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

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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 temperature 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 by using differential scanning microcalorimetry. Both the proteins' secondary structures, monitored by circular dichroism, were high in β-sheet content, but the spectra were slightly different in their crossover points as well as their minima. When the proteins were reconstituted and channel activity was assayed by 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 mV, than the wild-type sample. The closing events of the mutant porin were predominantly of monomer size. The channels detected by 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.

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. (1988) 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 that appear to alter pore properties (Benson & Decloux, 1985; Misra & Benson, 1988a,b; Benson et al., 1988). One such OmpF deletion mutant, PLB3255, produces stable, functional, wild-type OmpC porin dimers (Rocque & McGroarty, 1989).

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 (Schindler & Rosenbusch, 1978; Schindler & Rosenbusch, 1981; Xu et al., 1986; Dargent et al., 1986); however, other investigators have indicated that voltage gating of porins does not occur (Benz et al., 1978; Hancock, 1987; Lakey et al., 1985). Dargent et al. (1986) have reported that the PhoE protein trimers contain three pores that fluctuate between open and closed states and increasing membrane potential shifts the equilibrium to the closed state. In addition, Xu et al. (1986) 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 (1988a,b) 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 accommodate large maltodextrins, Misra and Benson (1988b) 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 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 (Benson & Decloux, 1985) and RAM122 (Misra and Benson, 1988a), both lacking the *lamB* gene. PLB3255 produces wild-type OmpC porin and an OmpF porin with a short deletion, Δ115–129; RAM122 lacks OmpF porin and produces an OmpC that contains an eight amino acid deletion, Δ103–110. The cells were grown in 1% tryptone, 2.4% yeast extract, 4% glycerol, and 0.1 M potassium phosphate, pH 7.0, at 37 °C and harvested in the late logarithmic phase. Porins were isolated by the method of Lakey et al. (1985) with slight modifications (Rocque & McGroarty, 1989). The final preparation was suspended in either 2% or 30% sodium dodecyl sulfate (SDS[1]) and 10 mM Tris-HCl,

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[1] Abbreviations: SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; LPS, lipopolysaccharide; BLM, bilayer lipid membrane; KDO, 2-keto-3-deoxyoctulosonic acid.

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pH 7.4. The protein was then applied to a Sephadex G-200 gel filtration column (2.5 × 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 by using the buffer system of Laemmli (1970). 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 (1982).

Temperature denaturation studies were performed by heating the proteins in 1% SDS and 10 mM Tris-HCl, pH 6.8, for 5 min 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 min prior to neutralization with 0.1 N NaOH. The proteins were then loaded onto 12% SDS gels and examined by 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, and 0.2% sodium azide, pH 6.8, and spun at 100000*g* for 30 min to remove contamination. Calorimetric analysis was performed on a Microcal 2 scanning microcalorimeter (Microcal Inc., Amherst, MA) at a scanning rate of 90 deg/h. Proteins were scanned from room temperature to 100 °C with the resuspension buffer as a reference. Thermodynamic characterization and deconvolution curve fitting were performed by using the Deconv. program (Microcal Inc.).

Circular Dichroism Spectroscopy. Circular dichroism measurements were made on a JASCO J-500C spectropolarimeter with a 0.5-mm path length quartz cell. Porin samples were suspended at 0.2 mg/mL in 1% SDS and 10 mM sodium phosphate, pH 7.0, and scanned from 350 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 (1986), using the X-ray structure matrix given by Manavalan and Johnson (1987).

Liposome Swelling Assays. The liposome swelling assay was performed as described by Nikaido and Rosenberg (1983) with slight modifications (Rocque & McGroarty, 1989). The internal liposome solution of 12 mM stachyose, 4 mM sodium NAD, and 1 mM imidazole–NAD buffer, pH 6.0, was replaced with 17% dextran (w/v), molecular weight 9400, in 5 mM Tris-HCl, pH 7.5, to accommodate 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 by using the procedure of Yoshihara et al. (1988).

BLM Analysis. Porins were prepared for BLM analysis as previously described (Rocque & McGroarty, 1989). Briefly, porins were suspended in 1% octyl glucoside or 1% SDS and 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 diphytanoylphosphatidylcholine (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, and adjusted to defined pH values. A constant voltage of 25, 50, or 75 mV was applied across the membrane using a 1.5-V 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 angstroms, versus 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 for LPS-depleted porins suspended in 1% octyl glucoside.

Miscellaneous Chemical Assays. Protein was quantitated by the BCA protein assay (Pierce Chemical Co.). Lipopolysaccharide was quantitated either by KDO analysis using the thiobarbituric acid assay (Droge et al., 1970) or by phosphate analysis according to Ames and Dubin (1960).

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, and 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 (Rocque et al., 1987; 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 (Rocque & McGroarty, 1989). 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 strained for LPS (Rocque et al., 1987), showed no free LPS.

Temperature and pH Stability of the Porin. The temperature stability of the mutant and wild-type porins was examined by two different methods, SDS–PAGE and differential scanning microcalorimetry. Unheated OmpC porins retain their trimeric structure in SDS–PAGE (Yu et al., 1979); therefore, protein denaturation can be monitored by heating the porin to different temperatures and observing the formation of monomers. The results from both techniques showed that for LPS-depleted samples the mutant OmpC porin was less heat stable and denaturated 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

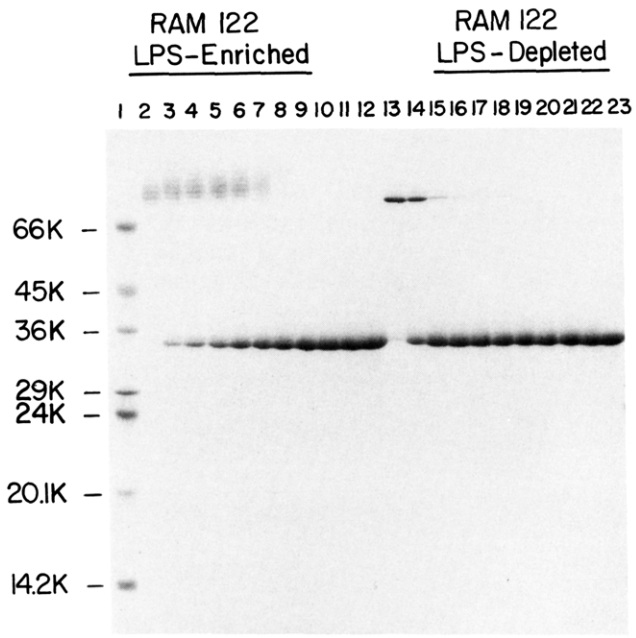

FIGURE 1: A 12% acrylamide–SDS gel depicting the thermal stability of RAM122 porin. The porin was heated for 5 min at each temperature and then cooled to room temperature before loading onto the gel. Lane 1 contains the molecular weight markers. Lanes 2–12 contained LPS-enriched porin and lanes 13–23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

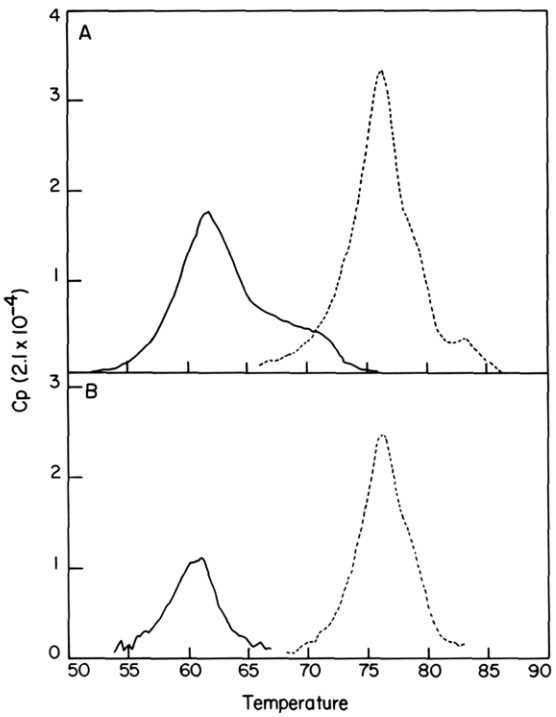

FIGURE 2: Differential scanning microcalorimetry showing temperature (°C) versus heat capacity (C_p, cal K^{-1} mol^{-1}) for (A) wild-type OmpC porin enriched in LPS (---) and RAM122 porin enriched in LPS (—) and (B) PLB3255 wild-type OmpC porin depleted of LPS (---) and RAM122 porin depleted of LPS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

addition, the overall enthalpy of the denaturation of the LPS-depleted porins was almost 2-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 and 2). Also, the enthalpy of denaturation was higher for both proteins if LPS was bound, although the main transition temperature was unchanged (Figure 2).

The stability of LPS-enriched and LPS-depleted porins in 1% SDS to low pH values was also analyzed by 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 unit when the proteins were solubilized in 1% octyl glucoside instead of SDS (data not shown).

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 (Rosenbusch, 1974; Markovic-Housley & Garavito, 1986). However, there were slight differences in both the minimum ellipticity and crossover point between the two proteins. The wild-type porin had a crossover point at 204 nm compared to the mutant porin at 207 nm. In addition, the minimum ellipticity 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 by the method of Compton and Johnson (1986) but with the X-ray structure matrix given by Manavalan and Johnson (1987). 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 (Rosenbusch, 1974); 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 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 3 min. 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 Da (daltons) whereas that of the wild-type porin was 350 Da. These results, for the wild-type OmpC porin, are consistent with those obtained by Nikaido and Rosenberg (1983). 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

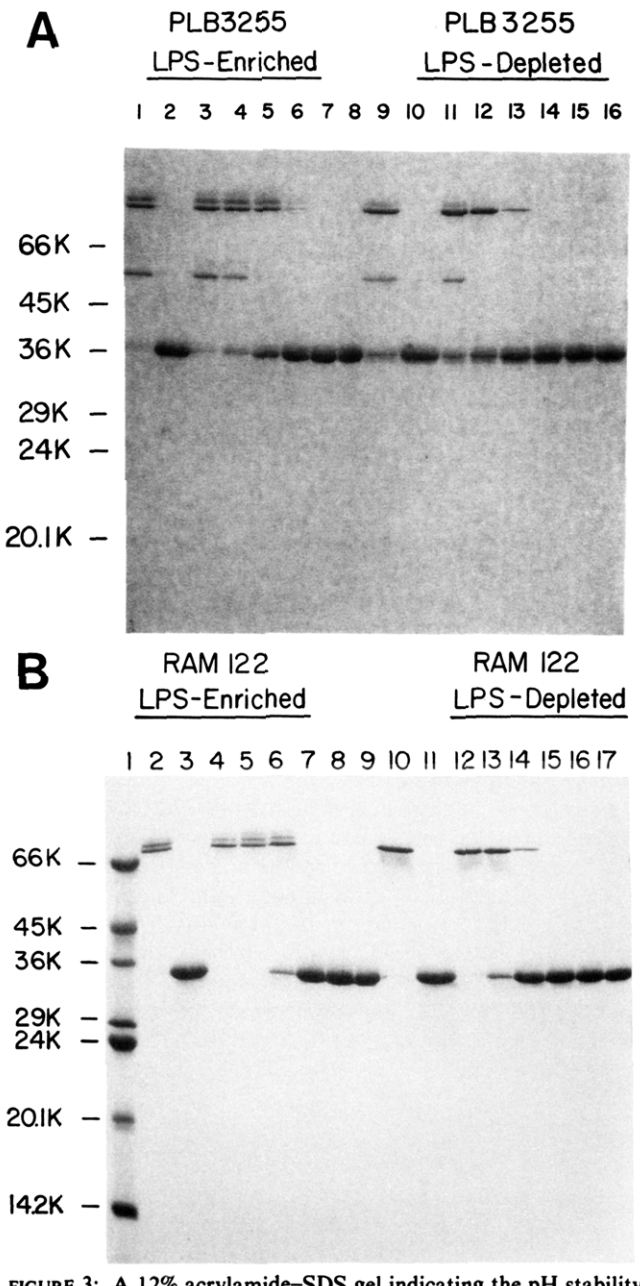

FIGURE 3: A 12% acrylamide–SDS gel indicating the pH stability of wild-type OmpC porin and RAM122 OmpC porin enriched and depleted in LPS. (A) Lanes 1–8 contain LPS-enriched wild-type OmpC and lanes 9–16 contain wild-type OmpC, depleted of LPS. Lanes 1 and 9 contain unheated porin, pH 6.8; lanes 2 and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

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 angstroms), is a function of the ionic strength of the bathing solution. The size parameter is plotted against the probability, P, of an event

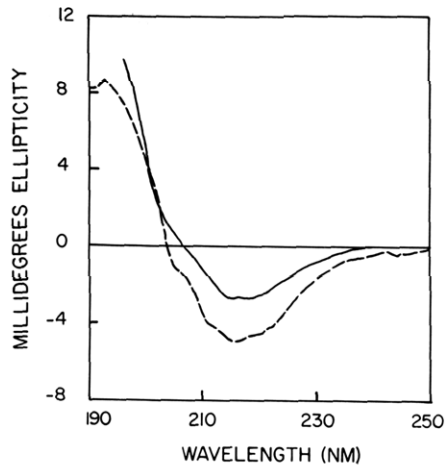

FIGURE 4: Circular dichroic spectra of LPS-depleted wild-type (---) and RAM122 (—) OmpC porins. Samples at 0.2 mg/mL were suspended in 10 mM sodium phosphate, pH 7.0, containing 1% SDS. The measurements were performed at room temperature on a JASCO J-500C spectropolarimeter with a 0.5-mm path length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

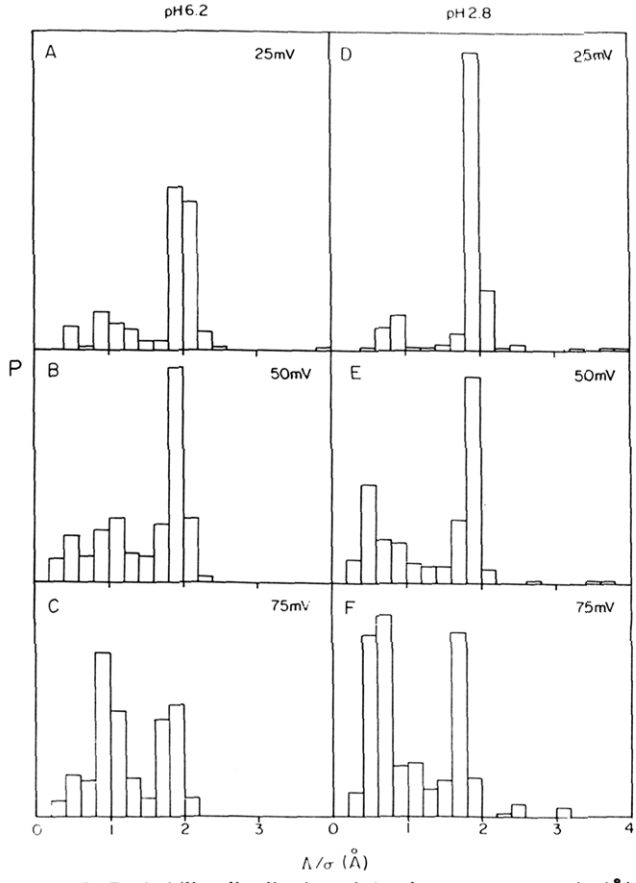

FIGURE 5: Probability distribution of the size parameter, Λ/σ (Å), for wild-type OmpC porin as measured in bilayer lipid membranes. The LPS-depleted porin solubilized in 10 mM Tris-HCl and 1% octyl glucoside, pH 7.5, was added to the bathing solution at a final concentration of 50 ng/mL. Electrical conductance across the membrane was measured by 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.

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 aggre-

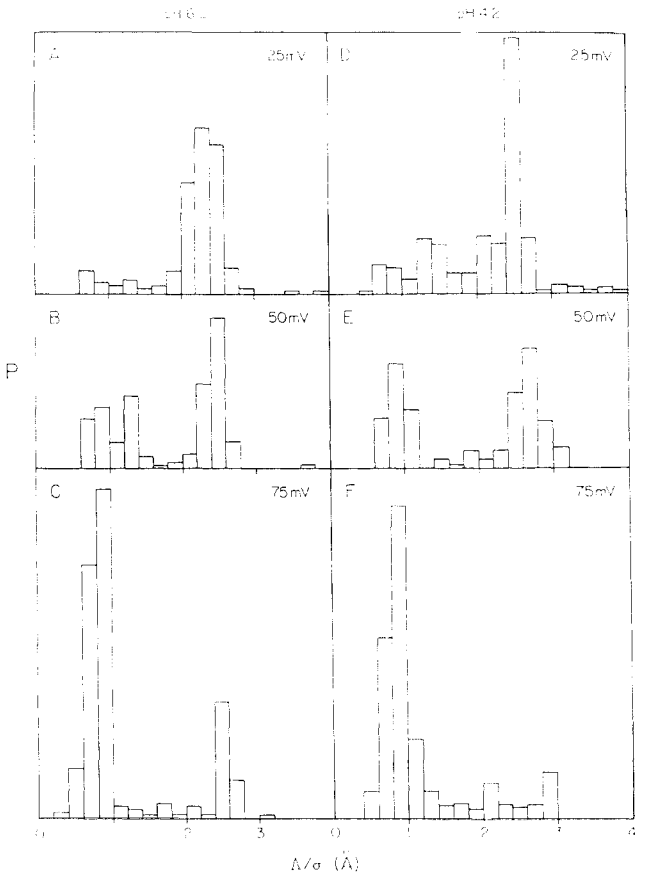

FIGURE 6: Probability distribution of the size parameter for RAM122 OmpC porin as measured in bilayer lipid membranes. All parameters are similar to those in Figure 5 except that for D, E, and F porins were suspended in a bathing solution of pH 4.2.

gate. For the wild-type protein this main peak was centered at approximately 1.9 Å (Figure 5A,D) with a 0.5 M NaCl bathing solution, whereas for the mutant porin this value was 2.5 Å (Figure 6A,D). This larger channel size is consistent with the results obtained with the liposome swelling assay.

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 5-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,F), 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 I). 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 monomeric size. The loss of cooperativity of trimeric channel activity appears to be voltage dependent and conditions that stabilize the closed state of the porins, i.e., low pH and high voltage, also decrease the cooperativity of the conformational changes.

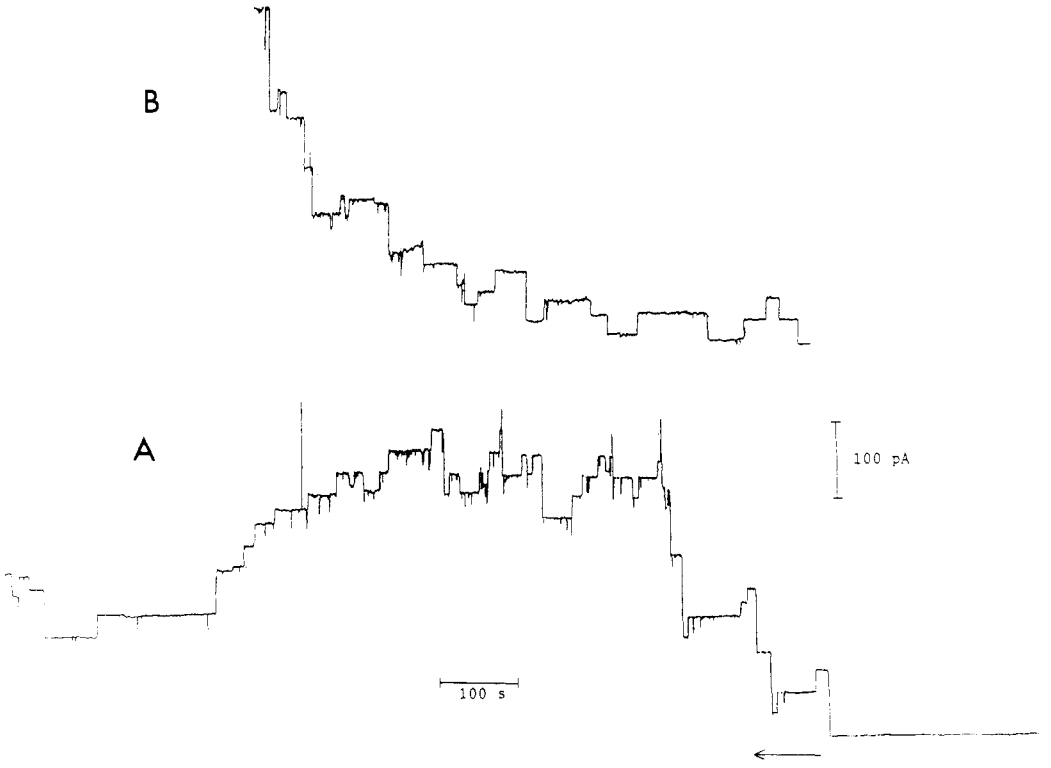

FIGURE 7: Scan of the conductance across a bilayer lipid membrane containing RAM122 OmpC porin in a bathing solution of pH 4.2 and measured at a transmembrane potential of 75 mV. The arrow at the bottom right indicates the direction of the scan. The scan in panel B is a continuation of the scan in panel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

Table I: Stability of the Closed State of LPS-Depleted Porins as a Function of Voltage and pH

porin	pH	voltage (mV)	no. of events measd	% closings[a]
	6.2	25	159	4
		50	210	10
		75	221	9
wild-type OmpC	2.8	25	163	5
		50	188	14
		75	279	28
	6.2	25	193	4
		50	185	24
		75	289	45
deletion mutant	4.2	25	239	7
		50	189	28
		75	531	51

[a] Number of closing events/total number of events × 100.

Discussion

RAM122 OmpC porin, containing an eight amino acid deletion in its primary sequence, appears to have a larger channel size than the wild-type porin. This protein was isolated, and the structural and functional properties were compared to those of 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 (Rocque et al., 1987). 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 (1980) 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 (1989), analyzing the electron density of PhoE trimers, has suggested that an LPS molecule is located at the axis of 3-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 at a pH between 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 (1987) and Schindler and Rosenbusch (1984) 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 (Markovic-Housley & Garavito, 1987).

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 (1974). Both OmpC proteins contained similar amounts of β-structure. Porins in general are proposed to exist either in a β-barrel configuration or as stacked sheets and no large segments of α-helix are thought to be present (Nabedryk et al., 1988; Kleffel et al., 1985). Analysis of our circular dichroism spectra using the X-ray structure matrix given by Manavalan and Johnson (1987) 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 by using two artificial membrane systems. The liposome swelling technique of Nikaido and Rosenberg (1983) was used to determine the size-exclusion limit of the channels of the two porins. Misra and Benson (1988b) 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 accommodate 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 (1988b). 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 octyl glucoside 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 (1988b) 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 (Misra & Benson, 1988b) as well as the PhoE channel (Tommassen et al., 1985).

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 (Markovic-Housley & Garavito, 1986; Xu et al., 1986). Similarly, the PhoE (Dargent et al., 1986) and OmpF (Schindler & Rosenbusch, 1981; Xu et al., 1986) 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 in 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 those of 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 (Stock et al., 1977), are too low to induce gating. In addition, Sen et al. (1988) 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. (1977) 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. (1988) 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 that 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 that may be critical for voltage gating.

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Limited Proteolysis of Synapsin I. Identification of the Region of the Molecule Responsible for Its Association with Microtubules

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ABSTRACT: Synapsin I is a highly asymmetric neuronal structural phosphoprotein implicated in the regulation of neurotransmitter release probably by the multiple interactions it can contract with membranous and cytoskeletal elements of the neuronal cell. In order to locate the region(s) of synapsin I responsible for its association with microtubules, we have first studied synapsin I limited digestion by trypsin. The resulting polypeptides were localized in the synapsin I molecule by using three different criteria: their kinetics of appearance, their collagenase sensitivity, and the presence of the synapsin phosphorylation site 1 (cyclic AMP dependent). Synapsin I digestion kinetics are not affected by phosphorylation at this site. Analysis of the ability of various synapsin I tryptic fragments in mixture to cosediment with microtubules shows that a 44-kDa fragment corresponding to the NH_2-terminal hydrophobic head of the molecule contains a binding site for polymerized tubulin. This fragment competes with native synapsin I for binding on microtubules. None of the polypeptides belonging to the tail region of synapsin I (COOH-terminal half of the molecule) were found to cosediment with microtubules.

Synapsin I is a neuronal phosphoprotein implicated in the regulation of neurotransmitter release [for a review, see DeCamilli and Greengard (1986)]. It is concentrated in the presynaptic terminals where it is associated with the cytoplasmic surface of small synaptic vesicles (DeCamilli et al., 1983; Huttner et al., 1983; Navone et al., 1984). Synapsin I is a major substrate for cyclic AMP dependent and for (calcium, calmodulin)-dependent protein kinases which

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