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Biophysical Characterization of OprB, a Glucose-Inducible Porin of *Pseudomonas aeruginosa*

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OprB, a glucose-inducible porin of *P. aeruginosa,* was characterized by black lipid bilayer analysis and circular dichroism spectroscopy. Black lipid bilayer analysis of OprB revealed a single-channel conductance of 25 pS, the presence of a glucose binding site with a K_s for glucose of 380 ± 40 mM, and the formation of channels with a strong selection for anions. Analysis of *P. aeruginosa* OprB circular dichroism spectra revealed a high ß sheet content (40%) which is within the range of that determined for other porins. Values obtained from black lipid bilayer analysis were compared to those previously obtained for OprB of *P. putida* [Saravolac *et al.* (1991). *J. Bacteriol.* **173,** 4970–4976] and indicated extensive similarities in the single-channel conductance and glucose-binding properties of these two porins. Immunological and amino terminal sequence analysis revealed a high degree of homology. Of the first 14 amino terminal residues, 12 were identical. A major difference between the two porins was found in their ion selectivity. Whereas *P. aeruginosa* OprB is anion selective, *P. putida* OprB and other carbohydrate selective porins are known to be cation selective.

KEY WORDS: *Pseudomonas aeruginosa;* OprB; glucose porin; black lipid bilayer; circular dichroism.

INTRODUCTION

Pseudomonas aeruginosa possesses two inducible pathways for the uptake of glucose: a high-affinity pathway with a K_m for glucose of 8 μM and a low-affinity pathway with a K_m of approximately 2 mM (Midgley and Dawes, 1973). The low-affinity pathway is known to be induced by growth on glucose or glycerol and involves the extracellular conversion of glucose to gluconate and 2 ketogluconate by glucose dehydrogenase and gluconate dehydrogenase, respectively (Midgley and Dawes, 1973; Lessie and Phibbs, 1984). The high-affinity pathway is known to be induced by glucose, galactose, 2-deoxyglucose, and methyl-α-glucoside (Midgley and Dawes, 1973; Mukkada *et al.,* 1973). This pathway transports glucose directly into the cytoplasm with no chemical alteration of substrate. Both uptake systems are repressed by growth on TCA cycle intermediates such as succinate, citrate, or malate (Lessie and Phibbs, 1984).

Two proteins, potentially involved in the high-affinity glucose transport pathway, have been identified. OprB (formerly D1) is believed to form a water-filled channel spanning the outer membrane, facilitating the diffusion of glucose into the periplasm (Hancock and Carey, 1980; Trias *et al.,* 1988). Glucose binding protein (GBP) is a periplasmic protein believed to transport glucose across the periplasm to yet to be identified inner membrane transport proteins (Stinson *et al.,* 1977).

In the present study we have characterized the OprB protein of *P. aeruginosa.* Saravolac *et al.* (1991) previously purified and characterized a protein from *P. putida* which they called OprB based on evidence suggesting it to be homologous to OprB of *P. aeruginosa* (both proteins were glucose-inducible, showed similar heat modifiability proper-

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ties, and had similar amino acid compositions). However, the methodologies which have been used to examine the functional properties of the two OprB proteins differed considerably and it is difficult to assess the extent of similarity between the two channels. *P. putida* OprB was examined using black lipid bilayer analysis and characterized as a glucose-specific porin possessing a small constricted channel (Saravolac *et al.*, 1991). In contrast, Trias *et al.* (1988) proposed *P. aeruginosa* OprB to be a glucose-specific pore but based their results on the liposome-swelling assay. The results of their analysis, and a similar one by Hancock and Carey (1980), were consistent with the *P. aeruginosa* channel being quite large since substantial permeation of solutes larger than disarcharides was observed. This suggested that the relationship between the two OprB proteins could be similar to that between the maltodextrin-specific porin LamB and the sucrose-specific porin ScrY (Hardesty *et al.*, 1991). Both are sugar-specific porins found in enteric bacteria, share some amino acid homology, and possess a similarly constructed binding site with a length of 5 glucose residues, yet ScrY forms a larger channel and shows some characteristics of a general diffusion pore (Schülein *et al.*, 1991). These authors attributed the general diffusion properties of ScrY to an additional 70 amino acids at the N terminus of the protein. A similar situation could also occur with OprB of *P. aeruginosa* as the *P. aeruginosa* protein is 4500 Da larger than *P. putida* OprB (Saravolac *et al.*, 1991).

To resolve the potential differences between the two OprB proteins, we have examined *P. aeruginosa* OprB using methodology identical to that used to characterize *P. putida* OprB. This provided a more detailed characterization of *P. aeruginosa* OprB and allowed a direct comparison between the channel-forming properties of the two proteins.

MATERIALS AND METHODS

Bacterial Strains and Media

P. aeruginosa H673, *P. putida* ATCC 12633 (American Type Culture Collection, type strain), and *E. coli* LE392 (*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*) were used throughout. *P. aeruginosa* H673 is a PAO1 strain (Holloway *et al.*, 1979) containing a Tn501 insertion in a gene required for expression of outer membrane protein OprD and as a result fails to produce OprD (Huang *et al.*, 1992). We found that OprD can contaminate OprB preparations to some extent, and use of this strain simplified purification of OprB. Rich media for culturing cells was Luria broth (LB) (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl). LB media was supplemented with 15 μg/ml $HgCl_2$ when culturing H673. Basal medium 2 (BM2) minimal media (Hancock and Carey, 1979) supplemented with 0.4% (w/v) glucose was used to induce production of OprB in *P. aeruginosa* and *P. putida*. M63 media supplemented with 0.2% (w/v) maltose was used to induce production of LamB in *E. coli*.

OprB Purification

Purification of *P. aeruginosa* OprB was based on a protocol developed by Hancock and Carey (1980). Fourteen grams of cells (wet wt.) were routinely used for OprB purification. Cells were harvested by centrifugation at 10,000 × *g* at 4°C and resuspended in 10 mM Na_3PO_4 (pH 7.4). The cell suspension was French pressed at 15,000 PSI and intact cells removed by a subsequent centrifugation step at 3500 × *g* for 10 min. Cell envelopes were collected by centrifugation at 145,000 x *g* for 1 hr and resuspended in 8 ml H_2O. Resuspended cell envelopes were layered on a two-step sucrose gradient composed of 70% (w/v) sucrose and 54% (w/v) sucrose. Following centrifugation for 18 hr at 75,000 × *g*, the lower outer membrane band was collected, washed free of sucrose, and resuspended by sonication in 2% (v/v) Triton X-100, 20 mM Tris-HCl (PH 8.0) and left at 4°C for 30 min. Outer membranes were collected by centrifugation at 184,000 × *g* for 1.5 hr. This step was repeated. Finally, OprB was dissociated from outer membrane fragments by resuspension in 8 ml 2% (v/v) Triton X-100, 10 mM EDTA, 20 mM Tris-HCl (pH 8.0). Outer membrane fragments were pelleted by centrifugation at 184,000 x *g* for 1.5 hr and the supernatant containing OprB was loaded onto a 1 × 20 cm DEAE-Sephacel column equilibrated with 0.1% (v/v) Triton X-100, 10 mM EDTA, 20 mM Tris-HCl (pH 8.0) (TTE). The column was washed with 20 ml TTE, then 25 ml of 0.1 M NaCl in TTE, and OprB eluted using 80 ml of a 0.1–0.25 M NaCl gradient in TTE. Fractions were screened by Coomassie Blue-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing OprB were pooled and concentrated in an ultrafiltration cell with a 30,000-Da cutoff

membrane (Diaflo PM30, Amicon, Beverley, Massachusetts). OprB purified in this manner was used for black lipid bilayer analysis. For circular dichroism (CD) analysis, concentrated OprB was precipitated with ice-cold acetone (60% final concentration), washed with 80% (v/v) ethanol, and resuspended overnight at 4°C in TNS (0.1% SDS [w/v], 100 mM NaCl, 10 mM Tris-HCl [pH 8.0]) (Schlaeppi *et al.*, 1985). For polyclonal antibody production, OprB was further purified by electroelution from SDS-PAGE gels with the Bio-Rad 422 electroeluter (Bio-Rad Laboratories, Richmond, California) using a 12,000-Da cutoff membrane cap.

Black Lipid Bilayer Analysis

Black lipid bilayer analysis was conducted using previously described protocols (Benz *et al.*, 1985, 1987). Membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidycholine in *n*-decane. For single-channel experiments OprB was used at a concentration of approximately 1 ng/ml. For zero-current membrane potential measurements, membranes were formed in a 100 mM salt solution and OprB added so that conductance increased approximately 100- to 1000-fold within 20 to 30 mins. The voltage was then switched off and the salt concentration was raised on one side of the membrane. The zero current membrane potential was measured after 5–10 mins. Macroscopic inhibition experiments were initiated as for zero-current membrane potentials. Once the conductance had increased 100- to 1000-fold inhibition following titration with glucose was measured.

Polyclonal Antibody Production and Immunodetection Experiments

OprB electroeluted from SDS-PAGE gels was injected intramuscularly into female New Zealand white rabbits. OprB was in a partially unfolded form following electroelution as it showed the same mobility on SDS-PAGE gels as heat-modified OprB (data not shown). The first injection consisted of 60 μg of OprB suspended in 400 μl of 0.1% SDS (w/v), 192 mM glycine, 25 mM Tris-base and mixed with 1000 μl of Freund's complete adjuvant. A second injection, 3 weeks later, consisted of an identical quantity of OprB mixed with 600 μl of Freund's incomplete adjuvant. Serum was collected 1 week later and, prior to use, preadsorbed with *P. aeruginosa* heat-treated cell envelopes (100°C, 10 min) isolated from succinate-grown cells. This treatment removed antibodies reacting with LPS or other *P. aeruginosa* outer membrane proteins. Outer membrane proteins for immunodetection experiments were prepared using the procedure of Lugtenberg *et al.* (1975) and separated by SDS-PAGE. Western blotting of samples for immunodetection followed the procedure of Burnette (1981). Bound antibodies were detected with goat anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, Missouri) using the procedure of Towbin *et al.* (1979).

Circular Dichroism Spectroscopy

Spectra were recorded on a Jasco J-500A spectrophotometer. Cuvettes of 1 mm pathlength contained protein solutions at a concentration of 112 ug/ml. Analyses were based on an average trace from four scans. Baseline readings due to the solvent (0.1% SDS (w/v), 100 mM NaCl, 10 mM Tris-HCl [pH 8.0]) were subtracted. The instrument was calibrated with *d*(+)-1-camphorsulfonic acid as described by Hennessey and Johnson (1982). The concentration of OprB was determined using its extinction coefficient at 205 nm determined with the method of Scopes (1974).

The secondary structure content of OprB was determined using the convex constraint algorithm (CCA) of Perczel *et al.* (1992). This algorithm deconvolutes CD spectrum to its pure components consisting of α helix, ß sheet, ß turn, etc. Pure components are assigned to specific types of secondary structure by comparison with the CD spectrum characteristic for a given type of secondary structure. The CD spectrum of the protein to be analyzed is appended to the reference data set of 25 proteins supplied with the program. For our analysis, program input was in 1-nm intervals from 197 to 240 nm.

N Terminal Sequencing

The N terminal sequences of *P. aeruginosa* OprB and OprD and *P. putida* OprB were determined by S. Kielland at the Protein Microchemistry Centre, University of Victoria, Victoria, British Columbia. For *P. aeruginosa* OprB and OprD, outer membrane proteins were prepared by the method of Lugtenberg *et al.* (1975). Proteins were separated by SDS-PAGE and transferred by Western blotting onto PVDF membranes (Millipore Corp., Bedford, Massachusetts) using CAPS buffer (10% [v/v] methanol, 10 mM CAPS [pH 11] [Sigma Chemical Co., St. Louis, Missouri]) as the transfer buffer. Membrane strips containing the desired protein were cut out

		1	5	10	15
Pseudomonas aeruginosa	OprB	A E A F S P N S K W M L G D W G G			
P. putida	OprB	A E A F S s e S K W M T G D			
P. aeruginosa	OprD	d a f v S d q a e a k g f			
P. aeruginosa	OprP	g t v t T d g a d i v I k t k G G			
P. aeruginosa	OprF	q g q n S v e i e a f g k r y f t			
Escherichia coli	LamB	v D f h g y a r s g i g w t g s g			
E. coli	ScrY	q t d i S t i e a r l n a l e k r			

Fig. 1. Amino terminal amino acid sequence comparison of various bacterial porins. Each sequence was individually aligned by eye with *P. aeruginosa* OprB. For a given protein, identical or conserved amino acids in common with *P. aeruginosa* OprB. are capitalized. Conserved residues were considered to be I/L/V, S/T, and D/E. Sources for data are as follows: *P. aeruginosa* and *P. putida* OprB (this study); OprD (this study, Yoneyama *et al.*, 1992; Huang *et al.*, 1992); OprP (Worobec *et al.*, 1988); OprF (Duchêne *et al.*, 1988); LamB (Clément and Hofnung, 1981); and ScrY (Hardesty *et al.*, 1991).

following visualization of proteins with Ponceau S (Sigma Chemical Co., St. Louis, Missouri). Purified *P. putida* OprB for N terminal sequencing was supplied by N. F. Taylor and E. G. Saravolac (University of Windsor, Windsor, Ontario, Canada).

SDS-PAGE

Analysis of proteins by SDS-PAGE used the 11% (w/v) gel system of Lugtenberg *et al.* (1975). Gels were stained with Coomassie Blue. Samples of heat-unmodified protein were prepared by solubilization at 20°C in 0.2% SDS (w/v), 20% (v/v) glycerol, 125 mM Tris-HCl (pH 6.8) prior to loading on the gel. Heat modified proteins were prepared by solubilization in the same buffer containing 3% (v/v) dithiothreitol and heated for 10 min at 95°C.

RESULTS

Amino Terminal Amino Acid Sequence Analysis

The amino acid sequence was determined for the first 17 and 14 residues of *P. aeruginosa* OprB and *P. putida* OprB, respectively. These sequences were compared to *P. aeruginosa* porins OprD, OprF, and OprP, and *E. coli* LamB and ScrY, as illustrated in Fig. 1. OprF is believed to be the main nonspecific diffusion pore of *P. aeruginosa* (Hancock *et al.*, 1979; Nikaido *et al.* 1991), whereas OprD and OprP are substrate-specific pores recognizing basic amino acids (Trias and Nikaido, 1990) and phosphate (Hancock *et al.*, 1982), respectively. LamB and ScrY are respectively, maltodextrin-specific and sucrose-specific porins found in *E. coli* and several other enteric bacteria (Smelcman and Hofnung, 1975; Palva, 1978; Hardesty *et al.*, 1991). A high degree of homology was evident for the first 14 residues (equal to the extent of information avilable for *P. putida* OprB) of the OprB proteins of *P. aeruginosa* and *P. putida,* with 78.5% of the residues being identical. In contrast, little sequence homology was evident with other *P. aeruginosa* porins or LamB and ScrY of *E. coli.* This degree of similarity is not unusual for closely related porins. Although many porins are known to show relatively little sequence identity, homologous porins in closely related species of bacteria do show extensive homology [e.g., LamB (Francoz, 1990) and OprF (Ullstrom *et al.*, 1991)].

Immunoblot Analysis

To further examine the extent of homology between the OprB proteins, outer membrane proteins of glucose-grown *P. aeruginosa* and *P. putida,* and maltose-grown *E. coli* were electrophoretically transferred to nitrocellulose and exposed to antiserum specific for heat-modified *P. aeruginosa* OprB. The results are shown in Fig. 2. No cross-reaction was visible with heat-modified LamB, whereas both *P. aeruginosa* and *P. putida* OprB were recognized by

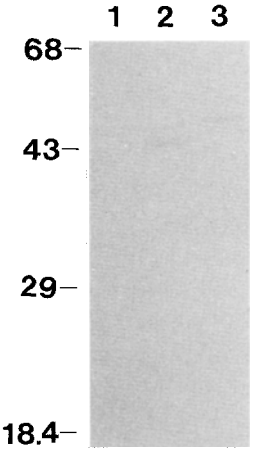

Fig. 2. Immunodetection of outer membrane proteins with antiserum specific for heat-modified *P. aeruginosa* OprB. Outer membrane proteins isolated from maltose-grown *E. coli* (Lane 1), glucose-grown *P. putida* (Lane 2), and glucose-grown *P. aeruginosa* (Lane 3). Outer membrane protein samples were solubilized at 95°C for 10 min prior to electrophoresis. Molecular weight standards are indicated on the left ($\times 10^3$).

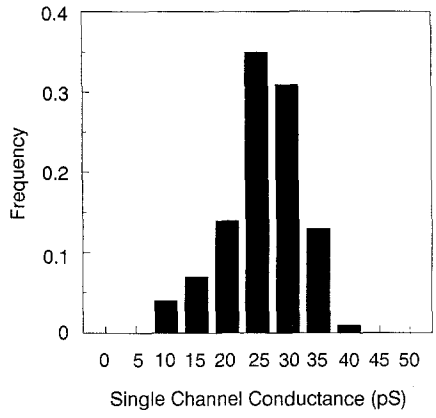

Fig. 3. Histogram of the conductance steps observed with diphytanoyl phosphatidylcholine-*n*-decane membranes in the presence of *P. aeruginosa* OprB. Average single-channel conductance was 25 pS for 102 steps. Aqueous phase contained 1 M KCl and applied voltage was 20 mV.

the antibodies under these conditions. We chose antibodies specific for heat-modified OprB as these antibodies would recognize predominantly linear epitopes rather than conformational epitopes and thus they would provide a better indicator of amino acid homology. Poole and Hancock (1986) demonstrated that antibodies specific for native OprP cross-reacted with phosphate-specific porins from other Pseudomonads and *E. coli* whereas antibodies specific for heat-modified OprP reacted only with *P. aeruginosa* OprP.

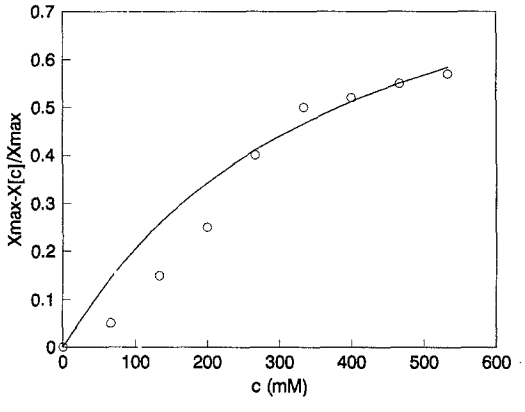

Fig. 4. Macroscopic conductance inhibition as a function of glucose concentration. OprB macroscopic conductance, $X[c]$, was measured following titration with increasing concentration of glucose (c). Membranes were formed from diphytanoyl phosphatidycholine-*n*-decane; aqueous phase contained 1 M KCl. The line was drawn from the equation, $X_{max} - X[C]/X_{max} = k \cdot c/(k \cdot c + 1)$ using a value of $K_s = 380$.

Single-Channel-Conductance Measurements of *P. aeruginosa* OprB

P. aeruginosa OprB was added to the aqueous phase bathing black lipid bilayer membranes. Stepwise increases in conductance were observed with an average single-channel conductance of 25 pS (Fig. 3). Values were concentrated within a narrow range, indicating no significant contribution from contaminating porins. Single-channel conductance for *P. aeruginosa* OprB was very close to that previously measured for *P. putida* [35 pS (Saravolac *et al.*, 1991)]. Significantly larger single-channel conductance values have been previously measured for other carbohydrate-specific porins; 160 pS for LamB (Benz *et al.*, 1986) and 1400 pS for sucrose-specific porin ScrY (Schülein *et al.*, 1991). Values for ScrY fall within the range characteristic of those for nonspecific diffusion pores (Benz *et al.*, 1985). The similarity in single-channel conductance between the OprB proteins, in comparison with the other carbohydrate-specific pores, suggests extensive conservation of the channel structure of these two proteins.

Glucose Binding to *P. aeruginosa* OprB

Previously, *P. aeruginosa* OprB has been proposed to act as a glucose-specific porin based on results from liposome swelling assays (Trias *et al.*, 1988). This assay, however, does not specifically verify the presence of a glucose binding site (Saravolac *et al.*, 1991), nor was the binding affinity for glucose determined. In this study the potential presence of a glucose binding site in *P. aeruginosa* OprB was examined by means of macroscopic conductance black lipid bilayer analysis (Benz *et al.*, 1987). Sufficient *P. aeruginosa* OprB for incorporation of 100–1000 channels was added to the solutions bathing black lipid membranes and allowed to reach a maximum conductance (approximately 30 min). Addition of glucose led to a dose-dependent inhibition of conductance, consistent with the presence of a saturable binding site for glucose within the channel (data not shown).

Based on the two-barrier, one-site model proposed for transport of maltodextrins through LamB (Benz *et al.*, 1987), the sugar-induced block of ion movement through OprB can be used to determine the stability constant, K_s, for sugar binding based on the equation

$$(X_{max} - X[c])/X_{max} = K \cdot c/(K \cdot c + 1)$$

where X_{max} is the conductance before addition of sugar, $X[c]$ is the conductance at a given sugar

concentration, c is sugar concentration, and K is the stability constant ($= 1/K_s$). K_s values for glucose binding to *P. aeruginosa* OprB are derived from a plot of the dose-dependent inhibition of conductance (Fig. 4) or by means of a Lineweaver–Burke plot. A value of 380 ± 40 mM (based on four experiments) was determined, which is within the same order of magnitude as the K_s for glucose binding to *P. putida* OprB (110 mM; Saravolac *et al.*, 1991), LamB (110 mM; Benz *et al.*, 1987), and ScrY (120 ± 40 mM; Schülein *et al.*, 1991).

Ion Selectivity

In order to determine the ion selectivity of *P. aeruginosa* OprB, zero-current membrane potentials were determined in the presence of salt gradients of KCl, LiCl, or KCH_3COO. These salts are commonly used for black lipid bilayer experiments to assess ion selectivity due to the differing aqueous mobilities of the various ions. K^+ and Cl^- ions possess approximately the same aqueous mobilities [limiting molar conductivities of 73.5 and 76.4 $S \times cm^2$ per mol, respectively (Castellan, 1983)]. The mobilities of Li^+ and CH_3COO^- are nearly equal to each other (38.7 and 40.9 $S \times cm^2$ per mol, respectively) but considerably smaller than K^+ or Cl^-. After insertion of approximately 100–1000 channels into the black lipid membranes, the salt concentration was raised in steps on one side of the membrane (from 100 to 500 mM). After each addition, the zero-current membrane potential was measured and the ratio P_{cation}/P_{anion} was calculated according to the Goldman–Hodgkin–Katz equation (Benz *et al.*, 1979). Results for *P. aeruginosa* OprB, in comparison to other carbohydrate-specific porins, are presented in Table I. In contrast to the cation selectivity of other carbohydrate-specific porins, the results indicate that *P. aeruginosa* OprB is anion selective.

Circular Dichroism

For circular dichroism analysis, *P. aeruginosa* OprB was transferred by acetone precipitation from a Triton X-100 buffer to one containing SDS. Triton X-100 absorbs strongly at several wavelengths and would have interfered with CD analysis. This treatment did not alter the native structure of OprB based on identical mobilities on SDS-PAGE gels before and after acetone treatment (Fig. 5). Furthermore, the acetone-treated protein was altered by heat treatment to the same extent as the Triton-solubilized protein. The property of heat modifiability is characteristic of porins and is related to their high ß sheet content. ß sheet structure is stable in the presence of SDS unless heated (Mizushima, 1974; Nakamura and Mizushima, 1976; Hancock and Carey, 1979). Heating these proteins in the presence of SDS results in a partial loss of secondary structure which alters their mobility on SDS-PAGE gels.

Table I. Permeability Ratios (P_{cation}/P_{anion}) for *P. aeruginosa* OprB, *P. putida* OprB, and *E. coli* LamB and ScrY

Salt	P_{cation}/P_{anion}			
	OprB[a]	OprB[b]	Lamb[c]	ScrY[d]
KCl	0.64	17	28	8.6
LiCl	0.29	14	12	5.8
KCH_3COO	—[e]	19	50	16

[a] *P. aeruginosa* OprB, zero current membrane potential measured using a fivefold gradient for each of the salts.
[b] *P. putida* OprB, data from Saravolac *et al.* (1991), salt gradient fivefold.
[c] Data from Benz *et al.* (1987), salt gradient tenfold.
[d] Data from Schülein *et al.* (1991), salt gradient tenfold.
[e] Due to the strong anion selectivity of *P. aeruginosa* OprB, no activity could be detected when KCH_3COO was used as the salt.

The CD spectrum of native unheated OprB and heat-modified OprB are presented in Fig. 6A and B. The spectra of both forms of OprB show a broad minimum with a negative peak at 208 nm. The CD spectra of OprB were analyzed using the CCA algorithm of Perczel *et al.* (1992). Prior to analysis the number of pure component curves (P) comprising a given data set are unknown and must be determined *a priori* (Perczel *et al.*, 1992). We conducted four analyses of the OprB data using a value of either 2, 3, 4, or 5 for P. In the case of $P = 2$, one of the pure curves produced was flat and featureless, whereas for $P = 4$ or 5 some curves corresponding to specific secondary structures were duplicated. (For $P = 4$ the random-structure curve was duplicated, while for $P = 5$ the α-helical curve and the ß sheet curve were duplicated.) As outlined by Perczel *et al.* (1992), these results are indicative of underestimation and overestimation, respectively, of the information content of the data set. Figure 6C shows the pure curves generated for $P = 3$ for native OprB. In this case, the curves generated were distinct and corresponded to α helix, ß sheet, and random structure [based on a comparison with spectra from Perczel *et al.* (1992) and Park *et al.* (1992), and references therein]. For heat-modified OprB, the

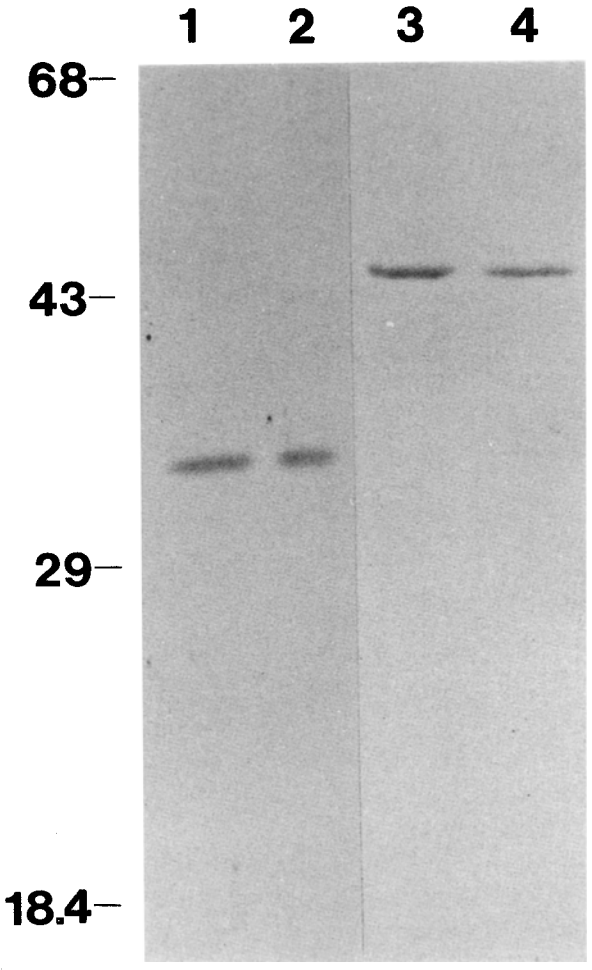

Fig. 5. SDS-polyacrylamide gel electrophoresis of purified OprB. Lanes 1 and 3, OprB in TTE buffer following purification by ion exchange chromatography; lanes 2 and 4, OprB in TNS buffer following acetone precipitation. Lanes 1 and 2, unheated, lanes 3 and 4 heated at 100°C for 10 min. Molecular weight standards are indicated on the left ($\times 10^3$).

curves generated for $P = 3$ were similar. The estimated amounts of these structures in native OprB were 25% α helix, 40% ß sheet, and 34% random structure. For heat-modified OprB, random structure increased to 42% at the expense of ß sheet (34%) and α helix (24%).

DISCUSSION

This paper characterizes the OprB protein of *P. aeruginosa* and describes to a greater extent the functional and structural homology between *P. aeruginosa* and *P. putida* OprB suggested by Saravolac *et al.* (1991). Previously, the OprB proteins from both species of *Pseudomonas* were proposed to form glucose-specific pores (Trias *et al.*, 1988; Saravolac *et al.*, 1991). The methodology used to reach this conclusion for the two porins, however, differed greatly, making direct comparisons of functional characteristics difficult. *P. aeruginosa* OprB was examined using the liposome-swelling assay (Trias *et al.*, 1988), which is performed at very high substrate concentrations. Results of this analysis, and a similar one by Hancock and Carey (1980), were consistent with *P. aeruginosa* OprB forming pores of quite

large diameter. In contrast, *P. putida* OprB was analyzed by black lipid bilayer analysis, which indicated formation of very small constricted channels (Saravolac *et al.*, 1991). Given the possible difference between the two OprB pores in what is their basic functional property, we undertook this study to more fully determine the extent of homology between these porins. Additionally we wanted to further characterize *P. aeruginosa* OprB as part of our ongoing analysis of the high-affinity glucose transport system of *P. aeruginosa.*

Results presented in this report indicate an extensive degree of homology in primary structure and channel properties of the *P. aeruginosa* and *P. putida* OprB proteins. Evidence for primary structure homology is based on the N-terminal sequences and the recognition of heat-modified *P. putida* OprB by antiserum specific for heat-modified *P. aeruginosa* OprB. The N-terminal sequence of *P. aeruginosa* OprB showed no obvious relationship to other *P. aeruginosa* porins or to other known carbohydrate-specific porins. In addition, both OprB channels also possess a glucose-binding site with a similar K_s. The single-channel conductance measurements indicate that *P. aeruginosa* OprB forms a channel with a constriction very similar to that found in *P. putida* OprB and thus does not show the characteristics of general diffusion pores demonstrated in ScrY. Earlier evidence suggesting that the pore of *P. aeruginosa* OprB was quite large can be related to the requirements of the liposome assays used in previous studies. These analyses require either high substrate concentrations or lengthy incubation times which permits considerable diffusion of all solutes, including those with only a very low rate of permeation through a given pore.

Although many similarities exist between *P. aeruginosa* and *P. putida* OprB, a marked difference was noted in the ion selectivity, as determined by black lipid bilayer experiments. *P. aeruginosa* OprB was found to be anion selective, rather than cation selective as determined for *P. putida* OprB and other carbohydrate-specific porins. Variability in ion selectivity between related porins has previously been demonstrated for some nonspecific porins by Benz *et al.* (1985). OmpF and OmpC of *E. coli,* although closely related to *E. coli* PhoE, show cation selectivity as opposed to the anion selectivity of PhoE. Similarly, OmpD of *Salmonella typhimurium* is cation selective, whereas the related porin of *E. coli,* NmpC, is anion selective. However, in the case of OprB, this is

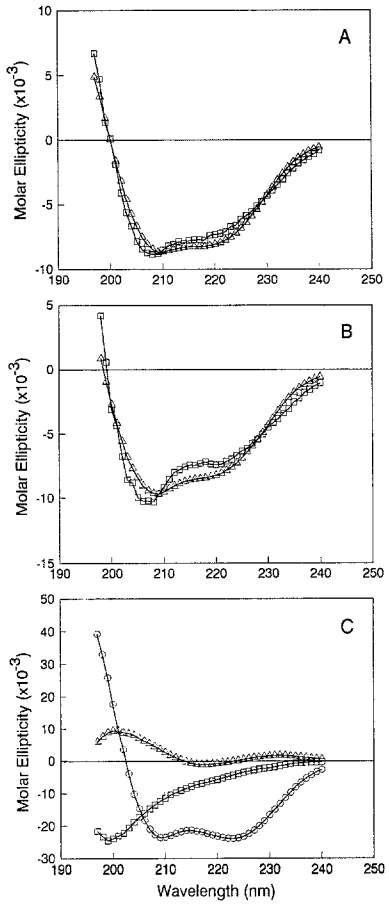

Fig. 6. Circular dichroism analyses of *P. aeruginosa* OprB. Circular dichroism spectra of native unheated OprB and heat-modified OprB are shown in (A) and (B), respectively: □, experimentally measured; △, calculated from the weighted pure component curves produced by convex constraint algorithm (CCA) analysis (Perczel *et al.*, 1992). (C) Pure component curves generated by CCA analysis of native unheated OprB: O, α helix; △, antiparallel ß sheet; □, random.

the first reported instance of closely related substrate-selective porins showing opposite ion selectivity. As such, future comparative studies may shed light on the mechanism of ion selectivity and substrate binding and whether any relationship exists between the two.

Previously, the high cation selectivity of LamB was proposed to be linked to the presence of a carbohydrate binding site (Benz *et al.*, 1987) and was assumed to be due to carbonyl groups inside the channel which could form hydrogen bonds to the hydroxyl protons of sugars. The formation of cation-selective channels in ScrY and *P. putida* OprB (Saravolac *et al.*, 1991; Schülein *et al.*, 1991) reinforced the correlation between the presence of a carbohydrate binding site

and cation selectivity. However, assuming a similar binding site is found in the two OprB proteins, the data presented here indicates that residues determining ion selectivity may be distinct from those forming the binding site of OprB. This may also be true of other carbohydrate-specific porins.

Chemical modification experiments have indicated that ion selectivity is determined by the number of positive vs. negative amino acid residues and their relative postions in the Channel (Benz *et al.*, 1984; Darveau *et al.*, 1984). In OprB most of the residues determining selectivity are presumably located at or near the exterior surface of the pore, away from the binding site. This location would be consistent with the difference in ion selectivity between the OprB proteins despite their structural and functional homology. Homologous porins are known to show variability in the residues exposed at the exterior of the outer membrane (Tommassen, 1988; Werts *et al.*, 1992). Relatively minor changes in the ratio of positive to negative residues at the mouth of the pore of the two OprB porins could be sufficient to alter the selectivity of the pore while not affecting the single-channel conductances or binding sites. Future site-directed mutagenesis experiments on OprB will be used to attempt to determine whether the residues determing selectivity are distinct from those involved in glucose binding.

The structure of *P. aeruginosa* OprB was analyzed by circular dichroism spectroscopy and the CCA algorithm of Perczel *et al.* (1992). To date, this is the most appropriate algorithm developed for analysis of the CD spectra of membrane proteins. Other methods require a large data set consisting of the CD spectra of proteins and their known secondary structures, usually deduced from X-ray diffraction analysis. In comparison to soluble proteins, information of this kind is lacking for membrane proteins (Park *et al.* 1992). Additionally, errors in interpretation of the X-ray data compound the errors associated with CD analysis. In contrast, the CCA algorithm extracts the common spectral components from a set of data without relying on previously determined secondary structures of the proteins composing the data set.

Analysis of native OprB yielded a secondary structural content of 25% α helix, 40% ß sheet, and 34% random structure. These values fall within the range of those determined for other porins analyzed with the CCA algorithm. Park *et al.* (1992) examined *E. coli* OmpF, LamB, and PhoE porins and *Rhodobacter capsulatus* porin and found values ranging from 15–39% α helix (sum of transmembrane and peripheral α-helical components [see Park *et al.* (1992) for details regarding these two types of α-helical components], 30–54% ß sheet, and 1–35% random structure. The deduced ß-sheet content seems lower than expected in comparison to the X-ray data of *R. capsulatus* (Weiss and Schulz 1992). These lower than expected values may be due to the inclusion of only a limited number of proteins with a high ß-sheet content in the data set. As additional CD spectra of porins becomes available, prediction of ß sheet content by CCA should improve. An underestimation of ß sheet would probably be reflected in an overestimation of random structure rather than an effect on α-helical content. Hennessey and Johnson (1981) note that α-helical content is accurately predicted using only the wavelengths above 200 nm. For OprB an α-helical content of 25% is much higher than the α-helical content previously determined for two other *P. aeruginosa* porins, OprP (3%) and OprF (9%) (Siehnel *et al.*, 1990). Although some error may be inherent in the CD analyses for each of these proteins, a qualitative assessment of the CD spectra of OprB and OprP is consistent with the apparent differences in structural content. The CD spectrum of OprB shows a definite negative peak at 208 nm (Fig. 6A), characteristic of α-helical structure, which is absent in the CD spectrum of OprP (Worobec *et al.*, 1988). Further analyses of *P. aeruginosa* porins are necessary to determine where these structural differences occur within the proteins and whether they are related to the differing functions of these porins.

We are currently determing the amino acid sequence of *P. aeruginosa* OprB. This information will serve as a basis for further studies centering on the identification of those residues which determine the ion selectivity and single-channel conductance properties of OprB and those which form the substrate binding site of this porin.

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