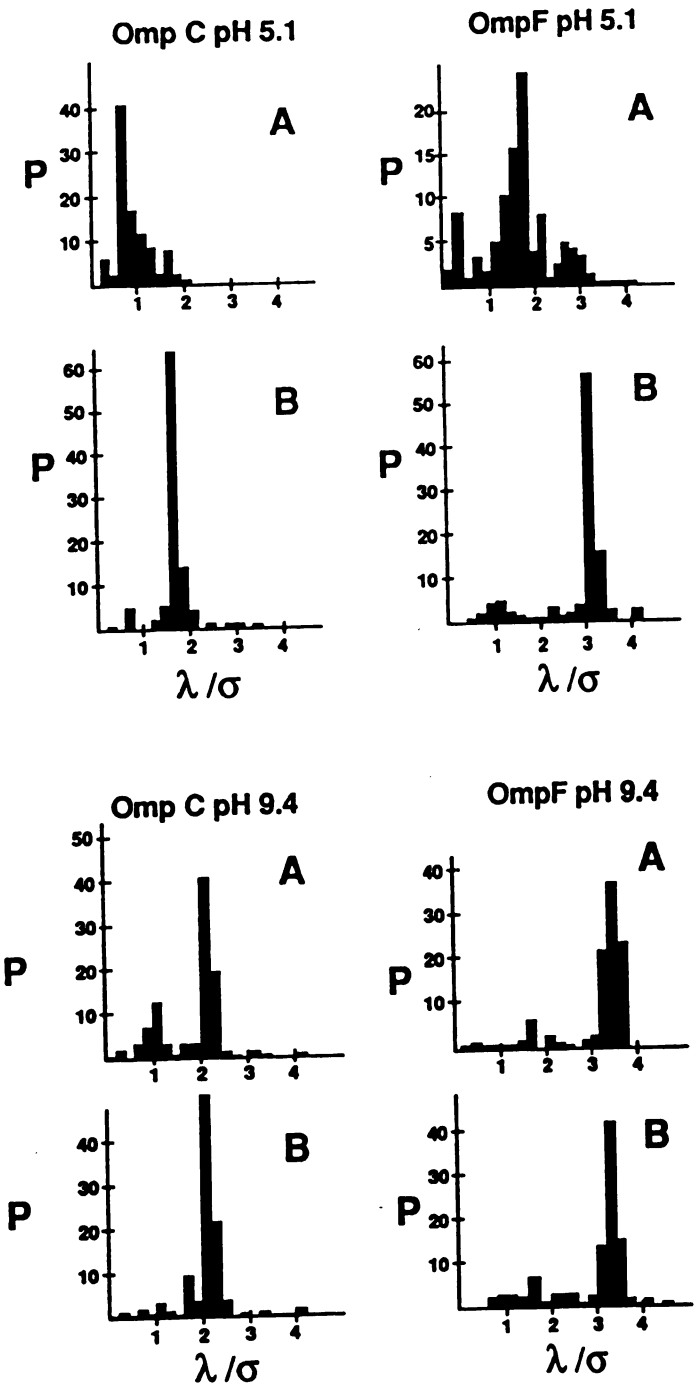

Figure 3

insertion of porin from one side of the membrane altered the membrane lipid, preferentially allowing the insertion or opening of porins with a large-size channel configuration. Sheetz and Singer (1974) found that changes in the contour of a bilayer lipid membrane could be induced by the addition of amphipathic molecules. They proposed that the preferential insertion of these amphipathic compounds into one leaflet of the bilayer would result in the expansion of that monolayer with repect to the other causing a "cupping" effect. Martinac and coworkers (Nature *in press*) have found that either the addition of amphipathic molecules or the application of pressure can activate *E. coli* mechanosensitive channels. Our experiments suggest that the pH-induced switch in porin channel size is "mechanosensitive" under *in vitro* conditions; presumably a reduction in surface pressure on the side of porin addition preferentially allows the insertion and opening of porins with the large-size channel configuration. Since the lipid bilayer present in cells does not have the same composition as in the BLM its debatable whether this is also true *in vivo* although Saimi and coworkers (Meth. Enz. *in press*) have found mechanosensitive channels in a wide variety of organisms under different conditions. In conclusion, we have found that the pH-induced switch in channel size is affected by several factors *in vitro* including MDO concentrations and the tension on the membrane into which porin inserts.

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Chapter 6

Analysis of Mutant OmpF Porins From *Escherichia Coli* K-12 Strains OC905, OC904, and OC901

ABSTRACT

Escherichia coli K-12 strains have been described which have point mutations in the OmpF gene allowing them to grow in maltodextrins in the absence of the maltodextrin-specific porin LamB (Dex^+ phenotype). Previous studies have indicated that these porins have enlarged channels. In this study, mutant OmpF porins from *Escherichia coli* strains OC901 (R82⇒C), OC904 (D113⇒G), and OC905 (R82⇒S) were isolated and their functions compared to that of wild type OmpF using the bilayer lipid membrane assay. At 25 mV, all the mutant porins showed a pH-induced switch in channel size similar to that of the wild type; as the pH was increased, there was a switch from a small-size channel to a set of larger-sized channels. There was no evidence from our *in vitro* studies of enlarged channels for the mutant porins as has been reported by others (using various *in vivo* analyses). This was due either to the fact that there was a shift in the pK_a of the channel-size switch which would not have been detected in these experiments, or that other factors (such as the amount of lipopolysaccharide bound to porin) affecting channel size *in vitro* are masking the effect responsible for the *in vivo* phenotype. The results also indicated that Asp113 may play a role in voltage gating.

INTRODUCTION

Porins such as OmpF and OmpC form channels in the outer membrane of gram-negative bacteria. They have an exclusion limit of ~600 Daltons; as a result, *Escherichia coli* can not grow on maltodextrins larger than maltotriose in the absence of the maltodextrin-specific porin LamB. It has been shown that certain mutations in *ompF* allow *E. coli* K-12 to grow on maltodextrins larger than maltotriose in the absence of LamB (Dex^+ phenotype) (Benson et. al., 1988; Benson & Decloux, 1985). Presumably, these mutations occur in positions in the gene sequence important in defining porin channel size. Specifically, Benson and coworkers have isolated Dex^+ porin mutants with point mutations resulting in changes in amino acid residues R42, R82, D113 or R132 (Benson et. al., 1988). They also found that small deletions between A108 and V133 gave a Dex^+ phenotype. Cells containing these point or deletion mutations had increased growth rates, [^{14}C]-maltose uptake, and antibiotic sensitivity (for antibiotics which enter via porins), suggesting increased pore size. However, only *E. coli* with porin containing deletions showed increased sensitivity to detergents and hydrophobic antibiotics, suggesting that these deletions may also affect the interaction of porin with other components of the membrane (Benson et. al., 1988). Benson and coworkers (1985) described *E. coli* containing point mutations in OmpC that also had the Dex^+ phenotype. Some of these OmpC mutant

porins had increased channel conductance and increased voltage dependence *in vitro* (using the bilayer lipid membrane assay; Lakey, 1991). In a previous study, we found that porin channel size could be increased by raising the pH (Todt et. al., 1992). In this study, we analyzed three OmpF porins which have Dex^+ point mutations to determine using *in vitro* techniques if either channel size or sensitivity to voltage is affected by these mutations.

MATERIALS AND METHODS

Cell Growth and Porin Isolation

OmpF was isolated from *E. coli* K-12 strain PLB 3261 ($ompC^-$, $lamB^-$; Benson & Decloux, 1985). Mutant OmpF porins were isolated from *E. coli* K-12 strains OC901 (OmpF Arg82 to Cys, $OmpA^-$), OC904 (OmpF Asp113 to Gly, $OmpA^-$), and OC905 (OmpF Arg82 to Ser, $OmpA^-$); each strain lacks OmpC and LamB proteins. Cells were grown in 1% tryptone, 0.5% yeast extract, pH 7.5 at 30°C and harvested in late logarithmic growth phase. Porins were isolated by the method of Lakey and coworkers (1985) with modifications (Todt et. al., 1992). The final porin preparation was resuspended in 1% SDS, 10 mM Tris-HCl, 0.02% sodium azide pH 6.8. Protein was quantitated using the bicinchoninic acid protein assay (Pierce Chemical Co.). Lipopolysaccharide (LPS) associated with porin was quantitated using the thiobarbituric acid assay (Droge et. al., 1970).

Bilayer Lipid Membrane (BLM) Assay

BLM analysis was performed as described previously (Todt et. al., 1992). Analysis of electrical conductance across a bilayer lipid membrane was used to measure porin channel size under various conditions. A small volume of porin was added to both sides of a membrane composed of diphytanoyl phosphatidyl choline (in decane). This membrane was bathed in a salt solution containing 0.5 M NaCl and 0.5

mM of an appropriate buffer; sodium succinate for pH 5.6 or 2-(N-cyclohexylamino)ethane sulfonic acid (CHES) for pH 9.2. Changes in current were reported as a size parameter λ/σ (channel conductance increment/bathing solutions specific conductance) versus the probability of the occurrence of an event with a particular size. A statistically significant number of channels (≥200) were analyzed for each condition.

RESULTS

The channel conductance of mutant OmpF porins from *E. coli* K-12 strains OC901 (R82⇒C), OC904 (D113⇒G), and OC905 (R82⇒S) was measured using BLM analysis and compared to that of wild type OmpF at 25 mV (Figure 1) and 75 mV (Figure 2). Porins were analyzed at pH 5.6 and 9.2. As found previously (Todt et. al., 1992), at 25 mV the wild type OmpF showed a pH-induced switch from small-size channels (~1.6 Å) to larger-sized channels (3.2-3.5 Å). OmpF porins from strains OC901 and OC905, both of which have a mutation in Arg82, showed a pH-induced switch in channel size similar to that of the wild type, although at pH 5.6 the channel size distribution for the mutant isolates was broader. At 25 mV, OmpF from strain OC904 had a similar size distribution to that of the wild type OmpF at pH 5.6. At 9.2, there was an increase in the number of small-size channels with OmpF from strain OC904 (compared to the wild type at pH 9.2). Therefore, at 25 mV, the mutant porins showed no obvious increase in channel size (compared to the wild type) but a pH-induced switch in channel size similar to wild type was detected.

At 75 mV under acidic conditions (pH 5.6), the wild type OmpF had an increase in monomer- and dimer-sized channels suggesting decreased cooperativity between the subunits of the trimer compared to wild type OmpF at 25 mV. There was also an increase in the percent closings (Table I)

Figure 1. Probability distribution histograms of the size parameter λ/σ(Å) for OC901 OmpF (A), wild type OmpF (B), OC904 OmpF (C), and OC905 OmpF (D), as measured in bilayer lipid membranes at 25 mV. Porins, solubilized in 1% SDS, 10 mM Tris HCl, 0.02% sodium azide pH 6.8 were added to both sides of the lipid membrane which was bathed in a solution containing 0.5 M NaCl with 0.5 mM of either sodium succinate (pH 5.6) or sodium 2-(N-cyclohexylamino)ethanesulfonic acid (pH 9.2). 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 histograms of $\geq$ 200 events.

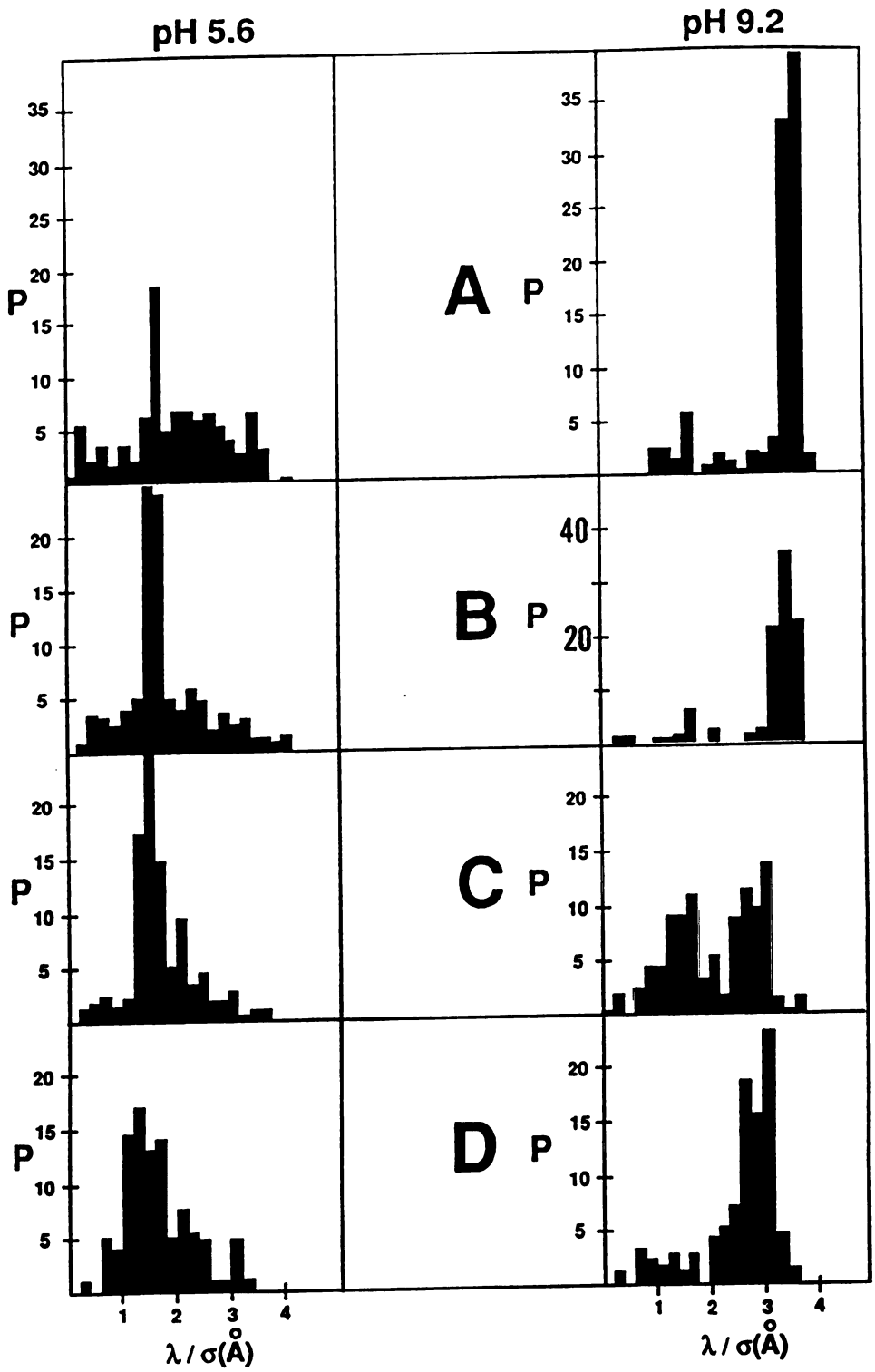
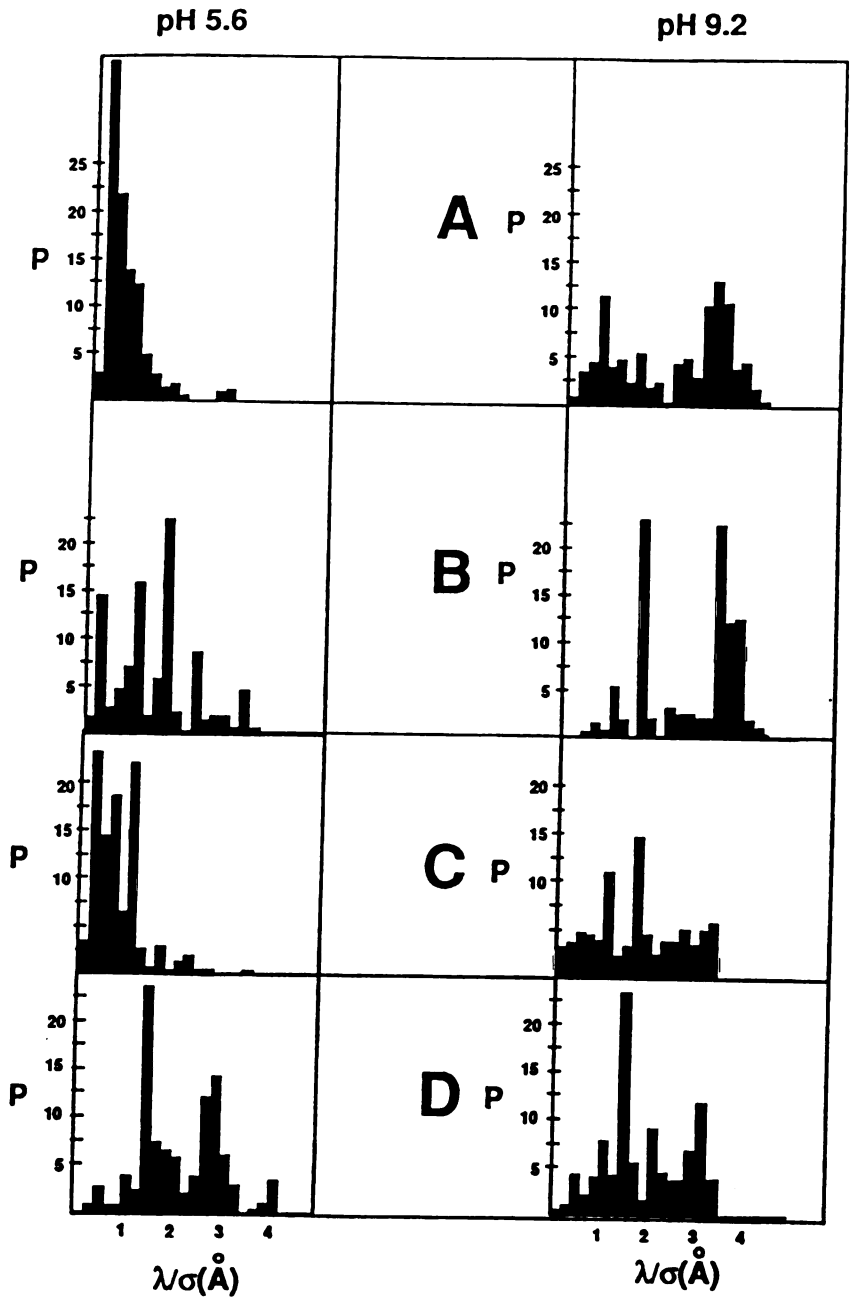


Figure 1

Figure 2. Probability distribution histograms of the size parameter λ/σ (Å) for, OC905 OmpF (A), wild type OmpF (B), OC901 OmpF (C), and OC904 OmpF (D) as measured in bilayer lipid membranes at 75 mV. The porins were added to bathing solutions and electrical conductance was measured as described in Figure 1.



grams of the si
ild type Ompf
asured in bilaye
added to bath
measured as

Figure 2

TABLE 1

Voltage Gating of Wild-type and Mutant OmpF Porins. Relative Number of Closing Events Expressed as a Percentage of Total Events Detected in the BLM at Different pH Values and at Different Voltages

OmpF From *E. coli* K-12 Strain:	25 mV		75 mV	
	pH 5.6	pH 9.2	pH 5.6	pH 9.2
PLB3261 (wild type)	5.4%	3.0%	30.3%	6.3%
OC901 (R82⇒C)	6.5%	2.9%	45.5%	15.9%
OC904 (D113⇒G)	1.4%	9.7%	3.4%	8.3%
OC905 (R82⇒S)	2.0%	2.5%	43.2%	15%

for wild type OmpF under acidic conditions at 75 mV (compared to 25 mV). Under basic conditions at 75 mV, OmpF had an increase in the number of small-size channels compared to 25 mV indicating a decrease in stability of the larger-sized channels at this voltage.

Comparing the OmpF mutants to the wild type at 75 mV under acidic conditions, both OC901 and OC905 porins had an increase in channels with extremely small channel sizes (although monomer and dimer sizes were not as clearly defined) suggesting decreased monomer cooperativity; there also was an increase in percent closings indicating increased voltage gating. With OC904 porin, there was no decrease in cooperativity or increase in channel closings at pH 5.6 and 75 mV; however, there was an increase in the number of large-size channels (compared to wild type OmpF). Under basic conditions at 75 mV, the mutant porins had a broader distribution of channel sizes (compared to the wild type OmpF) with the trend being toward the smaller channels (with either monomer-, dimer-, or trimer-type sizes). This suggests that the larger-size channel of the mutant porins had less stability than the larger-size channel of the wild type OmpF at high voltages under basic conditions.

DISCUSSION

Overall, these results are difficult to explain. The mutant porins showed no evidence of enlarged channels *in vitro* (except possibly with OmpF from OC904 at 75 mV and pH 5.6) even though Benson found using *in vivo* techniques that the channels appeared larger (Benson et. al., 1988). There also was no evidence of increased stability of the large channel configuration. Possibly the pKa of His21 (the amino acid presumed to be involved in the pH-induced channel size switch: Todt & McGroarty, 1992), is shifted by changes in these amino acids so that at physiological pH more channels are in the large-size channel configuration. However, in that case, mutations in Arg82 must result in several changes in porin function since porins with this mutation also showed decreased cooperativity among monomers and increased voltage gating. Another explanation for these results could be related to the amount of LPS bound to the porins; the level of bound LPS is known to modulate the channel size switch *in vitro* (Todt et. al., 1992). We found a difference in the amount of LPS bound to some of the mutant porins compared to the wild type OmpF. Wild type OmpF normally had 6-9 molecules of LPS bound per trimer; while OC905 OmpF had 16.7 LPS/trimer, OC901 OmpF had 9.0 LPS/trimer, and OC904 OmpF had 12.6 LPS/trimer. Another mutant OmpF, from *E. coli* K-12 strain OC915 (82R⇒H), contained very high amounts of bound LPS (23 LPS/trimer). This porin did not insert into

the BLM and appeared to be unstable since only monomers were detected on SDS-polyacrylamide gels (data not shown).

Arg82, according to three models for OmpF, is in an intramembranous β-strand (Welte et. al., 1991; Klebba et. al., 1990, Cowan et. al., 1992). Porins from all three strains (OC901, OC905 and OC915), containing mutations in Arg82, had reduced cooperativity among trimer subunits; however, it does not appear likely that this amino acid is involved in interactions between subunits in the trimer given its location in the channel lumen (Cowan et. al., 1992). Again, this contradiction may be explained by alterations in bound LPS present in the mutant porins. The reduced cooperativity among trimer subunits may be responsible for the observed increase in closings since these two phenomena seem to be coupled to each other (see also Todt et. al., 1992). Arg113 is in a large loop between strand β-5 and β-6 which also is part of the constricted zone in the channel lumen (Welte et. al., 1991; Weiss et. al., 1991; Cowan et. al., 1991). Since the porin with a mutation in Arg113 (OC904) showed very little gating (compared to the wild type OmpF at 75 mV), this amino acid may be necessary for voltage gating. In conclusion, these point mutations have a variety of effects on porin function *in vitro* none of which appear to explain the *in vivo* phenotype.

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CHAPTER 7

Acid pH Decreases OmpF and OmpC Channel Size *In vivo*

ABSTRACT

To be effective against gram-negative organisms, β-lactam antibiotics must be able to penetrate the outer membrane. For *Escherichia coli*, these compounds generally cross this barrier through non-specific channnels in porins OmpF and OmpC. *In vitro* studies have shown that increased pH induces a switch in the structure of OmpF and OmpC from a small channel conformation to a set of larger-sized channel conformations. In this study, the permeability of two cephalosporins into cells producing either OmpC or OmpF was examined at various pHs. The results suggest that the pH-induced switch in channel size observed *in vitro* also occurs *in vivo*.

INTRODUCTION

The outer membrane of gram-negative bacteria acts as a permeability barrier protecting the cells against deleterious agents such as antibiotics and bile salts while allowing the flux of small metabolites and nutrients. Pore-forming proteins, such as OmpF and OmpC in *E. coli*, are responsible for the permeability of small hydrophilic compounds across the outer membrane. The influx of antibiotics such as β-lactams through these porin channels is one determinate of β-lactam effectiveness; other determinates include the level of β-lactamase activity within the periplasmic space and affinity of β-lactams for penicillin-binding proteins present in the periplasm. Several studies have shown that the acidity of the media can affect porin function *in vitro* (Benz et. al., 1979; Schindler & Rosenbusch, 1978; Buehler et. al., 1991; Todt et. al., 1992). Specifically, we have found in *in vitro* studies that increased pH induces a switch from a small-size to a set of larger-sized channels for OmpC and OmpF (Todt et. al., 1992). The pK_a for this switch was 6.5 for OmpC and 7.2 for OmpF (Todt et. al., 1992). Other investigators have observed that acidic conditions reduce the effectiveness of certain hydrophilic antibiotics as measured by the minimum inhibitory concentration, bacteriocidal activity and post-antibiotic effect; however, the cause of this reduced effectiveness has not been determined

(Gudmundsson et. al., 1991; Sabath et. al., 1991; Laub et. al., 1989). In this study, we attempted to determine if the pH-induced switch in channel size observed *in vitro* could be measured in an *in vivo* system by assaying, at various pHs, the permeability of β-lactams into the periplasm of cells producing either OmpC or OmpF.

METHODS

Cell Growth

Escherichia coli K-12 strains ECB 621 ($ompF^-$, $lamB^-$; gift of S. Benson) and PLB 3261 ($ompC^-$, $lamB^-$; Benson & Decloux, 1985) were grown in 1% tryptone, 0.5% yeast extract, 0.4% NaCl, and 5 mM $MgCl_2$, pH 6.5. Cephalosporin C was added to a final concentration of 14 μg/ml to induce β-lactamase production. Cells were harvested in mid-logarithmic growth phase.

Assay for Cephalosporin Hydrolysis

Cells were collected by centrifugation at 23,000 x g (4°C) for 15 minutes. They were washed twice with cold 10 mM sodium phosphate, 5 mM $MgCl_2$, pH 6.5 (phosphate buffer) and resuspended in phosphate buffer at pH 5.95 or 7.4. Cells were kept on ice and aliquots were preincubated at room temperature for 30 minutes prior to drug addition. The rate of cephalosporin hydrolysis by intact cells or by crude β-lactamase (see below) was determined as described previously (Nikaido et. al., 1983). Briefly, intact cells (added at a final concentration of ~0.77 mg cell dry weight/ml) or crude β-lactamase (added at a final concentration of 0.46 mg protein/ml) were incubated in a 1 mm cuvette in phosphate buffer (at either pH 5.95 or 7.4) containing either 431 μM cephalosporin C or 323 μM cephaloridine. The rate of cephalosporin hydrolysis was

measured at room temperature for 20 minutes by the decrease in absorbance at 260 nm.

Crude β-lactamase preparation

Periplasmic β-lactamase was released from *E. coli* K-12 ECB 621 or PLB 3261 cells by spheroplast formation (Schnaitman, 1981). Briefly, logarithmically-growing cells (grown as described above) were centrifuged, washed as above and resuspended in 0.075 M sucrose and 10 mM Tris HCl, pH 7.8 at one-tenth the culture volume. Lysozyme was added to a final concentration of 0.1 mg/ml followed by the slow addition of two volumes of 1.5 mM (ethylenedinitilo)tetraacetic acid (EDTA). All steps were performed on ice. Spheroplasts were removed by centrifugation at 23,000 x g for 15 minutes. The β-lactamase in the supernatant solution was further purified by dialysis at 4°C first against distilled water for 24 hours and then against 1 mM phosphate, 0.5 mM $MgCl_2$, pH 6.5 for 48 hours. The dialyzed sample was centrifuged at 23,000 x g for 15 minutes to remove contaminants and lyophilized. The lyophilized "crude β-lactamase" was resuspended in phosphate buffer at pH 5.95 or 7.4. Protein was quantitated using the bicinchoninic acid protein assay (Pierce Chemical Co.).

RESULTS AND DISCUSSION

The rate of disappearance of antibiotic when added to intact cell suspensions can be used to determine outer membrane permeability since the enzymes involved in degradation of these drugs are located primarily in the periplasmic space (Zimmermann & Rosselet, 1977). In this study, we measured the rate of β-lactam hydrolysis at pH 5.95 or 7.4 using *E. coli* K-12 cells which contained either OmpC or OmpF as the main porin in the outer membrane (Table 1). We found that at the higher pH, the rate of β-lactam hydrolysis was significantly higher than at low pH for both strains tested. This increased rate was observed using either cephalosporin C or cephaloridine which are negatively-charged or neutral, respectively, in this pH range. The fact that the rate of cephaloridine hydrolysis was significantly lower than the rate of cephalosporin C hydrolysis (for either intact cells or crude β-lactamase) reflects the reduction in β-lactamase activity with the former substrate (Lindstrom et. al., 1970).

Since the higher rate of β-lactam hydrolysis at the elevated pH could result either from increased permeability of the outer membrane or from increased β-lactamase activity at high pH, the rate of β-lactam hydrolysis by β-lactamase in solution was measured at pH 5.95 and 7.4. The β-lactamase was isolated from OmpF- and OmpC-producing cells (Table 1). We found that β-lactamase activity increased

TABLE 1

Rate of Hydrolysis of Antibiotic by Cells Producing Either OmpF or OmpC and by Crude β-lactamase Isolates▲

Source of Hydrolytic Enzyme	ANTIBIOTIC HYDROLYSIS RATE▲			
	Cephalosporin C		Cephaloridine	
	pH 5.95	pH 7.4	pH 5.95	pH 7.4
Whole cells (OmpF)	13.0 ± 0.6	22.1 ± 0.9	7.8 ± 0.2	11.4 ± 0.6
Whole cells (OmpC)	5.3♦ ± 0.8	11.9♦ ± 0.9	0	1.1♦ ± 0.2
Released β-lactamase (OmpF)*	59 ± 3	71 ± 1	14 ± 0.8	18 ± 0.9
Released β-lactamase (OmpC)*	53 ± 2	76 ± 1	12 ± 0.6	20 ± 0.2

▲Measured by the decrease in absorbance at 260 nm per minute ($x10^4$)

*Hydrolysis rate using crude β-lactamase isolated from cells producing specific porin. An equivalent amount of enzyme was added in each experiment.

♦Corrected to amount of cells added to OmpF-whole cell assay.

with the increase in pH (20% for enzyme from OmpF-producing cells, 43% for enzyme from OmpC-producing cells using the substrate cephalosporin C). In comparison, Lindstrom and coworkers (Lindstrom et. al., 1970) examining chromosomal β-lactamase from various strains of *E. coli* K-12 reported that enzyme activity doubled with a pH increase from 6.5 to 7.4 (using the same buffer and substrate); the optimal pH was 7.3. The difference between our results and Lindstrom's may be explained as follows. First, the production of β-lactamases from *E. coli* K-12 strains ECB 621 and PLB 3261 was found to be inducible (data not shown) in contrast to the constitutive production of the *E. coli* K-12 chromosomal enzyme (Lindstrom et. al., 1970). Therefore, these β-lactamases might not be encoded by the same genes and thus may have different properties. Different β-lactamases have been reported to have different optimal pH's depending on the type and source of the enzyme (Bicknell et. al., 1983). Investigators have reported a change of ≤7% in TEM-type β-lactamase activity in the pH range 5.8-7.2 (Bellido et. al., 1983; Sen et. al., 1988). However, the fact that in our experiments, the pH-dependent change in the rate of β-lactam hydrolysis by crude β-lactamase was not as great as that by intact cells suggests that the increased rate of β-lactam hydrolysis by cells with pH was not the result of an increase in β-lactamase activity alone. Also, the change in pH in the periplasm is presumably smaller than in the cell media due to the high buffering capacity of components in

the periplasm (ie. MDO and peptidoglycan) (Stock et. al., 1977). The increased rate of hydrolysis of the β-lactams at high pH is also not likely to be the result of a change in Donnan potential since increased pH should increase the Donnan potential (negative inside) which would decrease the permeability of the negatively-charged cephalosporin C and have no effect on the permeability of neutral cephaloridine. Therefore, we propose that the increased rate of β-lactam hydrolysis at pH 7.4 is due to an enlargement of OmpF and OmpC channels resulting in an increase in cell permeability at higher pH. This is consistent with our *in vitro* studies which showed that porin channel size increases with pH. Also, the rate of β-lactam hydrolysis was significantly lower for OmpC-producing cells than for OmpF-producing cells when measured under identical conditions indicating that the OmpC-producing cells have smaller channels. This is in agreement with the smaller channel size measured for OmpC *in vitro* (Todt et. al., 1992).

In conclusion, these data suggest that the pH-induced switch in channel size observed using *in vitro* methods, also occurs *in vivo*. This phenomenon could explain the "intrinsic resistance" of gram-negative bacteria to some hydrophilic antibiotics at acidic pH which has been reported even in strains lacking β-lactamase (Laub et. al., 1989). The fact that there is a reduction in OmpF production and an increase in OmpC production at acidic pH (Heyde & Portalier, 1987) could also explain the reduction in β-lactam

effectiveness at acidic pH. However, a transcriptional control could not explain our results since permeability was measured over a very short time range and under conditions where cells were not actively metabolizing. This acid-induced reduction in porin channel size should be considered when developing new drugs; antibiotics used to treat bacterial infections may need to be active in acidic environs such as phagolysosomes, urine or abscesses, sites of bacterial infection and inactivation (Gudmundsson et. al., 1991; Laub et. al., 1989), and be able to penetrate a very small channel.

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Chapter 8

Summary and Perspectives

The effect of various environmental factors on the structure and function of bacterial porins has been debated in recent years due to the prevailing view presented by some investigators of porin as a static filter in the outer membrane of gram-negative bacteria. In this thesis, evidence is presented for a more active role for porin in the cell's response to changes in the environment. In particular, we have found that *E. coli* porins OmpF, OmpC, and PhoE exist in at least two open channel configurations, a small channel stable at acidic pH and a larger-size channel stable at basic pH. This pH-induced switch occurred over a very narrow range near physiological pH and appeared to be reversible. The cross-sectional area of the larger-size channel was approximately double the size of the small channel. This increased porin channel size under basic conditions appears to be physiologically relevant since it was found *in vitro* using the BLM assay and the LSA, as well as *in vivo* using the Zimmermann-Rosselet technique for measuring cell permeability. This "fast response" of channel size to alterations in pH is complemented by "slow" permeability changes found to be controlled at the level of porin synthesis; there is an increase in the synthesis of OmpC (ie. smaller channels) and a decrease in OmpF (ie. larger channels) with a drop in pH of the media (Heyde & Portalier, 1987). It's possible that both of these mechanisms for pH-induced changes in cell permeability protect the cell under parasitic conditions from antibiotics

produced by other organisms since fluid from sites of bacterial infection in humans has been found to be acidic (Gudmundssen et. al., 1991).

Other factors which could affect the transition *in vivo* between these channel configurations have been studied using BLM. In our experiments, LPS depletion of porin decreased the stability of the larger-size channel, decreased cooperativity among trimer subunits, and increased channel closings. In the most recent structural analysis of OmpF, Cowan and coworkers (1992) found a number of carboxyl groups on porin facing the lipid environment at the external surface which they proposed to interact with LPS. This interaction would explain the requirement for LPS in the insertion of porin into the membrane and in porin trimerization. Depletion of this bound LPS could result in decreased porin rigidity and stability decreasing the threshold for voltage gating and allowing decreased subunit interactions.

High voltage (up to 125 mv) and low pH were also found to decrease subunit cooperativity and to increase gating. The fact that increased gating and decreased cooperativity occurred under the same change in conditions (as with LPS deletion) indicated that decreased subunit cooperativity may cause the monomers to be more susceptible to gating. The small channel stabilized at low pH had a lower gating threshold than the larger channel stabilized at high pH. Also OmpC seemed to be more stable than OmpF since it showed

gating only at very high voltages. We found the number of charges involved in gating to be ≤1 for OmpF and OmpC. This number was not affected by pH suggesting that gating was a result of motion of a fixed charge rather than of a proton. Although the physiological relevance of gating has not been proven, the fact that voltage gating has been observed in a variety of systems has prompted investigators to propose that either 1) gating is an *in vitro* expression of a phenomena which occurs via another mechanism *in vivo* (Lakey & Pattus, 1989) or 2) gating is a mechanism whereby porins mistakenly inserted into the cytoplasmic membrane are closed (Nikaido, 1992).

We also found that MDO can affect the pH-induced switch in channel size. The MDO concentration is increased under conditions of low osmolarity. At low MDO concentration under acidic conditions, there was an increase in the percentage of large-size channels followed by a drop as the concentration of MDO was increased further. At pH 7.9, the effect was reversed. It's possible that there are two sites in porin to which MDO binds in a pH-dependent manner causing a conformational change. Interpretation of this effect *in vitro* is complicated by other factors such as 1) working with MDO concentrations lower than those present *in vivo*, 2) localized changes in membrane potential caused by MDO, or 3) alterations in the pressure of the membrane (see below). Delcour and coworkers (1992) found that MDO promotes closing of porins at membrane potentials opposite to that of the

Donnan potential and suggested that the depolarization of cells adapted to low osmolarity, which occurred when cells were suddenly placed in high-salt medium, caused the MDO-dependent closure of porin channels via phosphoethanolamine (a substituent of MDO). Since MDO is in high concentration in the periplasm (as high as 20 mM) and is part of a generalized response (along with control of porin synthesis) to changes in osmolarity, it probably plays a role in regulation of porin.

Another factor which can affect the pH-induced switch in channel size is the tension of the membrane into which porin inserts. We found that the addition of porin to only one side of the membrane in the BLM apparatus preferentially allowed the insertion or opening of porins with a large-size channel configuration. It's possible that the insertion of porin into one side of the membrane causes a change in the tension of the membrane as can occur with the application of pressure or the addition of amphipathic compounds to a bilayer membrane (Sheetz and Singer, 1974). Presumably a reduction in pressure on the membrane on the side of porin addition preferentially allowed the insertion or opening of large-size channels. In this regard, Martinac and coworkers (Nature, *in press*) have found mechanosensitive channels (channels activated by the application of pressure or by the addition of amphipaths) in a wide variety of organisms under different conditions. In conclusion, all the aforementioned factors, LPS content, voltage, MDO, membrane tension, and

acidity, can affect the equilibrium between the two porin substates *in vitro*; this indicates a complex mechanism for regulation of porin size *in vivo* which may allow quick short-term responses to environmental changes.

To complement these studies of alterations in porin function we examined various aspects of porin structure. Since the pKa of the pH-induced switch in channel size was close to that of a histidine, we chemically modified His21 in OmpF and OmpC and studied the effects on function using BLM. Our experiments suggested that this histidine is involved in the pH-induced switch in channel size since chemical modification eliminated this transition (larger-size channels were present at all pH's).

To determine whether a global change in structure was involved in porin functional changes with pH, we examined the amide I region of the FTIR spectra and detected no change in secondary structure comparing porin at pH 5.6 and 8.5. However, porin tertiary structure seemed to be altered in the same pH range evidenced by the small increase in H-D exchange (seen in the amide II region of the FTIR spectra) at the higher pH.

Finally, our tryptophan fluorescence studies indicated an alteration in structure of OmpF and PhoE with pH. Our studies suggested that the fluorescence of the common tryptophan to both these porins decreased at basic pH indicating a change in porin conformation. Two lines of evidence suggested that these changes in structure with pH

might be correlated to the functional changes we observed *in vitro* and *in vivo*. First, we observed a similar change in porin tertiary structure using FTIR analyses when comparing porins with modified His21 to unmodified porins, as that obtained when comparing unmodified porins at pH 8.5 to those at 5.6. Therefore, modification of His21 seemed to result in large-size channels at all pH's as determined by functional as well as structural analyses. Second, the pKa for the change in tryptophan fluorescence (ie. for a structural change) for OmpF was very close to that obtained using BLM (ie. for a functional change).

To explain these structural and functional changes in terms of the latest model for porin, we examined structural analysis of OmpF presented by Cowan and coworkers (1992). In this model, a large, negatively-charged loop between β strand 5 and 6 (L3) defines the eyelet or exclusion limit of the channel lumen. Since His21 is across from this loop, we propose that at acidic pH, this amino acid becomes protonated and causes L3 to come closer narrowing the channel. This would cause the small change in tertiary structure detected using FTIR. The tryptophan whose fluorescence is altered in PhoE (trp210) and OmpF (trp214) by elevated pH is in an intramembranous β-strand, faces the lipid environment, and is close to L3. Therefore, the movement of L3 during the pH-induced switch in channel size may cause an alteration in the polarity of the environment

of this tryptophan resulting in the change in its fluorescence.

Benson and coworkers have also detected large changes in cell permeability with small changes in structure by examining the permeability of cells with point mutations in porin (Dex^+ phenotype). These Dex^+ cells grew on maltodextrins in the absence of the maltodextrin-specific porin lamB and had mutations in R82, R42, D113, or R132 (for OmpF). Since all these amino acids are in the channel eyelet forming the cell's exclusion limit, these mutations caused increased cell permeability. In our experiments, porins OC901 (R82⇒C), OC904 (D113⇒G), and OC905 (R82⇒S) were isolated and their functions *in vitro* compared to wild type OmpF's using the BLM assay. We did not find any evidence for enlarged channel size with the mutant porins possibly due to differences in bound LPS which is know to modulate the pH-induced switch in channel size. Our experiments did seem to implicate D113 in voltage gating since the porin with a mutation in this amino acid showed very little gating (compared to wild type OmpF at 75 mV).

In combination, our structural studies seemed to complement our experiments examining porin function by showing a small change in porin tertiary structure with either an alteration in pH or modification of His21. With the availabiity of detailed structural analysis of OmpF, changes in structure under various environmental conditions can be examined further using molecular modeling techniques.

Further experiments suggested by these functional and structural studies of porin are 1) examining the effect of pH on porin heterotrimers since they are known to exist *in vivo*, 2) studying the effect of mutation of His21 on porin function and structure, and 3) continuing studies on the effect of such periplasm components as MDO and peptidoglycan on porin function and structure. These studies along with our current data expand our knowledge of porin function and structure allowing further refinement in such fields as antibiotic design, X-ray crystallographyic analysis of porin, and antimicrobial therapy.

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