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dissertation entitled

CHARACTERIZATION OF OMPC AND OMPF PORINS
FROM ESCHERICHIA COLI K-12

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date 4/19/90

CHARACTERIZATION OF OMPC AND OMPF PORINS FROM *ESCHERICHIA COLI* K-12

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A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1990

ABSTRACT

CHARACTERIZATION OF OMPC AND OMPF PORINS FROM *ESCHERICHIA COLI* K-12

By

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The structure and function of OmpC and OmpF porins from *Escherichia coli* K-12 strains were analyzed using several biochemical and biophysical techniques. Isolated porins migrated on sodium dodecyl sulfate (SDS) gels as trimeric aggregates in the absence of heating and contained greater than equal molar amounts of bound lipopoly-saccharide (LPS). Heating porins to 100°C in SDS released most of the LPS, but equal molar amounts of LPS remained tightly associated to porin. Anti-lipid A monoclonal antibodies reacted with heat denatured porin but failed to react with LPS bound to undissociated porin trimers, suggesting the lipid A portion of LPS was masked or buried and inaccessible to antibody binding. Greater than equal molar amounts of LPS stabilized the OmpF porin to high temperature and low pH. This stabilization was only observed at low pH with the OmpC protein. These results imply that LPS is important for the three dimensional structure and the stability of porins in the outer membrane.

To characterize the structure/function relationship of porins, an eight amino acid deletion mutation in the OmpC protein and two point mutations in the OmpF protein were analyzed and compared to their wild type counterparts. The OmpC mutant porin had a larger channel size, increased temperature sensitivity, and closed more frequently at high

transmembrane potentials when compared to the wild type OmpC. These data suggest that the amino acids deleted from the OmpC mutant are critical for maintaining the size and voltage sensing properties in the wild type OmpC protein. Similarly, two point mutations in OmpF, Arg82-Cys and Asp113-Gly each had larger porin channel sizes than the wild type OmpF. The Asp113-Gly substitution decreased the stability of the porin to high temperatures and low pH but had no effect on channel closing at high trans-membrane potentials. The amino acid substitution of Arg82-Cys had no effect on the temperature or pH stability but allowed for increased channel closing at high trans-membrane potentials. These data suggest that Asp 113 is important for OmpF porin stability and Arg 82 is important for the voltage-dependent opening and closing of the OmpF porin.

Finally, a 50 kDa dimer of OmpC porin was isolated from strains containing a wild type *ompC* gene and a small deletion in the *ompF* gene. The dimer was less stable to high temperature than the trimer but showed similar functional properties. These findings suggest that the formation of heterotrimers containing an unstable OmpF subunit and two OmpC subunits occur in these strains. Upon purification, the OmpF subunit dissociates from the heterotrimer, leaving a stable, functional OmpC porin dimer.

to my parents

ACKNOWLEDGEMENTS

A very special thanks to my major thesis advisor Dr. Estelle McGroarty for her support and patience to see me through these last 4 years.

I would also like to thank the following people:

The members of my thesis committee, Drs. Shelagh Ferguson-Miller, Alfred Haug, Jack Holland, Paul Kindel, and John Wang for their guidance and interest in my thesis project.

Dr. John Wilson and Tracy White for their help with the differential scanning calorimeter.

Mark Prairie for performing the circular dichroism studies, and Dr. William Krueger for assisting in the analysis of the CD spectra.

Dr. Richard Coughlin for providing the antibodies used in this study and for performing the radioimmunoassays, solid phase immunoassays, and *Limulus* amebocyte lysate tests.

Dr. Spencer Benson from whom I received the bacterial strains used in my thesis project.

Jan Wood for her excellent typing skills and patience in rearranging my thesis in an organized manner.

Ben Gardner for his technical assistance in setting up the BLM apparatus, for his knowledge and enthusiasm in my thesis project, and for his competitive spirit on the racquetball court.

Mildred Rivera and Barb Hamel for being good lab mates and for their advice and technical assistance in my thesis project.

Finally, I am extremely grateful to my wife, Pamela, for her love, support, and encouragement.

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LIST OF ABBREVIATIONS

BCA	Bicinchoninic acid
BLM	Bilayer lipid membrane
BSA	Bovine serum albumin
Cp	Heat capacity
CD	Circular dichroism
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetate
FTIR	Fourier transform infrared spectroscopy
H_{cal}	Calorimetric enthalpy
H_o	Total enthalpy
H_{vH}	van't Hoff enthalpy
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
K, kDa	Kilodalton
KDO	2-keto 3-deoxy octulosonic acid
LPS	Lipopolysaccharide
NaDOC	Sodium deoxycholate
Omp	Outer membrane protein
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tm	Melting temperature
VDAC	Voltage dependent anion channel
wt	Wild type
Λ	Conductance
σ	Specific conductance

INTRODUCTION

The outer membrane of Gram-negative bacteria forms a permeability barrier to small hydrophilic compounds important for cell growth and metabolism. At the same time, large molecules such as lysozyme, β-lysin and bile salts, which are toxic to Gram-positive bacteria, are excluded. The major outer membrane components responsible for this selective permeability are lipopolysaccharide (LPS) and porin proteins. Since LPS and porins are critical for protecting cells against noxious compounds, yet allowing cells to grow and reproduce normally, it is important to define the structure/function relationship of these compounds and to characterize the interactions between these molecules.

Two major goals of this thesis have been 1) to isolate and characterize the major outer membrane proteins, OmpC and OmpF, from *Escherichia coli* K-12, determining the effects of LPS on the structural stability and functionality of the porins, and 2) to characterize porins with known single amino acid substitutions or small deletions in their primary sequence, characterizing the importance of particular amino acid residues on the structural stability and functionality of porin isolates.

Porin proteins from *Escherichia coli* K-12 strains were purified and the level of LPS was determined using chemical, immunological and gel staining techniques. The stability of the isolated proteins was further characterized by monitoring porin denaturation to high temperatures and low pH using sodium dodecyl sulfate

polyacrylamide gel electrophoresis and differential scanning calorimetry. The functionality of the porins was determined by reconstituting porins into two different artificial membrane systems, liposome vesicles and planar bilayer lipid membranes. Each mutant protein's structure and function was compared to its wild type counterpart, and the effect of LPS on the protein's stability and functionality was measured.

The first chapter provides general background on outer membrane porins and LPS from *Escherichia coli*. Subsequent chapters detail the experiments involved in this project and the results and conclusions obtained. The final chapter summarizes the results found and proposes the physiological importance of these results as they relate to the organism. The effect of LPS on porin stability and functionality, and the involvement of specific amino acid residues in the structure and function of porins are emphasized.

CHAPTER 1

Literature Review

Cell Envelope Composition:

The Gram-negative bacteria's cell envelope consists of an outer membrane and an inner or cytoplasmic membrane separated by a cellular compartment known as the periplasm (Figure 1). The peptidoglycan layer, located between the inner and outer membranes, is comprised of a repeating disaccharide polymer crosslinked by tetrapeptides, and is important in maintaining the rod-like structure of the bacterium (1). This peptidoglycan structure is covalently linked to the outer membrane via a small lipoprotein (1). Also found within the periplasm are a wide variety of enzymes which process materials into molecules capable of transport across the inner membrane (2).

The inner or cytoplasmic membrane is a protein-phospholipid bilayer containing many transport proteins and enzymes (3). This membrane is critical in cellular division, in the active transport of materials across the bilayer, in the synthesis of cell envelope components, in the electron transport chain, and oxidative phosphorylation (3).

The outer membrane is an asymmetric bilayer with lipopolysaccharide (LPS) on the outer monolayer and phospholipids, almost exclusively phosphatidylethanolamine, comprising the inner monolayer lipid (4-7). The outer membrane contains only a few major protein species. The most abundant is the lipoprotein, 7,200 Da, at approximately 7×10^5 copies per cell (1). Another outer membrane protein which contributes to the mechanical rigidity of the cell is the outer membrane protein (Omp) A protein, often referred to as the "heat modifiable" protein because of its altered mobility on sodium dodecyl sulfate (SDS) gels after heating (8). Present in about 10^5 copies/cell (9) are a class of proteins called porins (10). These proteins form non-specific water-filled diffusion channels which can passively transport small hydrophilic molecules of less than

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 represented by *b*, the outer membrane porins *c*, phospholipids *d*, and lipoprotein *e*.

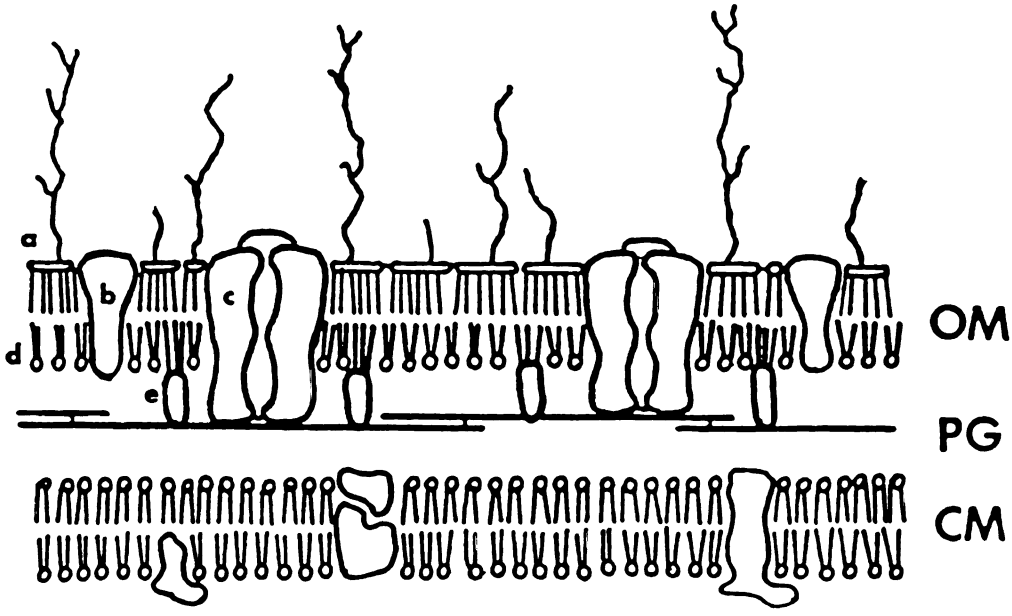

FIGURE 1

600 Da across the outer membrane (10,11). Two outer membrane porins constituitively expressed in *Escherichia coli* K-12 are the OmpC and OmpF porins (1). Only the OmpF porin is produced in *Escherichia coli* B cells (1). Two other conditionally expressed porins found in *Escherichia coli* K-12 are the LamB and PhoE proteins, which are specific for maltodextrin (12-14) and phosphate transport (15), respectively. Under conditions of phosphate starvation cells will produce PhoE (16,17). LamB production is derepressed by culturing cells in media containing 0.5% glucose (18).

Other minor transport proteins present in the *Escherichia coli* outer membrane include the BtuB protein required for Vitamin B_{12} uptake (1), Tsx protein necessary for nucleoside uptake (19), and TonA protein required for the transport of ferrichrome (20). Additionally, phospholipase A_1 (21) and a few proteases (22) have their enzymatic activity associated with the outer membrane. Only a few hundred copies per cell of these minor proteins are present in the outer membrane (1).

Gram-negative bacteria are resistant to a wide variety of proteases, antibiotics, surfactants, and bile salts because of the chemical structure and physical interactions of LPS on the outer surface of the cells (1,6). LPS creates an extremely hydrophilic cell surface, important for evading phagocytosis and for resistance to the actions of complement (1). At the same time, the bacteria are permeable to small hydrophilic compounds, including nutrients and waste products. This selective permeability can be attributed to the outer membrane porin proteins. Therefore, the outer membrane of Gram-negative bacteria forms a well-regulated permeability barrier due to the interactions of LPS and porins.

Lipopolysaccharide:

The structure of LPS from enteric bacteria can be divided into three distinct regions, the O-antigenic side chain, the core polysaccharide and the lipid A (Figure 2). The lipid A region comprises the hydrophobic component of the outer monolayer of the outer membrane. At the membrane surface is the lipid A headgroup, a β-(1-6) linked diglucosamine backbone that is phosphorylated at positions 1 and 4' (23,24). Most bacterial LPS contains six fatty acid residues. Usually the fatty acids are saturated and hydroxy fatty acids, mainly 3-OH-tetradecanoic acid in *Escherichia coli* (23). Four of the six fatty acids chains are linked via ester and amide bonds directly to the diglucosamine backbone. The other two fatty acids are ester linked to the hydroxy fatty acid producing a 3-acyl-oxy-acyl structure (1,23).

The polysaccharide core region is directly linked to the lipid A headgroup at position 6' via an unusual sugar present in most LPS molecules, 2-keto-3-deoxyoctulosonic acid (KDO; 5,25). Adjacent to KDO, heptose residues, and hexose and aminohexose sugars comprise the core oligosaccharide region (1). Within the core are a number of phosphorylation sites (25). The overall phosphate content varies among bacteria; for instance, *Escherichia coli* has 4-7 phosphate residues per LPS molecule (26,27) whereas *Pseudomonas aeruginosa* has 10 or more phosphates (28,29). In addition, in some enterobacterial strains the levels of substitutions with phosphate, ethanolamine, and rhamnose at specific sites is substoichiometric, which increases the structural heterogeneity of LPS molecules (25). Substitutions at selected sites has been reported to increase interactions between LPS molecules, increasing the resistance of the bacteria to noxious compounds (27,30).

Figure 2. The generalized structure of an *Escherichia coli* LPS. GLN-glucosamine, KDO-2-keto-3-deoxyoctulosonic acid, HEP-mannoheptose, GAL-galactose, GLC-glucose, COL-colitose (3,6-dideoxyglucose), ETH-NH_3^+-ethanolamine, P-phosphate.

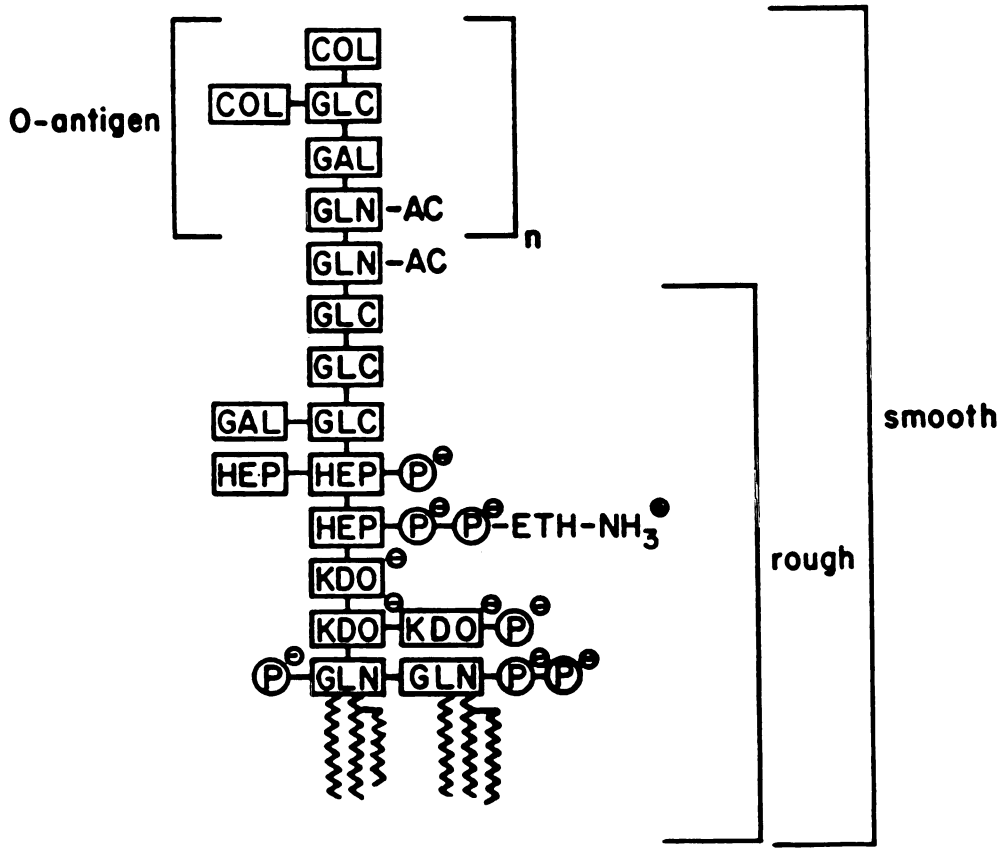
O-antigen
COL
COL
GLC
GAL
GLN-AC
n
GLN-AC
GLC
GLC
GAL
GLC
HEP
HEP
P
HEP
P
P
ETH-NH3
KDO
KDO
KDO
P
P
GLN
GLN
P
P
rough
smooth

FIGURE 2

Attached to the core polysaccharide is the O-antigenic side chain, comprised of a repeating oligosaccharide unit (31,32). This region gives the bacteria its antigenic specificity and is the least conserved structure among the Enterobacteriaceae LPS molecules (25). In *Escherichia coli* the O-antigenic side chain usually consists of neutral sugar residues (31,32). Colonies of strains containing an O-antigen often have a smooth appearance and are called smooth strains, while those lacking an O-antigen and having a rough appearance are termed rough strains (25). Strains in which no O-antigen is found but produce a complete polysaccharide core, such as *Escherichia coli* K-12 strains, are known as Ra chemotypes (25). Strains with only the lipid A and KDO sugars are referred to as Re chemotypes (25).

The acidic phosphates in the core and lipid A regions and the carboxyl groups on the KDO residues, create an anionic microenvironment at the surface of the cell. At physiological pH this net negative charge results in a strong negative surface charge on Gram-negative bacteria. In order to stabilize the surface and minimize charge repulsion between LPS molecules, the phosphates are cross-bridged by a variety of cations including Ca^{2+} and Mg^{2+} (33,34). These divalent cations can be removed by EDTA, which can result in the disruption of outer membrane organization (35). This cross bridging between neighboring LPS molecules by divalent cations provides the cell surface with an effective barrier to environmental perturbants (25).

Porins:

The major outer membrane proteins, porins, are also critical in determining this membrane's permeability. Porins (10) serve as passive transport pathways for small

hydrophilic solutes including nutrients and waste products. Large hydrophilic compounds and small hydrophobic materials are unable to pass across the channels because of their size and hydrophobicity, respectively (2). The first *in vivo* evidence that porins were involved in the uptake of nutrients across the outer membrane came from studies of mutant cells lacking porins in the outer membrane (36,37). These cells were severely limited in the uptake of nutrients and grew rather poorly. It was concluded that these proteins are critical for nutrient uptake.

The OmpC and OmpF porins are named for their structural genes. The synthesis of both proteins is regulated by temperature, pH, and the osmotic strength of the culture media (38-40). Cells grown at high temperatures, low pH, and high osmotic strength, preferentially produce the OmpC porin (38-40). Furthermore, the synthesis of the porins is modulated so that the total number of copies of the porins per cell remains constant (38,41,42).

The OmpC and OmpF porins have been isolated and chemically characterized by a number of laboratories. The proteins have been shown to span the outer membrane since they interact tightly with the underlying peptidoglycan layer (9), and act as bacteriophage receptors on the outer surface of the cells (1). The primary sequence has been determined from the gene sequence for both proteins (43,44) and they are approximately 60% homologous (45). Also, the PhoE porin has a primary sequence similar to OmpF and OmpC, and they are all probably derived from a common ancestral gene. The amino acid sequence of the OmpC and OmpF porin does not show any long stretches of hydrophobic residues, normally found in transmembrane proteins (43,44). Moreover, 67-69% of the amino acids in porins are polar residues and 21% are charged

amino acids. Since the protein forms a hydrophilic diffusion pore, presumably the charged and polar residues line the interior of the channel, while the hydrophobic residues extend into the bilayer.

The undenatured isolated proteins exist as trimers when extracted in sodium dodecyl sulfate (SDS; 9) and travel on SDS-gels with an aberrant mobility in the absence of heating due to the folded structure and low SDS binding (9,45). In the absence of heating, porins have been shown to be tightly associated with LPS as reflected by a distinct "ladder" of bands on SDS-gels (46-49). In addition to their resistance to SDS, isolated porins are also stable to a variety proteases, 8 M urea, high temperatures, and pH extremes (9,50,51). This stability has been attributed to strong protein:protein interactions and to the tight association with LPS (50-52). When heated to 100°C in SDS the trimeric complex dissociates, and the denatured monomers travel on SDS-gels with a molecular weight of 36 and 37 kDa for OmpF and OmpC, respectively (9,53,54). After heating, most of the LPS is removed from the porin (49,51); however, some LPS remains tightly associated with the denatured monomers (49). Gehring and Nikaido (55) have shown that a random distribution of OmpC/OmpF heterotrimers exists in cells that produce both the OmpC and OmpF porins. The sites of interaction between monomers within the trimeric complex are proposed to be conserved sequences between the two porins. The significance of heterotrimer formation is unclear at the present time.

In order to examine the structure and stability of isolated porins, a number of biophysical techniques have been used. Analyses of secondary structure by circular dichroism spectroscopy (9,45,50) and infrared spectroscopy (56,57) have shown that porins contain an unusually high percentage of β-sheet structure with no evidence of

α-helical segments. As much as 60-70% of the OmpF peptide backbone exists in a β-conformation as determined using Fourier transform infrared spectroscopy (56). Additionally, approximately 50% of the β-structure lies perpendicular to the plane of the membrane and 50% lies parallel to the plane (57). Since the β-sheet forms a much more extended conformation than the α-helix, it is thought that long hydrophobic stretches are not necessary to span the outer membrane (56,58). Once heated to 100 °C, the protein loses its β-structure and acquires both random coil and α-helical configurations (9,45,50).

Electron microscopic analysis and computer imaging of regular arrays of the OmpF porin have shown that the repeating unit corresponds to a trimer, each of which contains a triplet of negative stain-filled channels (59-62). Image reconstruction analysis of regular arrays of the PhoE protein has shown that the three channels converge near the center of the trimer but do not merge. The results suggest that there are three channels within a trimeric unit, each formed by a monomer subunit (63). Since the OmpF and OmpC porins have similar primary sequences to the PhoE porin it is assumed that three individual channels also exist in trimers of these two porins. This study contrasts with previous studies which indicated that three channels merge into a single pore (64-66). To clarify this discrepancy studies on the three dimensional structure of the OmpF are in progress but no detailed structure is available yet. The OmpF porin has been crystallized (47,64,67,68) and work is in progress on solving the three-dimensional X-ray crystallographic structure.

Directly related to the structural characterization of porins is their stability to thermal and pH denaturation. Several methods have been used to analyze the structural stability of porins including circular dichroism (9,45,50), intrinsic fluorescence

spectroscopy (50), gel electrophoresis (9,45), and differential scanning calorimetry (52,69). Results from several laboratories indicate that the OmpF porin is stable to high temperatures, > 60°C (9,50), as well as pH values between 4 and 10 (50,51). However, these results can vary by as much as 20°C and one pH unit depending upon the detergent used to solubilize the protein (50) and the amount of associated LPS (52). Porins have been shown to be more stable when suspended in nonionic detergents as opposed to ionic detergents (50) and when LPS is tightly associated (52). For these reasons, many investigators use nonionic detergents to perform *in vitro* functional assays.

Model Membrane Studies of Porins:

A number of functional assays have been used to examine the channel forming activity of porins *in vitro*. The two most common are liposome vesicles (10,13,14,70) and bilayer lipid membranes (BLM; 71-74). Liposome vesicles have been used to measure the relative rate of diffusion of solutes across porin channels (10,13,14,70). From these data an estimate of the size exclusion limit of the channel can be obtained (70,75). Using such an approach, Nikaido and Rosenberg (70) have shown that the OmpF channel size is slightly larger than OmpC and that small hydrophobic molecules are excluded from the channel interior. These results suggest that porins form channels with specific molecular weight cutoffs (70). Such liposome techniques have also been used to determine the size exclusion limits of porins from different bacterial strains (76-80).

A second *in vitro* system used to examine the functionality of porins is the planar BLM, which measures the conductivity of ions through porin channels (71,72,74,81,82). This technique is very powerful for looking at single molecular events, i.e. individual

porin channels (81). Information concerning the diameter of porin channels, cooperativity of channels, heterogeneity of porin channels, and ion binding sites or specificity of channels can be determined. Additionally, the regulation of channel opening and closing using changes in transmembrane potential, ionic strength, pH, and temperature, can be monitored (81). Studies from BLM analyses have indicated that the diameter of the OmpC porin is approximately 1.0 nm whereas that of the OmpF porin is 1.1 nm. (74). These are very similar to the diameters measured using the liposome assay (70) and electron microscopic techniques (62,63).

Using the BLM assay, Benz and coworkers (73) have shown that increasing the ionic strength of the KCl solution bathing the membrane from 0.03 M to 3 M results in a linear increase in the porin channel conductance. These results suggest that the flow of K^- and Cl^- ions through the porin channel is similar to the movement in free solution (73,82). Hence, the frictional forces of the ions against the sides of the wall are minimal. However, studies in which salts of larger ionic radius were used showed a slight cation selectivity of the OmpC and OmpF channels (83,84). Furthermore, none of the solutes tested could completely block the porin channels. On the other hand, the LamB porin channel conductance can be blocked by the addition of maltose or maltodextrins to the solution surrounding the BLM (85,86). Also, when LamB is inserted into proteoliposomes, the rates of influx of small solutes is blocked by small amounts of maltodextrins (14). These studies indicate that there is a specific maltose binding site within the LamB porin channel.

Additional BLM studies with OmpF and OmpC have shown that both porins exist is at least two different conformations, an open and a closed state (71,72,87,88). No

direct evidence exists for the closure of porins *in vivo* but the closed conformation has been suggested to exist in the outer membrane (1,89,90). Several issues have been raised regarding regulation of the opening and closing of porin channels *in vitro*. Opening and closing, i.e. gating, of porin *in vitro*, has been shown by a number of investigators to be regulated by increased transmembrane potentials (71,72,87,88,91-93); however, other investigators have failed to repeat these results (73,74,94). These differences may result from a number of factors including different assaying systems, differences in the methods of porin purification, and differences in the conditions of the assay (93,95). Recent work perfomed by Lakey and Pattus (93) using several assaying systems and purification procedures have shown that the channels are voltage regulated, and in some systems this regulation is more evident than others. However, in order to close porin channels large transmembrane potentials, > 100 mV are required (71,72). Several investigators suggest that high transmembrane potentials are not physiologically relevant (74,96) since the only potential difference across the outer membrane is a Donnan potential of appoximately 30 mV (97). However, short term membrane potentials of up to 130 mV, necessary to close porin channels, may result when external salt concentrations approach zero (95).

Recently, Dargent et. al. (87) showed that the PhoE porin opens as a cooperative unit of three channels at low membrane potentials. As the transmembrane potential was increased the cooperativity was lost, and monomer channel conductances were detected. In addition, at high membrane potentials the stability of the closed PhoE channel conformation was increased (87), similar to what has been observed with the voltage-gated Na^+ channel (98) and the mitochondrial voltage-dependent anion channel (VDAC; 99) of eukaryotes. The loss of cooperativity between trimer subunits at high membrane

potentials and the detection of monomers i.e. one-third the conductance of the trimer, substantiated the porin model of Jap (63) which showed three individual channels exist within a trimer.

The loss of cooperativity of channels upon increasing the transmembrane potential has also been detected using the OmpF porin (71,72,88). This voltage-dependent decrease in conductance size to one-third the size of the trimeric events was enhanced by low pH (88). Specific carboxyl groups responsible for ionic bonding may be neutralized at low pH. This titration appears to cause loss of cooperativity between subunits and increased channel closing (88). In general, the loss of cooperativity induced by high voltage and/or low pH is usually accompanied by an increase in the level of the closed porin conformation.

Porin : LPS Interactions:

Porins are very tightly associated with LPS when purified (9,72). Complete removal of LPS from porins is not possible without denaturing the proteins (49). LPS bound to porin can be detected using a number of techniques, including analysis for KDO (9,100) and phosphate (52), silver staining of SDS-gels (46,48,100-102), fatty acid analysis (46), and immunological assays (49,100). Several investigators have devised purification techniques to remove LPS; however, there appears to be some LPS tightly associated to these isolates (47,49,51). The inability to remove LPS from porins is one factor that has hampered progress in solving the X-ray crystallographic structure of porin (47,59).

Many investigators have attempted to determine if there is a functional role of the tightly bound LPS. Reconstitution of OmpC or OmpF porins with LPS results in an ordered hexagonal lattice structure, with a lattice constant of about 7 nm (9,103). Only one LPS molecule per trimer was needed to form the hexagonal array and this LPS molecule could be replaced by an equivalent amount of lipid A or fatty acids (103). Therefore, the lipid component appears important for porin aggregate structure. Although the exact interactions are unknown, the potential for hydrogen bonding, ionic bridging, and hydrophobic interactions between porin and LPS is great considering the chemical structure of the LPS molecule and the close proximity of LPS and porin on the outer membrane.

LPS is also critical for bacteriophage binding to porins (11,104). Studies of T4 phage binding to the OmpC porin using strains in which the LPS molecule was truncated to the heptose residues showed no bacteriophage binding. When an additional glucose residue was present on the LPS molecule, bacteriophage binding was restored (104). This study showed that the core polysaccharide region of LPS was necessary for bacteriophage binding to porin (104).

More controversial is the possible role of LPS in modulating the channel forming properties of porins. Schindler and Rosenbusch (71,72) and Nakae (10) have reported that LPS is critical for the proper functioning of porins as channels and that removing LPS from the porin isolates decreases their channel forming activity. In contrast, Hancock (65) and Benz *et al.* (73) have reported that LPS is not essential for channel forming activity. Additionally, Parr *et al.* (100) have indicated that LPS can be completely removed from the porins of both *Escherichia coli* and *Pseudomonas aeruginosa* and that

no change in channel activity is observed. However, to assay for LPS content they used an antibody to the core region of the LPS molecule which may have been cleaved and lost during electroelution of their protein from polyacrylamide gels; therefore, the lipid A portion of the LPS could still have been bound to the porin isolates. Nikaido and Rosenberg (70) have shown that excess LPS was not required for porin function; however, some LPS remained associated with the isolated porins upon vesicle formation.

Recently, it has been shown that even after denaturing porins by heat in SDS the lipid A portion of LPS remains tightly associated with the porin (49). Antibodies to the lipid A portion of LPS react with heat denatured porin but not to undissociated porin (49). These results indicate the lipid portion of LPS is not recognized or is buried and inaccessible to antibody binding when in an undissociated form. Jap (63) has proposed that an LPS molecule resides at the axis of three-fold symmetry among the subunits of the trimer in the PhoE complex since this region has an area of low density in the electron microscopic imaging. The positioning of the LPS molecule may account for the lack of reactivity with the LPS antibody until the protein is denatured. De Cock *et al.* (105) have shown that *in vitro* synthesized PhoE monomers require LPS and other outer membrane components for proper folding into a trimeric complex. The proper folding may occur around the central LPS molecule burying the LPS within the subunits. LPS may then act as a backbone and hold the trimer in the native configuration. The proper functioning of the channel may depend on the presence of LPS to stabilize this three dimensional structure.

Modification of Porin Channels Affect Permeability:

Comparing wild type and mutant or chemically modified proteins can help define critical residues important in function and/or structure. The OmpF porin has been chemically modified using acetic anhydride and succinic anhydride to alter amine groups, and glycinamide to modify carboxyl residues (106). *In vitro* modification of amine groups caused reduced diffusion rates of negatively charged solutes whereas modification of carboxyl groups caused decreased diffusion of positively charged solutes. However, neither modification significantly affected the rate of uncharged solute diffusion (106). It appears that ionic residues influence the diffusion of large charged molecules through the porin channels.

Similarly, Schindler and Rosenbusch (107) have chemically modified amine groups of the OmpF porin with eosin isothiocyanate, fluorescamine, and citraconic anhydride and showed that the number of residues modified by each chemical depended on the size and hydrophobicity of the reagent. Reactive probes of various sizes serve as indicators of the surface accessibility of reactive residues in the porin and can be used to probe the pore topology. Analysis of OmpF, OmpC and PhoE porins using planar BLM after lysine residue modification also indicated altered selectivity of large charged molecules (108).

Comparison of mutant proteins, with specific amino acid residue changes, to wild type proteins, provides information concerning residues or regions in the protein critical for structure and/or function. Benson and Decloux (109) have selected for spontaneous mutations in the structural genes for porin which increase the channel size. *Escherichia coli* K-12 cells, lacking the LamB porin, were grown on large maltodextrin. The wild type OmpC and OmpF channel's are too small to accommodate large maltodextrins so

bacteria were selected which were altered in the OmpC or the OmpF porin to allow for maltodextrin transport. Several *Escherichia coli* K-12 strains were selected for their ability to grow on large maltodextrins, and the mutations were mapped to the *ompC* or *ompF* gene (109-112). The gene sequences revealed that single point mutations or small deletion mutations allowed growth on maltodextrins. The mutations appear to alter the channel size of the porins allowing maltodextrin transport (111,112).

Several laboratories are involved in the isolation and characterization of such mutant porins. One such mutant strain, in which a wild type OmpC is expressed as well as OmpF with a 15 amino acid deletion, appeared to be altered in the assembly of the wild type OmpC porin. Not only were normal OmpC porin trimers isolated, but a stable, functional, OmpC dimer was also isolated (113). Porins are produced as monomers that fold and assemble into trimeric units after crossing the cytoplasmic membrane. A dimer intermediate has been detected during the assembly of the OmpF porin and the denaturation temperature of this dimer is similar to that of the isolated OmpC dimer (114). The accumulation of the OmpC dimeric intermediate in the OmpF mutant strain is not fully understood, but may involve the mutant OmpF monomer's inability to correctly associate with an OmpC dimer (to form a OmpC/OmpF heterotrimer) resulting in stable OmpC dimers (113). The assembly and export processes involved in porin synthesis are not fully understood at the present time.

Another porin that has been extensively studied by site-directed mutagenesis is the LamB porin. Three classes of mutants in LamB have been characterized; 1) single amino acid substitutions that did not alter maltose transport, 2) substitutions that altered transport by 20-30%, or 3) mutations that totally abolished maltose transport (115). An amino acid

substitution from glycine to valine at position 18 not only impeded the transport of maltose but also decreased the temperature stability of the trimer. A single amino acid change in the OmpF porin from an aspartic acid to a glycine at position 113 also decreases the temperature stability of the protein (52). The position of these mutations in the tertiary and quaternary structure of the porins is not known.

Tommassen (116) and Benson *et al.* (112) have constructed models for the folding of the PhoE and OmpF porins, respectively, in the outer membrane. Almost all of the amino acids which, when altered, produce larger porin channels (111,112), reside on the outer surface of the protein or within the transmembrane spanning region. Additionally, all of the mutations that affect channel size occurred within the first one-third of the polypeptide chain which has been shown to contain the channel forming domain of the porins (109,117). Therefore, if the models are correct, the mutations that arise to give larger porin channel sizes occur within the porin channel at the exterior opening of the pore and not at the site of greatest constriction, believed to be midway through the membrane (74). Future analysis of these mutants should provide information concerning amino acids critical for the stability and modulation of structure and function.

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CHAPTER 2

Lipopolysaccharide Tightly Bound to Porin Monomers and Trimers from *Escherichia coli* K-12

ABSTRACT

Lipopolysaccharide (LPS) bound to isolated porin was detected on polyacrylamide gels by using a carbohydrate-specific silver stain and on Western blots using anti-lipid A monoclonal antibodies. Porin was isolated from *Escherichia coli* JF733 (Ra chemotype). Isolated porin was separated from loosely associated LPS by polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS). Unheated porin traveled on gels as aggregates, presumably trimers, with an apparent molecular weight of 78,000 to 83,000. After heating to 100°C for 2 min in SDS, the porin traveled as a monomer with a molecular weight of 36,000. The unheated, high-molecular-weight trimer band reacted in the gel with the carbohydrate-specific silver stain, while the heated monomer band showed no staining. In contrast, lipid A-specific monoclonal antibodies showed reactivity on Western blots to the 36,000-molecular-weight band but not to the trimer. Finally, both monomer and trimer bands were isolated from gels and rerun in SDS-PAGE. LPS was released from the trimer preparation when the sample was heated, but the monomer band that was originally formed by heating the trimer isolate still reacted with anti-lipid A antibodies after a second heat treatment. Quantitative *Limulus* amebocyte lysate analysis revealed an approximately equal molar ratio of LPS to protein in the electroeluted porin monomer. Thus, some but not all of the LPS could be released from trimer complexes by boiling in SDS. The isolated monomer did not release more LPS on boiling in SDS a second time but still had LPS tightly bound, as detected by lipid A-specific monoclonal antibodies.

INTRODUCTION

The structure and interactions of the outer membrane porin proteins OmpF and OmpC from *Escherichia coli* are very similar. They have similar molecular weights and amino acid sequences (1), the native proteins are very high in beta structure (2,3), both are tightly associated with the peptidoglycan (3-5), and they are very tightly associated with lipopolysaccharide (LPS, 6-9). Porins are known to form water-filled channels (10,11) and are unusually stable to a wide variety of perturbants such as sodium dodecyl sulfate (SDS) detergent (2,12,13), pH extremes from 2 to 12 (14), and temperatures up to 70°C (3). Porins are also resistant to many proteases, perhaps because of their tight association with LPS (7); the proteins are thought to be functional only in the presence of LPS (7,11,15). Nakae *et al.* (12) have suggested that only the lipid component of LPS is essential for porin function. In contrast, Parr and co-workers (16) have reported that porin is functional in the absence of LPS. Thus, the importance of bound LPS for porin structure and function has yet to be resolved.

The OmpF porin exists as a trimeric complex (12,17) with three identical subunits with approximate molecular weights of 36,000 (3,18). Low SDS binding coupled with the tight, compact shape of the trimer results in more rapid migration on polyacrylamide gels than the aggregate molecular weight would suggest. Investigators have shown that, without heating, porin travels as aggregates with molecular weights from 75,000 to 85,000; and it has been suggested that this represents a trimer of porin (3,14,19,20). On heating the protein becomes denatured, loses its beta structure, and migrates on gels with a mobility that reflects its chemically determined molecular weight of 35,000 to 37,000.

Several investigators have described silver stains for carbohydrate-containing molecules (21-23) which are useful in measuring as little as 5 ng of LPS (23). In the purification of outer membrane porins, as much as 9 mol of LPS per mol of trimer has been reported (11), whereas others have detected only 0.2 to 0.3 mol LPS per mol of trimer (24,25). In recent reports it has been indicated that LPS can be completely removed from porin by electrophoresis on polyacrylamide gels (16) and by column chromatography (14) without loss of porin structure (14,16) or function (16). We found that unheated porin isolated from polyacrylamide gels still has tightly bound LPS and that heating dissociates some but not all of the LPS. Furthermore, purified porin heated to 100°C in 2% SDS still has tightly bound LPS, as detected with anti-lipid A monoclonal antibodies and quantitative *Limulus* amebocyte lysate analysis. This LPS could not be removed with additional heating and electrophoresis.

MATERIAL AND METHODS

Cell growth and porin isolation.

E. coli K-12 strains D21f2, an Re chemotype LPS which produces both OmpF and OmpC porins, and strain JF733, an Ra chemotype LPS which produces only the OmpF porin, were used in this study. The cells were grown in nutrient broth (1% tryptone, 0.2% yeast extract, 0.4% NaCl) at 37°C and harvested just before the stationary phase of growth. The porins were purified by the method of Lakey *et al.* (26), with slight modifications. Porin was extracted twice from the peptidoglycan with 0.5 M NaCl-0.7 M mercaptoethanol treatment. After extensive dialysis in 5 mM sodium bicarbonate (pH 7.2), the porin was precipitated with 90% acetone (vol/vol). The final preparation was resuspended in either 2% SDS, 10 mM Tris (pH 7.4), or 2% Triton X-100-5 mM EDTA-50 mM Tris hydrochloride (pH 7.2) and stored at -20°C.

Monoclonal antibodies and solid-phase immunoassays.

The production and partial characterization of the anti-lipid A monoclonal antibodies used here have been previously described (27,28). The antibodies were purified by protein A chromatography (29). Solid-phase immunoassays were performed on a screen machine (Pandex) by using a protocol similar to that described by Jolly *et al.* (30). Antigens were adsorbed directly onto polystyrene particles (diameter, 0.8 μm) in a 0.25% working solution of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) in 0.9% pyrogen-free saline at pH 7.2. All assays were performed in a forward sandwich format in which 0.02 ml of antigen-coated particle per well and 0.03 ml of

antibody per well were separated and washed through a filter (pore size, 0.2 μm) in the bottom of each well of the assay plate. A total of 0.02 ml of fluorescein-labeled goat anti-mouse $F(ab')_2$ per well, at 5 μg/ml in isotonic-buffered saline containing 1% normal goat serum, was incubated for 20 min and then separated and washed as described above. Antigen-bound antibody was then recorded in relative fluorescence units. All solid-phase immunoassays were performed in duplicate.

Gel electrophoresis and Western blots.

Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in the discontinuous buffer system described by Laemmli (31). Separating gels, containing 12% acrylamide and 0.1% SDS, were formed either with or without 4 M urea (final concentration). Electrophoresis of analytical gels was carried out at a constant current of 30 mA until the dye front reached the bottom. Molecular weight markers included bovine serum albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), and carbonic anhydrase (29,000). Three different staining procedures were used to visualize either LPS or protein. Coomassie brilliant blue was used to detect protein bands. Silver staining, which detected both protein and LPS, was carried out by glutaraldehyde treatment of the gels by the methods described by Oakley *et al.* (32). Finally, carbohydrate-specific silver staining with periodic acid oxidation of the LPS was performed by the method of Dubray and Bezard (21).

To detect LPS-containing bands with anti-lipid A monoclonal antibodies, bands from SDS-PAGE were electrophoretically transferred to nitrocellulose as described elsewhere (33). Western blots were developed either by the procedures described by

Otten *et al.* (34) or by using the following procedure. Nitrocellulose sheets were blocked with 2% fish gel in 0.9% saline-10 mM HEPES-0.1% azide for 2 h and then incubated with 10 μg of protein A-purified monoclonal antibody per ml in blocking solution for an additional 2 h. Following this the primary antibody was washed off and replaced with 100,000 cpm of ^{125}I-labelled goat anti-mouse F(ab')$_2$ per ml. After 2 h the excess secondary antibody was washed off with saline and the nitrocellulose sheet was allowed to develop on a sheet of X-ray film (XAR-5; Eastman Kodak Co., Rochester, N.Y.) overnight.

Protein electroelution and concentration.

Purified porin was subject to preparative gel electrophoresis in 3-mm-thick slab gels to accomodate large amounts of the protein isolates. The porin was either heated at 100°C for 2 min in 2% SDS to obtain monomers or left unheated to maintain the native trimeric configuration. Approximately 270 μg of protein was added to each lane. The 12% acrylamide gels were run at 50 mA until the tracking dye reached the separating gel, and the current was then increased to 120 mA. Two of the lanes were stained either with Coomassie brilliant blue or with silver to determine the location of the porin. The other lanes were cut and stored at -20°C until needed for electroelution.

Gels were thawed and bands were cut out according to the location of monomers and trimers seen on the stained lanes. The acrylamide was crushed, placed into an electroelution cup, and run at constant voltage (10 to 50 V) for 2 to 18 h at 4°C in the buffer system described by Laemmli (31). The eluted protein was further concentrated

either by lyophilizing dialyzed samples or by using a concentrator (Minicon B-15; Amicon Corp., Lexington, Mass.) with a 15,000-molecular-weight cutoff.

Protein concentrations were estimated with a protein assay (BCA; Pierce Chemical Co., Rockford, Ill.) by using bovine serum albumin as a standard. Approximately 5 to 6 μg of isolated porin trimer or monomer were added to a second gel and electrophoretically separated. The second analytical gel was stained for LPS or protein as described above.

Enzyme-linked immunoassay on thin-layer Chromatographs.

A total of 25 or 50 μg of each sample were spotted on precoated silica gel 60 aluminum-backed thin-layer chromatographic plates (20 by 20 cm; VWR Scientific, Philadelphia, Pa.). The plates were either developed in chloroform-methanol-water-concentrated ammonium hydroxide (50:25:4:2), as originally described by Chen *et al.* (35), or by using a variation of the ratio of the same solvents (40:35:11:0.2) to allow the movement of D21f2 Re chemotype LPS off of the origin. The enzyme-linked assay for lipid A was essentially identical to the Western blot detection protocol described above, except that an alkaline phosphatase-conjugated secondary antibody (Sigma Chemical Co., St. Louis, Mo.) was used. After the plates had developed and dried, the location of the protein was determined by scraping the silica in consecutive 1-cm sections from the origin to the solvent front of unreacted plates. The silica was suspended in 10 mM Tris-2% SDS (pH 7.4), sonicated, and centrifuged. The concentration of protein in the supernatant was measured as described above.

***Limulus* amebocyte lysate assays.**

OmpF porin samples were tested for residual LPS using a quantitative chromogenic *Limulus* amoebocyte lysate test (Whittaker, M.A., Bioproducts, Walkersville, Md.). Values were calibrated with *E. coli* D21f2 LPS and the *E. coli* 0111:B4 LPS standard provided in the kit. An empirically derived correction factor of 5.6 μg of *E. coli* 0111:B4 LPS per μg of *E. coli* D21f2 LPS was obtained from the calibration curve; this reflected the difference in the molecular weight of the two LPS samples. Control experiments indicated that neither residual SDS nor the OmpF protein itself interfered with the detection of equivalent amounts of exogenous LPS.

RESULTS

Porin isolation and LPS detection using SDS-PAGE:

Porin was isolated from either *E. coli* D21f2 or JF733 by the procedure described by Lakey *et al.* (26). The isolates contained only the major porin protein bands (molecular weight, 36,000), as seen when the isolate was heated, separated in analytical SDS-PAGE gels, and stained with the glutaraldehyde-silver stain (Figure 1, lanes 2 and 3). In contrast, the unheated porin isolated from strain D21f2 migrated as an aggregate with an apparent molecular weight of approximately 78,000 daltons (Figure 1, lanes 4 and 5). When these same samples were silver stained for LPS, only the trimeric aggregate band stained. The monomer band, produced by heating, did not stain (Figure 1, lanes 8 and 9). The carbohydrate-specific stain did detect free LPS with an apparent molecular weight of less than 14,000 that was migrating in the gel. Some LPS was released from the trimer without heating. These results indicate that the isolated porin contained detectable levels of LPS that could be removed by electrophoresis. In addition, the unheated trimeric aggregate still retained LPS that was detected by the carbohydrate-specific silver stain.

To obtain porin which was free of LPS, 220 to 270 μg of protein from D21f2 were applied to preparative polyacrylamide gels. Both heated and unheated samples were separated on the gels, and the location of the monomer and trimer complexes was detected either with the glutaraldehyde-silver stain, which can detect both protein and LPS, or with Coomassie blue, which stains only protein. Staining of the overloaded gels indicated that the porin isolate was contaminated with only very minor amounts of other

Figure 1. SDS-PAGE of purified porin from strain D21f2. Lanes 1 to 4 were silver stained after glutaraldehyde treatment, while lanes 5 to 8 were silver stained after periodate oxidation. Porin was either treated at 100°C for 2 min (lanes 2, 3, 8, and 9) or was not heated prior to electrophoresis (lanes 4 to 7). A sample of 1.5 μg of porin was added to lanes 2, 4, 7 and 9, while 3.0 μg was added to lanes 3, 5, 6, and 8. Lanes 1 and 10 contained the molecular weight protein standards (indicated to the left of the gels, in thousands).

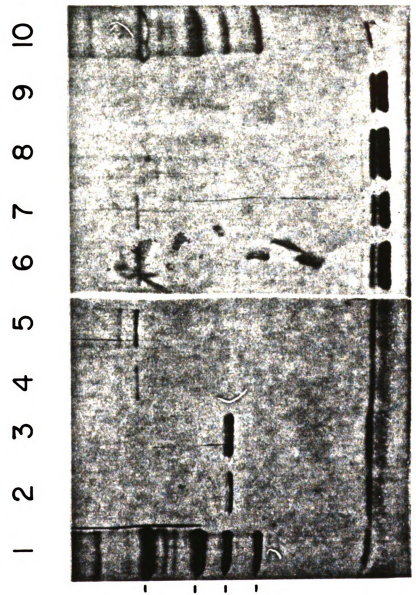

FIGURE 1

proteins (Figure 2). The monomer and trimer bands from the appropriate lanes in the unstained portion of the gel were cut out, electroeluted, and concentrated. Thus, these isolates were free from LPS which migrated to the dye front on the preparative gel. The preparations were then characterized on a second, analytical gel. The trimer isolates, when re-run on SDS-PAGE without heating, still stained with the carbohydrate-specific silver stain (Figure 3, lane 8). Furthermore, some additional LPS was released and was detected at the dye front. When the isolated trimer was heated to 100°C before it was run on the second gel, even more LPS was released, as detected by the carbohydrate-specific silver stain (Figure 3, lane 7); and the monomer band that formed (Figure 3, lane 3) did not react with the carbohydrate-specific silver stain (Figure 3, lane 7). The isolated monomer band, when heated a second time and rerun on SDS-PAGE, did not release detectable amounts of LPS to the dye front (Figure 3, lane 5).

To test whether urea might help dissociate LPS bound to trimeric complexes, gels containing 4 M urea were used. Yu and co-workers (13) have shown that OmpC trimers are stable in 8 M urea. We found that the banding patterns of the isolated complexes in the presence and absence of 4 M urea were very similar (Figures 3 and 4), although the trimeric complex, when loaded at high concentrations in preparative gels containing 4 M urea, showed two low-molecular-weight protein bands which were not detected in the absence of urea (data not shown). The trimeric complex in the analytical gel did not react strongly with the carbohydrate-specific silver stain (Figure 4, lane 12). However, trimers, when isolated in preparative gels containing 4 M urea and heated, released LPS that migrated to the dye front when the sample was run on a second analytical gel (data not shown). Thus, it appears that trimeric complexes contain tightly bound LPS which, in the

Figure 2. Separation of purified porin from strain D21f2 on a 3-mm-thick preparative gel stained with the glutaraldehyde silver stain. Lane 1 contained 270 μg of porin, and lane 2 contained an identical amount of porin heated to 100°C for 2 min. Molecular weights are indicated to the left of the gels, in thousands.

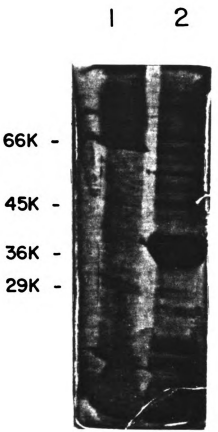

FIGURE 2

Figure 3. SDS-PAGE of electroeluted D21f2 porin from preparative SDS-gels. Porin from strain D21f2 isolated from a preparative gel by electroelution for 6 h at 50 mV was concentrated and then applied to the analytical gel. The right half of the analytical gel was silver stained for LPS after periodate oxidation, and the left half was stained for proteins and LPS with glutaraldehyde. Lanes 1 and 5 contained isolated monomer, which was heated after electroelution. Lanes 2 and 6 contained isolated monomer, which was not heated before it was applied to the second gel. Isolated trimer, which was heated after electroelution, was applied to lanes 3 and 7; and unheated trimer was applied to lanes 4 and 8. Approximately 5.0 μg was added to each lane. Molecular weights are indicated to the left of the gels, in thousands.

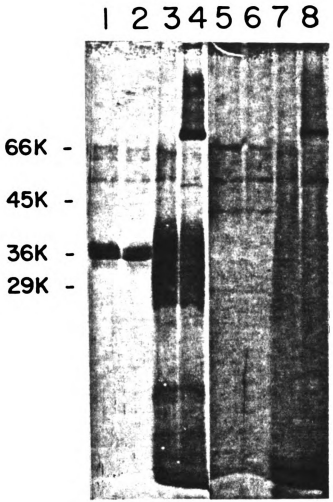

FIGURE 3

Figure 4. 4M urea-gel of D21f2 porin electroeluted from preparative SDS-gels. Porin from strain D21f2 isolated from a preparative gel by electroelution, as described in the legend to Figure 3, was then applied to an analytical gel containing 4 M urea. The left half of the gel was stained for proteins and LPS by using the glutaraldehyde procedure, while the right half was stained for LPS after periodate oxidation. Lanes 1 and 7 contained 1.5 μg of pure LPS from strain D21f2. Isolated monomer, which was heated after electroelution, was applied to lanes 3 and 9, while lanes 4 and 10 contained isolated trimer, which was heated after electroelution. In lanes 5 and 11 isolated monomer, which was not heated before it was applied to the analytical gel, was added; and in lanes 6 and 12 unheated isolated trimer was applied. Approximately 5 μg of protein was added to each lane. Molecular weights are indicated to the left of the gels, in thousands.

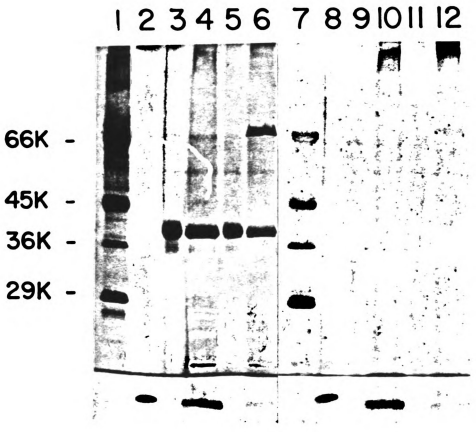

FIGURE 4

absence of urea, can be detected by the carbohydrate-specific silver stain and which can be, at least partially, released by heating the complex to 100°C.

Electroelution of the porin trimer bands for long periods of time (> 10 hr) at high voltages (50 V) appeared to cause a high-percentage breakdown of the trimers to monomeric units (Figure 4, lane 6). Similar patterns were observed in gels run without urea, thus eliminating the possibility that urea was denaturing the trimeric complex (data not shown). Lowering of the pH during extensive electroelution may have resulted in the denaturation of the porin trimer, or the release of tightly associated LPS.

LPS detection using lipid-A specific monoclonal antibodies:

A second approach to detect LPS bound to porin was to use lipid A-specific monoclonal antibodies. The antibodies 4A10, 1D4, and 8A1 all have approximately the same affinity for diphosphoryl lipid A (Figure 5A). In solid-phase immunoassays, both 4A10 and 1D4 were also reactive with purified OmpF trimer from *E. coli* D21f2. Although 1D4 has a higher apparent affinity for OmpF trimer than 4A10, the maximum level of 1D4 binding to OmpF trimer is less than 4A10 (Figure 5B). In contrast, the anti-lipid A antibody 8A1 was completely unreactive with OmpF trimer. None of these antibodies have significant reactivity with negative control proteins (myosin, albumin, lysozyme, etc.) or lipids (cardiolipin or sphingomyelin; data not shown).

Western blots of isolated trimer and monomer from strain D21f2 were developed by using the murine monoclonal antibodies 1D4 (Figure 6A and C) and 4A10 (Figure 6B). With or without heating, the isolated monomer was not visualized with the 1D4 antibody (Figure 6A, lanes 1 and 3; Figure 6C, lane 3), but the 36,000-molecular-weight

Figure 5. Reactivity of anti-lipid A monoclonal antibodies with isolated trimer and lipid A. Titers of the monoclonal antibodies 4A10 (◊), 1D4 (o), and 8A1 (□) were determined on either *S. minnesota* R595 diphosphoryl lipid A (A) or *E. coli* D21f2 trimer (B) as described in the text. rfu, Relative fluorescence units.

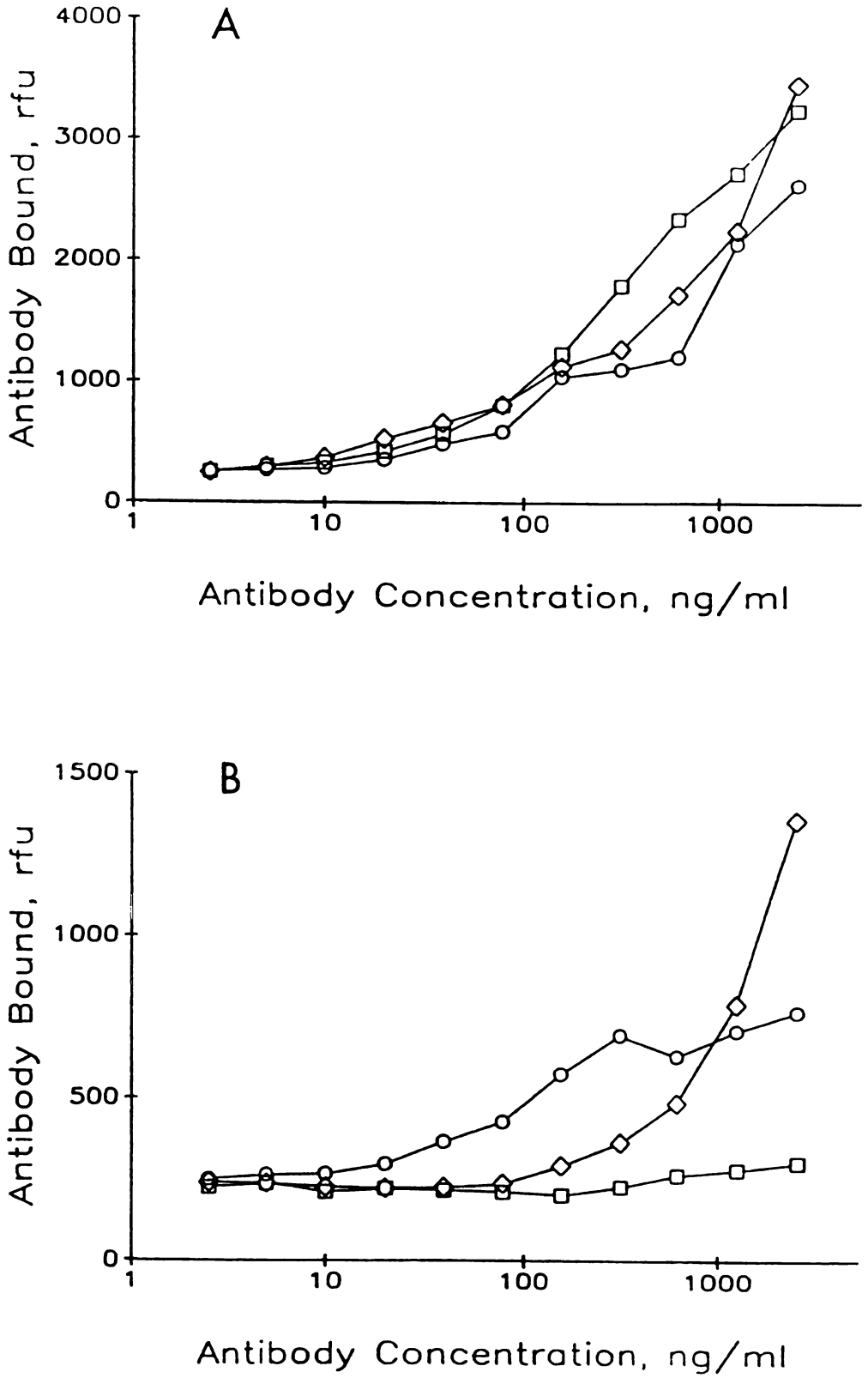
A
4000
3000
2000
1000
0
Antibody Bound, rfu
1
10
100
1000
Antibody Concentration, ng/ml
B
1500
1000
500
0
Antibody Bound, rfu
1
10
100
1000
Antibody Concentration, ng/ml

FIGURE 5

Figure 6. The selectivity of murine monoclonal antibodies 1D4 and 4A10 for the heated and unheated porin monomer and trimer. (A and B) A total of 0.5 μg of heated (lanes 3 and 4) or unheated (lanes 1 and 2) isolated monomer (lanes 1 and 3) or trimer (lanes 2 and 4) porins from strain D21f2 were separated on the gel and then electroblotted onto nitrocellulose. The isolated monomers and trimers were obtained by electroelution from preparative SDS-PAGE gels. The transblotted antigens from the analytical gel were detected by using the monoclonal antibodies 1D4 (A) and 4A10 (B), as described in the text. (C) Reaction of antibody 1D4 to Western blots of porin isolates by the method described by Otten *et al.* (27). In lanes 1 and 2, 9 and 6 μg, respectively, of electroeluted trimer reheated to 100°C for 2 min was applied. Lane 3 contained 6 μg of electroeluted monomer that was reheated to 100°C for 2 min. Lane 4 contained 5 μg of electroeluted monomer that was extrated from the origin of thin-layer chromatographic plates, and lane 5 contained 6 μg of electroeluted unheated trimer. Molecular weights are indicated to the left of the gels in each panel, in thousands.

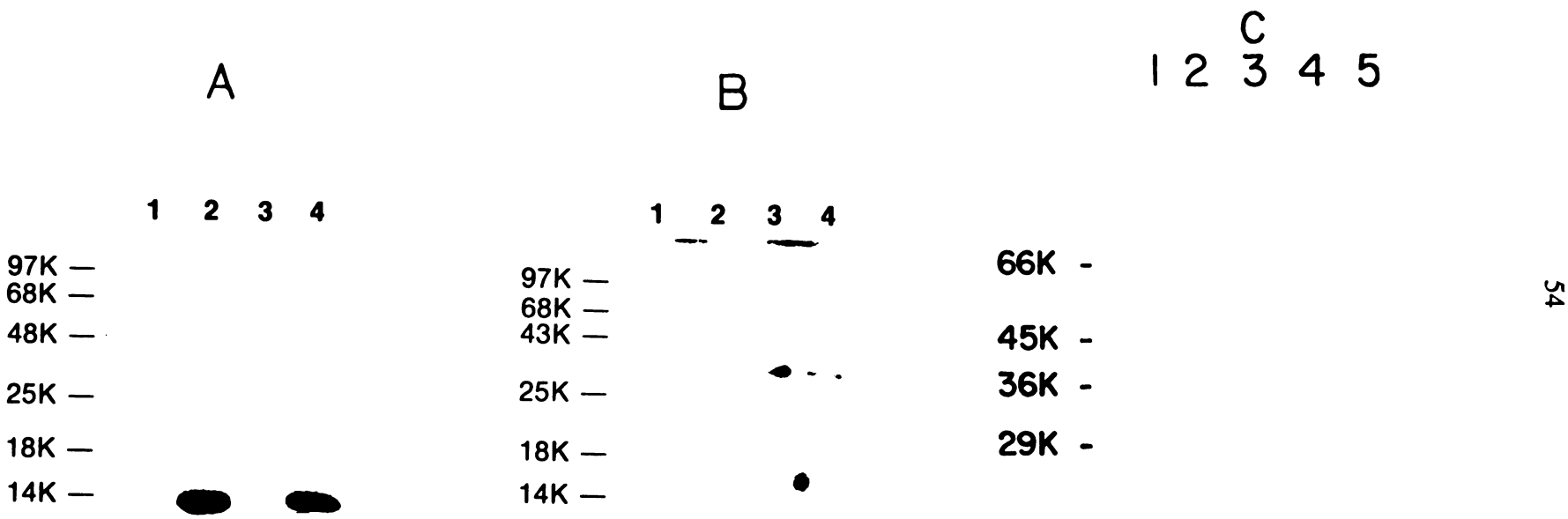
A
1 2 3 4
97K —
68K —
48K —
25K —
18K —
14K —
B
1 2 3 4
97K —
68K —
43K —
25K —
18K —
14K —
C
1 2 3 4 5
66K -
45K -
36K -
29K -

FIGURE 6

monomer was visualized with 4A10 (Figure 6B, lanes 1, 3 and 4). Also, the presence of free LPS in both the heated and unheated trimer was detected near the dye front with the 1D4 antibody (Figure 6A, lanes 2 and 4; Figure 6C, lanes 1, 2 and 5). The monomer that was produced by heating the trimer was reactive with antibody 1D4 (Figure 6C, lanes 1 and 2) as well as antibody 4A10 (Figure 6B, lane 4). However, the 78,000-molecular-weight trimer band did not appear to react with either monoclonal antibody. Finally, the monomer protein released very little LPS with heating, as detected by the absence or low level of reaction of the 1D4 antibody at the dye front (Figure 6A, lane 3; Figure 6C, lane 3). Several other murine anti-lipid A monoclonal antibodies were also tested in this way. All of them produced a pattern of reactivity similar to that of either 1D4 or 4A10 or were completely unreactive, as with 8A1 (data not shown).

Thin-layer chromatography and *Limulus* amebocyte lysate assays:

The results from the Western blots described above suggest that some anti-lipid A monoclonal antibodies can detect LPS bound to heat-denatured porin from strain D21f2, but this LPS was not detected by the carbohydrate-specific silver stain. It was important to confirm that the reactivity of 4A10 with porin was a direct result of 4A10 binding to LPS and not to protein itself. To accomplish this, porin trimer and monomer were chromatographed on aluminum-backed thin-layer silica plates (see above) in a solvent system which resolves rough LPS, but leaves protein at the origin (Figure 7). Approximately 64% of the monomer and 50% of the trimer protein was recovered from the origin after scraping the silica gel from the plates. No protein was detected beyond the origin.

Figure 7. Reaction of murine monoclonal antibodies 4A10 (A and B) and 1D4 (C) to thin-layer chromatograms. (A) The solvent system was as described in the text (40:35:11:0.2). Lane 1, 50 μg of *S. minnesota* R595 LPS; lane 2, 40 μg of *E. coli* D21f2 crude porin (unheated porin isolate before electroelution); lane 3, 50 μg of D21f2 monophosphoryl lipid A, lane 4, 50 μg of D21f2 disphosphoryl lipid A; lane 5, 50 μg of D21f2 LPS; lane 6, 30 μg of D21f2 electroeluted trimer; lane 7, 20 μg of D21f2 electroeluted monomer. For the gels shown in panels B and C, the solvent system was as described in the text (50:25:4:2). Lane 1, *S. minnesota* R595 4'-monophosphoryl lipid A; lane 2, *S. minnesota* R595 diphosphoryl lipid A; lane 3, *S. minnesota* R595 LPS; lane 4, *E. coli* D21f2 LPS; lane 5, *E. coli* D21f2 electroeluted monomer; lane 6, *E. coli* D21f2 electroeluted trimer. A total of 25 μg was added to each lane, except for lane 6, to which was added 15 μg.

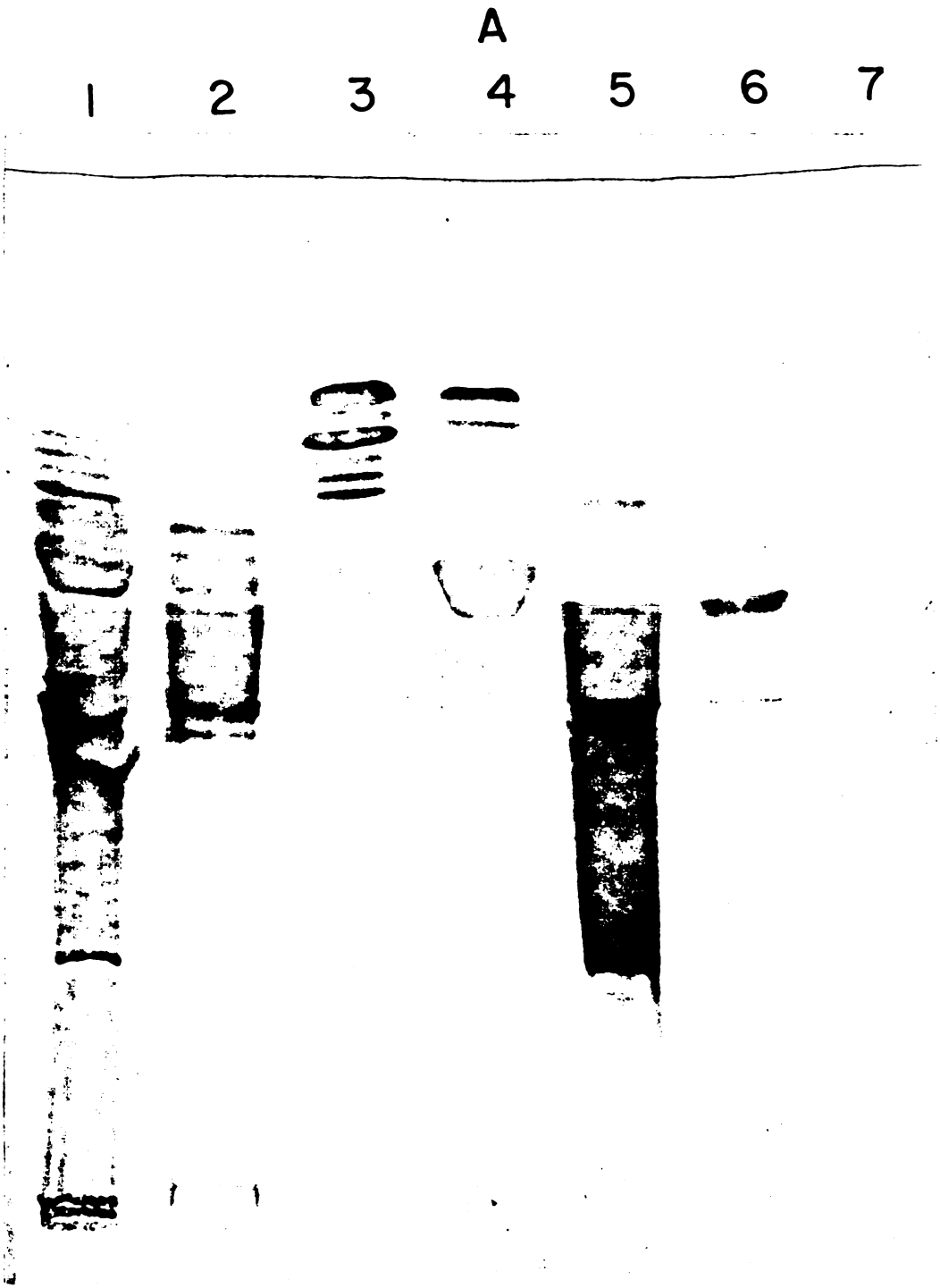

FIGURE 7

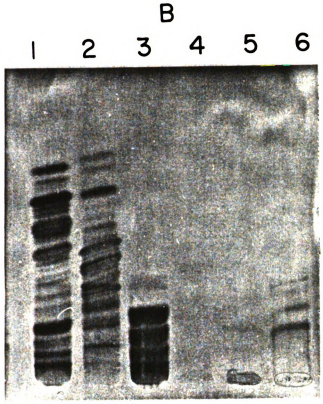

FIGURE 7

Enzyme-linked immunoassays on thin-layer chromatographs on these plates revealed the presence of 4A10-reactive material in the trimer lanes (Figure 7A, lane 6; Figure 7B, lane 6) corresponding to the mobility of D21f2 LPS (Figure 7A, lane 5) and *Salmonella minnesota* ReLPS (Figure 7A, lane 1; Figure 7B, lane 3). A faint reactive band was also seen in the monomer lanes on both plates migrating with the same R_f as the major *E. coli* D21f2 diphosphoryl lipid A band. Similar results were obtained with 1D4 on porin trimer; however, porin monomer reactivity was undetectable (Figure 7C).

Quantitative chromogenic *Limulus* amebocyte lysate tests confirmed the presence of LPS in electroeluted preparations of monomer. Monomer contained 0.047 ± 0.021 μg of *E. coli* D21f2 LPS per μg of monomer (average of three samples). Since the molecular weight of deep rough LPS is about 2,000 g/mol, an equal molar ratio of LPS to porin would produce a weight ratio of 0.057 μg LPS per μg of monomer. The level of residual LPS was determined by using a calibration curve generated with *E. coli* D21f2 LPS and confirmed by using the *E. coli* 0111:B4 LPS standard provided in the kit.

Ra chemotype LPS detection using SDS-PAGE:

The core of LPS from *E. coli* JF733 is more complete than that from strain D21f2; it contains eight or nine additional sugars (36,37). We tested whether the more complex Ra chemotype LPS also has a high affinity for the trimeric porin aggregates. We found that the trimer of OmpF protein from strain JF733 showed multiple protein bands with apparent molecular weights of 78,000 to 83,000 (Figure 8). All except the fastest migrating band stained with the carbohydrate-specific silver stain. Two-dimensional electrophoresis of porin, which was heated before the second dimension was run, showed

Figure 8. Separation of purified OmpF porin from strain JF733 using SDS-PAGE. Heated (lanes 1, 3, and 6) and unheated (lanes 2, 4, and 5) porin isolates were separated on analytical SDS-PAGE gels and stained with the glutaraldehyde silver stain (lanes 1 and 2), the carbohydrate-specific silver stain (lanes 3 and 4), or Coomassie brilliant blue (lanes 5 and 6). Approximately 5 μg of protein was added to lanes 1 to 4, and 10 μg was added to lanes 5 and 6.

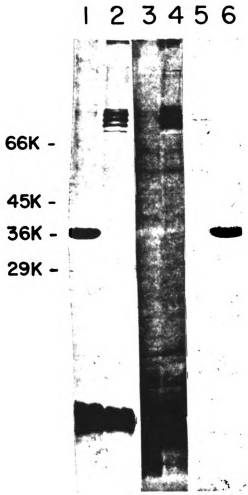

FIGURE 8

that the bands with the slower mobilities released more LPS to the dye front after heating (data not shown). The resulting monomer bands from all of the trimeric complexes travelled with identical mobilities which reflected their molecular weight of 36,000. Thus, it appears that the slower the mobility of the trimeric band, the higher the amount of LPS bound. When unheated porin from strain JF733 was separated in SDS-PAGE in the presence of 4 M urea, multiple bands were also resolved, although the resolution was not as good as in the absence of urea (data not shown). Interestingly, several of these bands reacted with the carbohydrate-specific silver stain. Because the anti-lipid A monoclonal antibodies have a lower affinity to Ra chemotype LPS compared with that of Re chemotype LPS, the Western blots of these bands were not analyzed.

DISCUSSION

The trimeric porin complex is thought to be stabilized by strong hydrogen bonding (38) and electrostatic and hydrophobic interactions (20). These same interactions are thought to stabilize the association of LPS with the porin. The combined strength of these interactions is thought to account for the unusual stability of the porin (7), and alterations in these interactions could modulate porin function (12). It has been reported that when LPS is removed from the complex, porin maintains its structure and activity (14,16). We report here that, using similar techniques, we were unable to completely remove LPS without denaturing the protein. Crude porin was readily separated from loosely bound LPS by electrophoresis (Figure 1). The trimer band detected in SDS-PAGE was, however, shown to contain LPS by two methods. First, a carbohydrate-specific silver stain was able to react with this band, but not with the heat-denatured monomer band. Second, when the trimer band was removed from the gel and heated, loosely bound LPS was released and could be separated from the porin band on a second SDS-PAGE. Unheated trimer, which was isolated from the gels, also released some LPS on a second separation on SDS-PAGE, but the trimer band still stained with the carbohydrate-specific silver stain (Figure 3). We propose that the LPS that is tightly bound to the trimer can be partially but not completely removed by repeated rounds of electrophoresis.

As has been reported by others (8), addition of urea to the gels does not appear to significantly increase the dissociation of LPS from trimers during electrophoresis. Trimeric porin complexes from either Ra or Re chemotype strains, which were

electroeluted from gels containing 4 M urea, retained LPS. This was confirmed by the carbohydrate-specific silver stain (Ra and Re chemotype) and by the detection of free LPS (Re chemotype) on Western blots following heat denaturation. Apparently, the presence of a complete core polysaccharide on LPS does not dramatically affect the affinity of LPS for the trimer.

The presence of multiple bands of trimers in the gels of porin from strain JF733 was striking. These bands presumably represent trimers which contain different amounts of LPS. The fastest migrating band did not stain with the carbohydrate-specific silver stain, and all of the protein-containing bands collapsed into a single monomer band when the protein was heated (Figure 8). Heating to 100°C in SDS apparently released all of the LPS that was accessible for silver staining. Perhaps the trimer from the Re chemotype strain did not show multiple bands because of the smaller size of the Re chemotype LPS. When the trimer from strain D21f2 was separated on longer gels, very closely spaced multiple bands were resolved (data not shown).

Thus, it appears that trimeric complexes of OmpC and OmpF porins retain variable amounts of LPS when isolated by electrophoresis. Some of the trimer-bound LPS can be removed by a second electrophoretic separation, and more is released by heat denaturing the trimer. However, none of the LPS bound to the trimers could be detected by using any of the anti-lipid A monoclonal antibodies. Presumably, the lipid A present in the nondenaturated trimeric complex either is inaccessible or the conformation of the lipid A in this complex is not recognized by these antibodies. Recently, Strittmatter and Galanos (8) have also reported LPS bound to porin complexes.

Heating of the trimers resulted in a breakdown of the complexes, releasing LPS and denatured monomers. The monomer traveled with an apparent molecular weight of 36,000 as reported by others (4,8,39-41). The monomers from our gels no longer reacted with the carbohydrate-specific silver stain, but could be detected by some anti-lipid A antibodies. Presumably, unfolding of the protein on heat denaturation exposed the lipid A for reaction to the antibodies. Several anti-lipid A monoclonal antibodies, in addition to 4A10, showed this binding on Western blots. This class of antibodies has a much weaker affinity for free LPS at the dye front and detected free LPS only at high concentrations. We stress that these anti-lipid A monoclonal antibodies did not bind to an epitope on the porin polypeptide. This was shown by thin-layer chromatographic separation of the LPS and porin. The polypeptide did not move from the origin on the plates; the antibodies only reacted with the LPS which migrated off the origin. These thin-layer chromatography results also show that LPS detected by the monoclonal antibodies is not covalently bound to the protein but can be removed by organic solvents. Interestingly, Strittmatter and Galanos (8) also were able to isolate LPS-free porin monomers by specific organic extraction methods.

The LPS bound to monomers did not react with the carbohydrate-specific silver stain, either because the amounts of LPS were too low to detect or the reaction groups in the LPS were masked or missing. Detection of LPS-specific staining with higher concentrations of monomeric protein would not be conclusive since proteins, such as the molecular weight markers in Figure 1, stained weakly in this protocol when at a high concentration. The groups in the LPS necessary for reacting with the silver stain are thought to be in the core polysaccharide since lipid A does not stain (23). Thus, in the

LPS from strain D21f2, the 2-keto-3-deoxyoctulosonic acid groups, on oxidation with periodate, induce silver precipitation.

Quantitive *Limulus* amebocyte lysate analysis of the electroeluted monomer showed that it contained nearly an equivalent molar ratio of LPS to porin. Thus, a detergent-resistant, high-affinity LPS binding site exists on monomers. This observation may also extend the significance of the *Limulus* amebocyte lysate test itself. The pharmaceutical industry routinely uses the *Limulus* amebocyte lysate test to detect endotoxin contamination in injectable reagents. Since the LPS associated with monomeric porin could not be removed under the harsh conditions described here, the *Limulus* amebocyte lysate test apparently can detect LPS that is tightly bound to proteins.

We conclude that isolated porin trimers contain at least two classes of LPS. One class, detected by the LPS-specific silver stain, can be removed, at least partially, by extensive electrophoresis. Removal of this loosely associated LPS may not result in a breakdown of the trimer complex since the fastest migrating trimer band from the Ra chemotype strain did not react with the carbohydrate-specific silver stain but still moved on gels as an aggregate. Presumably, all of the loosely associated LPS is removed by heat denaturing the protein in SDS and by separating the monomer from the LPS by electrophoresis. A second class of very tightly bound LPS is also present on porin. Heating and electrophoresis does not remove this LPS. We were able to remove at least some of this very tightly associated LPS by thin-layer chromatography in organic solvents.

Finally, in the original reports on the specificity of these anti-lipid A antibodies, it was observed that 1D4 not only had the highest average titer on a panel of heat killed

gram-negative bacteria (27) but is also had the greatest range of gram-negative bacteria with which it could bind (28). We are presently exploring whether the ability of an anti-lipid A antibody to bind to lipid A in a complex with major outer membrane proteins is relevant to its ability to bind to live bacteria.

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CHAPTER 3

Isolation and Preliminary Characterization of Wild-Type OmpC Porin Dimers from *Escherichia coli* K-12

ABSTRACT

Escherichia coli K-12 strain PLB3255 contains a mutation in the *ompF* gene that results in a 15 amino acid deletion in the porin protein. The mutation (*dex*) appears to increase the OmpF channel size, allowing the PLB3255 cells to grow on maltodextrins in the absence of a functional maltoporin. Porin isolated from strain PLB3255, which contains a wild type *ompC* gene, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and shown to contain 50-60% trimer aggregates and 35-40% of a 50-kDa "dimer." When the porin isolate was heated to 100°C and separated on SDS gels containing 8 M urea, both the trimer and the "dimer" were recovered in a single band at 36 kDa that corresponded in mobility to wild type OmpC porin. An analysis of the temperature stability of the isolate showed that the OmpC "dimer" was less stable and denatured at 66°C compared to 81°C for the trimer. To separate these aggregates, the unheated porin was suspended in 30% SDS, applied to a Sephadex G-200 gel filtration column, and eluted with 0.5% sodium deoxycholate. Two peaks were recovered containing separated trimers and "dimers." Circular dichroism spectra of isolated dimers and trimers indicated similar amounts of β-structure. The isolated dimers and trimers were reconstituted into artificial membranes. Electrical conductance across planar bilayer lipid membranes and liposome swelling assays showed that the two isolates had similar channel-forming activity. Four other *ompF* deletion mutants of the same phenotype were also shown to produce 50-kDa OmpC porin "dimers." In contrast, an *ompC* deletion mutant and an *ompF* point mutant, each of which produced a porin with an expanded channel, showed no evidence of "dimer" formation, indicating that dimers are not

involved in forming the expanded channel. These studies indicate that specific changes in the structure of OmpF porin can alter the assembly of the OmpC protein. Furthermore, we found that the "dimer" is a stable porin configuration, and this "dimer" may represent the smallest functional, channel-forming aggregate of the OmpC protein.

INTRODUCTION

The outer membrane of *Escherichia coli* K-12 contains two major channel-forming proteins, the OmpC and OmpF porins. These porins form water-filled diffusion channels with a size-exclusion limit of 600 Da. Electron microscopy, chemical cross-linking, and sedimentation equilibrium studies have shown that porins exist in the outer membrane as trimers of 36-kDa polypeptide subunits. Both porins are tightly bound to the peptidoglycan (1,2) and to lipopolysaccharide (LPS; 3,4), and they are unusually stable to a wide variety of perturbants including high temperature (1), SDS (5,6), and urea (6).

Previous studies have indicated that the trimer is the smallest functional channel-forming unit for all *E. coli* (5) and *Salmonella typhimurium* porins (7,8). Monomers obtained by denaturing the isolates show no channel-forming activity (5). However, monomers and dimers within trimeric complexes appear to be functional in planar bilayer lipid membrane studies when the protein subunit interactions are weakened by low pH and high voltage (9). Although many of the physical properties of porins are well characterized, very little is known about the assembly and export mechanisms involved in targeting these proteins to the outer membrane. The kinetics of OmpF porin synthesis, processing, and assembly have recently been described by Reid *et al.* (10). This study reported that a 50-kDa dimer was an intermediate in the assembly of OmpF trimers. Other studies have shown that overexpression of the OmpC porin results in an almost complete inhibition of transport of OmpA and LamB proteins to the outer membrane (11). Furthermore, a mutation within the *ompC* gene has been shown to affect the expression of many outer membrane proteins (12). The transport of these mutant or overexpressed

porins to the outer membrane appears to jam the export machinery, preventing the export of other outer membrane proteins.

Analysis of specific mutations which alter porin structure can give information of the assembly and transport of these proteins as well as define the regions of the protein critical for function. Recently, several *E. coli* K-12 *ompF* deletion and point mutations have been characterized which allow for cells to grow on maltodextrins in the absence of the maltoporin, which is normally required for uptake of these larger polymers (13). Since the OmpC and OmpF wild-type porin channels cannot accommodate these larger maltodextrins, the *dex* mutations in the *ompF* gene result in an increase in the OmpF pore size. In this study, we have analyzed the structure and function of porins isolated from these *ompF dex* mutant strains. We show that *E. coli ompF dex* deletion mutants containing a wild-type *ompC* gene produce stable, functional wild-type OmpC "dimers."

MATERIALS AND METHODS

Cell Growth and Porin Isolation:

The strains used in this study were PLB3252, PLB3255, PLB3256, OC200, OC208, OC209, and ECB5084 all derivatives of *E. coli* MC4100 and were the generous gift of S. Benson. The first three strains have been described previously (14). Strains OC200, OC208, OC209, and ECB5084 are derivatives of strain MCR106 lacking the *lamB* gene but containing a wild-type *ompC* gene and the mutant *ompF* gene of strains OC1500, OC1508, OC1509 and OC5104, respectively (13). Cells were grown in nutrient broth (1% tryptone, 0.2% yeast extract, and 0.4% NaCl) at 37°C and harvested in late logarithmic phase. Outer membrane porins were isolated by the method of Lakey *et al.* (15) and the final preparations were suspended in 10 mM Tris-HCl, pH 7.4, containing either 2% or 30% SDS.

Column Chromatography and Gel Electrophoresis:

Porin from strain PLB3255 was applied to a Sephadex G-200 gel filtration column (2.5 x 100 cm) and eluted either with 10 mM Tris-HCl, 0.5% SDS, 0.02% NaN_3, pH 8.0, or with 10 mM Tris-HCl, 0.5% NaDOC, 0.2 M NaCl, 0.02% NaN_3, 1 mM EDTA, pH 8.0. Protein absorbance of the eluted fractions was monitored at 280 nm. Fractions were analyzed by SDS-PAGE in a discontinuous buffer system (16). Separating gels contained 11% acrylamide and 0.1% SDS with or without 8 M urea. Molecular weight markers included bovine serum albumin (66K), egg albumin (45K), glyceraldehyde-3-P dehydrogenase (36K), carbonic anhydrase (29K), trypsinogen (24K), trypsin inhibitor

(20.1K), and α-lactalbumin (14.2K). Protein was detected either by a glutaraldehyde-silver-staining method (17) or by Coomassie Brilliant Blue staining.

Bilayer Lipid Membrane Analysis:

To prepare porins for BLM analysis the proteins were precipitated with 10 volumes of ice-cold 90% acetone (v/v). The protein was centrifuged at 7700 x *g* for 20 min at 4°C and washed twice with 90% acetone to remove excess detergent. The porin was then suspended in 1% octyl glucoside and diluted to the appropriate concentration (1-10 μg/mL) for BLM analysis. Membranes were formed by applying a solution of oxidized cholesterol and egg phosphatidylcholine (1:1, w/w) in *n*-decane to a hole in a Teflon partition. This partition separated two chambers containing an aqueous solution of 0.5 M NaCl, pH 5.7.The membranes thinned spontaneously until they formed an optically black bilayer. Silver-silver chloride electrodes (Microelectrodes Inc.) were placed in both sides of the chamber, and a constant voltage was applied across the membrane using a 1.5-V battery. A small amount of porin (20-60 ng/mL) in octyl glucoside was then added to one side of the membrane. Stepwise conductance fluctuations were monitored with a chart recorder using a Keithley model 614 electrometer for signal amplification.

Liposome Swelling Assay:

The liposome swelling assays were performed essentially as described previously (18). Briefly, approximately 6.2 μmol of acetone-extracted egg phosphatidylcholine and 0.2 μmol of dicetyl phosphate were dried onto the bottom of a test tube with a stream of

nitrogen. The film was suspended in 0.2 mL of solution containing 20 μg of purified porin suspended in 0.1% octyl glucoside or a solution of 0.1% octyl glucoside without porin for control experiments. The suspension was sonicated in a bath sonicator until the suspension became translucent. The suspension was then dried in a vacuum over $CaSO_4$ overnight. The film was suspended in 0.4 mL of a solution containing 12 mM stachyose, 4 mM sodium NAD, and 1 mM imidazole-NAD buffer, pH 6.0. The suspension was left at room temperature without mixing for 2 h and then shaken by hand. The liposome suspension was passed through a 8-μm nucleopore filter to remove large aggregates, and 40 μL of the suspension was diluted into test solutions. The rate of swelling at 25°C was monitored by measuring light scattering at 400 nm with a Gilford Response II spectrophotometer. The test solutions contained 1 mM Na-NAD, 1 mM imidazole-NAD buffer, pH 6.0 and one of the following sugars: D-glucose, D-mannose, D-arabinose, D-galactose, sucrose or lactose, at a concentration of 18 mM.

Circular Dichroism Spectroscopy:

Circular dichroism measurements were made using a JASCO J-500C spectropolarimeter with a 0.5-mm path-length quartz cell. Porin samples at 0.2 mg/mL were suspended in 1% SDS/10 mM sodium phosphate, pH 7.0, and scanned from 350 to 190 nm. The spectra were obtained by subtracting the buffer blank. All measurements were performed at room temperature.

Miscellaneous Chemical Assays:

Protein was quantitated using the BCA protein assay (Pierce Chemical Co.).

RESULTS

Isolation of PLB3255 Porin:

Porins were isolated from *E. coli* K-12 strain PLB3255, which produces a mutant OmpF protein (15 amino acid deletion, Δ114-129) and a wild-type OmpC protein (14). When the unheated samples were separated by SDS-PAGE and the gel scanned using a Bioimaging scanning densitometer, approximately 50-60% of the isolate migrated as a trimer (72 kDa) with a typical ladder pattern indicating the presence of tightly bound LPS. In addition, between 35 and 40% of the protein migrated as several closely spaced bands with apparent molecular weights of approximately 50 kDa. The remaining 10-15% migrated as proteins of between 35 kDa and 37 kDa molecular weight (Figure 1, lane 1). After the isolate was heated to 100°C, the trimers and the 50-kDa bands collapsed into a single band at 36 kDa (Figure 1, lane 2). To remove excess LPS, the unheated protein was passed through a Sephadex G-200 gel filtration column, eluted with 0.5% SDS, and concentrated in an Amicon PM-10 concentrator. The concentrated protein peak when run on an SDS gel showed a reduction in the number of bands in the trimer "ladder" as well as in the 50-kDa aggregate, indicating removal of LPS (Figure 1, lane 3). After heating the LPS-depleted sample to 100°C, both aggregates were again resolved as a single band at 36 kDa (Figure 1, lane 4).

Porin Isolation from *ompF*(*dex*) and *ompC*(*dex*) Deletion and Point Mutants:

To determine if other *E. coli dex* mutant strains produced this unique 50-kDa protein aggregate, a set of strains containing either a deletion or a point *dex* mutation in

Figure 1. Separation of porin from strain PLB3255 on a 12% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue. Lanes 1 and 3 contain porins kept at room temperature before applying to the gel. Samples in lanes 2 and 4 were heated for 3 min at 100°C before applying to the gel. Lanes 1 and 2 contain crude porin preparations before separating on a Sephadex G-200 gel filtration column. Lanes 3 and 4 contain LPS-depleted porin recovered after fractionation on a Sephadex G-200 column eluted with 0.5% SDS. Molecular weight markers are those described in the Materials and Methods.

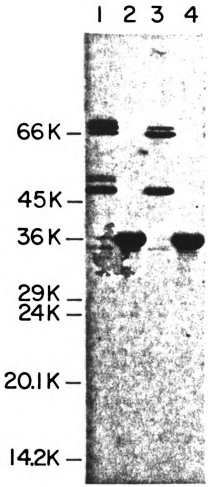

FIGURE 1

the *ompF* or *ompC* structural gene was examined (13,19). The strains, PLB3252, OC200, OC208 and OC209 contained different *ompF*(*dex*) deletion mutations. Strain PLB3256 contained an *ompC*(*dex*) deletion mutation, and strain ECB5084 contained an *ompF*(*dex*) point mutation. Porins were isolated from each of these strains and characterized by SDS-PAGE. The porin isolated from the five *ompF*(*dex*) deletion mutants all contained the 50-kDa aggregate (Figure 2A, lanes 5, 7-10). However, the *ompF* point mutant, ECB5084, did not produce the 50-kDa complex further indicating that dimers are not forming the expanded channel (Figure 2A, lanes 13, 14). In addition, strain PLB3256, carrying an *ompC*(*dex*) deletion mutation and the wild-type OmpF porin gene, did not produce a 50-kDa complex. These porin isolates were also heated and run on an 11% SDS gel containing 8 M urea. The major band detected in the isolates from all of the *ompF*(*dex*) deletion mutants migrated identical to wild-type OmpC porin (Figure 2B, lanes 4, 6-9). The mutant *ompF*(*dex*) gene product was not recovered in large amounts from any of these strains except OC209, (Figure 2B, lane 9). Porins from both the wild-type *ompF* and the *ompC*(*dex*) genes were recovered from strain PLB3256 in nearly equal amounts (Figure 2B, lane 5). Similarly, high amounts of the mutant *ompF* gene product were isolated from the peptidoglycan of the *ompF*(*dex*) point mutant ECB5084 (Figure 2B, lanes 12, 13). Since the 50-kDa OmpC aggregate was isolated in the highest amounts in strain PLB3255 with the smallest contamination by the mutant OmpF protein, further characterization of the OmpC dimer was performed using this strain.

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Figure 2. 11% SDS-polyacrylamide gels of crude porin isolates from several *E. coli* K-12 strains. (A) Separation of porins kept at room temperature before applying to the gel. Lane 1 contains the molecular weight markers. Lane 2 contains porin from strain D21f2, an Re LPS chemotype. Lanes 3 and 4 contain porin from JF733, which only produces the OmpF porin. Lanes 5, 11, and 12 contain porin from strain PLB3255. Lane 6 contains porin from strain PLB3256. Lane 7 contains strain PLB3252 porin; lane 8, OC200 porin; lane 9, OC208 porin; lane 10, OC209 porin; lanes 13 and 14 contain porin from strain ECB5084. (B) Separation of crude porin isolates on an 8 M urea gel after the porin preparations were heated to 100°C for 3 min. Lane 1 contains porin from strain D21f2. Lanes 2 and 3 contain strain JF733 porin; lanes 4, 10, and 11, PLB3255 porin; lane 5, PLB3256 porin; lane 6, PLB3252 porin; lane 7, OC200 porin; lane 8, OC208 porin; lane 9, OC209 porin; and lanes 12 and 13 contain strain ECB5084 porin. Only the relevant portion of the gel is shown. Both gels were stained with Coomassie Brilliant Blue.

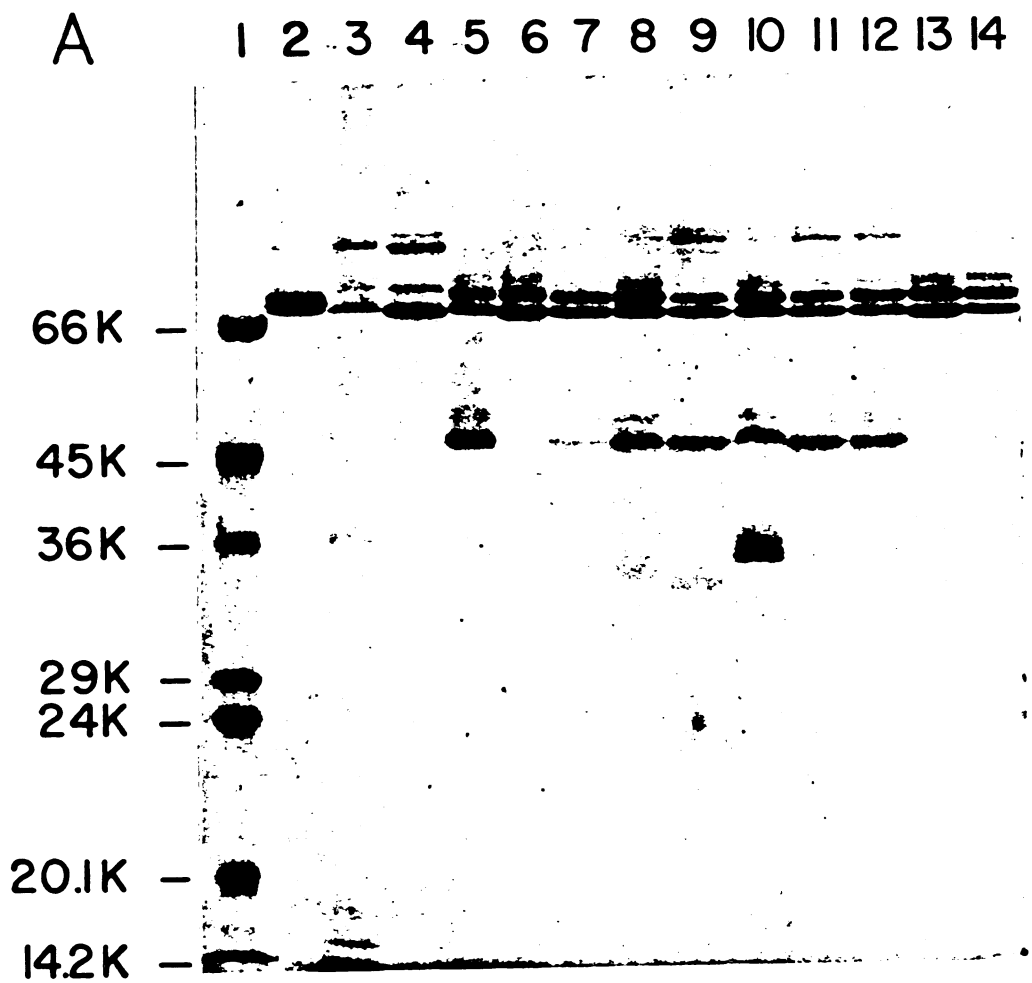
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
66K
45K
36K
29K
24K
20.1K
14.2K
B
1 2 3 4 5 6 7 8 9 10 11 12 13
omp C
omp F

FIGURE 2

Temperature Stability of the 50-kDa Dimers:

LPS-depleted porin, following Sephadex G-200 purification of the PLB3255 isolate, was used to determine the heat stability of the trimer and the 50-kDa dimer. The results showed that the 50-kDa complex, when heated in SDS, is less stable than the trimer, and denatures between 60-66°C (Figure 3, lanes 7-9). The heat stability of the OmpC trimer in SDS is slightly higher than that of wild-type OmpF trimer of *E. coli* (data not shown) and denatured only above 78°C (Figure 3, lanes 13, 14). In addition, removal of excess LPS from the OmpC dimers and trimers did not affect their heat stabilities (data not shown). A 50-kDa OmpF dimer, shown to be an intermediate in the assembly of wild-type trimers, has also been reported to denature at lower temperatures, i.e., between 45-58°C (10).

Dimer Purification by Gel Filtration Chromatography in Sodium Deoxycholate:

Dimer and trimer aggregates of OmpC porin, isolated from strain PLB3255, were solubilized in 30% SDS, applied to a Sephadex G-200 column, and eluted with 0.5% sodium deoxycholate. This column removed large porin aggregates and excess LPS. The majority of the protein was recovered in two distinct peaks, corresponding to the OmpC trimer and OmpC dimer (data not shown). When the column fractions were run on SDS-PAGE the leading edge of the first peak contained purified OmpC trimers, and the trailing edge of the second peak contained purified dimers (Figure 4). These results further indicate that the dimer is a smaller complex of the OmpC porin than the trimer isolate. That the 50-kDa bands migrate in SDS gels midway between the trimer and the monomer also suggests that the protein is a dimer of porin.

Figure 3. Temperature stability of LPS-depleted porin isolated from strain PLB3255. The porin was heated at different temperatures for 5 min and then applied to a 12% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. Lane 1 contains the molecular weight markers. Lane 2 contains sample heated at 25°C, lane 3 at 37°C, lane 4 at 45°C, lane 5 at 50°C, lane 6 at 55°C, lane 7 at 60°C, lane 8 at 63°C, lane 9 at 66°C, lane 10 at 69°C, lane 11 at 72°C, lane 12 at 75°C, lane 13 at 78°C, lane 14 at 81°C, lane 15 at 85°C, and lane 16 at 100°C.

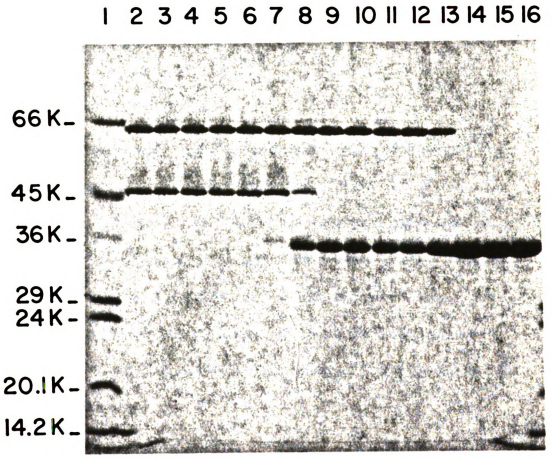

FIGURE 3

Figure 4. A 12% SDS-polyacrylamide gel of fractions of PLB3255 porin eluted from a Sephadex G-200 column with 0.5% sodium deoxycholate/10 mM Tris pH 8.0. The samples in lanes 2, 4, 6, 8, 10, and 12 were left at room temperature before application to the gel. Samples in lanes 1, 3, 5, 7, 9, 11, and 13 were heated to 100°C for 3 min before applying to the gel. Lanes 2 and 3 contain column fraction 43, lanes 4 and 5 contain fraction 45, lanes 6 and 7 contain 47, lanes 8 and 9 contain fraction 49, lanes 10 and 11 contain fraction 50, and lanes 12 and 13 contain fraction 51. The gel was stained with Coomassie Brilliant Blue.

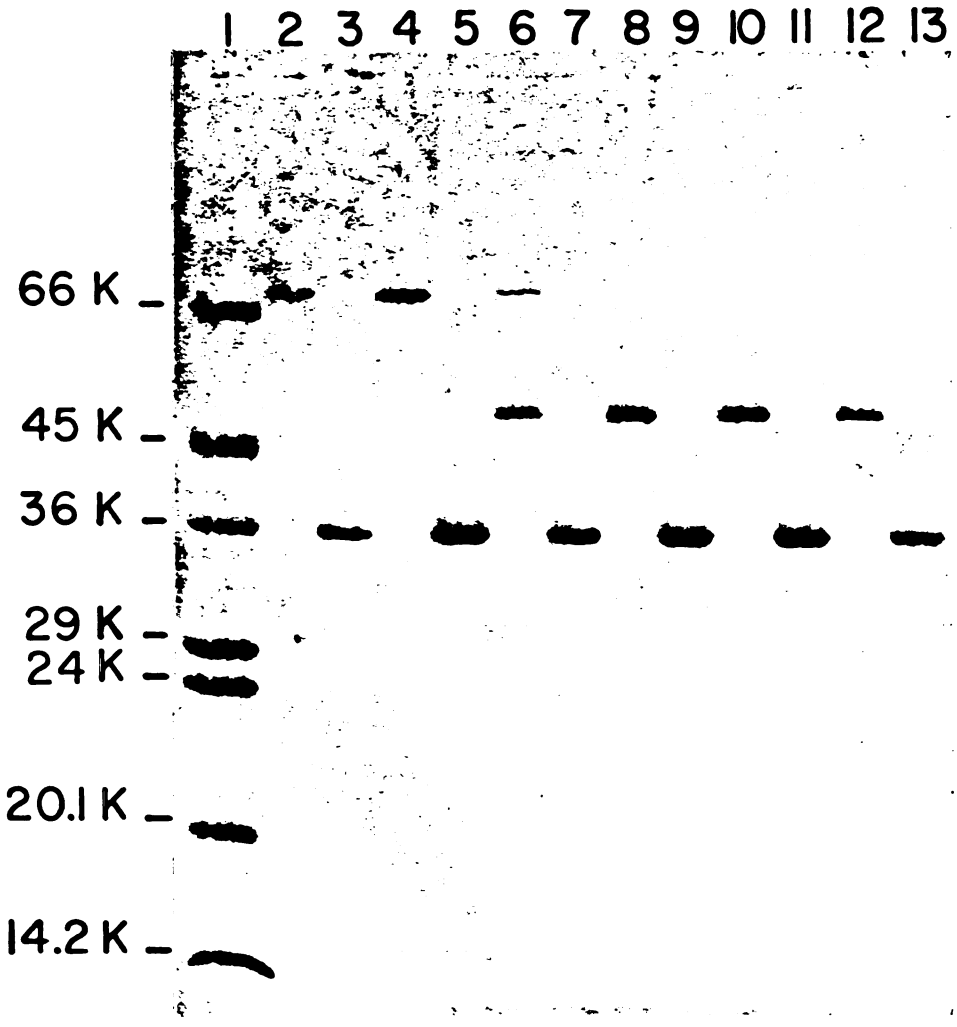

FIGURE 4

Circular Dichroism Spectra of Dimers and Trimers:

The secondary structure of the OmpC dimer and trimer isolates was determined by measuring their circular dichroism spectra. The spectra of the two isolates were similar in shape, and both exhibited a minimum ellipicity at 216 nm with a crossover of 204 nm for the trimer and 205 nm for the dimer (Figure 5). The spectra indicate that both dimers and trimers contain a large amount of β-structure similar to what has been reported by others (1,20). The spectrum of the trimer sample, after heating to 100°C for 2 min, showed a loss of β-structure and an increase in α-helix and random coil.

Assays of Porin Activity:

To measure the channel-forming activity, the isolated porin complexes were reconstituted into two different artificial membrane systems, liposomes and planar BLM's. The liposome swelling assay that was used is outlined under Materials and Methods (18). The rate of liposome swelling was followed spectrophotometrically for 3 min for monosaccharides and between 20 and 180 min for disaccharides. Solutes unable to penetrate the porin channels caused no change in the osmotic equilibrium, and no swelling was observed. This was also true for control liposomes without porin and for liposomes containing non-porin proteins. It is assumed that the initial rate of uptake of solutes into the liposomes, i.e. the initial rates of optical density decrease, reflects the solute penetration rate into the outermost layer of the liposome (18). The results in Table I show that both the dimer and the trimer allowed all solutes tested to enter the liposome; not surprisingly, the larger disaccharides, sucrose and lactose, diffused at a much slower rate than did the monosaccharides. In addition, the relative diffusion rates and

Figure 5. Circular dichroism spectra of OmpC dimers (---), trimers (——) and monomers obtained by heating OmpC trimers (●–●). Samples at 0.2 mg/mL were suspended in 10 mM sodium phosphate, pH 7.0, containing 1% SDS.

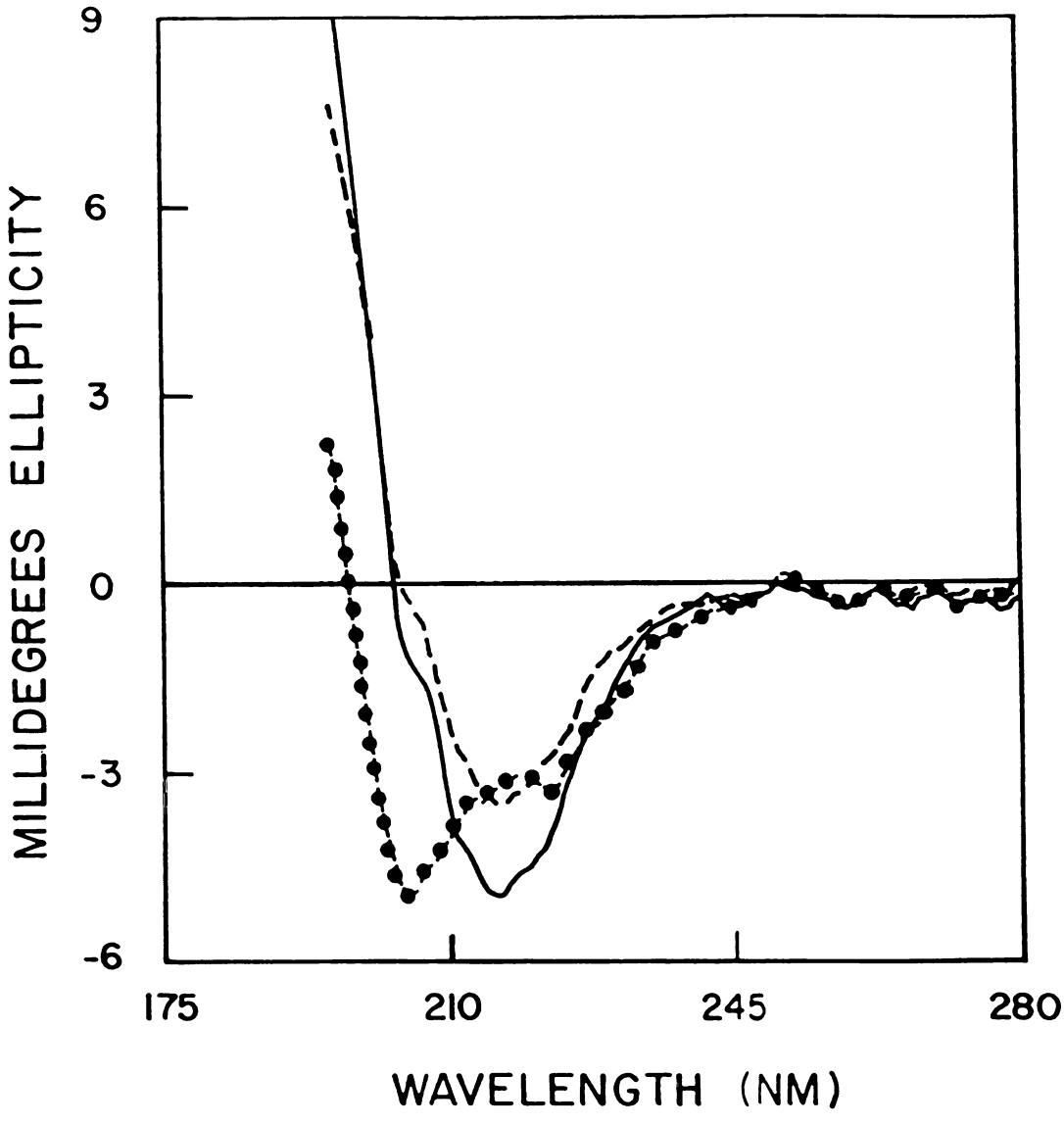
9
6
3
0
-3
-6
MILLIDEGREES ELLIPTICITY
175
210
245
280
WAVELENGTH (NM)

FIGURE 5

TABLE I. Diffusion Rates of Saccharides through Porin Channels[a]

Solutes	Mol. Wt.	Initial Swelling Rates of Liposomes Containing[b]	
		PLB3255 Dimer	PLB3255 Trimer
D-arabinose	150	0.96	0.97
D-mannose	180	1.0	1.0
D-galactose	180	0.88	0.88
D-glucose	180	0.90	0.94
lactose	342	0.08	0.11
sucrose	342	0.04	0.03

[a]In each experiment 20 μg of protein was added to liposomes as outlined under Materials and Methods.

[b]The swelling rates were normalized to the fastest permeating sugar, D-mannose.

size-exclusion limits of the isolated dimers and the trimers were similar; therefore, the two aggregates appear to have a similar channel size. SDS-solubilized liposomes containing either the OmpC dimers or the trimers were applied to SDS-polyacrylamide gels, and their mobility was measured to determine whether the dimers (and trimers) maintained their aggregate nature in the liposomes used in the swelling assay. The results in Figure 6 show each of the porins tested maintained their aggregate nature when placed into phospholipid liposomes (lanes 3 and 4). The slight contamination of trimer in the dimer-containing liposomes (lane 4) was present in the dimer isolate prior to liposome incorporation and does not appear to be significant.

The results from the BLM analysis of dimer and trimer isolates also indicated that both porin samples had channel-forming activity. Stepwise current increases and decreases across the bilayer membranes in the presence of OmpC dimers and trimers were analyzed at a membrane potential of 25 mV. The conductance change across the membrane, Λ, was measured and divided by the specific conductance of the 0.5 M NaCl bathing solution, σ. In this study, the conductance changes are reported as a size parameter, Λ/σ, in angstroms, plotted as a function of the probability, P, of a channel of a given size. The size distribution histogram was then derived for each porin isolate (Figure 7). The results indicate that the channel sizes were similar for the two porins; 134 events were observed for the OmpC trimer and 152 events for the OmpC dimer. The average specific conductance for the trimer was 1.78 ± 0.73 Å whereas for the dimer, this measurement was 1.43 ± 0.57 Å.

Figure 6. A 12% SDS-polyacrylamide gel of isolated OmpC dimers and trimers from strain PLB3255 before and after addition to liposomes. Lanes 1 and 3 contain porin trimers. Lanes 2 and 4 contain OmpC dimers. Lanes 1 and 2 contain porins that were used to form liposomes. Lanes 3 and 4 contain the porins recovered from liposomes, by solubilizaton in 4% SDS prior to electrophoresis. The gels were silver stained as described in the Materials and Methods.

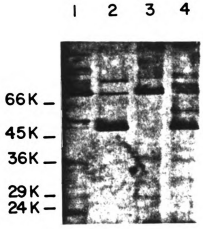

FIGURE 6

Figure 7. Distribution of the size parameter, Λ/σ, (Å), for porin trimer (A) and dimer (B) isolates of strain PLB3255 measured in BLM's. The porin at 50 ng/mL was added to the bathing solution. Electrical conductance across the membrane was measured by using a transmembrane potential of 25 mV. Λ is the conductance change and σ is the specific conductance of the bathing solution. *P*, in arbitrary units is the relative number of events with the given size parameter values. Purified trimer and dimer were isolated from a Sephadex G-200 gel filtration column eluted with 0.5% NaDOC. The isolates were resuspended in 0.1% octyl glucoside after acetone precipitation. Both opening and closing events are included in the histogram.

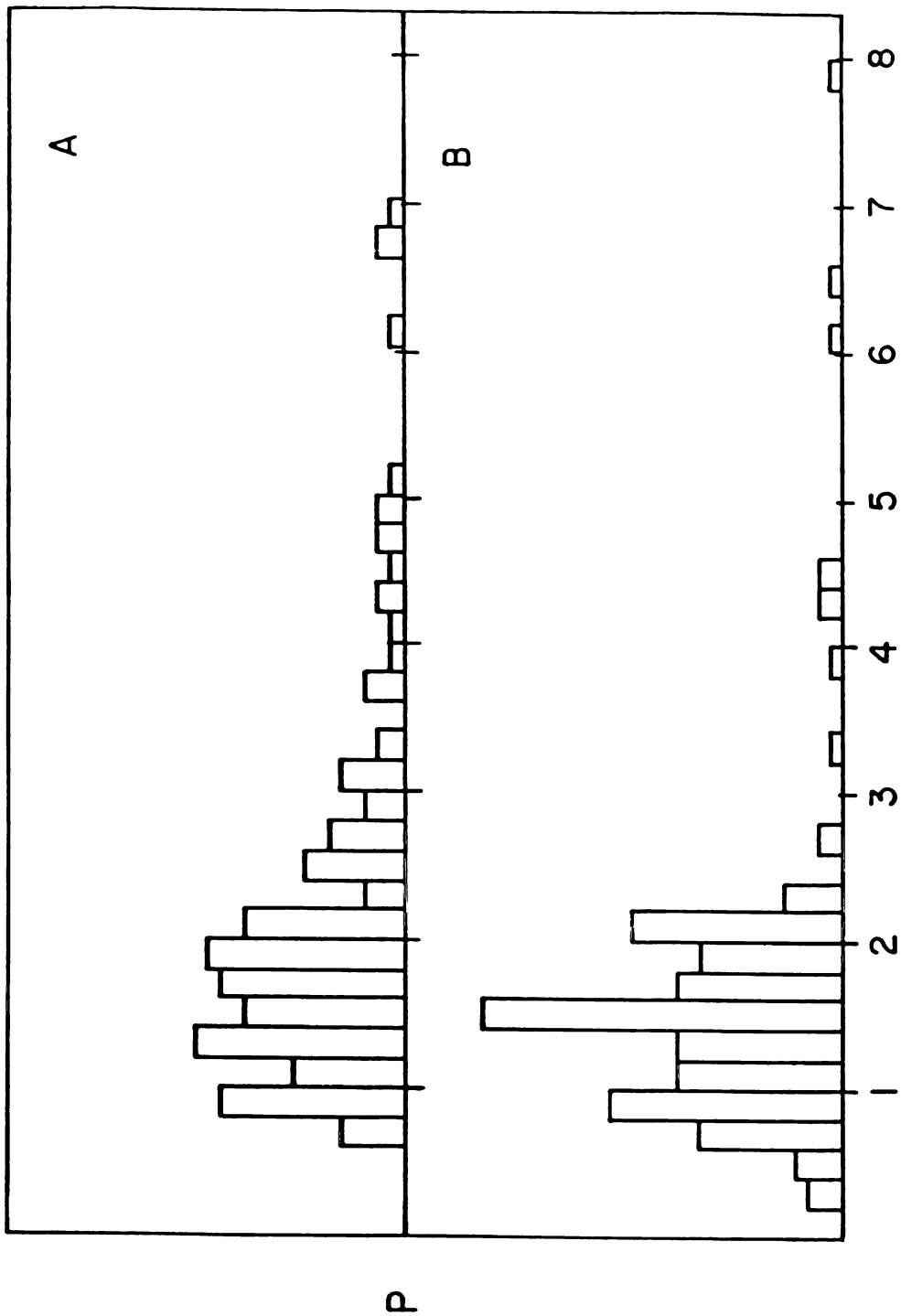

FIGURE 7

DISCUSSION

E. coli K-12 strain PLB3255 can grow on maltodextrins in the absence of a LamB porin due to a mutation (*dex*) that was mapped to the *ompF* gene (14). The mutation was shown to result in an OmpF protein with a deletion of 15 amino acid residues (13). Supposedly, the deletion increases the size of the porin channel. Presumably, the PLB3255 strain can grow on maltodextrins because the mutant OmpF(dex) porin is functional in the outer membrane. However, the OmpF(dex) porin appears unable to form stable trimers and may be functional either as unstable trimers or as monomers. When we set out to examine this OmpF(dex) porin from strain PLB3255, we found that the mutant OmpF porin did not associate tightly with the peptidoglycan layer and could not be recovered in large amounts using standard protocols. The 15 amino acid deletion in the polypeptide chain appears to alter the peptidoglycan-OmpF porin interactions. In addition, we observed a typical 72-kDa trimer and an unusual 50-kDa "dimer" in the unheated porin isolates of this strain which travelled as a 36-kDa polypeptide when heated to 100°C. Careful analysis of the 50-kDa protein indicated that it was an aggregate of the wild-type OmpC porin.

Surprisingly, we found that all of the *ompF*(*dex*) deletion mutant strains produced the 50-kDa aggregate. Furthermore, the larger the deletion in the OmpF protein, the lower the amount of mutant OmpF porin that was recovered on the peptidoglycan and the greater the level of OmpC "dimer" that was isolated. All of these deletions changed the overall charge of the mutant OmpF porin due to the loss of one or more aspartic acid residues. Perhaps this alteration in charge modified the OmpF conformation and

interaction with components such as the peptidoglycan or other porin subunits, in turn affecting the assembly of other outer membrane proteins. The *ompF* point mutant, ECB5084, which also produced a larger pore, contained no detectable dimers, and while this mutant porin lost a negatively charged aspartic acid residue, which was substituted by glycine, this mutant porin bound tightly to peptidoglycan. Thus, loss of this single negatively charged amino acid does not appear to be critical in the assembly of the trimer or binding of the porin to peptidoglycan. It is known that the electrochemical potential of the inner membrane, the signal sequence of the secreted protein, and the three-dimensional protein configuration are important factors in determining the final destination of outer membrane proteins. Altering one or more of these essential determinants could jam the assembly and export of porin. Mutant OmpF(dex) porin, containing the same 15 amino acid deletion as the OmpF porin in PLB3255, produced by a strain lacking an *ompC* gene does not form stable trimers or dimers in the presence of SDS (data not shown). This mutant OmpF porin also does not bind well to the peptidoglycan. This mutation in the *ompF* gene in the PLB3255 strain could be affecting the aggregation of the wild-type OmpC porin by jamming porin assembly, resulting in the accumlation of dimer intermediates which are exported to the outer membrane. Surprisingly the *ompC*(*dex*) mutation in strain PLB3256 did not alter the OmpF porin assembly. This suggests that the OmpC and OmpF porins may have different assembly/export pathways. Either the OmpC(dex) porin adopts a different conformation than the analogous OmpF(dex) porins or they are recognized differently by assembly/transport systems.

Another explanation for the production of OmpC dimers in *ompF*(*dex*) deletion mutants could be that the dimer is a result of the breakdown of a heterotrimer consisting

of two wild-type OmpC subunits and a single mutant OmpF polypeptide. Heterotrimers have been shown to exist in cross-linking studies between OmpC and OmpF monomers (21). Because of the altered structure of the OmpF porin, the heterotrimer might readily break down to OmpF monomer and a stable OmpC dimer. At this time we have no strong evidence to support either model for OmpC dimer formation.

In our initial characterization of the OmpC dimer, we found that this aggregate is a stable porin complex that is tightly associated with the peptidoglycan. Its secondary structure appears to be very similar to that of the trimer. The OmpC dimer is not the component which confers on the PLB3255 cells the ability to grow on maltodextrin or dye and detergent sensitivity since the mutant *ompF*(*dex*) strains lacking an *ompC* gene have the ability to grow on maltodextrins and have similar dye and detergent sensitivities. Upon analyzing the functioning of the 50-kDa aggregate, we found that the dimer has a channel size similar to that of the wild-type OmpC trimer. It is not known if the dimer is functional on the outer surface of the intact cell. Yet, the PLB3255 cells grow at a rate similar to wild-type *E. coli* K-12 cells. So perhaps the dimers, comprising 35-40% of the total ompC porin, are active in forming channels in intact cells.

The OmpC dimer when reconstituted into liposomes and BLM's appeared to have channel properties similar to that of the trimer, but there are some differences between the two isolates. One important difference, other than their mobilities on SDS-PAGE, may be the amount and affinity of LPS bound to each. Crude wild-type OmpF porin from *E. coli* K-12 strain JF733 forms a typical ladder pattern on SDS-PAGE, indicating the presence of different amounts of bound LPS (4). Most of this LPS can be removed by solubilizing the porin in high detergent concentrations, e.g., 30% (w/v) SDS.

Preliminary evidence suggests that the 50-kDa dimer either binds less LPS than trimer, since fewer bands were seen, or binds LPS less tightly, since the multiple bands of the dimer ladder pattern disappeared more readily during the purification on Sephadex G-200. LPS bound to porin may have several critical roles including stabilization of porin structure, regulation of porin activity (22,23) and contribution to the structure necessary for bacteriophage recognition (24,25). We have found that removal of excess LPS from porin dimers and trimers does not affect their heat stabilities. However, little is known about LPS interactions which may be necessary for porin assembly and export to the outer membrane. Perhaps LPS is essential for the porins to be properly folded and transported to the outer membrane. It has been suggested that LPS influences the rate of oligomerization and porin insertion into the outer membrane (10).

Previously, the trimer was thought to be the smallest functional unit of *E. coli* porins (5). Using chemical cross-linking, porin protein F from *Pseudomonas aeruginosa* has been shown to exist as a trimer in the outer membrane (26), but upon purification this aggregate breaks down into monomers that are still functionally active in planar bilayer membranes (27) and liposomes (28). In addition, porin from *Rhodopseudomonas sphaeroides* exists as a functionally active oligomer of 67-kDa, but upon addition of EDTA or heat, the porin breaks down to functionally active "monomers" of 47 kDa which retain their secondary structure (29). Unlike the porins from *Pseudomonas* and *Rhodopseudomonas*, isolated porin monomers of *E. coli* are not functional. However, within trimeric complexes, each monomer appears to contain a diffusion channel (9).

In conclusion, the results presented here show that the strains carrying *ompF*(*dex*) deletion mutations and a *ompC*(wt) gene are altered not only in the structure of the OmpF protein but also in the assembly of OmpC subunits. These strains appear to produce OmpC dimers which accumulate and are transported to the outer membrane. These dimers are stable and exhibit channel-forming activity in reconstituted membrane systems. We are presently attempting to determine if these *ompF*(*dex*) deletion mutations can also alter the assembly of other outer membrane porin proteins.

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CHAPTER 4

Structure and Function of an OmpC Deletion Mutant Porin from *Escherichia coli* K-12

ABSTRACT

Escherichia coli K-12 strain RAM122 contains a mutation in the *ompC* gene that results in an eight amino acid deletion, Δ103-110, in the porin protein. Since this strain is capable of growing on maltodextrins in the absence of a functional *lamB* gene, the mutant protein is thought to have a larger channel size. The stability and structure/function properties of the mutant OmpC porin were investigated and compared to wild type porin. Isolated unheated RAM122 porin was characterized as a trimer on sodium dodecyl sulfate polyacrylamide gels. The RAM122 trimer was less stable to both temperature and low pH when compared to the wild type porin. In addition, the overall enthalpy for thermal denaturation was lower for the mutant than the wild type porin as determined using differential scanning microcalorimetry. Both the proteins' secondary structure monitored by circular dichroism, was high in β sheet content, but the spectra were slightly different in both their crossover points as well as their minima. When the proteins were reconstituted and channel activity assayed using a liposome swelling technique, the size exclusion limit of the mutant porin was twice that of the wild type porin. Conductance measurements across bilayer lipid membranes showed that the mutant porin was voltage gated at much lower membrane potentials, 50 and 75 millivolts, than the wild type sample. The closing events of the mutant porin were predominantly of monomer size. The channels detected using the mutant protein were larger in size than those measured for the wild type porin monomer. These data suggest that the OmpC mutant porin has a channel size capable of allowing maltodextrins to enter and that this channel is highly voltage regulated.

INTRODUCTION

The outer membrane of *Escherichia coli* K-12 contains two general diffusion porins, OmpC and OmpF, as well as selective porins, such as LamB and PhoE specific for maltodextrin and phosphate transport respectively. The structure and function of wild type *E. coli* porins have been extensively studied with little recent progress in understanding the regulation of porin activity or the assembly/export process. Isolation of mutations in the structural genes for porin which alter structural and functional properties has allowed investigators to examine regions of the protein critical for channel activity. Dargent *et al.* (1) have reported that specific point mutations in *lamB* alter transport properties of the encoded protein whereas other mutations have no effect on transport. In addition, several *E. coli* K-12 deletion and point mutations in *ompF* and *ompC* have been described which appear to alter pore properties (2-5). One such OmpF deletion mutant, PLB3255, produces stable, functional, wild-type OmpC porin dimers (6).

In an attempt to analyze the regulation of porin activity, *in vitro* reconstitution techniques have been used. It has been shown that porins exist in at least two different conformations, an open and a closed state. Voltage-dependent regulation of porin channel opening and closing *in vitro* has been reported (7-10); however, other investigators have indicated that voltage gating of porins does not occur (11-13). Dargent *et al.* (10) have reported that the PhoE protein trimers contain three pores which fluctuate between open and closed states and increasing membrane potential shifts the equilibrium to the closed state. In addition, Xu *et al.* (9) suggested that at low pH and high transmembrane

potentials the protein monomers of the OmpF trimeric unit are less tightly associated and open and close as independent subunits.

Recently, Misra and Benson (3,4) have isolated several strains with *ompC* deletion and point mutations that are capable of growing on maltodextrins in the absence of a functional *lamB* gene. Since the wild type OmpC pore is too small to accomodate large maltodextrins Misra and Benson (4) have proposed that the altered OmpC has a larger channel diameter. We have extensively characterized the porin from the deletion mutant, RAM122, and shown that this porin is less stable than the wild type OmpC trimer to acid and heat and that it contains a channel that is twice as large as the wild type porin channel.

MATERIALS AND METHODS

Cell Growth and Porin Purification:

The strains used in this study were PLB3255 (2) and RAM122 (3) both lacking the *lamB* gene. PLB3255 produces wild type OmpC porin and an OmpF porin with a short deletion, Δ115-129; RAM 122 lacks OmpF porin and produces an OmpC which contains an eight amino acid deletion, Δ103-110. The cells were grown in 1% tryptone, 2.4% yeast extract, 4% glycerol, 0.1 M potassium phosphate pH 7.0 at 37°C and harvested in late logarithmic phase. Porins were isolated by the method of Lakey *et al.* (1985) with slight modifications (6). The final preparation was suspended in either 2% or 30% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl pH 7.4. The protein was then applied to a Sephadex G-200 gel filtration column (2.5 x 100 cm) and eluted with 0.5% sodium deoxycholate, 10 mM Tris-HCl, 0.2 M NaCl, 0.02% sodium azide and 1 mM EDTA pH 9.0. Protein absorbance of the column fractionations was monitored at 280 nm.

Gel Electrophoresis:

SDS-PAGE was performed using the buffer system of Laemmli (14). Separating gels contained 12% acrylamide and 0.1% SDS. Molecular weight markers included bovine serum albumin (66K), egg albumin (45K), glyceraldehyde 3-phosphate dehydrogenase (29K), trypsinogen (24K), trypsin inhibitor (20.1K), and α-lactalbumin (14.2K). Protein was detected by Coomassie Brilliant Blue staining. LPS was monitored on gels after periodate oxidation and silver staining according to the method of Dubray and Bezard (15).

Temperature denaturation studies were performed by heating the proteins in 1% SDS, 10 mM Tris-HCl, pH 6.8 for five minutes at different temperatures prior to running the gel. The pH stability was determined by incubating unbuffered porins in 50 mM sodium citrate pH 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, or 6.8 for 20 minutes prior to neutralization with 0.1 N NaOH. The proteins were then loaded onto 12% SDS gels and examined using Coomassie Brilliant Blue. In all pH denaturation experiments the protein concentration was minimized to prevent porin buffering effects.

Differential Scanning Microcalorimetry:

Proteins were prepared for microcalorimetry by precipitating porin with cold 90% acetone (v/v) and washing the samples two times with 90% acetone. The proteins were resuspended in 10 mM Tris-HCl, 1% SDS, 0.02% sodium azide pH 6.8 and spun at 100,000 x g for thirty minutes to remove contamination. Calorimetric analysis was performed on a Microcal 2 scanning microcalorimeter (Microcal Inc., Amherst, MA) at a scanning rate of 90° per hour. Proteins were scanned from room temperature to 100°C using the resuspension buffer as a reference. Thermodynamic characterization and deconvolution curve fitting was performed using the Deconv. program (Microcal Inc.).

Circular Dichroism Spectroscopy:

Circular dichroism measurements were made using a JASCO J-500C spectro-polarimeter with a 0.5 mm path length quartz cell. Porin samples were suspended at 0.2 mg/ml in 1% SDS, 10 mM sodium phosphate, pH 7.0 and scanned from 350 nm to 190 nm. All measurements were performed at room temperature and spectra were obtained

by subtracting a buffer blank. Secondary structure analysis was performed according to Compton and Johnson (16) using the X-ray structure matrix given by Manavalan and Johnson (17).

Liposome Swelling Assays:

The liposome swelling assay was performed as previously described (6) with slight modifications. The internal liposome solution of 12 mM stachyose, 4 mM sodium NAD, 1 mM imidazole-NAD buffer pH 6.0 was replaced with 17% dextran (w/v), molecular weight 9,400, in 5 mM Tris-HCl pH 7.5 to accomodate the large RAM122 porin channel. The test solutes were suspended in 5 mM Tris-HCl pH 7.5 and included either D-glucose, D-mannose, D-arabinose, D-galactose, sucrose, lactose, maltotriose, or stachyose at concentrations of between 75 and 90 mM. The rate of swelling at 25°C was monitored with a Gilford Response II spectrophotometer by measuring light scattering at 400 nm in a 1 cm cuvette. The molecular weight cutoff of porin channels was determined using the procedure of Yoshihara *et al.* (18).

BLM Analysis:

Porins were prepared for BLM analysis as previously described (6). Briefly, porins were suspended in 1% octyl glucoside or 1% SDS, 10 mM Tris-HCl pH 7.5 and diluted to 0.1-1.0 mg/ml before adding to the solutions used in the BLM measurements. Membranes were formed by applying a 1% solution of diphytanoyl phosphatidylcholine (w/v) in n-decane to a hole in a teflon partition. Silver-silver chloride electrodes were placed in both sides of the chamber, bathed in 0.5 M NaCl adjusted to defined pH values.

A constant voltage of 25, 50 or 75 millivolts (mV) was applied across the membrane using a 1.5 volt battery. Stepwise conductance fluctuations were monitored with a chart recorder using a Keithley model 614 electrometer for signal amplification. The opening and closing events were monitored and reported as the size parameter, Λ/σ, in Å, vs. the probability, P, of the occurrence of an event with a particular size conductance. The bilayer measurements were ohmic over the entire range used for the experiments. In studies using LPS-enriched porins, samples were suspended in 1% SDS instead of 1% octyl glucoside to facilitate protein incorporation into the membranes. However, the SDS had a destabilizing effect on the porins in the membranes so all comparisons between the wild type and mutant porins are reported using LPS-depleted porins suspended in 1% octyl glucoside.

Miscellaneous Chemical Assays:

Protein was quantitated using the BCA protein assay (Pierce Chemical Co.). Lipopolysaccharide was quantitated by either KDO analysis using the thiobarbaturic acid assay (19) or by phosphate analysis according to Ames and Dubin (20).

RESULTS

Isolation of RAM122 porin:

Porin was isolated from *E. coli* K-12 strain RAM122, which produces a mutant OmpC protein with an eight amino acid deletion (Δ103-110) and lacks the OmpF protein. The porin was solubilized in 2% SDS and passed over a Sephadex G-200 gel filtration column, eluted with 0.5% SDS, 10 mM Tris-HCl, 10% glycerol pH 8.0. When the sample was run on SDS-PAGE the protein migrated as a trimer with a ladder pattern, indicating the presence of tightly associated LPS (21; Figure 1, lane 2). However, when the protein was solubilized in 30% SDS and passed over the same column only a single band was observed on SDS-PAGE indicating the removal of most of the LPS (Figure 1, lane 13). Similar results were obtained with the wild type OmpC trimer (6). KDO and phosphate analyses on LPS-depleted porins showed that there was less than one molecule of LPS per molecule of trimer in both the wild type and RAM122 porins after solubilizing the proteins in 30% SDS, whereas, LPS-enriched porins contained at least three molecules of LPS per porin trimer. In addition, LPS-depleted samples, when electrophoresed and silver stained for LPS (21) showed no free LPS.

Temperature and pH Stability of the Porin:

The temperature stability of the mutant and wild type porins was examined using two different methods, SDS-PAGE and differential scanning microcalorimetry. Unheated OmpC porins retain their trimeric structure in SDS-PAGE (22); therefore, protein denaturation can be monitored by heating the porin to different temperatures and

Figure 1. A 12% acrylamide SDS gel depicting the thermal stability of RAM122 porin. The porin was heated for five minutes at each temperature and then cooled to room temperature before loading onto the gel. Lane 1 contains the molecular weight markers. Lanes 2-12 contained LPS-enriched porin and lanes 13-23, LPS-depleted porin. Lanes 2,13, were treated at 25°C; Lanes 3,14, at 57°C; Lanes 4,15, 60°C; Lanes 5,16, 63°C; Lanes 6,17, 66°C; Lanes 7,18, 69°C; Lanes 8,19, 73°C; Lanes 9,20, 76°C; Lanes 10,21, 79°C; Lanes 11,22, 82°C; Lanes 12,23, 100°C.

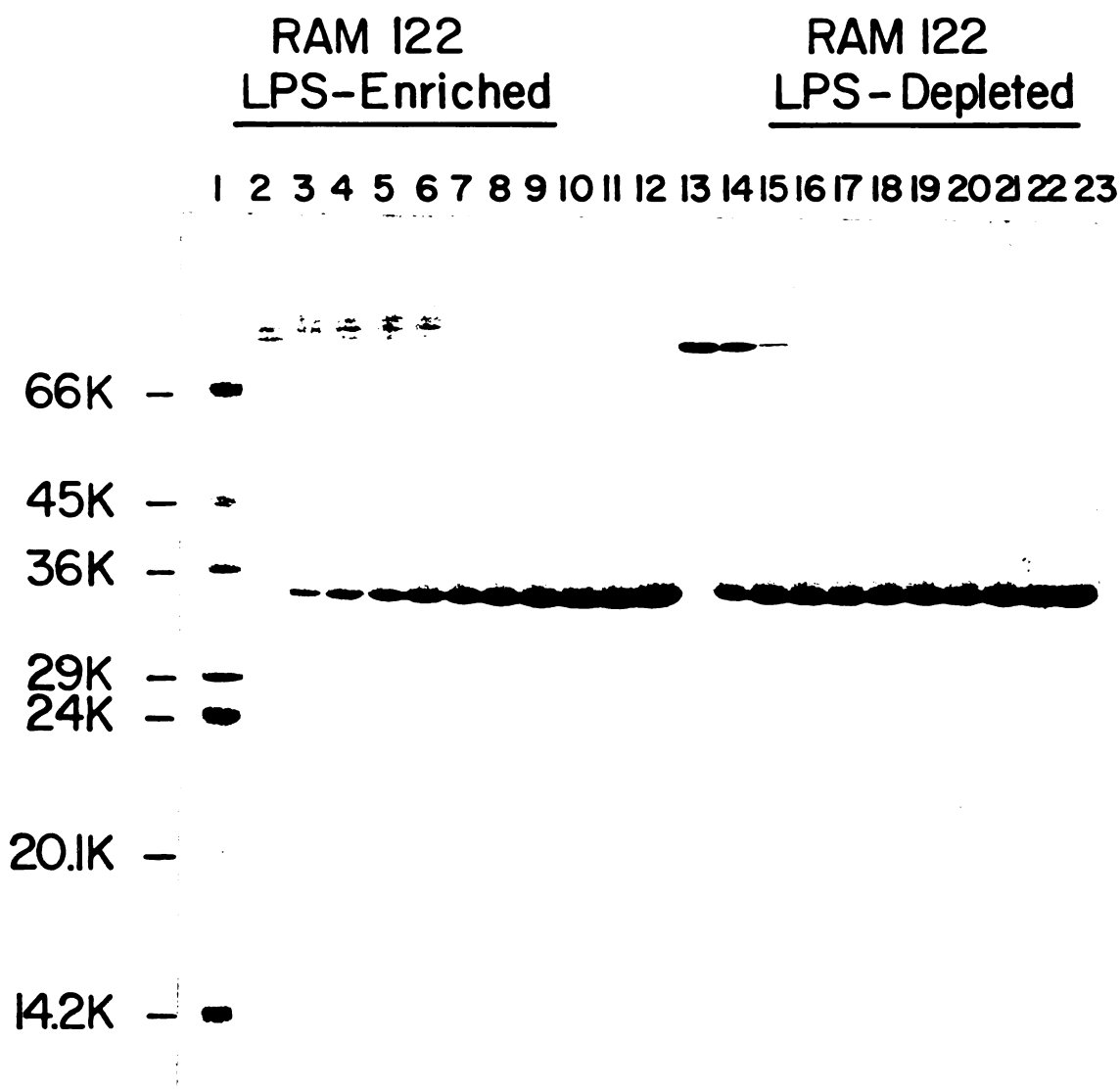
RAM 122
LPS-Enriched
RAM 122
LPS-Depleted
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
66K
45K
36K
29K
24K
20.1K
14.2K

FIGURE 1

observing the formation of monomers. The results using both techniques showed that for LPS-depleted samples the mutant OmpC porin was less heat stable and denatured at approximately 60°C in 1% SDS (Figures 1 and 2A), whereas the wild type OmpC porin denatured near 76°C (Figure 2A). Rescanning the porins after heat denaturation in the calorimeter showed no additional transitions indicating the absence of protein refolding. In addition, the overall enthalpy of the denaturation of the LPS-depleted porins was almost two fold lower for the mutant porin compared to the wild type. The presence of bound LPS did not seem to affect the wild type porin's transition temperature measured by SDS-PAGE (data not shown) or calorimetric analyses (Figure 2B); however, an increase in stability of the mutant OmpC porin bound with LPS was observed with both techniques (Figures 1, 2A, and 2B). Also, the enthalpy of denaturation was higher for both proteins if LPS was bound although the main transition temperature was unchanged (Figures 2A and 2B).

The stability of LPS-enriched and LPS-depleted porins in 1% SDS to low pH values was also analyzed using SDS-PAGE. The results showed that both the wild type and deletion mutant porins were slightly stabilized by the presence of bound LPS and denature between pH 4.0 and 3.5 (Figure 3A, lanes 5 and 6, Figure 3B, lanes 6 and 7). When the LPS was removed from the protein only a trace of the trimer band was detected after treatment at pH 4.0 for both of the isolates (Figure 3A, lane 13, Figure 3B, lane 14). The eight amino acid deletion does not appear to destabilize the mutant protein to low pH; therefore, the interactions disrupted at low pH appear to be similar for both proteins. In addition, both porins were more resistant to acid by 0.5 pH units when the proteins were solubilized in 1% octyl glucoside instead of SDS (data not shown).

Figure 2. Differential Scanning Microcalorimetry showing temperature (°C) vs. heat capacity (Cp, cal $°K^{-1}$ mol^{-1}) for wild type OmpC and RAM122 porins. A) wild type OmpC porin enriched in LPS (---) and RAM122 porin enriched in LPS (——) and B) PLB3255 wild type OmpC porin depleted of LPS (---) and RAM122 porin depleted of LPS (——). The proteins were suspended in 10 mM Tris-HCl, 1% SDS, pH 6.8. Measurements were made using a Microcal 2 scanning microcalorimeter at a scan rate of 90°C/h using 10 mM Tris-HCl, 1% SDS, pH 6.8 as the reference.

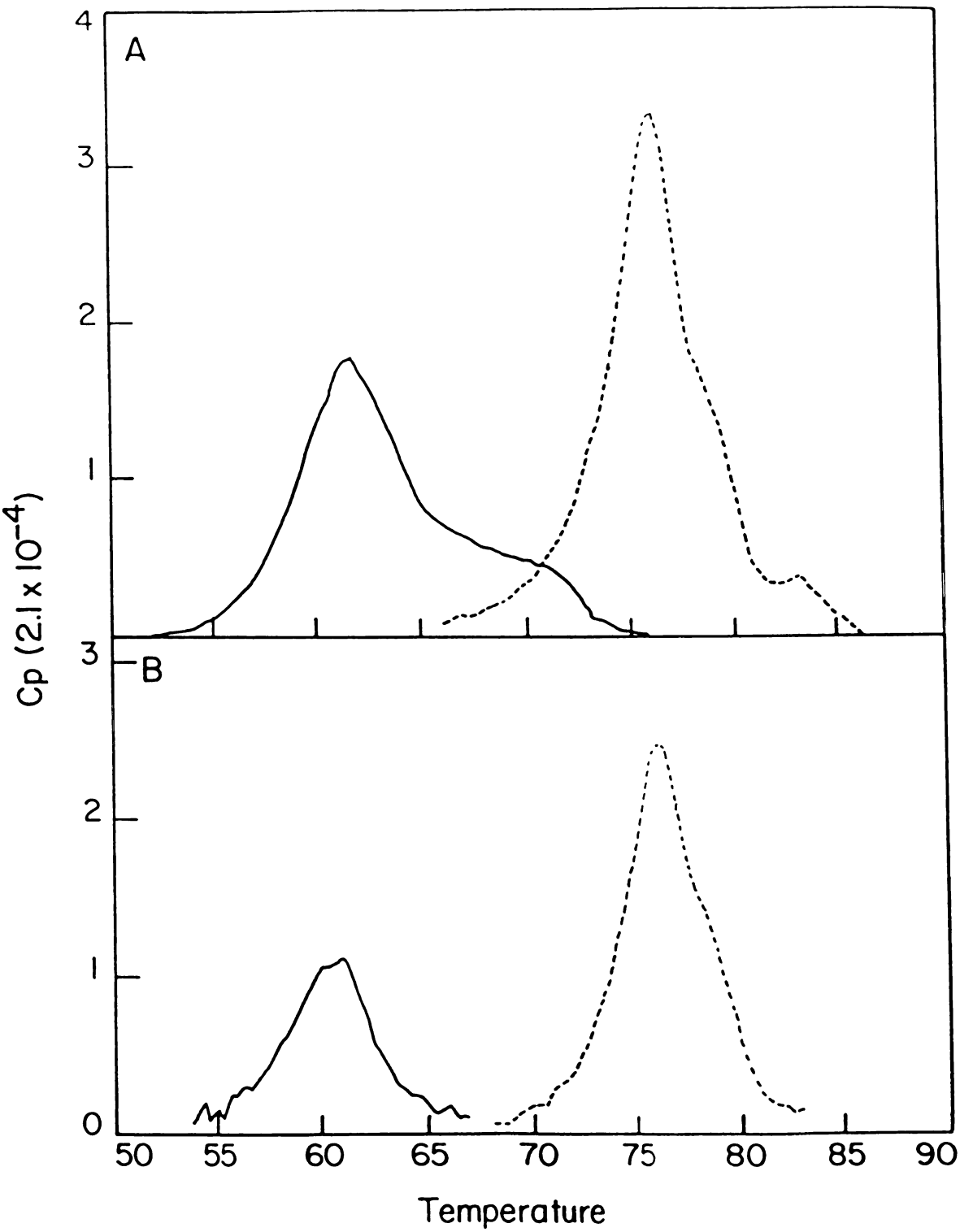

FIGURE 2

Figure 3. A 12% acrylamide SDS gel indicating the pH stability of wild type OmpC porin and RAM122 OmpC porin enriched and depleted in LPS. A) Lanes 1-8 contain LPS-enriched wild type OmpC and lanes 9-16 contain wild type OmpC, depleted of LPS. Lanes 1,9, contain unheated porin pH 6.8; Lanes 2,10, porin heated to 100°C, pH 6.8; Lanes 3,11, unheated porin treated at pH 5.0; Lanes 4,12, pH 4.5; Lanes 5,13, pH 4.0; Lanes 6,14, pH 3.5; Lanes 7,15, pH 3.0; Lanes 8,16, pH 2.5. B) Lanes 2-9 contain LPS-enriched RAM122 porin and lanes 10-17 contain LPS-depleted RAM122 porin. Lane 1 contains the molecular weight markers. Lanes 2,10, contain unheated porin pH 6.8; Lanes 3,11, porin heated to 100°C, pH 6.8; Lanes 4,12, unheated porin pH 5.0; Lanes 5,13, pH 4.5; Lanes 6,14, pH 4.0; Lanes 7,15, pH 3.5; Lanes 8,16, pH 3.0; Lanes 9,17, pH 2.5.

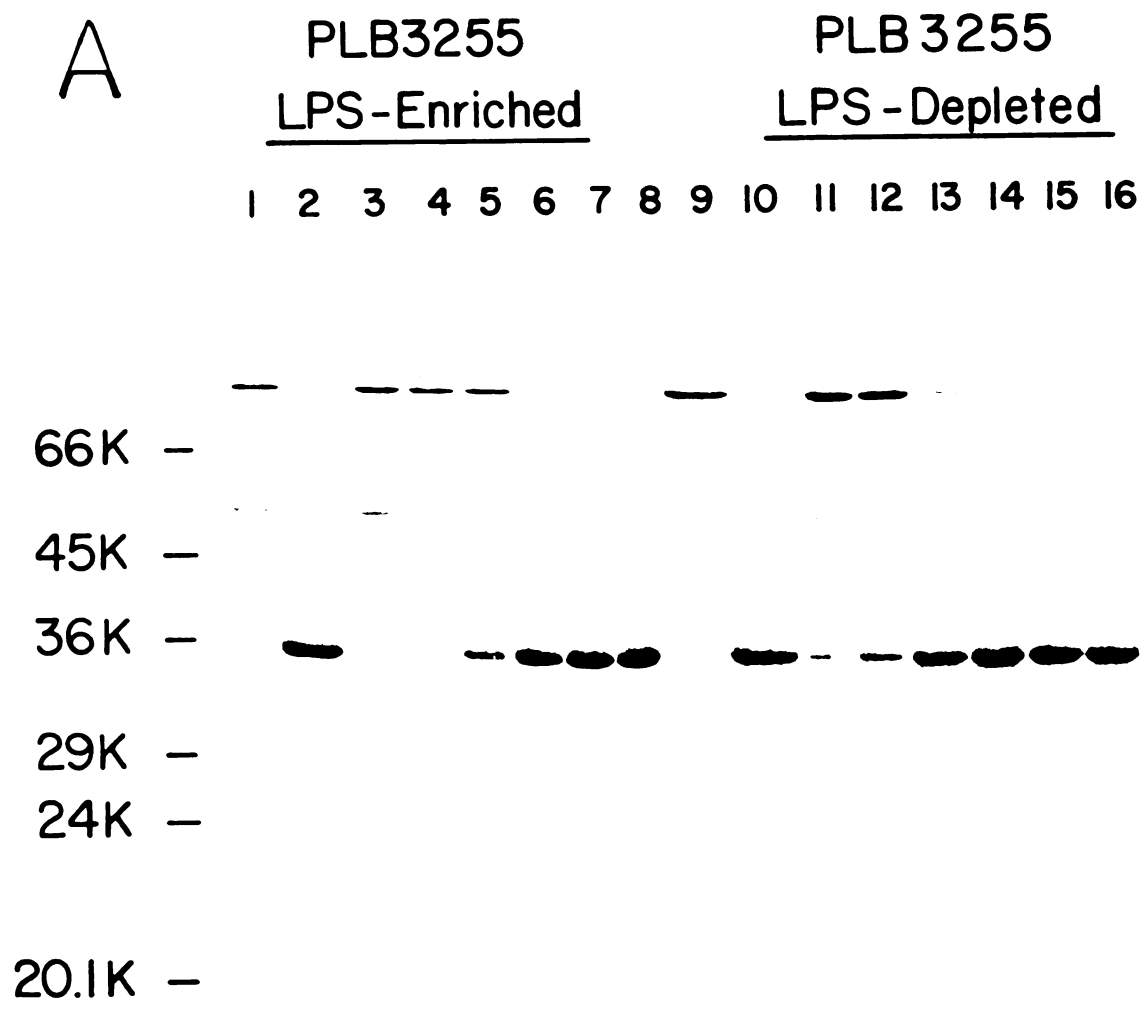
A
PLB3255
LPS-Enriched
PLB3255
LPS-Depleted
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
66K
45K
36K
29K
24K
20.1K

FIGURE 3

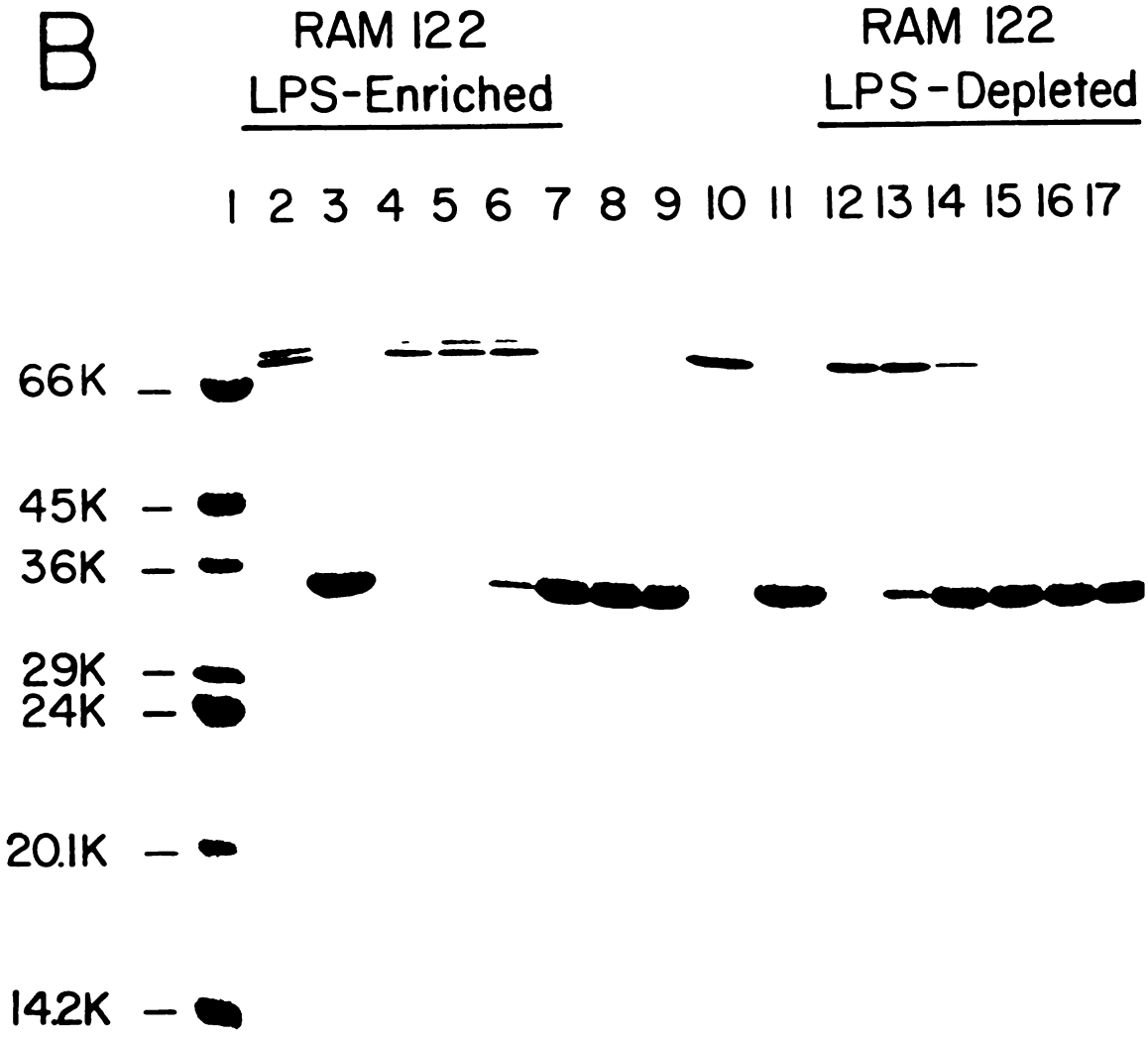
B
RAM 122
LPS-Enriched
RAM 122
LPS-Depleted
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
66K
45K
36K
29K
24K
20.1K
14.2K

FIGURE 3

Secondary Structure Analysis Using Circular Dichroism:

The secondary structure of the OmpC wild type and mutant porins was determined by measuring their circular dichroism spectra. The results of the analysis showed that the mutant and wild type porin spectra were similar in shape and indicated a large amount of β-structure (Figure 4), similar to what has been reported for the OmpF porin (23,24). However, there were slight differences in both the minimum elipticity and cross over point between the two proteins. The wild type porin had a cross over point at 204 nm compared to the mutant porin at 207 nm. In addition, the minimum elipticity occurred at 216 nm for the wild type and 217 nm for the mutant. Upon heating, the wild type trimer showed a loss of β-structure and an increase in α-helix and random coil. Analysis of the differences in secondary structure was performed using the method of Compton and Johnson (16) but with the X-ray structure matrix given by Manavalan and Johnson (17). The amount of β-structure does not appear to be significantly different between the two proteins, but, the analysis suggested that the α-helical content is less in the mutant porin compared to the wild-type. OmpF is reported to lack significant amounts of membrane spanning α-helices in its secondary structure (23); thus the apparent decrease in α-helix may not be significant.

Assays of Porin Activity:

Two different artificial membrane systems, liposomes and planar bilayer lipid membranes, were used to measure the channel-forming activity of LPS-depleted porins. In each assay system both the wild type and mutant porins showed strong channel-forming activity. Liposome swelling rates of both LPS-enriched and LPS-depleted

Figure 4. Circular dichroic spectra of LPS-depleted wild type (---) and RAM122 (——) OmpC porins. Samples at 0.2 mg/ml were suspended in 10 mM sodium phosphate, pH 7.0, containing 1% SDS. The measurements were performed at room temperature using a JASCO J-500C spectropolarimeter with a 0.5 mm path length quartz cell.

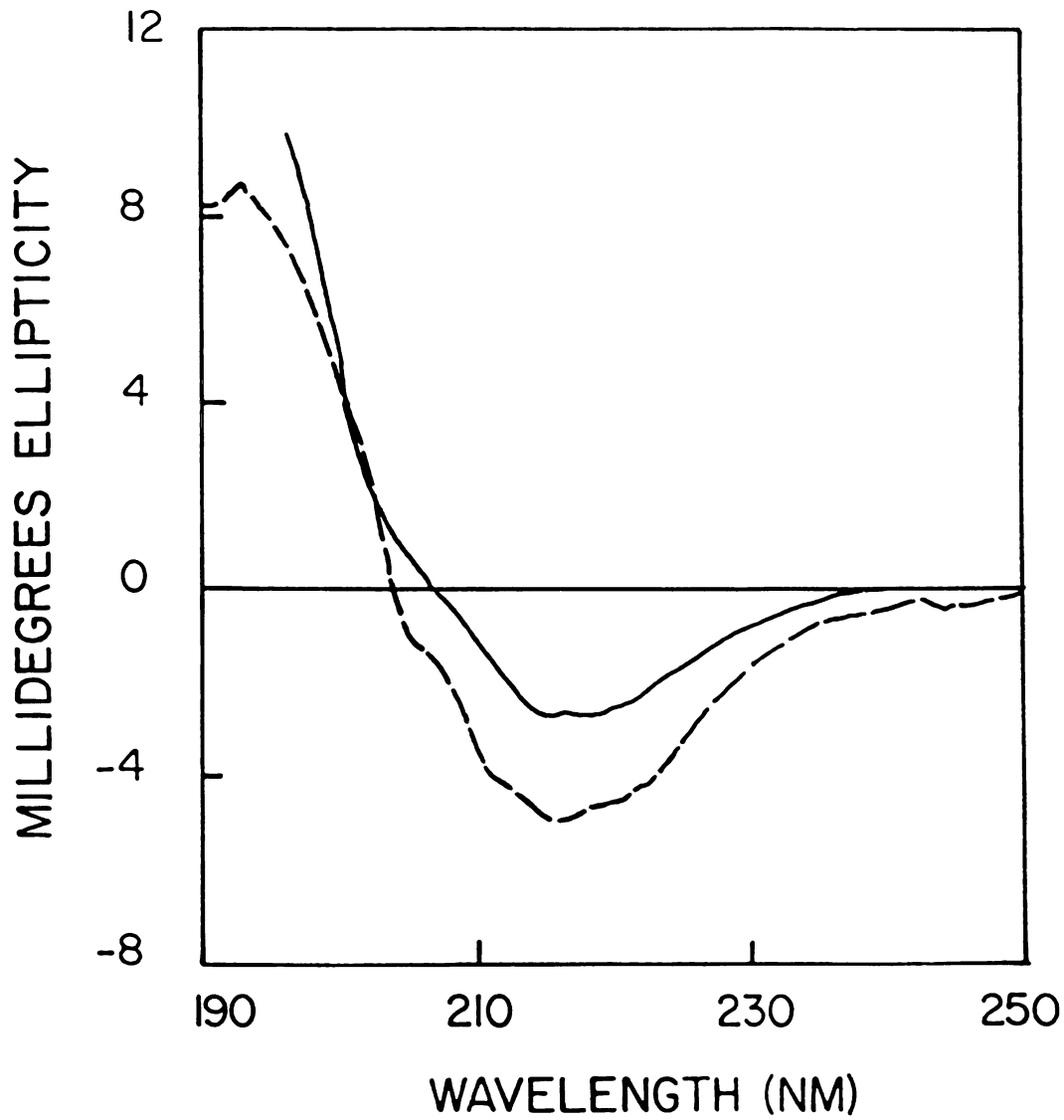
12
8
4
0
-4
-8
MILLIDEGREES ELLIPTICITY
190
210
230
250
WAVELENGTH (NM)

FIGURE 4

isolates were used to determine the size exclusion limit of the porin channels. The rate of swelling of liposomes was followed spectrophotometrically at 400 nm for three minutes. The assays were performed on the same batch of liposomes and the average of three separate runs was analyzed. When the swelling rates were plotted against the molecular weight of the saccharide, the size exclusion limit for both LPS-enriched and LPS-depleted mutant porin was 680 daltons whereas that of the wild type porin was 350 daltons. These results, using the wild type OmpC porin, are consistent with those obtained by Nikaido and Rosenberg (25). No swelling was observed with control liposomes without porin.

To further define the functioning and regulation of the wild type and mutant porins, conductance fluctuations across bilayer lipid membranes, following porin insertion, were measured. Stepwise current increases and decreases detected for each of the porin samples were measured at transmembrane potentials of 25, 50, and 75 mV. No channel activity was observed when the detergent 1% octyl glucoside alone was added to the bathing solution. In these experiments the channel size, reported as a size parameter, Λ/σ (in Å), is a function of the ionic strength of the bathing solution. The size parameter is plotted against the probability, P, of an event of a given size range. At low membrane potentials, the main conductance peak for each porin is assumed to be the cooperative opening of all three subunits within a trimeric aggregate. For the wild type protein this main peak was centered at approximately 1.9 Å (Figure 5A and 5D) using a 0.5 M NaCl bathing solution, whereas for the mutant porin this value was 2.5 Å (Figure 6A and 6D). This larger channel size is consistent with the results obtained with the liposome swelling assay.

Figure 5. Probability distribution of the size parameter, Λ/σ, (Å), for wild type OmpC porin as measured in bilayer lipid membranes. The LPS-depleted porin solubilized in 10 mM Tris-HCl, 1% octyl glucoside, pH 7.5 were added to the bathing solution at a final concentration of 50 ng/ml. Electrical conductance across the membrane was measured using a transmembrane potential of 25, 50, or 75 mV. Λ is the conductance change, and σ the specific conductance of the 0.5 M NaCl bathing solution. P, in arbitrary units is the relative number of events with the given size parameter range. A, B, and C present analyses of porins using a bathing solution pH of 6.2. D, E, and F present analyses of porins using a pH of 2.8. A and D were measured at 25 mV. B and E were measured at 50 mV. C and F were measured at 75 mV. Both opening and closing events are included in the histogram.

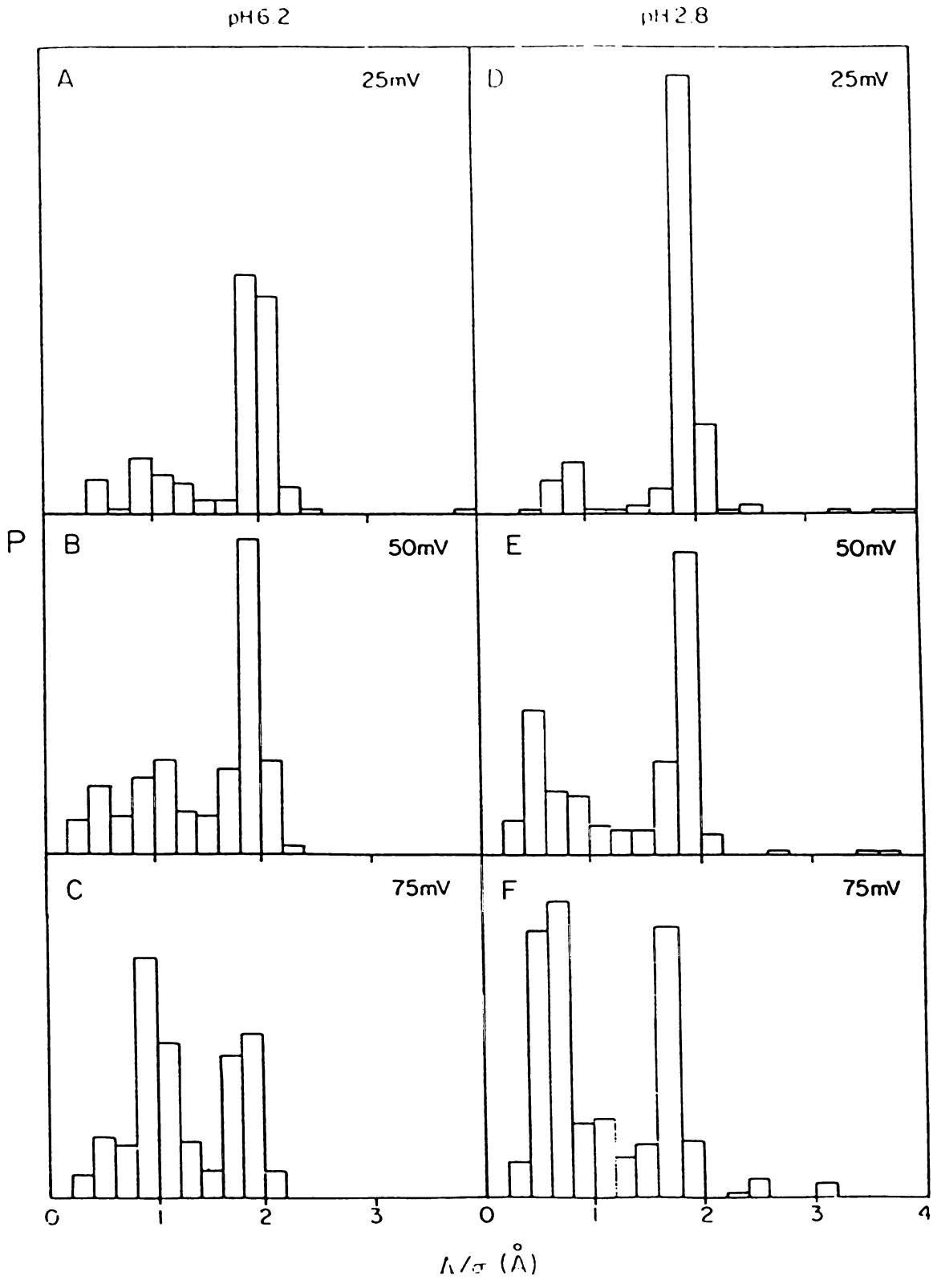
pH6.2
pH2.8
A
25mV
D
25mV
P
B
50mV
E
50mV
C
75mV
F
75mV
0
1
2
3
0
1
2
3
4
Å/σ (Å)

FIGURE 5

Figure 6. Probability distribution of the size parameter for RAM122 OmpC porin as measured in bilayer lipid membranes. All parameters are similar to those in Figure 5 except that for D, E and F porins were suspended in a bathing solution pH of 4.2.

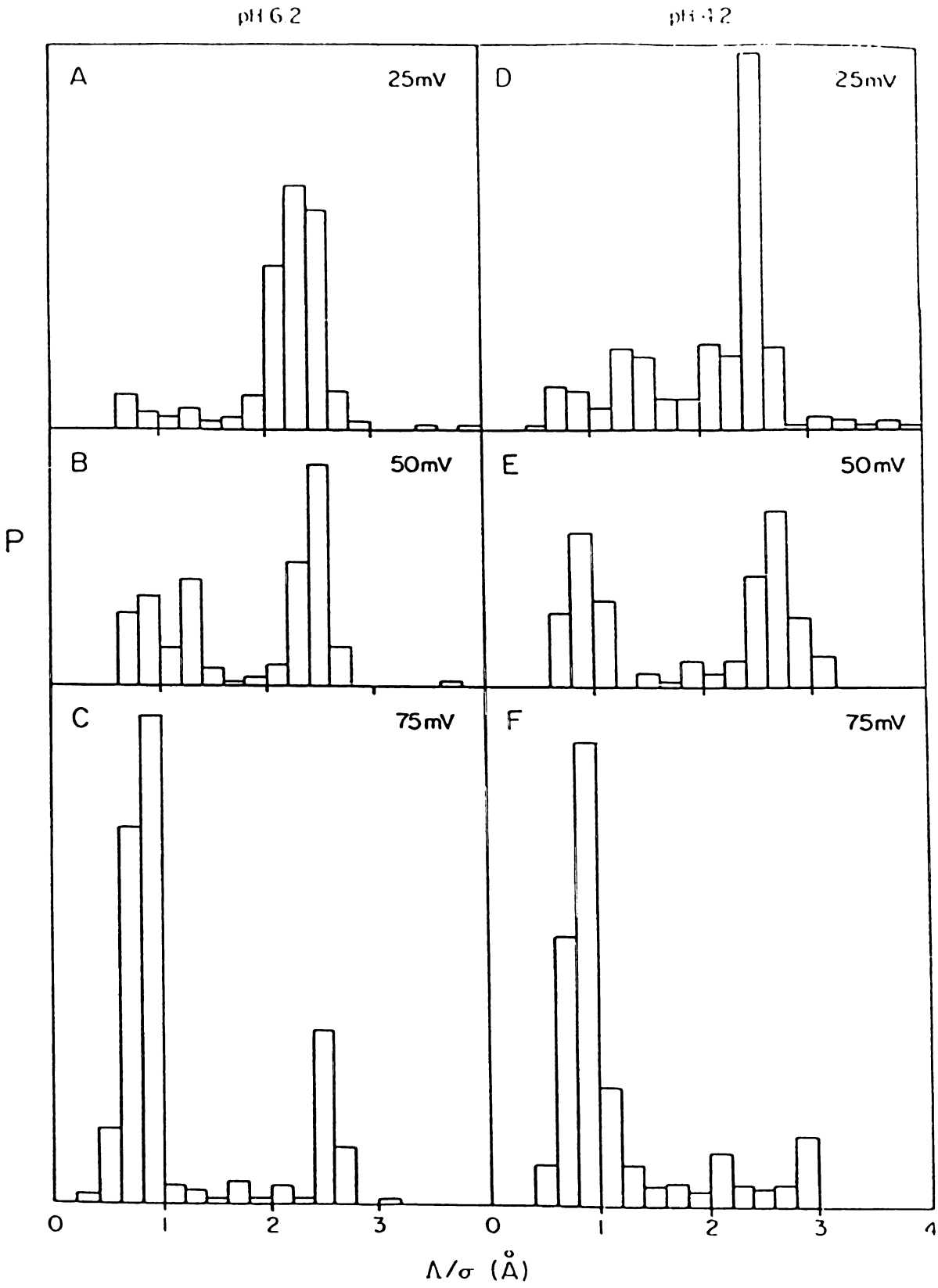
pH 6.2
pH 4.2
A
25mV
D
25mV
B
50mV
E
50mV
C
75mV
F
75mV
P
0
1
2
3
0
1
2
3
4
Λ/σ (Å)

FIGURE 6

When the potential across the membrane was increased to 50 and 75 mV and the pH of the bathing solution maintained above the denaturing point, the wild type porin showed very little voltage gating. However, when the protein was analyzed at low pH and at 75 mV, the percentage of porin closing events increased five fold, indicating pH-induced voltage gating (Table I). Also, the main conductance peak, detected at 75 mV, was approximately one-third the size seen at 25 mV (Figure 5C and 5F), indicating independent conformational changes of the subunits within the trimer. Apparently for both porins, not only is the channel voltage-regulated, but the cooperativity of opening and closing is lost at low pH and high voltage.

The main conductance peak of the mutant porin at 25 mV at high and low pH was about 1.3 times greater than the major conductance peak of the wild type porin. In addition, the RAM122 OmpC porin channel activity was extremely sensitive to membrane potentials of 50 and 75 mV at the two pH's used (Figure 6). The channels fluctuated between an open and closed state when the membrane potential was raised to 75 mV and the pH of the bathing solution dropped to 4.2 (Figure 7, Table 1). The percentage of closing events at 75 mV was seven to ten times greater than the level of closings at 25 mV. The mutant porin also showed a more dramatic shift in the size of the porin channels at 75 mV than the wild type. Again, the main conductance peak at 25 mV was shifted to approximately one-third the size detected at 50 and 75 mV. Very few trimeric channels (large conductances) were evident at low pH and high membrane potential, and almost all of the channels were of a monomeric size. The loss of cooperativity of trimeric channel activity appears to be voltage dependent and conditions which stabilize

TABLE I. Stability of the Closed State of LPS-depleted Porins as a Function of Voltage and pH

Porin	pH	Voltage (mV)	# Events Measured	% Closings*
		25	159	4
	6.2	50	210	10
		75	221	9
Wild type OmpC				
		25	163	5
	2.8	50	188	14
		75	279	28
		25	193	4
	6.2	50	185	24
		75	289	45
Deletion Mutant				
		25	239	7
	4.2	50	189	28
		75	531	51

*Number of closing events / Total number of events x 100

Figure 7. A scan of conductance across a bilayer lipid membrane containing RAM122 OmpC porin in a bathing solution of pH 4.2 and measured at a transmembrane potential of 75 mV. The arrow at the bottom right indicates the direction of the scan. The scan in panel B is a continuation of the scan in panel A.

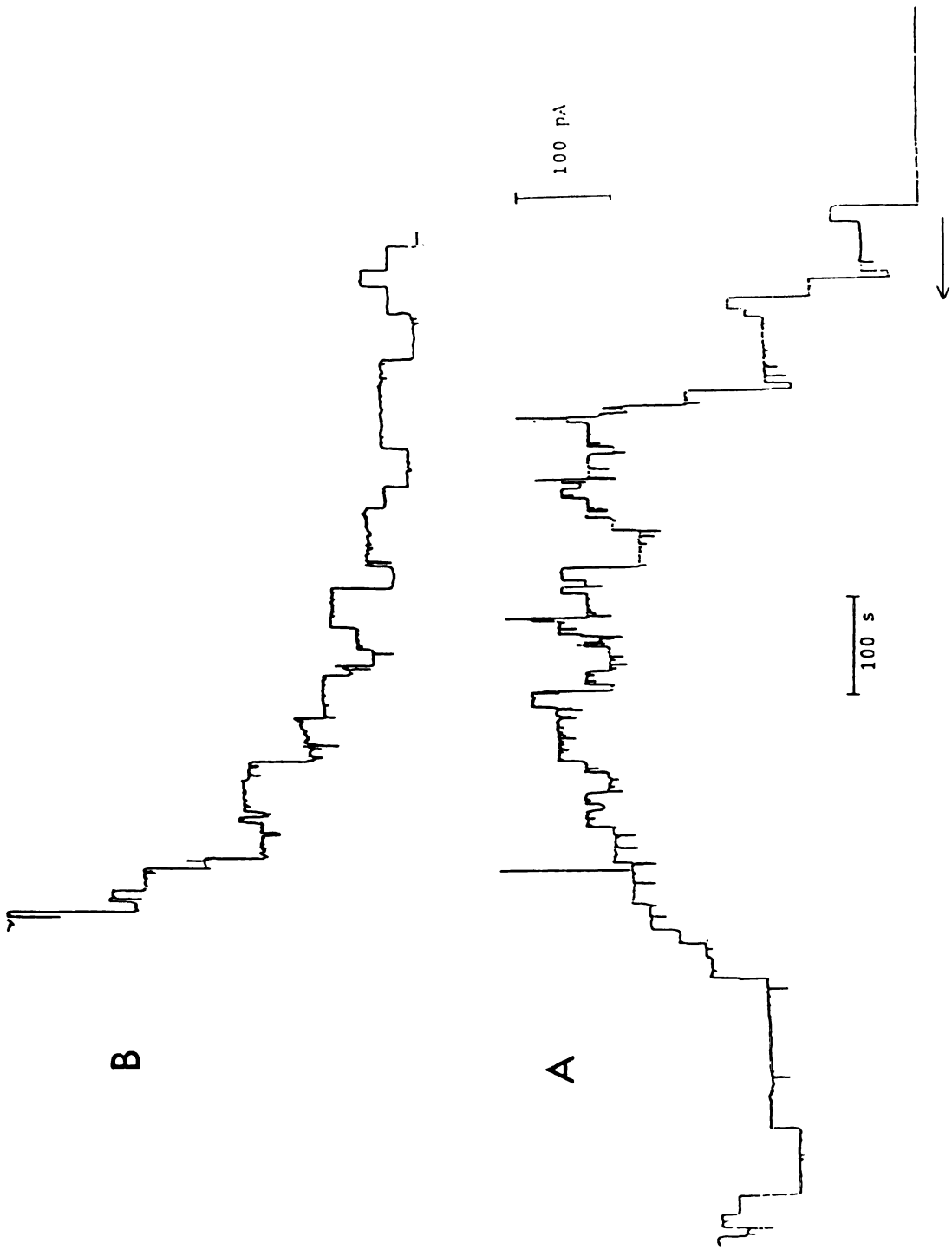

FIGURE 7

the closed state of the porins, i.e. low pH and high voltage, also decrease the cooperativity of the conformational changes.

DISCUSSION

RAM122 OmpC porin, containing an eight amino acid deletion in its primary sequence, appears to have a larger channel size. This protein was isolated, and the structural and functional properties compared to the wild type OmpC protein. To define critical changes in the structure of the mutant protein we characterized its stability to factors such as pH and temperature. The data on the temperature stability of the porins obtained from SDS-PAGE and differential scanning microcalorimetry were similar and indicated that the RAM122 protein denatured at a lower temperature and that it was stabilized by LPS. The deletion of eight amino acids results in the loss of amino acids capable of ionic and hydrogen bonding. Decreasing these interactions appears to destabilize the mutant protein to high temperatures. The decrease in the overall enthalpy of denaturation for the mutant protein also indicates that RAM122 porin lacks specific interactions that are important in stabilizing the native trimer. Whether these interactions are within the subunits or between the monomers has not been determined.

Lipopolysaccharide stabilized the mutant porin to high temperature but did not increase the stability of the wild type porin. These results suggest that the deletion may affect protein-LPS interactions since the presence of LPS increased the thermal stability only of the mutant isolate. When LPS was removed from the RAM122 protein, the protein was less stable apparently because of the loss of interactions with the LPS.

To remove LPS from both of the proteins, high concentrations of SDS were used. The SDS appears to compete for LPS binding sites on porin, and yet the trimers were stable to the high detergent concentrations. After gel filtration in high detergent

concentrations, the LPS bound to the eluted protein was quantitated. Some evidence suggests that only the lipid A portion of LPS remains associated with porin trimers and monomers after this purification (21). In the study reported here lipid A was assayed by measuring the levels of phosphate, assuming two to three phosphates per lipid A. The results indicated that less than one molecule of lipid A (LPS) was bound to the purified trimers. Yamada and Mizushima (26) reported that as little as one LPS molecule per OmpC trimer was required to form a hexagonal lattice structure, and this LPS could be replaced by an equivalent amount of fatty acid. Also, Jap (27) analyzing the electron density of PhoE trimers, has suggested that an LPS molecule is located at the axis of three fold symmetry. This would explain how as little as one LPS molecule could stabilize the porin in its trimeric configuration and affect channel activity.

Since LPS has been shown to stabilize the mutant porin to high temperatures, low pH stabilization studies were performed to determine if LPS also altered the protein's acid lability. Both the wild type and mutant porins showed similar acid stabilities, and we conclude that critical acid-labile regions involved in acid denaturation are not found within the deleted region. Both porins denatured between a pH of 4.5 and 4.0 which indicated that ionic bonds lost by titrating carboxyl residues are critical for structural stability. Our results on the pH denaturation of the OmpC proteins are similar to those of Markovic-Housley and Garavito (24), and Schindler and Rosenbusch (28) for the OmpF protein, which has a similar pI as OmpC.

LPS stabilizes both porins to acid denaturation. Again, protein-LPS charge and hydrophobic interactions probably contribute to this stabilization. Porins solubilized in the nonionic detergent octyl glucoside showed an increase in stability to low pH. This

may be attributed to the nonionic detergent being less able to unfold the protein. The OmpF porin solubilized in octyl glucoside instead of SDS also has been shown to have increased stability to low pH (24).

The eight amino acid deletion interrupts the primary sequence of the protein. To determine whether the secondary structure has also been altered circular dichroism spectra were obtained for both proteins. LPS-depleted porins were used because the samples were more dispersed. The mutant porin showed a secondary structure similar to the wild type porin, and resembled the secondary structure of the OmpF protein described by Rosenbusch (23). Both OmpC proteins contained similar amounts of β-structure. Porins in general are proposed to exist in either a β-barrel configuration or as stacked sheets and no large segments of α-helix are thought to be present (29,30). Analysis of our circular dichroism spectra using the X-ray structure matrix given by Manavalan and Johnson (17), indicated that the mutant porin may contain 5% lower α-helical content compared to the wild type protein. The significance of this decrease in α-helix is unknown at the present time. Further analysis of the difference in structures of these proteins using fourier transform infrared spectroscopy is in progress.

Since the RAM122 porin is less stable than the wild type porin the functionality of the proteins was also expected to be different. The channel-forming activity of the porins was analyzed using two artificial membrane systems. The liposome swelling technique of Nikaido and Rosenberg (25) was used to determine the size exclusion limit of the channels of the two porins. Misra and Benson (4) have indicated that the mutant porin channel may be larger since the cells can grow on maltodextrins in the absence of the LamB protein. Our results support their proposal since the isolated mutant porin channel

had a size exclusion limit approximately twice as large as the wild type. Since the swelling assay is measured over a short time span it is possible that, over longer time spans during growth, the RAM122 OmpC porin will accomodate large maltodextrins. Therefore, *in vivo* this mutant protein may allow for the uptake of molecules as large as maltohexose as demonstrated by Misra and Benson (4). The increased sensitivity of RAM122 cells to antibiotics does not appear to be due to altered porin-LPS interactions since the affinity of LPS for the isolated mutant porin appeared similar to that of the wild type. In addition, LPS levels recovered from RAM122 and PLB3255 cells were similar, and the amount of KDO and phosphate on the LPS isolated from both strains was the same. Also, the levels of LPS bound to either porin did not affect channel size. Thus, the changes in permeability of the RAM122 strain do not appear to be due to any changes in LPS. The results suggest that the alteration of the RAM122 protein is the basis for alterations in cell permeability allowing maltodextrins and antibiotics to cross the membrane. However, we cannot rule out the possibility that increased antibiotic permeability across the outer membrane is due to altered interactions of the porin with other outer membrane components.

The other artificial membrane system used to measure channel forming activity was the planar bilayer lipid membrane. LPS-depleted porins suspended in octylglucoside were reconstituted into such membranes and increases and decreases in the conductance were monitored. This technique provided information concerning individual porin channels and the effects of environmental parameters on channel activity. The larger channel conductance size for the mutant porin substantiated our hypothesis that the amino acids deleted from this protein are critical for defining the porin channel size. Since little is

known about the folding of the primary sequence in the tertiary and quaternary structure, we do not know where in the wild type protein these deleted amino acids normally reside. Misra and Benson (4) have proposed a model for the transmembrane spanning regions of the OmpC porin where the deletion is located at the beginning of a transmembrane spanning segment at the outer surface of the porin. This site could be in or near the channel since it has been shown that the first one-third of the primary sequence comprises the OmpC (4) as well as the PhoE channel (31).

The effect of environmental factors such as voltage and pH on porin channel activity in reconstituted systems has been used by many investigators to probe the protein structure/function relationships. Low pH values have been shown to decrease the protein-protein interactions between subunits of the OmpF porin *in vitro* (9,24). Similarly, the PhoE (10) and OmpF (8,9) trimers reconstituted into bilayer lipid membranes at high membrane potentials show a conductance that is one-third the size of the main conductance peak at lower voltages; hence, voltage also appears to decrease the protein-protein subunit interactions. It appears that the trimer undergoes cooperative conformational changes and at neutral pH the three channels within the aggregate open as a single unit. Upon decreasing the pH or increasing voltage, the protein loses its cooperativity and each subunit acts independently of the other two. When both low pH and high voltage are introduced into the system at the same time the wild type OmpC trimer showed decreased subunit interactions and a shift in conductance to one-third the channel size of the main conductance peak at 25 mV. Voltage and pH seem to act synergistically in decreasing the cooperativity of OmpC wild type trimer conformational change since the number of small conductance readings was considerably higher at low

pH and 75 mV than at neutral pH and 75 mV. The specific amino acids that are critical for voltage- and pH-dependent conformational changes have not been determined but are presumed to include charged residues.

Interestingly, the RAM122 porin was extremely sensitive to voltages greater than 50 mV at either pH, and therefore, the deleted residues may be critical for damping the voltage sensitivity of the wild type protein. The shift in conductance to one-third the size of the main conductance peak at 25 mV occurred more frequently in the mutant porin than the wild type. The lowered pH did not have a significant effect on the size of conductance changes; so voltage alone appears to be altering the cooperativity of the mutant channel. We propose that the wild type porin is a more rigid structure than the mutant and harsher conditions are needed to destabilize the protein.

The destabilization of the porin by pH or voltage is correlated not only with loss of cooperativity of trimers but also with increased closing of porin channels; i.e. increased stability of the closed conformation. We propose that as the protein subunit interactions are weakened by environmental factors, the subunits interact less tightly and undergo conformational changes independent of each other. The mutant porin subunit interactions appear to be decreased compared to the wild type since the loss of cooperativity is more easily triggered, and the closed state more readily stabilized. We also suggest that the OmpC porin has a voltage sensor and that deleting eight amino acids from the protein enhances the sensitivity of the gating mechanism to voltage.

Many investigators question the physiological relevance of voltage gating at high membrane potentials because Donnan potentials across the membrane, measured at 30 mV in minimal salts media *in vivo* (32), are too low to induce gating. In addition, Sen *et al.*

(33) have reported that changing the Donnan potentials from 5 to 100 mV had no effect on the *in vivo* permeability of either OmpC and OmpF porin channels toward a zwitterionic compound, cephaloridine. But Stock *et al.* (32) suggest that the potential across the outer membrane could significantly affect the function of surface organelles and rapid small changes in potential could be physiologically significant. Porins are not surface organelles, but it may be possible that porins can "sense" microenvironmental pH and voltage changes near or within the channel which may induce the protein to undergo a conformational change to the closed state. Where the voltage sensor is located within the protein is still under investigation.

The absence of aspartic acid 105 may play an important role in the voltage gating of the mutant porin. Benson *et al.* (5) have isolated a number of porin point mutant strains and have found that only four charged amino acids can be altered in both the OmpF and the OmpC porins to increase maltodextrin uptake *in vivo*. All four amino acids are conserved between the two general diffusion pores and the PhoE porin and therefore, appear to be critical in maintaining pore size, stability and function. The aspartic acid residue 105 missing from the RAM122 OmpC porin may be critical in ionic bridging with a lysine or arginine within the channel or between the porin subunits; deleting the segment of protein containing this amino acid structurally destabilizes the porin. The destabilization may also increase the sensitivity of this porin to voltage perturbations. This amino acid residue then may be important in the opening and closing mechanism of the wild type OmpC porin *in vivo*.

In conclusion, the RAM122 OmpC porin, with an eight amino acid deletion, contains a channel which is larger than that of the wild type porin and is capable of

allowing maltodextrins into cells. In addition, the mutant porin is less structurally stable than the wild type and is also more voltage sensitive, closing more readily at high potentials. The closings are of a conductance size one-third the size of a trimeric opening. These deleted amino acids are presumed to be critical in maintaining the wild type trimer as a tight aggregate structure. Work is in progress using point mutants to analyze specific amino acids which may be critical for voltage gating.

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CHAPTER 5

A Comparative Study of *Escherichia coli* K-12 OmpF Wild Type and Point Mutant Porin Structure and Function

ABSTRACT

OmpF porins from two *Escherichia coli dex* mutant strains were isolated and their structures and functions compared to that of the wild type isolate. Strains OC901 and OC904, selected for producing porins with larger channel sizes, have been shown to synthesize OmpF with a single amino acid substitution, Arg 82 to Cys and Asp 113 to Gly, respectively (Benson, S.A., Occi, J.L., and Sampson, B.A. (1988) *J. Mol. Biol.* **203**, 961-970). All three porin samples travelled as trimers on sodium dodecyl sulfate polyacrylamide gels. The OC904 porin was less stable to high temperature and low pH than either the wild type or OC901 porins. All three porins were stabilized to high temperature and low pH by the presence of bound lipopolysaccharide. The secondary structure of each porin, as measured by circular dichroism spectroscopy, indicated a similar shape and a high percentage of β-structure. When the proteins were assayed for channel forming activity using a liposome swelling assay, the size exclusion limit of the two mutant porins was larger than the wild type OmpF porin. Also, conductance measurements across bilayer lipid membranes indicated that the OC901 porin was highly voltage regulated in the presence and absence of lipopolysaccharide, whereas the OC904 porin was not voltage regulated. These data suggest that the amino acid Arg 82 plays a role in the voltage gating mechanism, whereas Asp 113 is important for protein stability.

INTRODUCTION

The outer membrane of Gram-negative bacteria forms a selective permeability barrier that allows the influx and efflux of small hydrophilic molecules via a class of proteins called the porins. Two major porins of *Escherichia coli* K-12 that have been studied extensively are the OmpC and OmpF proteins. These porins form nonspecific diffusion channels that allow solutes of less than 600 Da to passively diffuse across the membrane. Porins are stable to sodium dodecyl sulfate (SDS), urea, high temperatures and low pH and interact very strongly with LPS.

Porins, isolated as trimeric aggregates from the outer membrane, contain a high percentage of β-structure (1,2). Each monomer within the trimeric aggregate forms a channel and can function independently within a trimeric complex (3,4). Structural and functional analyses of porins *in vitro* have shown that the proteins exist in at least two conformations, an open and a closed state. Voltage-dependent regulation of porins has been shown to occur *in vitro* (3-7), but the significance of *in vivo* voltage regulation is still disputed (8).

Recent advances in the analyses of porin structure and function have come from the characterization of mutant porins with altered channel properties. Several *E. coli* K-12 deletion and point mutations in *ompC* and *ompF* have been characterized which appear to produce porins with larger channel sizes (9-12). Rocque and McGroarty (4) have purified one of these mutant OmpC porins which contains an eight amino acid deletion and have reported that the channels are larger in size and highly voltage regulated. In addition, Dargent and coworkers (13) have shown that specific amino acid substitutions

in the LamB porin alter its transport properties *in vitro*, whereas other substitutions have no effect.

In this report, we describe our characterization of two point mutant OmpF porins from strains that were selected on maltodextrins for larger porin channel sizes (12). We show that the amino acid substitution Arg 82 to Cys alters the voltage gating properties of the OmpF, whereas the substitution Asp 113 to Gly alters the protein's stability.

MATERIALS AND METHODS

Cell Growth and Porin Purification:

The strains used in this study were PLB3261, which produces a wild type OmpF, and point mutant strains OC901, derived from OC5101 (OmpF Arg 82 to Cys, $OmpA^-$) and OC904, derived from OC5104 (OmpF Asp 113 to Gly, $OmpA^-$); each strain lacks the OmpC and LamB proteins (9,12). The cells were grown in 1% tryptone, 0.5% yeast extract, pH 7.5 at 30°C, and harvested in late logarithmic phase. Porins were isolated by the method of Lakey *et al.* (14) with slight modifications (15). The final preparation was suspended in 1% SDS, 10 mM Tris-HCl, 0.02% sodium azide, pH 6.8. To remove excess lipopolysaccharide, protein was suspended in 30% SDS, applied to a Sephadex G-200 gel filtration column (2.5 x 100 cm), and eluted with 1% sodium deoxycholate, 10 mM Tris-HCl, 0.2 M NaCl, 1 mM EDTA, 0.02% sodium azide, pH 9.0. Absorbance of the column fractionations was monitored at 280 nm.

Gel Electrophoresis:

SDS-PAGE was performed using the discontinuous buffer system of Laemmli (16). Separating gels contained 12% acrylamide and 0.1% SDS. Protein molecular weight markers included bovine serum albumin (66K), egg albumin (45K), glyceraldehyde 3-phosphate dehydrogenase (29K), trypsinogen (24K), trypsin inhibitor (20.1K), and α-lactalbumin (14.2K). The pH denaturation studies were performed as previously described (4). Protein was detected using Coomassie Brilliant Blue staining.

Physical Characterizations:

Differential scanning microcalorimetry was performed on a Microcal 2 scanning microcalorimeter (Microcal, Inc., Amherst, MA) at a scanning rate of 90°C per hour. LPS-enriched and depleted samples, at 1.1 mg/ml, were solubilized in 1% SDS, 10 mM Tris-HCl, 0.02% sodium azide, pH 6.8, and scanned from room temperature to 100°C with the solubilization buffer in the reference cell. Thermodynamic characterization was performed using the Deconv. program (Microcal, Inc.), assuming a single two state transition and a change in heat capacity, Δ Cp.

CD spectroscopy was performed at room temperature on LPS-enriched samples using a JASCO J-600 spectropolarimeter with a 0.5 mm path length quartz cell. Proteins were suspended in 1% SDS, 10 mM sodium phosphate, pH 7.0, at 0.3 mg/ml, and scanned from 260 nm to 190 nm.

Liposome Swelling Assays:

Liposome swelling assays were performed as previously described (15) using D-glucose, maltose, and maltotriose as test solutes at concentrations between 75 and 90 mM. The swelling rates were measured as a decrease in light scattering at 400 nm using a 1 cm cuvette and a Gilford Response II spectrophotometer. The swelling rates were determined as a percentage of the initial absorbance reading and adjusted for the rate of swelling of control liposomes lacking porin.

Bilayer Lipid Membrane (BLM) Analysis:

For BLM analysis, porins were diluted to 0.18 mg/ml in 1% SDS, 10 mM Tris-HCl, 0.02% sodium azide, pH 6.8. Membranes were formed by applying a 1% solution of diphytanoyl phosphatidylcholine (w/v) in n-decane to a hole in a teflon partition bathed in a 0.5 M NaCl solution, pH 6.5. Silver-silver chloride electrodes were placed in the aqueous solution on each side of the membrane. A constant voltage of 25 or 75 mV was applied across the membrane using a 1.5 V battery. The changes in current were amplified with a Keithley model 614 electrometer and monitored with a chart recorder. The increases in current (opening events) and decreases in current (closing events) are reported as the size parameter Λ/σ, in Å, versus the probability, P, of the occurrence of an event with a particular size. The measurements were ohmic over the entire range used for the experiments.

Miscellaneous Chemical Assays:

Lipopolysaccharide was quantitated by KDO analysis using the thiobarbaturic acid assay (17) or by phosphate analysis according to Ames and Dubin (18). Lipid A was detected by electrotransferring to nitrocellulose (19) and reacting with a lipid A specific monoclonal antibody, 1D4, as previously described (20,21). Protein was quantitated using the BCA protein assay (Pierce Chem. Co.).

RESULTS

Isolation and Physical Characterization

Lipopolysaccharide Detection:

The OmpF porin was isolated from *E. coli* K-12 strains OC901 and OC904 containing point mutations, Arg 82 to Cys and Asp 113 to Gly, respectively, and from strain PLB3261 which produces wild type OmpF. The proteins were solubilized in 1% SDS for LPS-enriched samples or in 30% SDS and passed over a Sephadex G-200 gel filtration column to remove excess LPS. When the samples were solubilized in 1% SDS and run on SDS polyacrylamide gels, the proteins migrated as trimers with a distinct ladder pattern indicating the presence of tightly associated LPS (20). The LPS in the samples was quantitated by KDO and phosphate analysis, and the results showed that there were 2-3 moles of LPS per mole of porin monomer. After solubilizing the samples in 30% SDS and removing the LPS on a Sephadex G-200 gel filtration column, no KDO or phosphate was detected in any of the protein peaks. In addition, no free LPS was detected when the proteins were electrophoresed and silver stained for LPS. However, there was a lipid A-containing moiety bound to the trimer and heat denatured monomer which could be detected when the proteins were electrotransferred to nitrocellulose and reacted with a lipid A-specific monoclonal antibody (data not shown). The anti-lipid A antibody does not react with the intact LPS molecule, eliminating the possibility that the reactivity associated with the monomer and trimer was from the intact LPS molecule. Whether this lipid A-like material is present on the outer surface of the bacteria or is a degradation product formed during isolation has not been determined.

Temperature Stability:

The temperature stability of the wild type and mutant porins was examined using differential scanning microcalorimetry. Figure 1 indicates the heat capacity scans of LPS-enriched and LPS-depleted porins normalized for concentration. The scans were analyzed assuming a single two state transition and a change in heat capacity after the transition. The results showed that the LPS-enriched wild type and OC901 porins were similar in temperature stability, denaturing around 78°C, but that the OC904 porin denatured at 71°C. Similar results were observed using SDS-PAGE (data not shown). When LPS was removed from the proteins, the temperature denaturation profiles shifted down approximately 10°C for each of the proteins, and the heat capacity peaks became sharper. The overall enthalpy of denaturation also decreased between 26-28% for all of the samples when LPS was removed (Table 1), indicating a decrease in stability of the proteins. In addition, the ratio of the van't Hoff enthalpy to the calorimetric enthalpy, which indicates the size of the cooperative unit in protein unfolding (22), increased dramatically when LPS was removed; thus, depleting porins of LPS appears to induce porin aggregation. Also, the change in the heat capacity upon heat denaturation was greater for the LPS-depleted samples. These results suggest that more hydrophobic sites become exposed after denaturation of the LPS-depleted samples compared to LPS-enriched porin (23).

pH Stability:

The acid stability of LPS-enriched and LPS-depleted porins in 1% SDS was analyzed using SDS-PAGE. The OmpF porins retain their trimeric configuration on SDS

Figure 1. Differential scanning microcalorimetry heating endotherms measuring temperature (°C) vs. heat capacity (Cp, cal $°K^{-1}$ mol^{-1}) for wild type OmpF porin (____), OC901 porin (-----), and OC904 porin (-•-•-). The scans of LPS-depleted porins are labelled with (- LPS), while the scans of LPS-enriched samples are unlabelled in the figure. The proteins were suspended in 1% SDS, 10 mM Tris-HCl, pH 6.8. Scans were run at 90°C/hr using 1% SDS, 10 mM Tris-HCl, pH 6.8, in the reference cell.

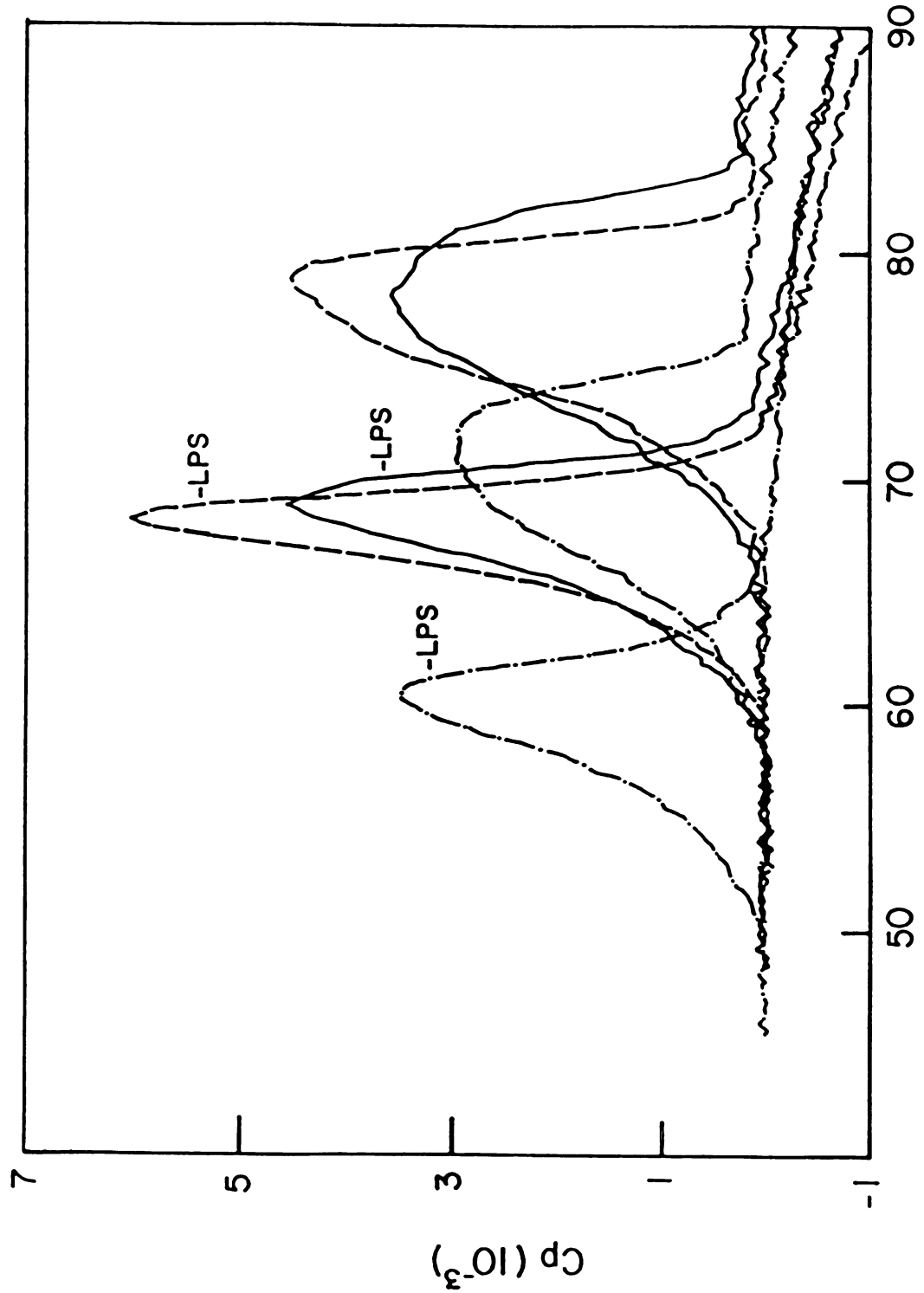

FIGURE 1

TABLE 1: Thermodynamic Parameters of Heating Endotherms for LPS-Enriched and LPS-Depleted Porins

Porin	Tm(°C)		ΔH_o(kcal/mol)		$\Delta H_{vH}/\Delta H_{cal}$		ΔCp(kcal/K°*mol)	
	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS
OmpF wt	78.1	68.8	59.9	44.6	3.1	34	1.2	1.3
OC901	78.8	68.2	61.7	46.1	4.8	253	1.3	1.7
OC904	70.8	60.4	50.7	36.1	6.1	174	1.0	1.3

gels unless they are denatured prior to electrophoresis (1,4,24,25). Thus, denaturation can be followed by lowering the pH of the porins to defined values before electrophoresis and observing the formation of monomers. The results showed that all three porins were slightly stabilized to acid by the presence of bound LPS. The wild type and OC901 porins denatured between pH of 4.0 and 3.5 (Figure 2A), whereas the OC904 porin denatured between pH 4.5 and 4.0. When LPS was removed, only a single trimer band was evident on gels (Figure 2B). The LPS-depleted wild type OmpF and OC901 porins denatured between 4.5 and 4.0, whereas the OC904 porin denatured between pH 5.0 and 4.5. The amino acid change from an aspartate to a glycine at position 113 decreases not only the temperature stability, but the low pH stability. Since the overall stability of the OC901 porin is similar to the wild type, the change at position 82 from arginine to cysteine does not appear to alter structural stability.

Circular Dichroism Analysis:

The secondary structures of LPS-enriched OC901 and OC904 OmpF porins were compared to that of wild type OmpF using CD spectroscopy. The spectra of all three porins were very similar and indicated a high percentage of ß-structure (Figure 3), similar to what has been reported for the wild type OmpF porin from *E. coli* B (1,2). The crossover point of each protein was at 206 nm and the minimum was at 218 nm. It appears that the two different single amino acid changes do not alter the overall secondary structure of either mutant porin.

Figure 2. Twelve % acrylamide SDS gels showing the acid stability of LPS-enriched and LPS-depleted OmpF wild type, OC901 and OC904 porins. A) LPS-enriched porins: Lanes 1-8 contain wild type OmpF porin. Lanes 9-16 contain OC901 porin. Lanes 17-24 contain OC904 porin. Lanes 1, 9, and 17 contain unheated porin pH 6.8. Lanes 2, 10, and 18 contain porins heated to 100°C, pH 6.8; Lanes 3, 11, and 19, unheated porins treated at pH 5.0; Lanes 4, 12, and 20, pH 4.5; Lanes 5, 13, and 21, pH 4.0; Lanes 6, 14, and 22, pH 3.5; Lanes 7, 15, and 23, pH 3.0; Lanes 8, 16, and 24, pH 2.5. B) LPS-depleted porins: Lanes 1-6 contain wild type OmpF. Lanes 7-12 contain OC901 porin. Lanes 13-18 contain OC904 porin. Lanes 1, 7, and 13 contain unheated porins treated at pH 6.8. Lanes 2, 8, and 14 contain porins heated at 100°C, pH 6.8. Lanes 3, 9, and 15 contain unheated porin treated at pH 5.0. Lanes 4, 10, and 16, pH 4.5; Lanes 5, 11, and 17, pH 4.0; and Lanes 6, 12, and 18, pH 3.5. Five μg protein added to each lane. Both gels are stained with Coomassie blue.

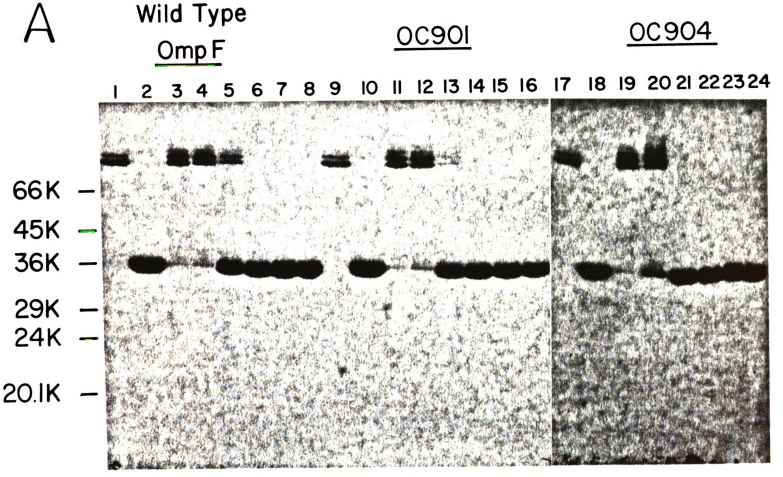
A
Wild Type
Omp F
OC901
OC904
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
66K
45K
36K
29K
24K
20.1K

FIGURE 2

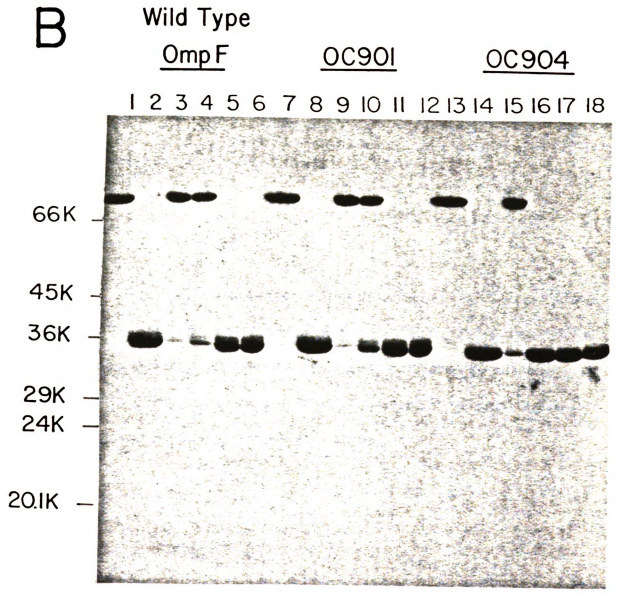
B
Wild Type
Omp F
OC901
OC904
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
66K
45K
36K
29K
24K
20.1K

FIGURE 2

Figure 3. Circular dichroic spectra of LPS-enriched wild type OmpF (____), OC901 (-----), and OC904 (-•-•-) porins. Samples were suspended at 0.3 mg/ml in 1% SDS, 10 mM sodium phosphate, pH 7.0. Measurements were performed at room temperature using a JASCO J-600 spectropolarimeter with a 0.5 mm path length quartz cell, and the spectra are corrected to the same protein concentration.

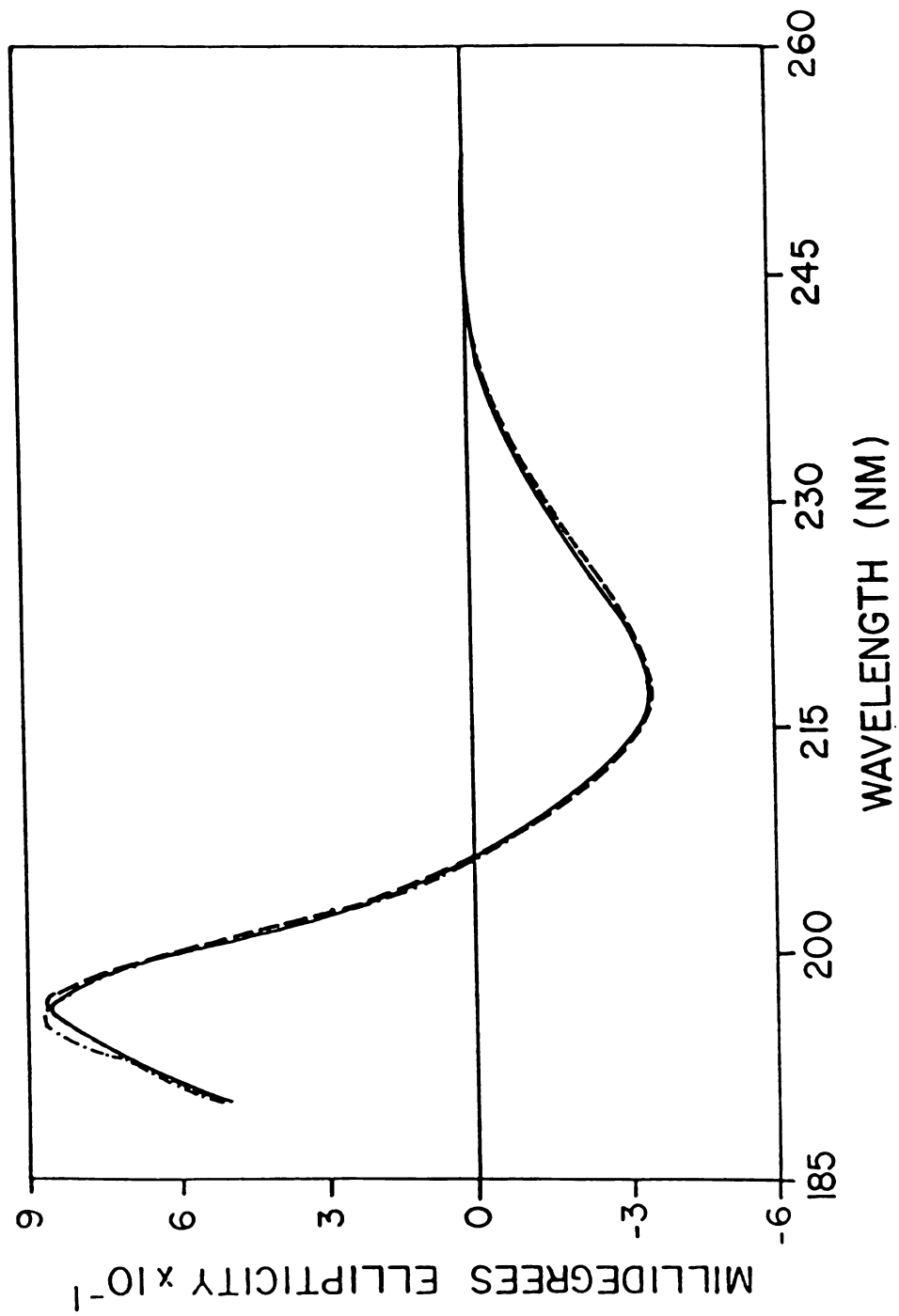
MILLIDEGREES ELLIPTICITY x 10⁻¹
6
3
0
-3
-6
185
200
215
230
245
260
WAVELENGTH (NM)

FIGURE 3

Porin Functional Analysis

Liposome Swelling Assays:

The liposome swelling assay used in this study was similar to that of Nikaido and Rosenberg (27). The rate of liposome swelling was followed by measuring light scattering for three minutes for each assay, and the average of three assays was used for rate analysis. Swelling rates were determined after 30 seconds as a percentage of the initial absorbance values (Table 2). LPS-enriched and LPS-depleted wild type OmpF porin allowed diffusion of solutes as large as maltose; however, the maltose diffusion rates were significantly lower than the diffusion rates using the two mutants. There was no significant difference between the diffusion rates of maltotriose using the wild type OmpF compared to the mutant porins. For the mutant porins, the rate of diffusion of maltose was similar to that of glucose; however, using the wild type OmpF glucose had a significantly greater diffusion rate than maltose. Furthermore, the level of LPS did not significantly affect the diffusion rates of solutes through any of the porin channels.

Bilayer Lipid Membrane Analysis:

The functionality of the OmpF porins were further characterized by monitoring conductance fluctuations across BLMs, following porin insertion. Stepwise increases and decreases in current were measured at transmembrane potentials of 25 and 75 mV. The size of the current changes were plotted as size distribution histograms (Figures 4 and 5). The main peak of each histogram at low membrane potentials is assumed to represent the cooperative opening of all three subunits within a trimeric aggregate (4). The main peak

TABLE 2: Relative Swelling Rates of Liposomes Containing LPS-Enriched and LPS-Depleted Porin in Different Test Sugar Solutions[1]

Porin	Glucose		Maltose		Maltotriose	
	LPS-enriched	LPS-depleted	LPS-enriched	LPS-depleted	LPS-enriched	LPS-depleted
WTOmpF	10.6 ± 0.9	12.4 ± 0.6	5 ± 1	3.4 ± 0.6	1 ± 2	-2 ± 2
OC901	9 ± 1	12.3 ± 0.3	10 ± 1[2]	9.8 ± 0.4[2]	2 ± 1	0.3 ± 0.3
OC904	9 ± 1	9.8 ± 0.3	11.1 ± 0.5[2]	10.4 ± 0.7[2]	3 ± 1	0.6 ± 0.5

[1]Swelling rates, presented as the percentage decrease in the initial absorbance after 30 sec, is the average from three assays (±SD)

[2]The swelling rate is significantly different from that of the wild type porin ($P < 0.01$).

Figure 4. Probability distribution histograms of the size parameter Λ/σ, in Å, for LPS-enriched OmpF porins as measured in bilayer lipid membranes. LPS-enriched porins were solubilized in 1% SDS, 10 mM Tris-HCl, pH 6.8, and added to the bathing solution at a final concentration of 45 ng/ml. Electrical conductance across the membrane was measured using a transmembrane potential of either 25 mV (A,C,E) or 75 mV (B,D,F). Λ is the conductance change, σ is the specific conductance of the bathing solution, and P, in arbitrary units, is the relative number of events within a given size conductance. A and B depict results with the wild type OmpF porin, C and D show OC901 porin, and E and F represent OC904 porin. The bathing solution was 0.5 M NaCl, pH 6.5, for all experiments. Both opening and closing events are included in the histogram.

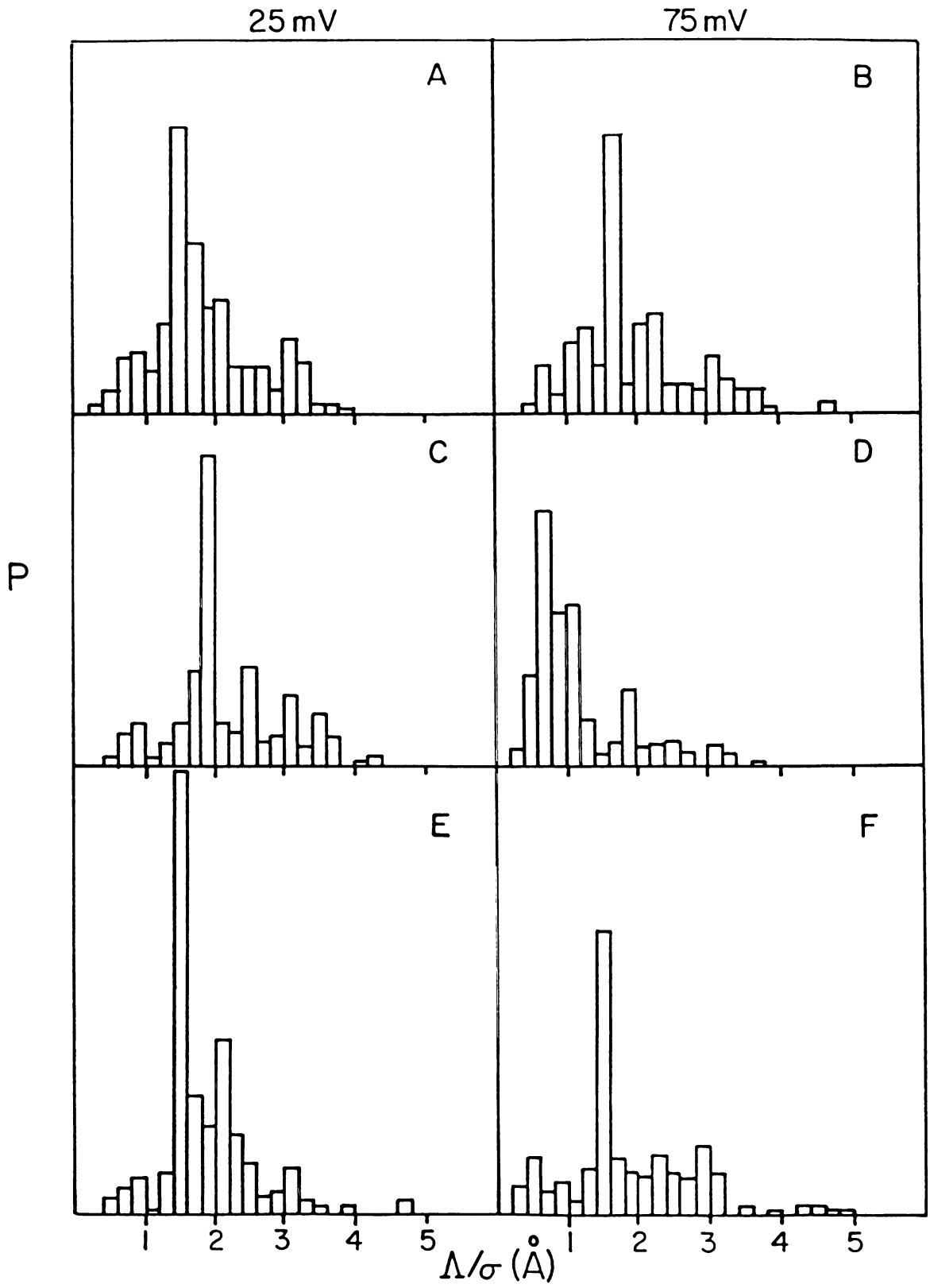

FIGURE 4

Figure 5. Probability distribution histogram of the size parameter Λ/σ, in Å, for LPS-depleted OmpF porins. All samples and protocols were similar to those described in Figure 4, except that LPS-depleted porins were added to the bathing solution at a final concentration of between 45 and 80 ng/ml.

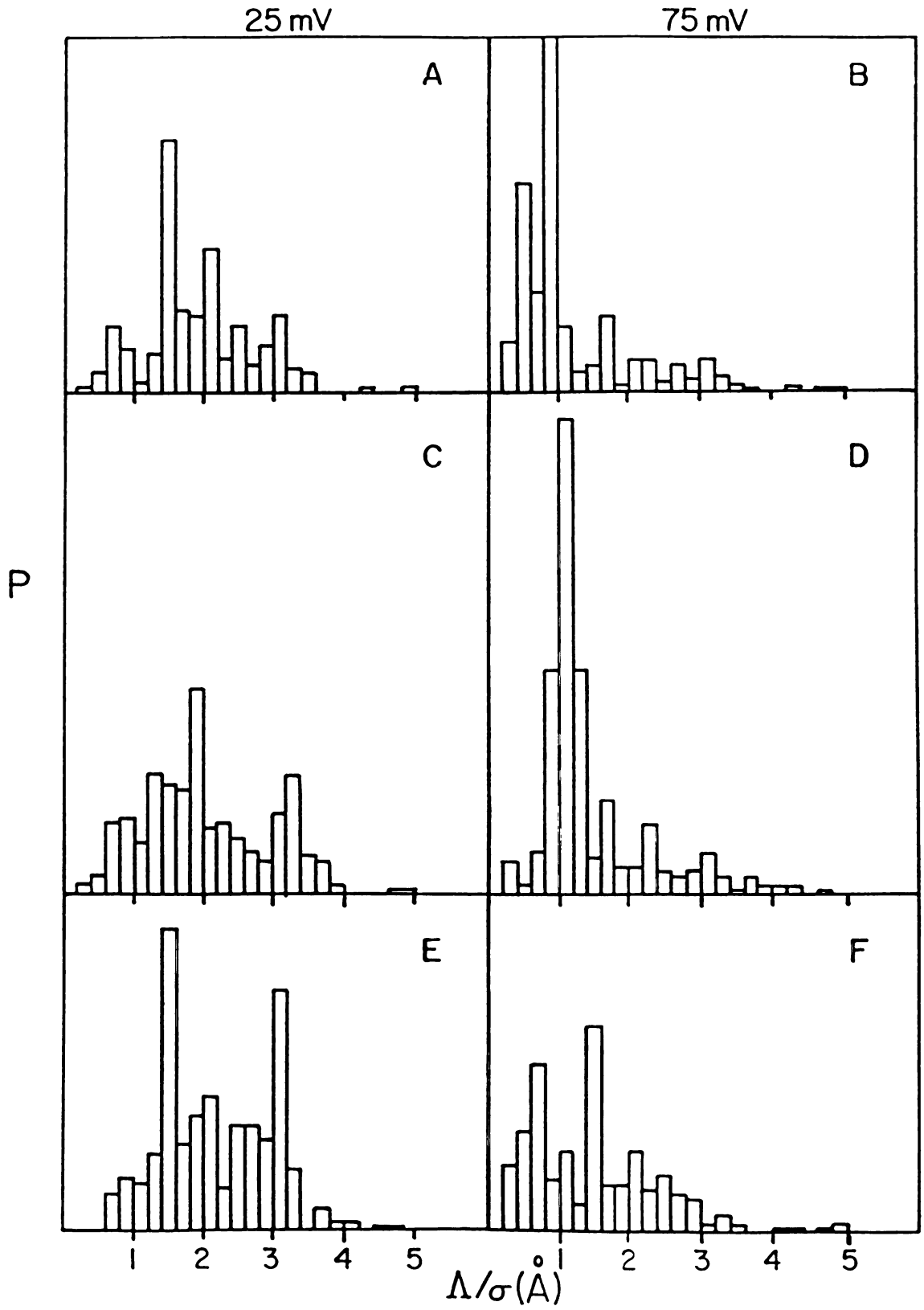

FIGURE 5

of LPS-enriched OC901 porin had a larger conductance than the LPS-enriched wild type OmpF (Figure 4), consistent with the liposome swelling assay results. The main conductance peak of OC904, however, was similar to that of the wild type OmpF porin and, thus, not consistent with the liposome swelling data. This discrepancy is not fully understood. Finally, at low voltage, the percentage of closing events measured was similar for all three porins indicating similar stabilities of the open porin conformation at low potentials (Table 3).

The sizes of the main conductance peaks at 25 mV were very similar between LPS-enriched and LPS-depleted porins; however, the size distribution histograms were broadened in the LPS-depleted samples (Figure 5). The LPS-depleted OC901 porin peak again had a slightly larger conductance than the wild type and OC904 porins. In addition, the LPS-depleted OC901 mutant porin had an increase in the percentage of closing events measured at 25 mV compared to the wild type OmpF porin (Table 3). The LPS-depleted porin samples occasionally showed very large conductance changes. These large conductances are thought to indicate the insertion and cooperative opening of large aggregates whose presence was suggested by the calorimetry results.

At a transmembrane potential of 75 mV, the size distribution histograms of the LPS-enriched wild type and OC904 porins looked very similar to the histograms at 25 mV (Figure 4). The main conductance peaks were almost identical, indicating the absence of voltage-induced changes in channel size or cooperativity. However, the OC901 porin showed a decrease in the main conductance peak to approximately one-third the size of the peak at 25 mV. These results are similar to those found using a deletion mutant OmpC porin (4) and suggest that the trimeric aggregate of the OC901 protein no

TABLE 3: Stability of the Closed State of LPS-Enriched and LPS-Depleted Porins as a Function of Voltage

Porin	Voltage (mV)	# Events Measured	% Closings*
OmpF Wild Type			
+ LPS	25	273	7
	75	214	12
- LPS	25	220	6
	75	525	38
OC901			
+ LPS	25	212	6
	75	403	47
- LPS	25	283	14
	75	311	44
OC904			
+ LPS	25	259	4
	75	200	15
- LPS	25	340	9
	75	243	27

* Number of closing events / Total number of events x 100

longer acts as a cooperative unit, but at high voltage, each unit within the aggregate appears to act independently. Furthermore, the percentage of closing events observed at 75 mV was dramatically increased for the OC901 sample, indicating voltage-induced closing of LPS-enriched OC901 porin channels at high membrane potentials (Table 3). Although the number of closing events for LPS-enriched wild type and OC904 porins also increased with potential, the change was not as great as with the OC901 porin. Hence, the amino acid change, Arg 82 to Cys, in the OmpF porin from OC901 increases the voltage gating, i.e. increases the stability of the closed state of the channels at high voltage.

When LPS was removed from the porins and channel activity monitored at 75 mV, the size distribution histograms were significantly different from the LPS-depleted porin histograms at 25 mV and the LPS-enriched porin histograms at 75 mV (Figure 5). The conductances were of smaller values than at 25 mV, yet the peaks were not one-third the size of the main peak at 25 mV. The altered size of the conductances suggests that the combination of replacing LPS with SDS and increasing transmembrane potential changes porin conformation. Further studies on LPS-porin interactions are in progress to characterize this unusual LPS-dependent change in conductance.

Interestingly, the LPS-depleted OC904 porin, which was less stable than the LPS-depleted OC901 and wild type porins, showed an increased stability of the open porin conformation at 75 mV. These results suggest that decreased structural stability does not always enhance voltage induced changes in porin conformation. Unlike OC904 porin, the ability of the more structurally stable OC901 porin to close at 75 mV is enhanced. In addition, the percentage of closing events at 75 mV for OC901 porin is not affected by

the presence or absence of LPS, indicating voltage-induced gating of OC901 porin is not influenced by LPS. This is not true for the wild type OmpF porin. The LPS-depleted wild type porin showed a significant increase in the percentage of closing events at 75 mV when compared to LPS-enriched porin (Table 3). This increased stability of the closed conformation of the wild type porin is thought to be due to altered protein-protein interactions within the porin complex upon removing LPS.

DISCUSSION

Analysis of porins with specific structural mutations has allowed investigators to define regions of the protein critical for channel activity. In this study, two OmpF point mutant proteins of *Escherichia coli* K-12 were isolated and their structure and function compared to that of the wild type protein. Strains OC901, OmpF Arg 82 to Cys; and OC904, OmpF Asp 113 to Gly, appear to have altered structure and function. The thermal stability of the OC904 OmpF porin, either in the presence or absence of bound LPS, was lower than that of the wild type and OC901 porins. The single amino acid change from aspartic acid to glycine at position 113 presumably disrupts ionic interactions that are critical for maintaining either the monomer folding or trimer interactions. If the aspartic acid residue is critical for interactions either between or within porin subunits, then replacing this amino acid residue with an uncharged amino acid would decrease the stability of the trimer complex due to the lack of that specific ionic bond. It is believed that the presence of ionic and polar residues within the membrane boundary form a tight network of hydrogen bonding and ionic bridging (27). Therefore, disrupting critical hydrogen bonds or ion pairs could destabilize the protein. Interestingly, the OmpF porin from OC901 was as thermally stable as the wild type OmpF porin. Since the stabilities were similar, the substitution of Cys for Arg at amino acid 82 is probably not as critical in ionic interactions, but it may be involved in hydrogen bonding within the aggregate. The Cys replacement could substitute in hydrogen bond interactions.

The effect of LPS on OmpF porin temperature and acid stability was examined using differential scanning microcalorimetry and SDS polyacrylamide gels. The binding

of LPS to porins has been reported by some investigators to be critical for the functioning of the porins (7,28), whereas others believe LPS is not necessary for porin activity (29-31). Several research groups have reported that LPS can be removed from porins, but they did not test for the presence of lipid A or fatty acids (30,32). We have found that bound LPS is critical for the thermal and acid stability of all the OmpF porins studied; therefore, it seems likely that LPS is important in the outer membrane for stabilizing OmpF porins against harsh environmental conditions. When tightly bound LPS is removed, the proteins become less stable, apparently due to the loss of hydrophobic and hydrogen bonds. Also, these LPS-depleted samples aggregate into large complexes in the presence of SDS. The LPS-porin interactions appear to be more critical for structural stability of OmpF porin than OmpC porin, since removing LPS from OmpC does not decrease the denaturation temperature (4). It also appears that the lipid A portion is the main component of LPS important for maintaining the trimeric configuration of the porin since lipid A cannot be completely removed from porin without denaturing the protein. We propose that porin folds around lipid A during synthesis; this would be consistent with the PhoE porin model of Jap (33) who proposes that an LPS molecule is present at the axis of three-fold symmetry of the porin trimer. Thus, LPS, or at least the lipid A portion of LPS, may be essential for porin structure and channel-forming activity.

To further define the stability of the LPS-enriched and depleted mutant porins, acid denaturation studies were performed. Our results using wild type OmpF porin in 1% SDS agree with previous reports (24,25). The LPS-enriched OC904 porin had a lower stability to acid pH than the wild type. The pH denaturation results obtained with OC904 porin were similar to the temperature denaturation studies and indicated that the glycine

substitution at position 113 alters the structural stability of the porin. The decreased pH and temperature stability of the OC904 isolate is not a result of an altered protein secondary structure since all three porins had essentially identical circular dichroic spectra. Presumably, the loss of an ionic bond in the OC904 porin decreases the pH stability. The porin from OC901 also had a slightly decreased stability to low pH compared to wild type porin, but the destabilization was not as significant as for OC904. When LPS was removed, all of the proteins were less stable to low pH, and this may be attributed to either the loss of charged residues on the LPS molecule or the loss of tight hydrophobic interactions.

Since the OC904 mutant porin was more labile than the wild type, we expected the channel-forming activity of this porin would also be different. To measure the channel-forming activity of the wild type and mutant porins, the proteins were reconstituted into two different artificial membrane systems. First, liposome swelling studies were performed according to the method of Nikaido and Rosenberg (26), and the rate of solute diffusion determined. Second, the conductance of porins inserted into BLM's was measured as a function of membrane potential. Benson *et al.* (12) have suggested that the *dex* mutant porins (e.g. OC901 and OC904 isolates) contain a larger channel than the wild type OmpF since these mutants can grow on maltodextrins in the absence of a functional *lamB* gene. Liposomes containing wild type OmpF showed a lower swelling rate in the presence of maltose compared to glucose presumably due to increased frictional forces of maltose against the sides of the channel. However, for both of the mutant porins the maltose diffusion rates were as fast as glucose. We believe this is due to an increased channel size in the mutant proteins and not to changes in selectivity of

the porins for maltodextrins since maltotriose diffusion was significantly slower than maltose diffusion. The OmpF swelling rates were determined over short time spans; over longer time spans of cell growth for the OC901 and OC904 strains, maltodextrins as large as maltotetraose may be accommodated, since these cells can grow on the tetrasaccharide (12). Our results suggest that maltodextrin passage thorugh the outer membrane occurs through the mutant OmpF porins and not by an alternate route. Additionally, there appeared to be no significant difference in the swelling rates of liposomes using porins enriched or depleted in LPS; therefore, excess LPS does not significantly affect channel-forming activity in these assays.

The second method used to determine channel-forming activity was planar BLMs. At low potentials, the LPS-enriched OC901 porin had a main conductance peak larger than the wild type; however, the OC904 porin was similar to the wild type. The OC904 porin results are not consistent with the liposome swelling results, which may reflect differences in the two techniques used. The BLM technique records single channel activities and requires the passage of ionic species through the channels while the liposome assays are performed using uncharged molecules. One assumption made in the BLM experiments is that ions passing through the channel travel as if they were in solution and have no interaction with the walls of the channel. Other differences make comparisons between techniques difficult (31). The OC904 porin's main conductance peak may not accurately reflect the actual channel size for uncharged molecules or for samples in low ionic strength; however, this method is extremely useful for studying the effect of environmental factors on the activity of individual porin channels.

The effect of environmental parameters on the channel-forming activity of the three porins was measured using the BLM technique. The influence of elevated membrane potential on porin channels has been examined by a number of investigators (3,4,6,7,13-15,29). Our results using LPS-enriched porins indicated that at 75 mV, the OC901 porin's main conductance peak was decreased to one-third the size of the main peak measured at 25 mV. The shift to one-third the conductance is similar to results obtained with an OmpC deletion mutant porin (4), and is presumed to represent the decrease in cooperativity of trimeric channels and the independent opening and closing of monomers within the aggregate. These results are also similar to those obtained by Dargent *et al.* (13) for the PhoE porin. They showed that PhoE functions as monomers and not trimers at high membrane potentials. The OmpF and OC904 porins did not show a shift to smaller size and loss of cooperativity with increased potential. We conclude that the amino acid change from Arg to Cys in the OC901 OmpF porin decreases the cooperativity of channel activity and may decrease subunit interactions.

Not only is the cooperativity of channel activity altered in the OC901 porin, but the stability of the open conformation is changed. The LPS-enriched OC901 porin is as stable to denaturation as the wild type OmpF porin, but its stability in the closed state at high voltages is greater than the wild type. However, when LPS was removed, the number of closing events for the wild type OmpF increased, but there was no change in the number of closings for the OC901 porin. We propose that the loss of LPS sensitizes the OmpF wild type porin to gating while gating of the OC901 porin is maximal even in the presence of LPS. The alterations induced by removing LPS from the wild type porin indicate that the OmpF porin structure and function are influenced by the presence of LPS.

One other parameter which may affect the stability and channel-forming activity of the porins is the detergent bound to the proteins. In all stability and structure/function experiments, porins were solubilized in 1% SDS. Markovic-Housley and Garavito (25) have shown that, in the presence of SDS, the OmpF porin from *E. coli* B has a decreased stability to high temperature and low pH when compared to protein in the non-ionic detergent β-octyl glucoside. The OmpF porins used in this study also showed a decreased temperature stability when solubilized in SDS compared to β-octyl glucoside. Solubilizing porins in high SDS concentrations has been shown to remove excess LPS on porin (4), presumably by SDS competing for LPS binding sites on the protein. When LPS is removed and replaced by SDS, the protein's stability to high temperature and low pH appears to decrease due not only to the loss of LPS, but also because of the presence of SDS. Therefore, the altered channel activities of LPS-depleted porins may also reflect the presence of SDS bound to porins. We are presently looking at LPS-depleted porins solubilized in β-octyl glucoside to determine whether the altered channel sizes and activities are a result of removing LPS or are due to the presence of SDS.

Another factor which influences the activity of porin channels *in vitro* is an increased voltage potential across the membrane. Whether porins are voltage gated *in vivo* and what the physiological significance of gating might be is still debated. We have shown for two different mutant porins, OmpC Δ103-110 (4) and OmpF Arg 82 to Cys, that channels exist in a closed conformation more often at high membrane potential than at low potentials. Voltage gating may not be significant under laboratory culture conditions, but under extremely harsh environmental conditions the ability to open and close porin channels may be a survival mechanism for the bacteria. We propose that a

voltage sensor exists within the structure of the bacterial pore and that this sensor involves charged amino acid residues. Recently, Mirzabekov and Ermishkin suggested that the mitochondrial porin contains a number of negative and positive charges which are part of a sensor, since modifying charged residues alters the voltage dependence of channel gating (34).

Although OC901 porin is more stable to high temperature and low pH than the OC904 porin, it is able to open and close more frequently and exhibits decreased cooperativity of channel activity at high membrane potentials. The OC904 porin, which is structurally less stable, remains in the open porin conformation and shows little voltage gating. We believe that the Arg residue at position 82 is important for voltage gating of the porin and may be a component of the voltage sensor in the protein, whereas the Asp residue at position 113 is not important for voltage gating, but is important for the stability of the protein. Amino acids 82 and 113 are presumed to be located on the porin's exterior surface and not in a transmembrane segment according to two different models of OmpF structure (12,35). Thus, the regulation of channel size and voltage gating may occur at the entrance to the channel and not in the interior. We are presently analyzing other mutant porins to determine whether other external residues are important for voltage regulation, for determining channel size, and for stability.

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CHAPTER 6

Summary and Perspectives

From the onset of this research project there has been speculation and debate concerning the structure/function relationship of porins. One critical concern was the importance of the interactions between porin and LPS. Previous studies had described the complete removal of LPS from porins without loss or alteration in channel forming activity (1). Attempts to reproduce these findings lead to the discovery of a lipid A-like component tightly associated with the proteins (Chapter 2). The lipid A recovered from heat denatured trimers was stoichiometric, but when the trimeric complex was not denatured, the lipid A component was masked or buried and inaccessible to anti-lipid A antibody detection. These findings indicated that LPS, or more specifically the lipid portion of LPS, could not be completely removed from the trimeric complex while in an aggregate. Therefore, LPS may be essential for the three dimensional structure.

Whether greater than stoichiometric amounts of LPS enhance or affect channel forming activity is still in question. LPS and other outer membrane components are required for proper porin trimerization *in vitro* (2). Evidence suggests that LPS stabilizes the isolated OmpF porin to high temperatures and low pH, and also affects OmpF gating at high voltages in the BLM (Chapter 5). Removing LPS physically destabilizes OmpF allowing for increased channel closing at high transmembrane potentials. How LPS modulates these activities is unknown. Characterization of the lipid A-like component tightly associated to porin has yet to be completed. An understanding of the chemical composition of the molecule(s) involved in porin:LPS interactions will be required to model the sites of interaction.

Both wild type OmpC and an altered OmpC with an eight amino acid deletion showed a slight pH and temperature stabilization when greater than stoichiometric

amounts of LPS were present (Chapter 4). However, the midpoint of DSC denaturation curves of the OmpC porins were not altered by LPS removal as were the OmpF porins' curves. These data indicate that OmpC, in the absence of LPS, is more stable than OmpF. The stability could be a result of the larger overall charge on the OmpC porin compared to OmpF. Stronger protein:protein interactions between OmpC subunits may allow for a more rigid structure. Higher temperatures are required to break these tight inteactions, and LPS provides no increased stability.

Amino acids critical for the structural stability and functionality of both porins were examined using deletion and point mutations. The OmpC porin with the eight amino acid deletion showed three properties distinct from the wild type protein (Chapter 4); 1) the protein was less stable to high temperatures, 2) the porin had an increased channel size, and 3) at 50 and 75 mV the porin was voltage regulated, with a channel conductance one-third the conductance size at 25 mV. The results suggest that deleting eight amino acids unmasks or increases the sensitivity of a voltage sensing mechanism in the porin channel since the wild type porin was not voltage regulated at this low of a voltage. A voltage sensor comprised of charged amino acid residues has been proposed for the sodium channel (3) and VDAC (4,5) from eukaryotes. The deleted region from this OmpC contains two negatively charged amino acid residues which, when removed, could increase the sensitivity of the voltage gating mechanism.

The OmpF point mutants showed properties similar to the OmpC deletion mutant. The diffusion rates of maltose in liposome swelling assays was significantly higher with the mutant isolates than the wild type isolates suggesting that the mutations stabilize an increased porin channel size (Chapter 5). The amino acid substitution, Asp113-Gly,

altered the porin's temperature and pH stability, whereas the Arg82-Cys substitution increased voltage gating, compared to the wild type OmpF. The decrease in stability upon removing a charged residue implies a loss of protein:protein interactions. Therefore, Asp 113 may help stabilize the tertiary or quaternary structure of porin. Alternatively, the Arg82-Cys mutation is not destabilizing but the channels of the mutant close more frequently at high transmembrane potentials, indicating the involvement of Arg 82 in the voltage gating mechanism. A similar change in voltage sensitivity was found with an Arg82-Ser mutation (data not shown). Furthermore, Delcour *et al.* (6) have reported increased voltage regulation in OmpC when Arg 37 was substituted with Cys. These modifications provide information concerning residues critical in the structure/function modulation of porin channels. Future experiments of importance include isolating and characterizing porins with point mutations at different amino acid residues to further define residues that are critical for porin structure and function.

Environmental factors that may trigger gating of porins or alterations in channel size can be monitored using the BLM apparatus. Preliminary evidence suggests that altering the pH of the bathing solution surrounding porins in a BLM from 6.5 to 8.5 increases the conductance size two-fold for the wild type OmpF porin. Titration of a histidine residue has been implicated as a possible mechanism for the stabilization of this increased channel size. The OmpC, OmpF, and PhoE porins all have one conserved His residue at position 21. This residue is proposed to be on the outer surface of the channel forming domain of the porins (7); thus, changes in pH within a physiological range may have dramatic consequences on the activity of the channels. In collaboration with Dr. Spencer Benson, at the University of Maryland, we propose to change this His residue,

by site-directed mutagenesis, to either a Gly or Cys residue. This will enable us to determine whether His is important for pH induced alterations in porin structure and function. We propose using a His-Gly substitution since this essentially eliminates the side chain on the amino acid, and this type of substitution has already been found in the spontaneous mutants, e.g. Asp113-Gly (8). Alternatively, a His-Cys substitution would increase the pKa of the side chain approximately 2 pH units. A Cys mutation, as shown before, can substitute for Arg at positions 82 and 132 (8). Determining amino acids important for voltage sensing and channel activity will be important for understanding the regulation of these proteins *in vivo*.

The presence of multiple species of porins with similar structural and functional properties raises important questions concerning the role porins play in the physiology of the organism. Since OmpC has a narrower channel than OmpF, the bacteria may regulate the synthesis of these porins to create a low level of resistance to toxic compounds by reducing their rates of uptake (9). This "long-term" defense mechanism decreases the permeability of toxic compounds while maintaing the permeability of nutrients. Investigators have shown that mutant strains lacking the OmpF porin are slightly more resistant to chloramphenicol, tetracycline, and β-lactams due to the lower diffusion rates through the OmpC porin (9). Additionally, porins are expressed under different culture conditions as the bacteria "sense" their environmental surroundings (10,11). Under "harsh" conditions of the mammalian gut, where temperatures and osmotic strengths are high, the more stable OmpC porin is produced (9). The narrower channel protects the bacterium from inhibitory substances in the body, but allows for bacterial growth since nutrients are abundant. Under conditions of low temperature and osmolarity, typical

environments found outside the body, the wider diameter OmpF porin would be beneficial for scavenging nutrients from dilute solutions in the environment (9). The periplasmic concentration of nutrients is kept low due to efficient active transport systems in the cytoplasmic membrane (9). Under these circumstances the larger OmpF is necessary to allow efficient nutrient uptake for bacterial survival.

A "short-term" regulatory defense mechanism that may be critical for bacterial survival is regulated channel closing. Since porins have been shown to exist in an open and closed conformation *in vitro*, this primary defense mechanism warrants consideration. Although this mechanism has not been proven *in vivo*, regulated porin closings may allow bacteria to move away from toxic compounds without being adversely affected. Bacteria have been shown to have both chemotactic and osmotactic properties, swimming away from noxious compounds at high, deleterious concentrations (12,13). If concentrations of toxic compounds remains high, then regulation of OmpC/OmpF expression may limit the rate of diffusion of the toxic compounds.

The regulation of OmpC/OmpF expression appears to be physiologically relevant, and the mechanism is being analyzed; however, the assembly of porin trimers into the outer membrane is quite complex. Ichihara and Mizushima (14) have shown that OmpC and OmpF monomers can be crosslinked to form heterotrimers of porin. Recently, Gehring and Nikaido (15) have shown that heterotrimers of OmpC, OmpF, and PhoE can be isolated and appear to form as a random mixing of subunits. The sites of interaction between heterotrimers are thought to be at conserved amino acid residues in porins. We have recently isolated porin from a strain producing a wild type OmpF protein and a mutant OmpC containing an eight amino acid deletion, in which no heterotrimers were

formed (data not shown). The results suggest that the two porins have different subunit conformations that are not readily recognized by each other.

On the other hand, strains which produce a wild type OmpC protein and a small deletion in OmpF appear to be altered in OmpC porin assembly, producing OmpC dimers (Chapter 3). This finding implies that OmpC/OmpF heterotrimers were formed in the outer membrane but were unstable to the purification procedures. The instability of heterotrimers suggests improper assembly of porin because of the lack of OmpC/OmpF interactions between subunits. We propose that the mutant OmpF porin subunit dissociates from an OmpC dimer in the heterotrimer, resulting in a stable and active OmpC dimer. Dimers have been shown to be intermediates in the synthesis pathway of OmpF *in vivo* (16) and PhoE *in vitro* (2); furthermore, the OmpF dimer is stable to high temperature, similar to the isolated OmpC dimer.

The physiological importance of heterotrimer formation is not known. Production of OmpC/OmpF heterotrimers may allow bacteria to limit the rate of diffusion of materials into cells through OmpC, yet allow larger nutrients into cells through OmpF. Under harsh environmental conditions the OmpF protein would be unstable, but in the presence of OmpC, these OmpF subunits in heterotrimers may be stabilized. Heterotrimer production then may allow for a range of responses to extreme stress conditions.

In collaboration with Dr. Spencer Benson we plan to construct bacterial strains which produce a wild type and mutant OmpF porin or a wild type OmpC and mutant OmpC porin to determine if heterotrimers are formed. Using gel electrophoresis and DSC we can determine whether homotrimers or heterotrimers are being produced. Additionally, we would like to see if dimers of OmpF or OmpC are produced in any of these

strains, as they are in OmpC wild type/OmpF deletion mutants. Furthermore, analysis of heterotrimers will allow us to look at the influence of different subunits on each other. Since the mutant porins should have larger channel sizes, we can readily determine heterotrimer formation using the BLM. Also, the cooperativity and gating of heterotrimers can be determined. These studies will allow us to answer questions concerning the assembly of porins and the stability and functionality of porin heterotrimers.

Finally, other important future studies include raising site-directed antibodies to particular regions of the porins that are thought to be exposed on the outer surface. By adding the antibodies to live cells or to porins incorporated into BLM, we can determine whether the channels can be altered or closed. Also, the structure of the mutant porins can be analyzed with FTIR and compared to the wild type to quantitate differences in the percentages of α-helix and β-structure. Since porin normally lacks Cys, the recent isolation of an OmpF mutant porin Arg82-Cys will enable investigators to heavy metal label this site for high resolution X-ray crystallographic structure analysis. However, analysis of different porin conformations stabilized by environmental conditions is not accessible to X-ray studies but can be analyzed using FTIR.

Although some aspects of porin structure and function have been revealed in this study, many questions remain concerning porin:porin and porin:LPS interactions. Further analysis of porins with known amino acid substitutions and the comparison of their structure and function to wild type porins will be critical for a more complete understanding of the functioning of this channel-forming protein.

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