Characterization of the Structure, Function, and Conformational Stability of PorB Class 3 Protein from Neisseria meningitidis

A PORIN WITH UNUSUAL PHYSICOCHEMICAL PROPERTIES*

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PorB proteins constitute the vast majority of channels in neisserial outer membranes and can be subdivided within meningococcal strains into two distinct and mutually exclusive families that are designated as class 2 and class 3 proteins. We recently characterized the functional activity and conformational stability of a PorB class 2 protein from Neisseria meningitidis (Minetti, C. A. S. A., Tai, J. Y., Blake, M. S., Pullen, J. K., Liang, S. M., and Remeta, D. P. (1997) J. Biol. Chem. 272, 10710–10720). To evaluate the structure-function relatedness among the PorB proteins, we have employed a combination of electrophoretic and spectroscopic techniques to assess the conformational stability of zwitverter-solubilized class 3 trimers. The functional, physicochemical, and structural properties of the meningococcal class 2 and class 3 proteins are comparable with the notable exception that the latter exhibits a significantly higher susceptibility to SDS. The SDS-induced dissociation and partial unfolding of PorB class 3 is characterized by a single two-state transition with a midpoint at 0.35% SDS. The native trimeric assembly dissociates reversibly, forming partially folded monomers that retain the characteristic β-sheet content of the transmembrane domain with a concomitant increase in random coil structure arising from unfolding the rigid surface loops. These results provide new insight into the elucidation of porin folding pathways and the factors that govern the overall structural stability of meningococcal proteins.

Bacterial porins are integral outer membrane channel-forming proteins that function as molecular sieves by mediating the exchange of nutrients and waste products with the environment (1, 2). The majority of these porins assemble as trimers of a single polypeptide and exhibit an unusually high resistance to harsh conditions such as low pH, elevated temperatures, and the presence of detergents. As a consequence of such properties, porin oligomeric structures may be visualized in SDS-PAGE.1 Elucidation of the crystal structures of bacterial porins from Rhodobacter capsulatus (3), Rhodopsseudomonas blasica (4), and the Escherichia coli porins OmpF, PhoE (5), and LamB (6) reveals the characteristic trimeric structure, each subunit consisting of 16–18 antiparallel β-strands. Recent studies employing circular dichroism spectroscopy indicate that the meningococcal PorB class 2 protein retains physicochemical properties similar to other porins, including the predominance of β-sheet structure and retention of native trimeric conformation at high temperature or increased concentrations of denaturants (7).

Meningococcal class 2 and class 3 proteins are gene products of two alleles of the single-copy porB gene locus (8) and are thus mutually exclusive within different Neisseria meningitidis strains (9, 10). Among the various class 2 and class 3 proteins sequenced to date, similarities in primary structure are on the order of 60–70%. The complete amino acid sequences of N. meningitidis PorB class 3 proteins derived from cDNA sequence analyses (11–13) reveal an average polypeptide molecular weight of approximately 34,000. Details regarding specific structural features of meningococcal porins have been gleaned primarily from indirect methods of analysis, including algorithmic generated topology models, epitope mapping, and gene sequence variability, whereby a significant amount of information has been acquired (14–17).

Van der Ley et al. (14) have demonstrated that the PorA class 1 and PorB class 2 meningococcal porins contain surface-accessible linear epitopes that have been identified using monoclonal antibodies and overlapping peptides. As far as meningococcal PorB class 3-expressing serotypes are concerned, limited success has been achieved in determining surface-exposed linear epitopes, leading to the assumption that the surface epitopes in class 3, if present, are mainly conformational (15–17). In fact, Mandrell and Zollinger (18) have described a procedure for restoration of binding electroblotted meningococcal outer membrane (OM) proteins to specific monoclonal antibodies generated against the respective native OM proteins. Recently, however, a linear B-cell epitope on class 3 has been identified within the putative loop I (residues 19–30) based on the finding that post vaccination human sera react with denatured class 3 protein in immunoblots (19).

Meningococcal porins have been studied extensively in terms of their genetic and immunological aspects, which have provided valuable information regarding their basic structural features. Recent investigations have furnished physicochemical evidence that PorB class 2 proteins behave as typical porins not solely in terms of function but also with respect to their overall resistance to detergents, chaotropes, and high temperatures (7). The focus of the present study is to characterize the structural and functional properties of the PorB class 3 meningococcal porin family to determine their relatedness with the corresponding PorB alleles (i.e. class 2 proteins). This knowledge should shed significant light on the role that different

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¶ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MES, 2-(N-morpholino)ethane sulfonic acid; NBS, N-bromosuccinimide; OM, outer membrane; rclass2, recombinant class 2 protein; rclass3, recombinant class 3 protein; bia-Tris, 2-[bis(2-hydroxyethyl)amino]-2-[bis(2-hydroxyethyl)-propane-1,3-diol.
domains play within these molecules and their resultant influence on functional and structural characteristics. The rather unusual SDS sensitivity observed for class 3 protein suggests that this porin represents an interesting model to evaluate the conformational stability of membrane proteins for which limited information is available to date (20). The results derived from such studies should facilitate elucidation of the structural and physicochemical properties which dictate the unusually high stability of porins as a general rule.

We have selected a wild type class 3-expressing meningococcal M981 strain that naturally lacks PorA class 1 expression and a genetically engineered mutant strain (i.e., 44/76 Δ1/Δ4) in which null gene replacements have been performed for PorA class 1 and reduction modifiable protein, both reported to interfere in the characterization of inherent functional and structural properties of PorB (21). Since the use of DNA recombinant technology affords several advantages (22, 23), including higher expression levels and the elimination of interfering components from meningococcal OMVs, we have characterized the functional activity and conformational stability of recombinant PorB class 3 protein after ensuring equivalence to its native counterpart.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Reagents—**Meningococcal strain M981 (serotype 4a) was obtained from Dr. Carl E. Frasch (Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD). The mutant of meningococcal strain 44/76 (i.e., 44/76 Δ1/Δ4) was provided by Dr. Lee Wetzler (Maxwell Finland Laboratory, Boston City Hospital, Boston, MA), and the preparation procedure has been described previously (24). The solid typing and liquid growth media for all the meningococci have been described elsewhere (25). The growth media and conditions for the preparation of E. coli strain BL21 (DE3)ompA harboring the expression plasmid pNV15 were similar to that of Qi et al. (22). All reagents not described specifically were of the highest grade possible as supplied by Sigma.

**Isolation and Purification of Porins from Meningococci—**PorB class 3 protein from both meningococcal strains (i.e., 44/76 Δ1/Δ4 and M981) was isolated employing the zwittergent-CaCl2 extraction procedure described by Wetzler et al. (26). Following initial extraction, the class 3-expressing strains were loaded onto and co-purified from a Hiload Q Sepharose HP column (Amersham Pharmacia Biotech) using a gradient of 0.2–1.0M NaCl (pH 8.0) and purified class 3 protein eluted as the major peak. Protein concentration was determined by plotting second derivative peak-trough absorbance differences as a function of chemical denaturant concentration as described elsewhere (7, 29). In the case of PorB class 3 protein in the native state, the ratio of \( \Delta A_{285} - \Delta A_{291} \) to \( \Delta A_{291} + \Delta A_{285} \) was employed based on the spectral characteristics of this protein under native conditions. N-Acetyltirosinamide and N-acetyltryptophanamide (Sigma) were employed as model compounds at a molar ratio of 15:4 to approximate the total content of tyrosine and tryptophan residues in class 3 protein. The aromatic amino acids were dissolved either in 100% anhydrous ethylene glycol (Aldrich) or in 6.0M guanidinium HCl prepared from an 8.0 M stock solution (Ferch) for calculation of \( 0.1 \) and 100% tyrosyl exposure, respectively. The degree of tryptophan exposure was also evaluated by monitoring the susceptibility of PorB class 3 tryptophan residues to oxidation by N-bromosuccinimide (NBS) according to Bray et al. (30). Aliquots of native PorB class 3 protein (0.25 mg/ml) were incubated with a 100-fold molar excess of NBS under native conditions (i.e., 25 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, and 0.05% zwittergent 3-14) at 25 °C and in the presence of SDS at different temperatures. Absorption spectra were acquired on a Hewlett-Packard Model 8453 UV-visible rapid scan spectrophotometer equipped with a diode array detector and Peltier temperature-controlled sample compartment. Exposure of tryrosyl residues was determined by plotting second derivative peak-trough absorbance differences as a function of chemical denaturant concentration as described elsewhere (7, 29). In the case of PorB class 3 protein in the native state, the ratio of \( \Delta A_{285} - \Delta A_{291} \) to \( \Delta A_{291} + \Delta A_{285} \) was employed based on the spectral characteristics of this protein under native conditions. N-Acetyltirosinamide and N-acetyltryptophanamide (Sigma) were employed as model compounds at a molar ratio of 15:4 to approximate the total content of tyrosine and tryptophan residues in class 3 protein. The aromatic amino acids were dissolved either in 100% anhydrous ethylene glycol (Aldrich) or in 6.0M guanidinium HCl prepared from an 8.0 M stock solution (Ferch) for calculation of 0.1 and 100% tyrosyl exposure, respectively. The degree of tryptophan exposure was also evaluated by monitoring the susceptibility of PorB class 3 tryptophan residues to oxidation by N-bromosuccinimide (NBS) according to Bray et al. (30). Aliquots of native PorB class 3 protein (0.25 mg/ml) were incubated with a 100-fold molar excess of NBS under native conditions (i.e., 25 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, and 0.05% zwittergent 3-14) at 25 °C and in the presence of SDS at different temperatures. Absorption spectra were acquired on a Hewlett-Packard Model 8453 UV-visible rapid scan spectrophotometer equipped with a diode array detector. The resultant data were recast as the fourth derivative (d^4A/dt^4), and changes in the magnitude of the tryptophan specific trough were monitored at 294 nm (30).

**Circular Dichroism Spectroscopy—**The oligomeric state of PorB class 3 protein—Since PorB class 3 trimers are highly sensitive to conventional SDS-PAGE analysis and dissociate under such conditions, we have employed modifications to the procedure such as varying the temperature, ionic strength, and SDS concentration. The modified protocol permits visualization of class 3 trimers and consists of preincubating porin samples (1 mg/ml) with 0.1% SDS following a 1:1 dilution with a 2× Laemmli’s native buffer (Novex). The sample is immediately loaded onto a Tris-glycine 8–16% gradient gel precast gel and heated to 95 °C prior to SDS-PAGE analysis. SDS-PAGE was performed using 10% pre-cast gels in his-Tris buffer (NuPAGE, Novex) and MES-SDS (pH 7.3) as running buffer at 200 V constant voltage.

**Circular Dichroism Spectroscopy—**The oligomeric state of PorB class 3 protein—Since PorB class 3 trimers are highly sensitive to conventional SDS-PAGE analysis and dissociate under such conditions, we have employed modifications to the procedure such as varying the temperature, ionic strength, and SDS concentration. The modified protocol permits visualization of class 3 trimers and consists of preincubating porin samples (1 mg/ml) with 0.1% SDS following a 1:1 dilution with a 2× Laemmli’s native buffer (Novex). The sample is immediately loaded onto a Tris-glycine 8–16% gradient gel precast gel and heated to 95 °C prior to SDS-PAGE analysis. SDS-PAGE was performed using 10% pre-cast gels in his-Tris buffer (NuPAGE, Novex) and MES-SDS (pH 7.3) as running buffer at 200 V constant voltage.

**SDS-induced Conformational Changes of PorB Class 3 Protein under Equilibrium Conditions—**Preliminary experiments revealed that low ionic strength sodium phosphate buffer in the presence of zwittergent 3-14 (pH 8.0) represented the optimal choice for evaluating the effects of the isolated proteins and the various sugars. Accordingly, concentrated samples dialyzed against 10 mM NaPO4 and 0.05% zwittergent 3-14 (pH 8.0) were diluted to 1 mg/ml and incubated in the presence of different SDS concentrations for a minimum of 24 h to ensure that equilibrium was achieved prior to electrophoretic and spectroscopic measurements. The oligomeric state of class 3 protein and its protease susceptibility as a function of SDS were monitored by CD spectroscopy in the far UV (180–250 nm) and near UV (250–350 nm) regions, respectively. Concentrated stock solutions of protein were dialyzed exhaustively against a buffer system comprised of 10 mM NaPO4 and 0.05% zwittergent 3-14 (pH 8.0). Spectra of samples containing 1.0 mg/ml protein were recorded at 0.1-nm wavelength intervals on a UV-visible rapid scan spectrophotometer equipped with a diode array detector and Peltier temperature-controlled sample compartment. Exposure of tryrosyl residues was determined by plotting second derivative peak-trough absorbance differences as a function of chemical denaturant concentration as described elsewhere (7, 29). In the case of PorB class 3 protein in the native state, the ratio of \( \Delta A_{285} - \Delta A_{291} \) to \( \Delta A_{291} + \Delta A_{285} \) was employed based on the spectral characteristics of this protein under native conditions. N-Acetyltirosinamide and N-acetyltryptophanamide (Sigma) were employed as model compounds at a molar ratio of 15:4 to approximate the total content of tyrosine and tryptophan residues in class 3 protein. The aromatic amino acids were dissolved either in 100% anhydrous ethylene glycol (Aldrich) or in 6.0M guanidinium HCl prepared from an 8.0 M stock solution (Ferch) for calculation of 0.1 and 100% tyrosyl exposure, respectively. The degree of tryptophan exposure was also evaluated by monitoring the susceptibility of PorB class 3 tryptophan residues to oxidation by N-bromosuccinimide (NBS) according to Bray et al. (30). Aliquots of native PorB class 3 protein (0.25 mg/ml) were incubated with a 100-fold molar excess of NBS under native conditions (i.e., 25 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, and 0.05% zwittergent 3-14) at 25 °C and in the presence of SDS at different temperatures. Absorption spectra were acquired on a Hewlett-Packard Model 8453 UV-visible rapid scan spectrophotometer equipped with a diode array detector. The resultant data were recast as the fourth derivative (d^4A/dt^4), and changes in the magnitude of the tryptophan specific trough were monitored at 294 nm (30).
electrophoresis and subsequent densitometric analysis of the Coomasie Blue-stained gels using an IS1000 Alpha Imager. SDS-induced conformational changes in the secondary and tertiary structure of PorB class 3 were monitored by analyzing the far UV CD and second derivative UV spectra, respectively (29).

RESULTS

Isolation and Purification of Native and Recombinant PorB Class 3 (rclass 3) Protein—PorB class 3 proteins have been purified to homogeneity from meningococcal strain M981 (serotype 4a) or from a mutant strain (i.e. 44/76 D1/D4) engineered as described under “Experimental Procedures.” Isolation and purification of rclass 3 from E. coli as washed inclusion bodies, followed by protein refolding using a combination of gel filtration and ion exchange chromatography, yields highly purified and refolded class 3 protein as described recently for PorB class 2 protein (7). The refolded and purified protein exhibits a native-like structure, as determined by its functional activity and solution structure. Although non-SDS-resistant under conventional electrophoretic procedures, the purified native and recombinant class 3 proteins may be visualized in their trimeric state by a modified PAGE developed in the present study. The protease observed for PorB class 3 proteins support the contention that these porins adopt a native trimeric structure.

Functional Characterization: Liposome Swelling Assay—Since stachyose (M₃, 666) does not permeate at significant rates through class 3 protein channels, this oligosaccharide was employed for determination of isosmotic concentrations as described previously for class 2 protein (7). Inspection of the relative permeability rates to L-arabinose and plotted on a logarithmic scale. The sugars used are: arabinose (M₁, 150), galactose (M₂, 180), rhamnose (M₂, 182), N-acetylglucosamine (M₂, 221), sucrose (M₃, 342), raffinose (M₃, 504), and stachyose (M₃, 666).

from E. coli (31), Salmonella typhimurium (32), and meningococcal PorB class 2 protein (7). The minima observed at 217 nm may be correlated with the native conformation of class 3 protein which is comprised predominantly of β-sheet structures. It has been suggested that secondary structure prediction methods based either on the primary amino acid sequence or deconvolution of the CD spectrum should be used with caution when applied to membrane proteins (2). Nevertheless, recent studies have reported that accurate determination of secondary structure can be obtained for porins using the CONVEX constraint algorithm for secondary structure predictions (33). Deconvolution of the PorB class 3 far UV CD spectrum employing this algorithm reveals a β-sheet content of approximately 37%, a value entirely consistent with that determined for PorB class 2 protein (7). Inspection of the near UV CD spectra of native and rclass 3 proteins in Fig. 2, B and D, respectively, reveals unique profiles characterized by a broad positive ellipticity, which is comparable to that obtained for PorB class 2 protein (7). The similarity in spectral features observed for class 2 and class 3 proteins suggests that the aromatic residues of these proteins experience similar asymmetric environments in solution.

Exposure of Tyrosyl Residues—Second derivative UV analysis has been used widely for the determination of aromatic residue exposure of proteins (7, 29). The results reveal that class 3 tyrosyl residues are essentially buried and nonaccessible to solvent under native conditions. These results are identical irrespective of the protein source and differ from those obtained for PorB class 2 protein in which 1–2 tyrosyl residues appear to be both surface- and solvent-exposed (refer to Table 1). In general, aromatic amino acid exposure is relatively low for other porins in detergent solutions such as E. coli (34, 35) or in the membrane-embedded state as determined previously for meningococcal PorB proteins (36).

Thermal Stability of Class 3 Protein in the Presence of Zwittergent 3-14—Near and far UV CD spectra of rclass 3 protein have been monitored as a function of temperature (Fig. 3), and the respective thermal denaturation profiles recorded at 290 and 217 nm (upper and lower panels, respectively). The biphasic thermal denaturation profiles observed for PorB class 3 protein are characterized by a broad low temperature transition at 67 °C followed by a cooperative unfolding transition at 92 °C. Rescans of class 3 protein following cooling to 20 °C
TABLE I

Comparison of surface-exposed tyrosyl residues in PorB class 2 and class 3 proteins

Solvent exposure of tyrosyl residues in PorB class 2 and class 3 proteins is calculated from second derivative analysis of the corresponding UV spectra (refer to "Experimental Procedures") according to Ragone et al. (29).

| Protein strain (serotype) | Total no. | Loops I–VIII | Loop no. | Empirical value |
|---------------------------|-----------|--------------|-----------|----------------|
| C12-M986 (2a)             | 18        | 6            | I 1 II 1 III 1 IV 1 V 1 VI 1 VII 1 VIII 0 | 1–2<sup>a</sup> |
| C13–8765 (15)             | 15        | 3            | I 1 II 1 III 1 IV 1 V 1 VI 1 VII 1 VIII 0 | 0               |
| C13–44/76 (15)            | 15        | 3            | I 1 II 1 III 1 IV 1 V 1 VI 1 VII 1 VIII 0 | 0               |
| C13-M981 (4)              | 16        | 5            | I 1 II 1 III 1 IV 1 V 1 VI 1 VII 1 VIII 0 | 0               |

<sup>a</sup>The sequences used in the present study are derived from Murakami et al. (58) (PorB class 2 protein, serotype 2a, strain M986); GenBank<sup>TM</sup> accession no. V02314 (PorB class 3, serotype 15, strain 8765); Delvig et al. (19) (PorB class 3, serotype 15, strain 44/76); Ward et al. (13) (PorB class 3, serotype 4, strain M981).

<sup>b</sup>Data from Minetti et al. (7).

Fig. 2. Circular dichroism spectra of native and recombinant PorB class 3 protein. Far UV (panels A and C) and near UV (panels B and D) circular dichroism spectra of native strain 44/76 Δ1/Δ4 (panels A and B) and recombinant (panels C and D) PorB class 3 proteins. Spectra are recorded at 25 °C under native solution conditions in 10 mM sodium phosphate and 0.05% zwittergent 3-14 (pH 8.0).

Fig. 3. Thermal denaturation profiles of PorB class 3 protein studied by circular dichroism spectroscopy. Thermal melting profiles of PorB class 3 recorded at 290 nm (panel A) and 217 nm (panel B) to monitor the disruption of tertiary and secondary structure, respectively. The samples are in a buffer system comprised of 10 mM sodium phosphate and 0.05% zwittergent 3-14 (pH 8.0).
Characterization of Meningococcal PorB Class 3 Protein

FIG. 4. Oligomeric state of class 3 protein as a function of SDS preincubation concentrations. Upper panel, SDS-PAGE analysis performed using a modification of the conventional procedure (as described under “Experimental Procedures”). Aliquots of class 3 samples loaded into the gel following overnight incubations with varying amounts of SDS (0.15–1.0%) and electrophoresed for 3 h at 90 V constant voltage. Lower panel, densitometric analysis of the relative areas of Coomassie Blue-stained protein bands corresponding to monomeric amounts of SDS (0.15–1.0%) and electrophoresed for 3 h at 90 V constant voltage.

Characterization of the SDS Sensitivity of Class 3 Protein—Normal SDS-PAGE analysis performed by preincubating class 3 protein samples with 0–1% SDS at ambient temperatures failed to demonstrate the presence of slow migrating bands that are characteristic of SDS-resistant trimeric porin species. A systematic evaluation of electrophoretic conditions has been performed by varying the SDS concentration (0–0.02%) in the running buffer that normally comprises 0.1% Class 3 trimers are observed under conditions that employ low concentrations of SDS in both the sample and running buffers under controlled voltage and temperature (refer to “Experimental Procedures”). Since optimal migration profiles for both trimers and monomers are obtained using 0.01% SDS in the running buffer and 0.1% SDS in the sample buffer, these conditions have been employed to monitor the native oligomeric state of class 3 protein (Fig. 4, upper panel, lane 1). Moreover, this technique has proven useful in the study of the concentration-dependent SDS-induced dissociation of class 3 trimers as depicted in Fig. 4 with a corresponding transition midpoint of 0.35% SDS (Fig. 4, lower panel).

FIG. 5. Limited proteolysis of recombinant PorB class 3 protein as a function of SDS concentration. Aliquots (5 µg) of PorB class 3 protein incubated overnight with varying amounts of SDS (0–1%) and digested with staphylococcal V8 protease (1 µg) for 30 min at 37 °C. The reaction mixtures are incubated 1:1 with 2× MES-SDS running buffer, loaded into a 10% bis-Tris polyacrylamide gel (NuPAGE, Novex), and electrophoresed for 35 min at 200 V constant voltage.

Use of Limited Proteolysis to Assess Tertiary Structure and Susceptibility to SDS—Frasch et al. (36) have demonstrated that class 3 proteins in their native conformation (i.e. within isolated meningococcal OMs) are resistant to trypsin, proteinase K, and chymotrypsin, even though the molecule contains numerous potential protease sites, particularly those located in the putative extracellular loop regions. Likewise, we have conducted proteolytic digestions of class 3 protein employing staphylococcal V8 protease, since this enzyme tolerates the presence of high concentrations of denaturing agents such as SDS. Our studies suggest that there are no significant changes in specific activity in the presence of this detergent (data not shown). Aliquots of class 3 are incubated with increasing concentrations of SDS over different time intervals after which the protease is added to the incubation mixture (at an enzyme:protein ratio of 1:10), and the reaction is allowed to proceed for 30 min at 37 °C. The results analyzed by SDS-PAGE reveal that class 3 is totally resistant under native conditions (i.e. in the presence of 0.05% zwittergent 3-14) as is evident when comparing lane 2 in Fig. 5 with the control sample in lane 1. Class 3 protein undergoes proteolysis in the presence of SDS in a concentration-dependent manner (lanes 3–8). The degree of susceptibility as monitored by averaging three independent experiments reveals a transition midpoint on the order of 0.35% SDS.

Structural Features of SDS-induced Unfolding of PorB Class 3 Studied by Spectroscopic Techniques—Inspection of the structural features of class 3 protein upon exposure to SDS reveals that this meningococcal porin assumes a partially unfolded intermediate state in the presence of high SDS concentrations, which is characterized by complete retention of β-sheet content and an increase in random coil structure (refer to Fig. 6). These findings contrast reports on a number of porins characterized to date (31, 32, 37, 38) including PorB class 2 protein (7) that describe superimposable CD spectra for porins incubated with either nonionic/zwitterionic detergents or SDS at concentrations as high as 1% and temperatures below 60 °C.
Moreover, the tertiary structure elements of class 3 are severely affected in the presence of this detergent, as monitored by tyrosyl exposure employing second derivative UV analysis (Fig. 7). SDS-induced changes in both secondary and tertiary structures are characterized by a transition midpoint at 0.35% SDS, which substantiates the SDS-dependent decrease in protease resistance as well as trimer dissociation.

The effects of SDS have also been investigated by monitoring the resultant susceptibility of tryptophan residues to oxidation by NBS. The fourth-derivative UV spectra presented in Fig. 8 reveals that the four tryptophan residues are protected under native conditions and the addition of SDS promotes a 78% decrease in the tryptophan-specific signal (as denoted by the arrow), which virtually disappears upon heating. These results indicate that three out of a total of four tryptophan residues within the molecule are exposed in the presence of 1% SDS at room temperature, undergoing complete exposure upon heating in the presence of this detergent.

The combination of spectroscopic, electrophoretic and gel filtration data reveal that the SDS-induced conformational changes of class 3 protein are characterized by partial unfolding and dissociation of the native trimeric assembly. Although tertiary interactions are disrupted in the presence of SDS, PorB class 3 retains its characteristic $\beta$-sheet conformation with a concomitant increase in random coil structure. The resultant conformer is less compact and exhibits increased susceptibility to proteases as well as solvent accessibility of aromatic residues, albeit to an extent which is only 30–40% of that generated by complete unfolding of the protein in the presence of 6.0 M guanidinium HCl.

**DISCUSSION**

Meningococcal PorB proteins are members of a distinguishable subgroup of the trimeric porin proteins in the OMs of Gram-negative bacteria. As noted by Jeanteur et al. (39), PorB proteins share limited sequence similarity with *E. coli* OmpF and PhoE and possess distinct domains which discriminate these neisserial porins from the latter. While such similarities are related to common structural and functional features, differences may account for some PorB properties that are rather unique among neisserial porins. The common features that are shared by apparently all bacterial porins include assembly and oligomerization into trimers resulting in the formation of aqueous channels through lipid membranes. Neisserial porins on the other hand (particularly those originating from pathogenic sources) possess an array of unique properties that in most
cases include (a) voltage gated pores at low membrane potentials (40), (b) the ability to vectorily transfer to a foreign membrane when in proximity to the latter (41), and (c) potential activity in both prokaryotic and eukaryotic cells, thus contributing to the pathogenicity of the porin-expressing microorganisms (42, 43).

Similar to other Gram-negative organisms, meningococci possess the ability to express at least two porin polypeptides that correspond to products of the porA and porB genes. However, as opposed to other bacteria such as E. coli in which the expression of OmpF, OmpC, and PhoE is controlled by external signals, no regulatory elements have been demonstrated for the neisserial porins that are therefore constitutive in nature. Consequently, the isolation and purification of PorB class 3 protein from meningococcal strains expressing both genes (e.g. wild type 44/76) represents a serious challenge since significant amounts of a PorA gene product (i.e. class 1 protein) are also present in the OM of such strains. In addition to the fact that these porins possess similar physical properties thereby imposing difficulties in separation and purification, it has been suggested that these two proteins may actually assemble as heterotrimers. In this regard, the use of mutant strains whereby only one of the porins is expressed becomes imperative.

Functional Characterization of PorB Class 3 Protein—The liposome swelling assay facilitates determination of pore sizes based on the relative permeability of porin molecules incorporated into liposomes to solutes of varying molecular weights (2). The fact that PorB proteins (i.e. class 2 and class 3 proteins) exhibit equivalent exclusion limits (i.e. 380–400 Da) indicates that their pore sizes are similar. Based on crystallographic studies (5–6), the region responsible for the channel properties in several different porins has been assigned to loop III, which is referred to as the pore eyelet. Studies by Rosenbusch and co-workers (44) employing site-directed mutagenesis indicate that either deletion or replacement of key residues in this loop dramatically influences channel properties. Sequence comparisons within class 3 proteins reveal that loops II–IV are the most conserved, and therefore are conceivably related to functional aspects of these porins. Comparison of class 2 and class 3 in the putative loop II and III regions (Fig. 9) reveals closer relatedness than that determined from sequence alignment comparisons involving the remaining loop regions (refer to Table II). Despite the limited similarity of such a segment (i.e. 48%) among the class 2 and class 3 sequences, putative loop III is approximately identical in length to all PorB proteins studied to date (16), suggesting a conserved function-related role within the protein structure. The conductivity properties, ion selectivity, and gating activity of class 3 channels have been characterized recently by Song et al. (40) in lipid bilayer experiments, providing new insight into the fine and specific channel properties of PorB proteins.

Topological Studies of PorB Class 3 Protein—Although high resolution x-ray crystallographic structures have not been determined for any neisserial porins to date, topological models proposed by van der Ley et al. (14) offer some indication as to how these porins are arranged within the outer membrane. In a recent study using a Gibbs sampler motif analysis, Neuwald et al. (45) detected significant similarities when comparing 10–13-amino acid residue stretches among porins from unrelated species including those of known structure. In such an analysis, the related sequences comprise transmembrane segments from the extracellular to the periplasmic space. This method lends support to the current neisserial porin topology models (14), since the selected sequences reside in putative transmembrane segments, all of which are assumed to be oriented from extra to intracellular spaces.

As far as PorB class 3 protein is concerned only genetic approaches have been utilized in proposing its topology. On the basis of sequence alignment comparisons and DNA-based techniques, a model for class 3 proteins has been reported (15–17). Secondary structure predictions utilizing the primary amino acid sequence (46) suggest that these proteins contain approximately 36% β-sheets, and conform to a β-sheet structure which is represented by the putative transmembrane regions. Our circular dichroism data on these PorB class 3 proteins are in reasonable agreement with such predictions in that approximately 37% of the native secondary structure of these molecules are comprised of β-sheets. The characteristic ellipticity of the PorB class 3 near UV CD profile reflects the asymmetry of Trp residues relative to the aromatic amino acid environment. Previous studies of PorB class 2 protein indicate that this meningococcal porin exhibits a positive near UV CD spectrum (7), a finding that has been confirmed herein for class 3 protein.
Regarded as a fingerprint for PorB proteins, these structural features have recently been observed for PorA class 1 protein yet are absent in P2 porin isolated from *Haemophilus influenzae* that lacks tryptophan residues and exhibits a negative ellipticity.

Surface exposure among class 3 serotypes has not been characterized to date since this requires a systematic study to identify linear surface epitopes. Although Delvig et al. (19) reported a linear epitope in putative loop I, limited proteolysis of class 3 in combination with evidence from the present study indicating that no tyrosyl residues are exposed in the native molecule suggests that class 3 protein assembles as a highly compact structure in the native trimeric state. Moreover, since a number of potential protease digestion sites and tyrosyl residues are present in each of the putative extracellular loops (refer to Table I), it is conceivable that these loops experience minimal exposure, since class 3 protein is totally resistant to a number of proteases (36) including staphylococcal V8 (present study). Aromatic amino acid exposure is relatively low for well-characterized porins such as *E. coli* (34, 35). The length of extracellular loops that are generally susceptible to the action of proteases and are therefore surface-exposed are reported to be on the order of 15–20 residues (47), which may explain both the overall protease resistance of class 3 as well as the low degree of exposure obtained by the various experimental procedures. Protease resistance of PorB class 3 proteins has also been observed in studies employing intact meningococci (36). Unlike PorB class 2 protein (7), there is no evidence of trypsin/chymotrypsin digestion sites under native conditions. The remarkable protease resistance of class 3 is striking when compared with other neisserial porins. It has been shown that gonococcal porins contain several distinct protease cleavage sites for various enzymes (48, 49). Likewise, PorA class 1 protein has also been reported to undergo limited digestion under controlled conditions (36).

**SDS-induced Conformational Changes of Class 3 Protein**—In contrast with class 2 and the majority of porins characterized to date, both purified native and recombinant PorB class 3 proteins exhibit reduced SDS resistance in SDS-PAGE. The latter has served as the basis for initial characterization of class 3 serotypes in conventional SDS-PAGE analysis of whole cell extracts as a function of temperature (50). In the present study, we have incorporated specific modifications to conventional electrophoretic procedures (e.g., reducing SDS concentration in the running buffer as well as decreasing temperature and voltage) that have afforded the possibility of studying the oligomeric state of this porin by electrophoresis. The lack of oligomeric resistance in normal SDS-PAGE indicates that low SDS concentrations (i.e., approximately one-tenth of the concentration tolerated by other porins) are sufficient to disrupt the trimeric assembly of this porin. Although relatively uncom-
Extracellular loops of PorB class 2 and class 3 proteins have been proposed based on secondary structure prediction, epitope mapping and polymerase chain reaction studies (14, 17). The position of a trypsin cleavage site (7) determined for class 2 protein (7, 36) is noted in Loop I. PorB class 2 loop surface exposure has been predicted using antibody binding to whole cells (14). The variable regions (VR) in class 2 protein sequences have been assigned using polymerase chain reaction and hybridization techniques (17). A B-cell epitope recently recognized (18) in class 3 protein (loop I) is underlined. Sequence similarity between the PorB class 2 and class 3 proteins has been evaluated and gaps (–) introduced into the class 3 loop sequences to improve overall alignment.

| Loop no. | Protein | Amino acid sequence* | Surface exposure | Region variability | Percent similarity |
|----------|---------|----------------------|------------------|-------------------|--------------------|
| I        | C12     | VEVSVRKGAQVQGKSKATATQI | +                | VR1               | 45                 |
|          | C13     | VETR................VHOFVOTVITATGKI |                |                   |                    |
| II       | C12     | ASIATGSSGNR           |                  |                   |                    |
|          | C13     | ASIGATDSGNR           |                  |                   |                    |
| III      | C12     | SGDNVWESSGNTEDVLG101GSVRES | TGD..NPWDSKK...DYLGVNK1AEFAE | 48                 |                    |
|          | C13     |                  |                  |                   |                    |
| IV       | C12     | PRNAVNDVXKYYKTSRR   |                  |                   |                    |
|          | C13     | LNDNAG..............RINS |                  |                   | 36                 |
| V        | C12     | KYADLNTAERVNTAMAHVVKDY | +                | VR2               | 13                 |
|          | C13     | RHRHQVQSLN.............IEKY |              |                   |                    |
| VI       | C12     | AAKINEVSGSTKNCXHEQTO |                  | VR3               | 29                 |
|          | C13     |                  |                  |                   |                    |
| VII      | C12     | VNGVKNANYQDOQ |                  | VR4               | 46                 |
|          | C13     | VDNADIGN.EDYQ |                  |                   |                    |
| VIII     | C12     | KQGKAGKVEQOTQ |                  | VR5               | 46                 |
|          | C13     | OEGKGENKVFTAT |                  |                   |                    |

* The sequences used in the present study are derived from Murakami et al. (58) (PorB class 2 protein, serotype 2a, strain M986) and GenBank™ accession no. V02914 (PorB class 3, serotype 15, strain 8765).

Critical analysis of these studies reveals that caution should be exercised in the interpretation of results in order to distinguish between a true monomeric state versus a trimeric albeit SDS-sensitive porin conformation. To gain insight into the SDS-induced conformational changes of class 3 protein, we have combined electrophoresis, limited proteolysis, UV, and CD spectroscopic techniques. The results presented herein reveal that the native trimeric conformation is disrupted in the presence of SDS, resulting in the formation of a partially folded monomer since no fully folded monomeric species are detectable under such experimental conditions. Therefore, the SDS-induced conformational changes of class 3 protein are characterized by the generation of a partially unfolded state as demonstrated by the SDS-dependent generation of a molecular species exhibiting faster electrophoretic migration than that observed for the native trimeric state. These results suggest that such species could populate the in vivo folding pathways of porins, particularly in the case of class 3 protein, and support the notion that porins may undergo folding/unfolding through monomeric intermediates as suggested before for other membrane proteins including porins (54). In fact, the effects of SDS may be envisioned as the initial step on the folding pathway of class 3 protein in which a partially unfolded intermediate inserts into the membrane and eventually assumes its final β-sheet oligomeric conformation. Several proteins have been proposed as possible in vivo chaperone-like molecules involved in the folding/assembly of outer membrane proteins (55). However, there are multiple lines of experimental evidence including those presented in our laboratory (7, 22, present study) that porins undergo complete folding from denaturant solutions solely in the presence of amphiphilic molecules such as detergents, thereby precluding assistance from molecular chaperones. Nevertheless, identification of an SDS-induced conformer that is intermediate between a fully folded native state and unfolded random coil conformation supports the notion that the folding of outer membrane proteins in vivo may also constitute a multistep mechanism as proposed by Surrey et al. (15).

Concluding Remarks—The overall results on the structural characterization of PorB proteins are noteworthy since a clear distinction is observed between class 3 and the recently characterized class 2 protein (7). The major differences include surface exposure and apparent conformational stability, both of which are reduced significantly in class 3 protein. A systematic study of the physicochemical properties of these two proteins should provide valuable information regarding the elucidation of functional as well as structural domains accounting for the unique properties of meningococcal porins. The lower stability of class 3 when compared with other porins deserves further investigation. Based on the assumption that the trimeric stability is governed by the interactions occurring at the interface which appear to comprise loops II–IV (5, 57), it is conceivable that the differential degree of susceptibility observed for PorB alleles may arise from differences encountered within this region (especially in loop IV). Fig. 9 illustrates the major differences in the amino acid sequence of PorB class 3 relative to class 2, the latter employed as a reference based on the proposed topology model of van der Ley et al. (14). We are currently studying the structural and thermodynamic stability of PorA and PorB proteins, as well as engineered mutants, in order to elucidate the mechanisms responsible for such differences. The resultant data should provide significant insights into the folding/unfolding pathways and overall conformational stability of neisserial porins.

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