FEBS Letters 392 (1996) 304–308 FEBS 17448

Purification and refolding of recombinant *Haemophilus influenzae* type b porin produced in *Bacillus subtilis*

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Received 20 May 1996; revised version received 15 July 1996

Abstract The major diffusion channel in the outer membrane of *Haemophilus influenzae* type b (Hib) is porin (341 amino acids; M_r 37 782). The *Hib* porin gene was cloned and overexpressed in *Bacillus subtilis*. Recombinant Hib porin (Bac porin), having aggregated into inclusion bodies, was purified under denaturing conditions and subsequently refolded. To compare Bac porin that is intrinsically devoid of lipooligosaccharides versus native Hib porin, the properties of Bac porin were assessed by the following four criteria: circular dichroism spectroscopy, channel formation in planar bilayers, resistance to trypsin digestion and formation of the conformational epitope recognized by an anti-Hib porin monoclonal antibody. We conclude that in the absence of lipooligosaccharides, Bac porin was refolded into a functional form which closely resembled the structure of Hib porin.

Key words: Porin; Membrane channel; Inclusion body; Refolding; *Haemophilus influenzae* type b

1. Introduction

Porins are water-filled, channel-forming proteins that span the bacterial outer membrane and that confer on the outer membrane the properties of a molecular sieve [1]. Structure–function relationships of porin have advanced significantly because of the determination to atomic resolution of the crystal structures of five different porins: *Rhodobacter capsulatus* porin [2], *Rhodopseudomonas blastica* porin [3] and three *Escherichia coli* porins, OmpF, PhoE [4] and LamB [5]. For all five porins, the crystal structures show the polypeptide folded into antiparallel β-strands with the N-terminus and C-terminus joining to form a β-barrel. Short β-hairpin turns are on the periplasmic side of the barrel and long loops of variable length are found at the extracellular face.

Based on the hydrophobicity, amphiphilicity and turn propensity of the amino acid sequence of Hib porin, we generated [6] a secondary structure model of the topological organization of Hib porin. Our model proposed that Hib porin is folded into a β-barrel with 16 antiparallel strands, eight short periplasmic loops and eight long surface-exposed loops. The extracellular location of two of the loops was confirmed by the epitope mapping of Hib porin-specific monoclonal antibodies (mAb) which reacted by flow cytometry with intact Hib cells.

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Abbreviations: Hib, *Haemophilus influenzae* type b; mAb, monoclonal antibody; LPS, lipopolysaccharide; CD, circular dichroism; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline

Porins of Gram-negative bacteria bind tightly to lipopolysaccharide (LPS) [7,8]. Purified OmpF of *E. coli* was shown to have up to nine molecules of LPS associated with each trimer [8,9]. While most of this LPS was loosely bound and easily dissociated, it was estimated that one LPS molecule remained tightly bound to each OmpF trimer [9]. FPLC-purified Hib porin was estimated to contain 10% (w/w) residual lipooligosaccharides [10]. Several reports have documented the effect of LPS on the biosynthesis, folding and function of porins. *E. coli* mutants expressing defective LPS molecules of the 'deep rough' type have very low amounts of OmpF and OmpC porin in their outer membrane [11]. Sen and Nikaido examined *E. coli* spheroplasts secreting water-soluble, monomeric OmpF. LPS bound to secreted OmpF and facilitated its renaturation, trimerization and incorporation into vesicles [12]. In contrast, Eisele and Rosenbusch [13] found that when OmpF was purified free of LPS, it could be denatured and then renatured in the presence of detergents and phospholipids into trypsin-resistant trimers so that its circular dichroism (CD) spectrum matched that of native OmpF. However, refolded OmpF was not capable of pore-forming activity in planar bilayers unless LPS, free lipid A or part of a lipid A were added [14].

Our laboratory reported [15] cloning of the Hib porin gene *ompP2* into an expression vector of *Bacillus subtilis*. In such an LPS-free background, Bac porin was produced in large quantities and it aggregated into intracellular inclusion bodies. The goals of this study were to purify Bac porin from inclusion bodies and to show that Bac porin can be refolded in the presence of detergents, but in the absence of LPS.

2. Materials and methods

2.1. Purification of Bac porin

Cloning of the *ompP2* gene from Hib ATCC 9795 into the expression vector pKTH288 of *B. subtilis* [16] has been described [15]. *B. subtilis* IH6140 cells (6 L) expressing Bac porin were grown at 37°C to stationary phase in 2×L broth containing kanamycin (30 μg/ml). Cells were harvested by centrifugation and inclusion bodies were prepared by amplifying the small-scale protocol [15]. Inclusion bodies were collected by centrifugation (7000×*g*, 15 min, ambient temperature) and proteins (Fig. 1, lanes 4–6) were solubilized by addition of an equal volume of 20 mM Tris-HCl, pH 8.0, 8 M urea, 5 mM EDTA, 10 μM phenylmethyl-sulfonyl fluoride. The mixture was centrifuged (15 000×*g*, 15 min, ambient temperature) to remove a minor amount of residue and diluted with 20 mM Tris-HCl, 5 mM EDTA to a final urea concentration of 6 M. The sample was dialysed against 50 mM Tris-acetate, pH 6.0, 6 M urea, a buffer in which Bac porin bound optimally to a cation exchange resin. For further purification, proteins from urea-solubilized inclusion bodies were subjected to fast protein liquid chromatography (FPLC) on an XK 26/206B column (Pharmacia) containing 80 ml CM-Sepharose CL-6B (Pharmacia). A sample of 1200 mg in 120 ml was loaded. The salt concentration of the elution buffer was raised from 0 to 0.5 M NaCl. A symmetrical peak was eluted at 0.2 M NaCl: it contained Bac porin

PII S0014-5793(96)00841-1

plus small amounts of other proteins (Fig. 1, lanes 7–9). Peak fractions containing Bac porin were pooled, dialysed against distilled water to precipitate the proteins, and centrifuged. To the pelleted material was added a minimum volume of 20 mM Tris-HCl, pH 8.0, 6 M urea, 5 mM EDTA giving a suspension of proteins at 40 mg/ml. Gel filtration chromatography on Sephacryl S-200 HR (Pharmacia) in 6 M urea required a column (90×2.6 cm) sufficient to accommodate a load of 250 mg. The leading fractions of the peak contained highly purified Bac porin (Fig. 1, lanes 10–12). Proteins were quantitated by the bicinchoninic assay [17], subjected to SDS-PAGE [18] and silver stained [19]. The yield of purified Bac porin per liter of culture was estimated as 50 mg.

2.2. Refolding of Bac porin

Purified Bac porin in 6 M urea was precipitated by dialysis against distilled water. Following centrifugation, the pellet was suspended in 50 mM Tris-HCl, pH 8.0, 1% SDS to a concentration of 1 mg/ml. The sample was boiled (10 min) and transferred to ice (15 min). Refolding was achieved by a three-step protocol that necessarily included Zwittergent Z-3,14, the same detergent in which Hib porin was originally isolated [20] and was found to be active in channel formation [21]: (1) SDS-denatured Bac porin was diluted 5-fold with a solution of 50 mM Tris-HCl, pH 8.0, 0.1% Zwittergent Z-3,14; (2) diluted Bac porin was lyophilized, resuspended in water to one-fifth its original volume and then dialysed for 12 h against 50 mM Tris-HCl, pH 8.0, 0.1% Zwittergent Z-3,14; (3) to ensure that the final Zwittergent concentration was 0.1%, Bac porin was bound to CM-Sepharose and eluted with 50 mM Tris-HCl, pH 8.0, 1 M KCl, 0.1% Zwittergent Z-3,14. The salt concentration was lowered by dialysing (12 h) against 50 mM Tris-HCl, pH 8.0, 0.1% Zwittergent Z-3,14.

In attempting to refold Bac porin, several protocols were devised. Many protocols yielded 'misfolded' Bac porin, a product which was included as a control for some experiments. Misfolded Bac porin was generated as follows: Bac porin, denatured in 50 mM Tris-HCl, pH 8.0, 2% SDS and at a concentration of 5 mg/ml, was diluted 5-fold with a solution of 50 mM Tris-HCl, pH 8.0, 0.1% Zwittergent Z-3,14. Dialysis (12 h) against 50 mM Tris-HCl, pH 8.0, 0.1% Zwittergent Z-3,14 served to reduce the amounts of SDS.

2.3. CD spectroscopy

CD spectra were recorded at room temperature on a Jasco-710 spectropolarimeter. Ten spectra were recorded, averaged and corrected for base-line contribution due to buffer. Measurements were performed in a cylindrical quartz cell of 0.5 mm path length. The protein concentration for each sample was determined by the bicinchoninic assay [17].

2.4. Lipid bilayer experiments

Planar bilayer studies were executed as previously described [22] but with the following change in instrumentation: an AXOPATCH-1D amplifier (Axon Instruments) was used to measure the ionic current across the membrane.

2.5. Trypsin digestion

Porin (2 μg) was digested for 1 h at 37°C with 200 ng of TPCK-treated trypsin (Sigma) in a total volume of 15 μl. The reaction was terminated by the addition of SDS-PAGE sample buffer (15 μl) and by boiling for 5 min. Trypsin cleavage products were resolved by SDS-PAGE and either silver stained or Western blotted.

2.6. Western blotting

Following SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose paper (Schleicher & Schuell). The nitrocellulose paper was blocked with 1% BSA in Tris-saline and incubated with affinity-purified mAbs diluted in Tris-saline. The developing antibody was 187-AP, a rat monoclonal that is specific for the mouse κ light chain and that is conjugated to alkaline phosphatase. Blots were developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium-nitro-blue tetrazolium (BioRad Laboratories).

2.7. ELISA

Purified porin was suspended in a solution of 10 mM ammonium acetate, 10 mM ammonium carbonate, pH 8.2 to a concentration of 10 μg/ml. A 50 μl aliquot of antigen solution was added to each well of a microtiter plate (Maxisorp F96, Nunc). Antigen was dried by allowing the volatile salts to evaporate during 12 h incubation at 37°C. Blocking or antibody incubation steps were done with phosphate-buffered saline (PBS) containing 5% skim milk and 0.5% skim milk, respectively. Washes were with PBS, 0.05% Tween 20 (Fisher Biotech). The secondary antibody was mAb 187-AP and was detected with *p*-nitrophenyl phosphate (2 mg/ml in 10 mM diethanolamine, pH 9.5, 0.5 mM $MgCl_2$; GIBCO BRL).

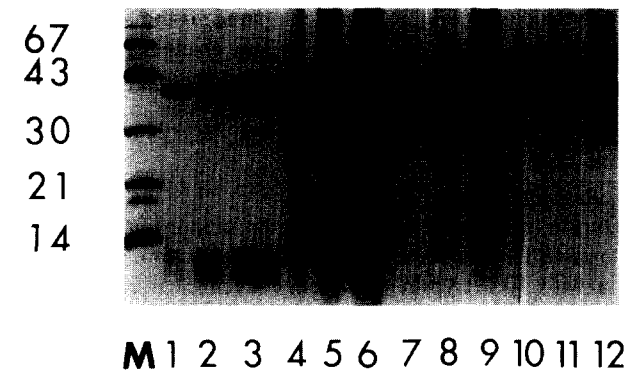

Fig. 1. Purification of recombinant Bac porin. Protein samples were loaded onto 15% SDS-PAGE and identified on the gel by silver staining. Lane M: molecular weight marker proteins are indicated according to their molecular mass in kDa. Lanes 1–3: porin purified from the outer membrane of Hib as previously described [10] (250, 500 and 1000 ng). Lanes 4–6: inclusion body proteins from *B. subtilis* cells expressing Bac porin (500, 1000 and 2000 ng). Lanes 7–9: proteins recovered following chromatographic purification of Bac porin on CM-Sepharose CL-6B (250, 500 and 1000 ng). Lanes 10–12: purified Bac porin after chromatography on Sephacryl S-200 (250, 500 and 1000 ng).

3. Results

3.1. CD spectroscopy

Secondary structures of Hib porin, refolded Bac porin and misfolded Bac porin were evaluated using CD spectroscopy (Fig. 2). The CD spectrum of Hib porin showed a CD minimum at 218 nm, a value close to 217 nm reported for OmpF [13]. Compared to Hib porin, refolded Bac porin showed a slightly altered CD spectrum: the CD minimum was at 220 nm, again indicative of β-sheet as the dominant secondary structure. Refolded Bac porin also showed a minor circular dichroism signal at approximately 210 nm, a region characteristic of α-helical structure. The CD spectrum of misfolded Bac porin, with a minimum at 208 nm and a shoulder at 220 nm, is typical for proteins adopting α-helical conformations [23]. However, the weak ellipticity of this spectrum suggests a small percentage of α-helical structure with much of the protein remaining structureless.

3.2. Channel-forming activity of Bac porin

The channel-forming properties of refolded Bac porin were tested in single channel experiments. To the 1 M KCl solution bathing the planar bilayer, refolded Bac porin was added at a concentration of 1 ng/ml. Stepwise increases in membrane conductance are attributed to the spontaneous insertion of Bac porin into the membrane. The amplitudes of the conductance steps were measured and data are summarized in a histogram (Fig. 3A). For references, the experiment was repeated with misfolded Bac porin (Fig. 3B) and Hib porin (Fig. 3C). Refolded Bac porin formed functional pores: 43% of the channels showed conductances between 0.6 and 0.8 nS. This

range was slightly shifted when compared to the single channel conductivities for Hib porin: 41% of channels were in the 0.7–0.8 nS range. Surprisingly, misfolded Bac porin was capable of forming channels: 30% of channels were in the 0.7–0.8 nS range.

3.3. Resistance to trypsin digestion

Trypsin is predicted to cleave Hib porin more than 40 times at Arg and Lys residues. However, after digestion (1 h) of Hib porin with 10% trypsin (w/w) only seven cleavage products were resolved by SDS-PAGE [10] suggesting that the native conformation of Hib porin protects most of the potential trypsin-sensitive sites. Hib porin, refolded Bac porin and misfolded Bac porin were subjected to trypsin digestion. The digestion products were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with either mAb POR.6 (Fig. 4A) or mAb POR.1 (Fig. 4B). While misfolded Bac porin was completely digested by trypsin, refolded Bac porin yielded a blotting pattern of cleavage products similar to that seen for Hib porin.

3.4. Reactivity of Hb-2 to refolded Bac porin

The mouse mAb Hb-2 was previously characterized as recognizing a conformational, surface-exposed determinant on Hib porin [24]. To determine whether refolded Bac porin regenerated this conformational epitope, Hib porin, refolded Bac porin and misfolded Bac porin were tested by ELISA for their reactivity with mAb Hb-2 (Fig. 5A) and with mAb POR.6 (Fig. 5B). Hib porin and refolded Bac porin showed similar reactivities to Hb-2 while misfolded Bac porin showed very poor binding by Hb-2. Importantly, all three antigens showed a similar reactivity to POR.6, a mAb which recognizes a linear epitope and serves as a positive control.

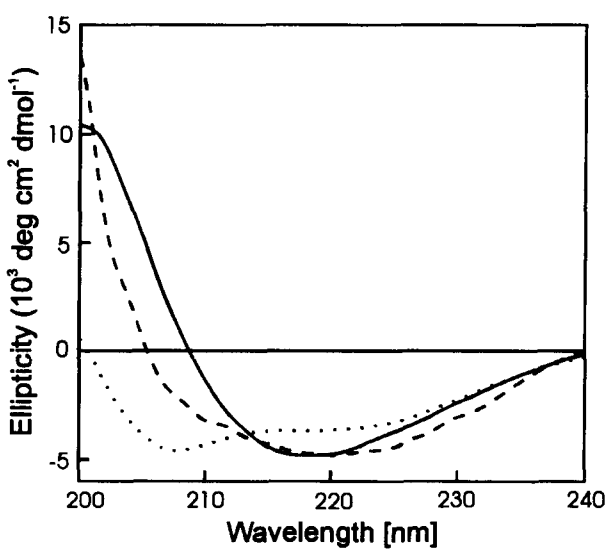

Fig. 2. CD spectra of refolded Bac porin (– – –), misfolded Bac porin (·····) and Hib porin (———). Each spectrum represents the average of 10 scans recorded at room temperature in a cell of 0.5 mm path length. The protein concentrations for refolded Bac porin, misfolded Bac porin and Hib porin were 0.1, 0.6 and 1.0 mg/ml, respectively. All three proteins were in buffer containing 50 mM Tris-HCl, pH 8.0, 0.1% Zwittergent Z-3,14.

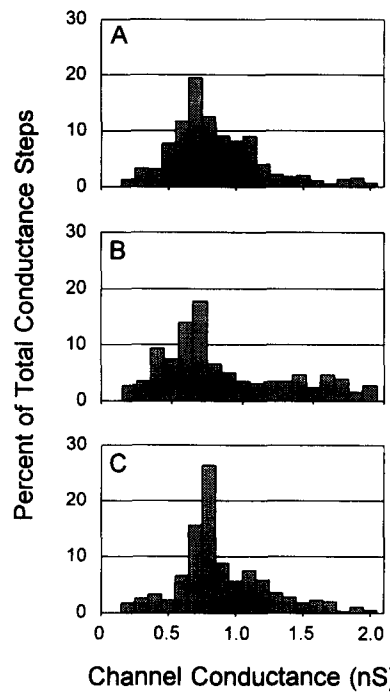

Fig. 3. Comparison of channel conductances as measured in planar bilayers for three porins: refolded Bac porin (A), misfolded Bac porin (B) and Hib porin (C). Conductance steps were recorded at a transmembrane potential of 10 mV and using 1 M KCl as the electrolyte. The different porins were diluted with 50 mM Tris-HCl (pH 8.0) to 5 ng/μl. Approximately 1 μl of this material was added to the 5 ml teflon chamber such that the final porin concentration in the chamber was 1 ng/ml. The total number of conductance steps analyzed was as follows: (A) 459; (B) 258; (C) 402.

4. Discussion

We previously described the high-level expression of Hib porin in *B. subtilis* and for immunological studies [15] used Bac porin from inclusion bodies. To exploit the Bacillus expression system for the production of large amounts of purified Bac porin, protocols were devised to refold Bac porin so that it adopts native conformation. The native state of refolded Bac porin was assessed according to four criteria: spectroscopic properties, channel formation in planar bilayers, resistance to trypsin digestion and reactivity by the mAb Hb-2.

The CD spectrum of refolded Bac porin indicated β-sheet as the major secondary structure along with some minor α-helical structure. The α-helical signal may have arisen from a small subpopulation of Bac porin that did not fold properly. Alternatively, differences between the CD spectra of Hib porin and refolded Bac porin may reflect some variance in the structure of these two proteins. Compared to Hib porin (341 amino acids), Bac porin has an amino terminal extension of 11 amino acids, five of which are positively charged [15]. This amino terminal extension may have prevented Bac porin from refolding into a conformation that is indistinguishable from that of Hib porin.

Channel formation in planar bilayers is a hallmark of bacterial porins [25] and experimental data lead to the calculation of channel conductances, values which are characteristic for any given porin. Refolded Bac porin efficiently formed channels in planar bilayers and these channels showed a conduct-

ance (0.6–0.8 nS) comparable to that of Hib porin (0.7–0.8 nS). These channel conductances contrast with our report [15] for Bac porin extracted with Zwittergent Z-3,14 directly from inclusion bodies: inclusion body-extracted Bac porin showed a conductance of 1.4–1.6 nS. Since inclusion body-extracted Bac porin was not subjected to any refolding protocols, and since our data indicated [15] that this preparation of Bac porin might be partially denatured, we propose that purified refolded Bac porin adopted a conformation closer to the native state than the crudely extracted porin.

Surprisingly, misfolded Bac porin, whose CD spectrum indicates some α-helical structure, was proficient at forming channels in planar bilayers. A subpopulation of the misfolded Bac porin sample may be in some native or near native conformation and was thus responsible for channel formation. Alternatively, misfolded Bac porin may have undergone a transition from an α-helical conformation to a β-barrel channel forming conformation either following dilution into the 1 M KCl solution that bathes the planar bilayer or during its insertion into the planar bilayer.

A previous report [10] from our laboratory showed that upon trypsin digestion of Hib porin, seven cleavage products were resolved by SDS-PAGE. Microsequencing of three N-terminal fragments of 18.9, 18.4 and 17.1 kDa, and two C-terminal fragments of 19.5 and 19.1 kDa identified cleavages between Lys_{161} and Ile_{176}. An additional cleavage after Lys_{32} accounted for the 15.0 and 14.0 kDa fragments. Since there are potentially over 40 trypsin-sensitive sites throughout Hib porin, it was apparent that the native conformation of porin rendered the majority of sites inaccessible to trypsin. As a diagnostic comparison of the conformations of Hib porin and refolded Bac porin, we subjected misfolded Bac porin, refolded Bac porin and Hib porin to trypsin digestion. Misfolded Bac porin was digested to peptide fragments too small to be resolved by SDS-PAGE; most if not all of the trypsin recognition sites were accessible to trypsin. In contrast refolded Bac porin yielded the same patterns of N-terminal and C-terminal fragments as seen for Hib porin, supporting our conclusion that refolded Bac porin adopted a tightly folded conformation similar to that of Hib porin. Finally, the reactivity of Hb-2 to refolded Bac porin indicates that the conformational epitope recognized by Hb-2 was regenerated in refolded Bac porin. We therefore conclude that in the absence of lipooligosaccharides, Bac porin was refolded into a functional form closely resembling the native state. In agreement with our findings, Schmid et al. reported [26] that *Rhodopseudomonas blastica* porin, overexpressed in *E. coli* and purified from inclusion bodies, was renatured in the absence of LPS. Refolded recombinant *R. blastica* porin formed crystals and X-ray diffraction studies indicated that the atomic structure of the recombinant porin was identical to that of the crystallized native porin.

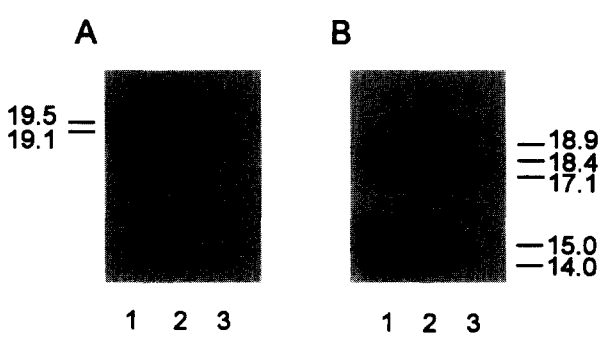

Fig. 4. Susceptibility of refolded Bac porin to trypsin digestion. Hib porin (lane 1), refolded Bac porin (lane 2) and misfolded Bac porin (lane 3) were digested with trypsin (10% w/w) for 1 h at 37°C. The tryptic fragments were separated on SDS-PAGE (15%), transferred onto nitrocellulose and probed with mAb POR.6 (A) or mAB POR.1 (B). The Hib porin epitopes recognized by POR.6 and POR.1 were previously mapped [6] to amino acids 318–325 and amino acids 116–126, respectively. The molecular masses (kDa) of three of the trypsin fragments are indicated in the left margin.

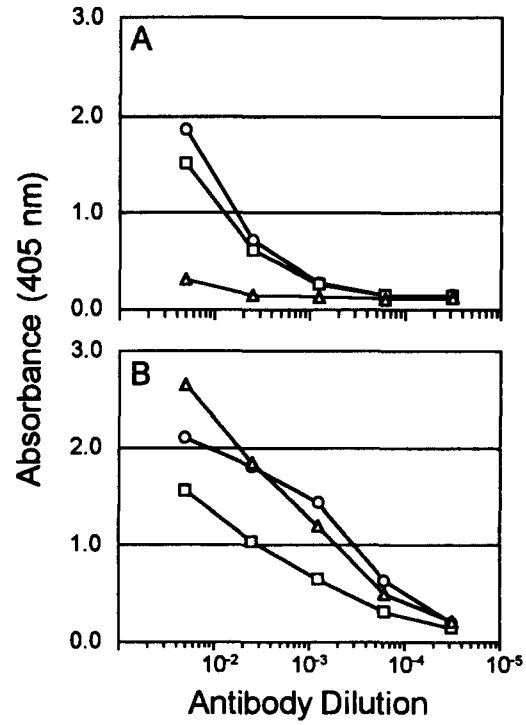

Fig. 5. Reactivity of mAbs to refolded Bac porin. Identical amounts (250 ng) of Hib porin, refolded Bac porin and misfolded Bac porin were added to a microtiter plate and subjected to an ELISA. Threefold dilutions (starting from 1/75) of anti-Hib porin mAbs were reacted with the antigens. The reactivities of Hb-2 in (A) and POR.6 in (B) to Hib porin (○), refolded Bac porin (□), and misfolded Bac porin (△) are shown. All the assays were performed in duplicates and the average absorbance for each mAb dilution was plotted. The difference between duplicate readings never exceeded 10% of the average absorbance.

Our first attempts at refolding Bac porin began with urea-denatured Bac porin; the denaturant concentrations were lowered by dialysis in the presence of different detergents and buffers. Although numerous conditions were evaluated, Bac porin either precipitated during dialysis or, when soluble, did not form channels in planar bilayers (data not shown). Refolding was achieved only when Bac porin was initially in the SDS-denatured state. Interestingly, the detergent refolding protocols described for OmpF [13] and OmpA [27] also began with these outer membrane proteins in the SDS-denatured state. Protein refolding is an empirical exercise and the rules for efficient refolding of SDS-denatured proteins are unknown. Since SDS remains during renaturation and it has a surface negative charge density comparable to LPS, Nikaido [1] suggested that SDS mimics LPS during refolding. Alternatively, renaturation to a β-barrel structure may be more efficient from the predominately α-helical conformation adopted

by SDS-denatured proteins [13,27] than from the random-coil conformation adopted by urea-denatured proteins [13].

Acknowledgements: Research was supported by Grant MT-6911 to J.W.C. from the Medical Research Council, Canada and by Grant OGP0171373 to R.L. from the Natural Sciences and Engineering Research Council, Canada. D.D. and R.S. were recipients of fellowships from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR), Quebec, Canada. We thank Dr. J.L. Turnbull (Concordia University, Montreal, Canada) for instruction and use of the CD spectropolarimeter. We appreciate the critical review of G.S. Moeck.

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