Pore Characteristics of Nontypeable *Haemophilus influenzae* Outer Membrane Protein P5 in Planar Lipid Bilayers

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ABSTRACT  The structure of outer membrane protein P5 of NTHi, a homolog of *Escherichia coli* OmpA, was investigated by observing its pore characteristics in planar lipid bilayers. Recombinant NTHi P5 was overexpressed in *E. coli* and purified using ionic detergent, LDS-P5, or nonionic detergent, OG-P5. LDS-P5 and OG-P5 could not be distinguished by their migration on SDS-PAGE gels; however, when incorporated into planar bilayers of DPhPC between symmetric aqueous solutions of 1 M KCl at 22°C, LDS-P5 formed narrow pores (58 ± 6 pS) with low open probability, whereas OG-P5 formed large pores (1.1 ± 0.1 nS) with high open probability (0.99). LDS-P5 narrow pores were gradually and irreversibly transformed into large pores, indistinguishable from those formed by OG-P5, at temperatures ≥40°C; the process took 4–6 h at 40°C or 35–45 min at 42°C. Large pores were stable to changes in temperatures; however, large pores were rapidly converted to narrow pores when exposed to LDS at room temperatures, indicating acute sensitivity of this conformer to ionic detergent. These studies suggest that narrow pores are partially denatured forms and support the premise that the native conformation of NTHi P5 is that of a large monomeric pore.

INTRODUCTION  Outer membrane protein P5 of NTHi is proposed to play a role in the initiation of infection by this opportunist pathogen, which is commonly associated with otitis media, chronic bronchitis, and other mucosal infections (1,2). NTHi P5 is a member of the *Escherichia coli* OmpA family of proteins. The amino acid sequences of *H. influenzae* NTHi P5 (353 residues) and *E. coli* OmpA (346 residues) are 50% identical and 64% similar (3). The C-terminal residues of NTHi P5 show even greater homology (65% identical, 77% similar) to periplasmic residues of OmpA (residues 165–325). Circular dichroism studies by Webb and Cripps (4) indicate a β-strand content of 49%, comparable to the 40% β-strand content reported for *E. coli* OmpA by Sugawara et al. (5). Moreover, anti-OmpA antisera react with NTHi P5 in Western blots. Although significant variation in the NTHi P5 gene has been noted during chronic infections (6), Webb and Cripps (7) found that the variable regions correspond to surface-exposed loops as predicted by alignment of P5 amino acid sequences with the topology model for OmpA proposed by Vogel and Jähnig (8).

Despite rigorous investigation by a variety of physical methods, the structure of this family of proteins remains controversial. The consensus opinion stated in a recent review (9) is that they exist in two forms: 1), a majority two-domain structure in which the N-terminal residues form a narrow low-conductance membrane pore and the C-terminal residues reside in the periplasm; and 2), a minority structure in which both domains combine and oligomerize to form trimeric, high-conductance membrane pores. The structure of the N-terminal membrane portion of the two-domain structure of *E. coli* OmpA has been well defined by two x-ray crystallography studies (10,11) and two NMR studies (12,13), and the structure of the C-terminal is depicted by the x-ray crystal structure of the OmpA-like C-terminal domain of RmpM from *Neisseria meningitidis* (14). However, the structure of the large pore is still unresolved. X-ray crystallography studies of other outer membrane porins indicate they typically form β-barrels with 16 strongly tilted strands (15–18). Stathopoulos (19) proposed a 16-stranded β-barrel large pore structure for OmpA, consistent with biochemical, immunochemical, and genetic topological data on proteins of this family. At present, there is little experimental information and there are conflicting hypotheses as to the structure of NTHi P5. Sirakova et al. (20) proposed a structure composed of coiled coils that assemble into fimbrae. The similarities of NTHi P5 with OmpA led Webb and Cripps (7) to suggest that NTHi P5 forms a two-domain structure in which the first 200 residues form a narrow 8-stranded β-barrel pore and the remaining 146 residues resided in the periplasm, where they bind to the peptidoglycan. The latter implies that NTHi P5 forms narrow low-conductance pores, but, to our knowledge, to date there have been no electrophysiological studies to test this postulate. The relative abundance of narrow and large pore forms reported for this family of proteins appears to differ

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Abbreviations used: NTHi, nontypeable *Haemophilus influenzae*; P5, outer membrane protein P5; OmpA, outer membrane protein A of *E. coli*; C₄Et₄, tetraethylene glycol monooctyl ether; LDS, lithium dodecyl sulfate; OG, n-octyl-b-D-glucopyranoside; SDS, sodium dodecyl sulfate; octyl-POE, octylpolyoxyethylene; DPhPC, diphytanoylphosphatidylcholine; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; I/V, current/voltage; IPTG, isopropyl-b-D-thiogalactopyranoside; PMSF, phenylmethylsulphonylfluoride.

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substantially with preparation history and incubation temperature. Sugawara and Nikaido (21) examined diffusion rates of proteoliposomes containing OmpA, which had been isolated using LDS and reconstituted with OG in the presence of urea buffer for 30 min at 37°C, and found only 2–3% large pores. Planar lipid bilayer studies by Arora et al. (22) of OmpA refolded into C6E4 micelles from urea-denatured protein and incubated overnight at 40°C showed the presence of ~55% narrow (50–80 pS) and ~45% large (260–320 pS) channels. Saint et al. (23) examined OmpA purified by preparative native-like electrophoresis and electroelution in octylpolyoxyethylene (octyl-POE) and observed only large pores (180 pS in 0.25 M KCl). Dé et al. (24) found that *Pseudomonas fluorescens* OprF, which shares sequence homology with the C-terminal domain of OmpA, formed large pores (180 pS in 0.25 M KCl). Dé et al. (24) found that *Pseudomonas fluorescens* OprF, which shares sequence homology with the C-terminal domain of OmpA, formed large pores (180 pS in 0.25 M KCl). Dé et al. (24) found that *Pseudomonas fluorescens* OprF, which shares sequence homology with the C-terminal domain of OmpA, formed large pores (180 pS in 0.25 M KCl). Dé et al. (24) found that *Pseudomonas fluorescens* OprF, which shares sequence homology with the C-terminal domain of OmpA, formed large pores (180 pS in 0.25 M KCl).

One may infer from the above studies that members of the OmpA family of proteins exist in vivo as two interconvertible conformations in ratios that vary with temperature, or they exist as a single large pore conformation which is labile to certain detergents and can be renatured by incubation at higher temperatures. Our recent studies of *E. coli* OmpA (26,27) tend to support the latter interpretation. Narrow pores were converted to large pores when maintained at temperatures ≥26°C, but the reverse transition was not observed when large pores were kept at low temperatures for extended periods. Large pores were converted to narrow pores only by denaturing agents. Accordingly, we here continue these studies by examining the influence of salient factors on the single-channel pore characteristics of *H. influenzae* NTHi P5 to better understand the physiological structure of this important outer membrane protein, as well as that of OmpA proteins in general.

**MATERIALS AND METHODS**

**NTHi P5**

Omp P5 from NTHi strain UC19 was originally recovered from the sputum of a chronic bronchitis patient and cloned in plasmid pCU28 by Webb and Cripps (26) with an N-terminal extension of 10 residues that included six histidines. This clone was generously provided by Dr. Dianne C. Webb (University of Canberra, Australia).

**Preparation of recombinant NTHi P5 protein**

Plasmid pCU28 containing His-tagged P5 was transformed into *E. coli* TG1 Z competent cells (Zymo Research, Orange, CA) and overexpressed by addition of 1 mM IPTG. After 2½ h, cells were collected and suspended in 50 mM K Hepes, pH 7.5, 100 mM KCl, 10 mM MgSO4, containing lysozyme (20 μg/ml), DNase (20 μg/ml), RNase (10 μg/ml), and protease inhibitors, leupeptin, pepstatin, and PMSF and lysed by ultrasonic disintegration. Unbroken cells were removed by low-speed centrifugation (Sorvall SS-34 rotor; 3000 rpm, 20 min; Sorval Products, Newtown, CT), and inclusion bodies were collected by centrifugation in the same rotor at 15,000 rpm for 30 min. The pellet was extracted with OG (1%; OG-P5) or alternatively with LDS (2%; LDS-P5) for different experimental purposes. SDS-PAGE of the two extracts showed a heavy band of P5 and minor components of numerous other proteins. Neither LDS-P5 nor OG-P5 bound to Ni-agarose beads, indicating that the His-tag was inaccessible. Consequently, each extract was further purified by chromatography on Superdex 5-200 (1.6 × 60 cm, HiPrep, Pharmacia, Piscataway, NJ) equilibrated with 0.4 M LiCl, 20 mM Tris-HCl, pH 7.5 containing 0.1% octylglucoside for OG-P5 or alternatively 0.1% LDS for LDS-P5. Fractions containing P5 were collected and rechromatographed on the same column using the same eluents. Purified P5 was concentrated using Microcon tubes (10 kD) to a final concentration of ~22 μg/ml.

**SDS-PAGE**

Samples were diluted (1:1, v/v) with loading buffer (0.125 M Tris-Cl, 4% SDS, 20% glycerol, 10% dithiothreitol, 0.2% bromphenol blue) and boiled for 5 min or left unheated. Proteins were electrophoretically separated on 12% SDS-PAGE gels (Bio-Rad, Hercules, CA) using Tris-glycine SDS buffer (Bio-Rad) at a constant voltage of 180 mV. Protein bands were visualized by staining with Coomassie brilliant blue R-250.

**Planar lipid bilayer measurements**

Planar lipid bilayers were formed from a solution of synthetic DPhPC (Avanti Polar Lipids, Birmingham, AL) in n-decane (Aldrich, Milwaukee, WI) (17 mg/ml). The solution was used to paint a bilayer in an aperture of ~150 μm diameter in a Delrin cup (Warner Instruments, Hamden, CT) between aqueous bathing solutions at 22°C of 1 M KCl, 10 mM Tris, pH 7.1. All salts were ultrapure (>99%) (Sigma-Aldrich, St. Louis, MO). Bilayer capacitances were in the range of 25–50 pF. After the bilayers were formed, 0.2–0.5 μl of NTHiP5 (2 μg/ml) in DPhPC vesicles were added to the cis compartment.

**Temperature studies**

For temperature studies, a Delrin cuvette was seated in a bilayer recording chamber made of a thermally conductive plastic (RCB-13T, Dagan Instruments, Minneapolis, MN). The chamber was fitted on a conductive stage containing a pyroelectric heating/cooler (HE 104R, Dagan Instruments). Deionized water was circulated through this stage fed by gravity to remove the heat generated. The pyroelectric heating/cooling stage was driven by a temperature controller (HCC-100A, Dagan Instruments). The temperature of the bath was monitored constantly with a thermoelectric device in the cis side, i.e., the ground side of the cuvette. Although there was a gradient of temperatures between the bath solution and conductive stage, the temperature within the bath could be reliably controlled to within ±0.5°C. After the bilayer membrane was formed, 0.2–0.5 μl of NTHiP5 (2 μg/ml) in DPhPC vesicles were added to the cis compartment and channels were observed after gentle stirring.

**Recording and data analysis**

Unitary currents were recorded with an integrating patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). The *trans* solution (voltage command side) was connected to the CV 201A head stage input, and the *cis* solution was held at virtual ground via a pair of matched Ag-AgCl electrodes. Currents through the voltage-clamped bilayers (background conductance) < 3 pS) were low-pass filtered at 10 kHz (~3 dB
cutoff, Besel type response) and recorded after digitization through an analog-to-digital converter (Digidata 1322A, Axon Instruments). Data were filtered at 100 Hz through an 8-pole Besel filter (902LPF, Frequency Devices) and digitized at 1 kHz using pClamp9 software (Axon Instruments, Haverhill, MA). Single-channel conductance events were identified automatically and analyzed by using Clampfit9 software (Axon Instruments).

RESULTS

Effect of preparation history on heat modifiability

NTHi P5, like other members of the OmpA family of proteins, is heat modifiable; i.e., the electrophoretic mobility in SDS-PAGE gels is comparable to the true MW after heating in SDS, but it migrates more rapidly and appears to have a lower MW protein when unheated (21–26). The convention has been to regard the slower moving form as denatured and the faster moving form as native. However, in the case of E. coli OmpA, we recently found that this method did not differentiate between the narrow pore and large pore forms, both of which migrated as ‘native’ protein (27). Nevertheless, the method is useful to determine whether completely denatured protein is present in a preparation of purified protein.

Recombinant NTHi P5 was overexpressed in E. coli and extracted from inclusion bodies by two methods: in one case with the ionic detergent LDS (LDS-P5) and in the second case with the milder nonionic detergent OG (OG-P5). Each extract was further purified by chromatography on Superdex S-200, using for each the same detergent as used in the extraction. The electrophoretic mobilities in SDS-PAGE gels of NTHi P5 in the two preparations were examined for samples left unheated in SDS-containing loading buffer and samples boiled in the same buffer for 5 min. As shown in Fig. 1, LDS-P5 and OG-P5 were both heat modifiable and indistinguishable by gel migration. By conventional criteria, neither preparation contained denatured protein—both unheated preparations migrated as native protein.

Effect of preparation history on single-channel characteristics in planar bilayers

The single-channel characteristics of LDS-P5 and OG-P5 were next compared in planar lipid bilayers. Each preparation was individually incorporated into vesicles of DPhPC and then into planar lipid bilayers of DPhPC between aqueous solutions of 1 M KCl in 10 mM Tris-Cl buffer, pH 7.1 at 22°C. Despite their identical migration on SDS-PAGE gels, LDS-P5 and OG-P5 formed pores with very different characteristics. As shown in representative current tracings in Fig. 2, LDS-P5 (bottom trace) formed low-conductance pores with major chord conductance of 58 ± 6 pS and minor chord conductance of 130 ± 15 pS. In contrast, OG-P5 (upper trace) formed high-conductance pores with chord conductance of 1.1 ± 0.1 nS and multiple subconductance states. The main conductance state (B) and three of the most frequently observed subconductance states (A,C,D) are labeled in Fig. 3. The conductance of these two subconductance states (A and C) were very close in magnitude, i.e., 1.17 ± 0.1 nS and 0.96 ± 0.1 nS, to that of the main conductance state (1.13 ± 0.1 nS), whereas the magnitude of the third subconductance state, D, was 0.50 ± 0.07 nS. The open probability was 0.99. The open pore was 80–90% of the time in the main conductance state, 10–20% in subconductance state A, and <1% in subconductance states C and D.

Both narrow and large pore forms were very stable in the bilayer at room temperatures. In numerous (>20) lengthy (>1 h) observations of several separate preparations of each conformer using negative and positive voltages up to 150 mV, large pores were not observed for LDS-P5 and narrow pores were not observed for OG-P5. LDS-P5 narrow pore were not affected by addition of OG; however, OG-P5 pores were converted to narrow pores by addition of LDS. Accordingly, incubation of LDS-P5 in DPhPC vesicles with 0.1% OG 

FIGURE 1 SDS-PAGE of NTHi OG-P5 and LDS-P5. Lane 1, LDS-P5 heated; lane 2, LDS-P5 unheated; lane 3, OG-P5 heated; and lane 4, OG-P5 unheated.

FIGURE 2 Representative single-channel current recordings of NTHi P5 incorporated in planar lipid bilayers formed from DPhPC/n-decane between symmetric bathing solutions of 1 M KCl in 10 mM Tris-Cl buffer, pH 7.1 at 22°C; 0.2–0.5 μl of 0.2 μg/ml NTHi P5 in DPhPC vesicles was added to the cis compartment (ground), and channels usually were observed within 5 min. Clamping potential was +30 mV. Data were filtered at 100 Hz. The closed state is delineated by a horizontal line under traces. Lower trace. LDS-P5. Upper trace, OG-P5. Data for histograms were summarized from 10 independent experiments for lower trace and 12 independent experiments for upper trace.
For measurements at increasing temperature, channels were inserted in the planar bilayer at 22°C and chord conductance was determined from I/V relationships. Temperature was increased in small steps of 2°C to 35°C and then maintained constant at each selected temperature for 10 min before chord conductance was measured, as shown in Fig. 5 A. Both OG-P5 (open circle) and LDS-P5 (solid upward triangle) displayed the expected gradual increase in conductance with increase in temperature for the entire range.

However, when LDS-P5 was incubated above 40°C for longer periods, narrow pores were gradually transformed to large pores that displayed the same gating mode and conductance as OG-P5. This transition from narrow to large pore was strongly temperature dependent. No transition was observed at 37°C, even when LDS-P5 vesicles were incubated in a constant-temperature bath for 4 days before observation in the planar bilayer. At 40°C, the transition in the planar bilayer required 4–6 h, but took only 40–45 min at 42°C. Fig. 5 A shows the conversion of narrow pores of LDS-P5 (solid upward triangle) to large pores (solid downward triangle) (arrow) after incubation in the bilayer at 42°C for ~45 min. Representative current traces that illustrate the effects of incubation at 42°C on single-channel currents of OG-P5 and LDS-P5 are shown in Fig. 5 B. OG-P5 channels displayed a chord conductance of 2.2 ± 0.2 nS after 30 min incubation at this temperature (upper trace), and conductance remained unchanged after longer incubation. LDS-P5 was still in the low-conductance state after 30 min (middle trace) with a chord conductance value of 166 pS ± 20 pS. However, after 45 min at 42°C, LDS-P5 converted to large pores (shown in lower trace) with chord conductance of 2.3 ± 0.2 nS, within the same range as OG-P5. The conversion of narrow LDS-P5 pores to large pores was not thermally reversible; i.e., large pores did not revert to small pores when temperatures were slowly decreased to 23°C in the planar bilayer, even when maintained at 23°C for several hours (Fig. 5 A, solid downward triangle).

The transition of LDS-P5 from narrow to large pore with gradual increase in temperature (~1° per min) is shown in Fig. 6. Representative currents in the upper trace display the steady increase in conductance of the pore with temperature in the range 23°C–42°C. The middle traces are representative of current records taken during incubation at 42°C. They are divided into three equal segments that demonstrate the beginning of the transition in the period 30–35 min, the noisy and disorganized channels of the transition at 35–40 min, and the formation of the mature large pore at 40–45 min. In four independent experiments, the time at which each segment began varied by <2 min. The large pore formed by LDS-P5 remained stable and conductance gradually decreased when temperatures were slowly lowered from 42°C to 23°C (lower trace of Fig. 6 shows the final 3 min of cooling). Continued incubation at 23°C or storage at refrigerator or freezer temperatures for extended periods did not result in reversion of large pores to narrow pores, demonstrating the irreversibility of the thermal transition.
DISCUSSION

Our studies indicate that recombinant NTHi P5 is a large pore that readily denatures to a narrow pore structure when exposed to ionic detergents, even at low temperatures. Using cells cultured under identical conditions, large pores (1.1 ± 0.1 nS) were observed when inclusion bodies were extracted with the milder nonionic detergent, OG, and narrow pores (58 ± 6 pS) when inclusion bodies were extracted with the more denaturing ionic detergent, LDS (Fig. 2). Narrow pore conformers were stable in either detergent at temperatures <40°C, but they were transformed into large pores when incubated at temperatures ≥40°C (Figs. 5 and 6). Conversely, large pore conformers, though acutely sensitive to LDS (Fig. 4), were insensitive to increases or decreases in temperature.

The conditions used for purification of membrane proteins are always case sensitive for reconstitution of the native form, and recognition of the native structure can be difficult. Electrophoretic mobility on SDS gels has been widely used to distinguish native and denatured forms of the OmpA family of proteins. However, we find here, as we did earlier for E. coli OmpA (27), that this method can be useful for identifying denatured protein, but it does not differentiate between narrow and large pore forms of NTHi P5, both of which migrate as native protein (Fig. 1). The two conformers can best be distinguished by single-channel observations in planar lipid bilayers.

The narrow to large pore transition in NTHi P5 required significantly higher temperatures than in E. coli OmpA (26,27). Accordingly, OmpA was converted from narrow to large pores after ~7 h at 26°C, 30 min at 37°C, and 13 min at 42°C, whereas the transition of NTHi P5 from narrow to large pores...
did not occur at 37°C, even after 4 days incubation, and required 4–6 h at 40°C and 35–45 min at 42°C. It is of interest in this regard that Webb and Cripps (4) reported that refolding of purified His-tagged NTHi P5 in vesicles containing OG required extended incubation at 42°C. The rapid conversion of large pores to narrow pores on exposure to ionic detergent at room temperatures explains the absence of large pores in the LDS extracts and also the comparable mobilities of LDS and OG preparations on SDS gels (Fig. 1).

The relatively low temperature for the transition from narrow to large pore in the case of E. coli OmpA led to the suggestion that the narrow pore conformer may be a folding intermediate (27). However, since the transition in NTHi P5 does not occur at physiological temperatures, it appears more likely that the narrow pore is a partially denatured form. By analogy with E. coli OmpA, one may suspect that ionic detergent removes the C-terminal segment from the bilayer, leaving the N-terminal polypeptide to form a narrow pore. In the case of OmpA, it has been proposed that the large pore is an oligomeric structure (9). However, repeated observations in the planar lipid bilayer of the transition of single molecules of OmpA (27) and NTHi P5 (Fig. 6) from the narrow pore to the large pore structure with increasing temperature indicates that the large pore, like the narrow pore, is a monomeric structure. By analogy with other large conductance porins (15–18), the large pore is likely a 16-stranded β-barrel structure.

In summary, one may conclude from these studies that the native conformation of H. influenzae NTHi P5 is a large monomeric pore. This information may be useful in evaluating its role in the initiation of infections. Moreover, we identify two factors that may elucidate the diverse reports citing differing ratios of narrow and large pore conformers in members of the OmpA family of porins (21–25), namely, the tendency of the large pore structure to undergo partial denaturation to a narrow pore form on exposure to harsh detergents and the ability of the narrow pore conformer to reassemble at incubation temperatures that are specific to each porin. It appears highly likely that the ability of the large pore conformers has resulted in great underestimation of their abundance and their contribution to outer membrane functions. Conversely, the physiological importance of the narrow pore structure, and perhaps even its physiological existence, are in question.

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