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Biochemical and biophysical characterization of in vitro folded outer membrane porin PorA of *Neisseria meningitidis*

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Abstract

Two subtypes of the outer membrane porin PorA of *Neisseria meningitidis*, P1.6 and P1.7,16, were folded in vitro after overexpression in, and isolation from *Escherichia coli*. The PorA porins could be folded efficiently by quick dilution in an appropriate buffer containing the detergent *n*-dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulphonate. Although the two PorA porins are highly homologous, they required different acidities for optimal folding, that is, a pH above the p*I* was needed for efficient folding. Furthermore, whereas trimers of PorA P1.7,16 were almost completely stable in 2% sodium dodecyl sulphate (SDS), those of P1.6 dissociated in the presence of SDS. The higher electrophoretic mobility of the in vitro folded porins could be explained by the stable association of the RmpM protein to the porins in vivo. This association of RmpM contributes to the stability of the porins. The P1.6 pores were moderately cation-selective and displayed a single-channel conductance of 2.8 nS in 1 M KCl. The PorA P1.6 pores, but not the PorA P1.7,16 pores, showed an unusual non-linear dependence of the single-channel conductance on the salt concentration of the subphase. We hypothesize that a cluster of three negatively charged residues in L5 of P1.6 is responsible for the higher conductance at low salt concentrations.

Keywords: Membrane protein; PorA; RmpM; Porin; *Neisseria meningitidis*; Black lipid membrane

1. Introduction

Neisseria meningitidis contains two major outer membrane porins, i.e. PorA and PorB. The PorB protein forms anion-selective pores [1,2], whereas the PorA pores were reported to be a cation-selective [3]. Topology models, which predict a β-barrel structure similar to those of the *Escherichia coli* porins [4], have been proposed for both porins [5]. Both porins, but especially the PorA protein, are considered as potential vaccine candidates. Furthermore, whereas the PorB protein was recently successfully isolated from *E. coli*, renatured and subsequently extensively characterized [2,6], detailed knowledge regarding the biochemical and biophysical properties of the PorA protein is lacking. For further immunological, biophysical and structural studies, large quantities of highly pure PorA porin would be desirable. How-

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ever, expression of the *porA* gene in *E. coli* is lethal [7], unless the expression level is very low [8]. A solution would be to express the neisserial porin, without its signal sequence, in high amounts as inclusion bodies in the cytosol of *E. coli*. However, such an approach should be combined with an efficient method to fold the protein in vitro, since the protein in the inclusion bodies is expected to be non-native.

The goal of the present study was to establish conditions for the efficient folding of two immunological subtypes of the porin PorA, i.e. PorA P1.6 and PorA P1.7,16, produced in large quantities as inclusion bodies in *E. coli*. This approach was already successfully performed by others [9,10]. However, the efficiency of the folding process was not previously studied. Furthermore, we characterized the in vitro folded PorA proteins biochemically and biophysically and compared their properties with those of the porins produced in vivo in the outer membrane of *N. meningitidis*, to ascertain that a native structure was obtained. The characterization of the in vitro folded porins revealed novel aspects of the PorA protein, which are being described.

2. Material and methods

2.1. Construction of the expression system

Plasmids pCF11 and 2-2/87 (P. van der Ley, National Institute of Public Health and the Environment, Bilthoven, The Netherlands) were used to amplify the DNA sequences encoding the mature domains of the PorA genes P1.6 and P1.7.16, respectively. Since the extreme 5′ and 3′ ends of the gene encoding PorA P1.6 have not been sequenced and since both termini of the *porA* genes are supposed to be highly conserved, the primers used (5′-GATGTCAGCATGTACGGCGAAATCAAA-3′ and 5′-TTAGAATTTGTGGCGCATACCGACGGAGGC-3′) were based on the DNA sequence of *porA* P1.16 of strain MC50 [11]. Mutations (underlined in primer sequence), which resulted in the substitution of two methionine residues at positions 4 and 369 of the mature protein sequence, respectively, for leucine residues, were introduced to facilitate radioactive labelling at the methionine residues in future studies. Polymerase chain reactions were performed in the buffer supplied by the manufacturer of the proofreading enzyme *Pfu* (Stratagene). The PCR fragments encoding mature PorA P1.6 and P1.7,16 were inserted into the *Nco*I site of pET11d [12], which was treated with the Klenow fragment of DNA polymerase to create blunt ends, resulting in the plasmids pCJ40 and pCJ41, respectively.

2.2. Bacterial strains and growth conditions

The *E. coli* strain BL21(DE3) contains a chromosomal copy of the T7 RNA polymerase gene under control of the *lac*UV5 promoter [13]. Plasmid-containing derivatives of this strain were grown at 37°C in LB medium, supplemented with ampicillin (50 μl/ml).

N. meningitidis strains M990 [14] and H44/76 [15] produce PorA proteins of the immunological subtypes P1.6 and P1.7,16, respectively. An *rmpM*-deficient mutant of strain H44/76, i.e. H44/76-Δcl4 [16], and a *porB* mutant derivative of strain H44/76, i.e. strain CE2002 [3], have been described. The strains were cultured overnight on GC agar plates (Difco) supplemented with Vitox (Oxoid) at 37°C in a humid 5% CO_2 atmosphere. Few colonies were resuspended in phosphate-buffered saline, and inactivated at 56°C for 30 min before further analysis. Alternatively, cells were grown overnight in Trypticase soy broth supplemented with Vitox and inactivated in this medium before further analysis.

2.3. Expression, folding and purification of PorA proteins

Cultures of strain BL21(DE3) containing appropriate plasmids, grown overnight at 37°C, were diluted 1/10 into fresh LB medium supplemented with 0.5% glucose. When the OD_{660} reached a value of 0.6, isopropyl-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After 3 h further incubation at 37°C, the cells were harvested and disrupted by sonication (2×5 min at level 7, 40% output, Branson sonifier 450), and the inclusion bodies were collected by centrifugation (15 min, 2400×*g*, SS34 rotor) and dissolved in 8 M urea, 50 mM glycine pH 8.0 to a final protein concentration of 10–20 mg/ml. Ultracentrifugation (2.5 h,

145 000×*g*, Ti-50 rotor) was used to remove residual membrane fragments. The supernatant was stored at 4°C. The presence of LPS was assayed by silver staining after sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) [17]. The protein concentration of the denatured protein stocks was determined by a Bradford protein assay [18].

The folding of PorA P1.6 was initiated by a 100-fold dilution of the urea-dissolved inclusion bodies in 20 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 14.8 mM *n*-dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulphonate (SB12; Fluka, purified as described by [19]) to a final concentration of 150–200 μg/ml protein and incubated overnight at room temperature. Small aggregates were observed and were removed by centrifugation (20 min, 8000×*g*, GSA rotor), and the folded protein was purified by ion-exchange chromatography on an SP-Sepharose HP column (Pharmacia), which had been equilibrated with 20 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 10 mM SB12. The protein was eluted with a linear gradient of 0–2 M NaCl in the same phosphate buffer. Fractions containing folded forms of PorA P1.6 were pooled and dialysed against 10 mM Tris-HCl pH 7.0, 3 mM SB12.

The PorA P1.7,16-urea stock was diluted 50–100-fold in 20 mM ethanolamine pH 10.8, 14.8 mM SB12 to a final protein concentration of 150–200 μg/ml and incubated overnight at room temperature. The folded proteins were loaded on a Q-Sepharose HP column (Pharmacia), which had been equilibrated with 20 mM ethanolamine pH 10.8, 10 mM SB12. The column was eluted using a linear gradient of 0–2 M NaCl in the ethanolamine buffer. Fractions were immediately neutralized with 200 mM Tris-HCl pH 7.8. Fractions containing folded forms of PorA P1.7,16 were pooled and dialysed against 10 mM Tris-HCl pH 8.0, 3 mM SB12.

The protein concentration was determined by absorbance measurements (A_{280} of 0.1% PorA P1.6 = 1.0605; A_{280} of 0.1% PorA P1.7,16 = 1.1093).

When indicated, PorA proteins were isolated after SDS-PAGE by extracting the proteins from the gels with 1% octyl-polyoxyethylene (OPOE) in 20 mM Tris-HCl pH 7.5 [20]. Subsequently, the extracted proteins of the in vitro folded and purified PorA P1.6 were concentrated by acetone precipitation.

2.4. Trypsin treatment, SDS-PAGE and Western blotting

Trypsin resistance of proteins was tested by incubating samples in 50 μg/ml trypsin at room temperature (in vitro folded porins) or at 37°C (whole cells) for 15 min, and digestion was terminated by adding 1 mM phenylmethylsulphonyl fluoride (PMSF) and incubation on ice.

Prior to electrophoresis, protein aliquots were incubated in sample buffer [21] containing either 0.05%, 0.5% or 2% SDS as a final concentration. Incubations were performed for 10 min at either room temperature, 56°C or 100°C. SDS-polyacrylamide gels were prepared according to [21], except that no SDS was added to the stacking and running gels. The gels were run at 20 mA in a temperature-controlled room at 4°C to prevent denaturation of various folded forms of the porins by heating of the gels during electrophoresis.

Western blotting was performed as described [22]. Prior to the blotting procedure, gels were heated under steam [23] to denature folded proteins, unless otherwise indicated. The monoclonal antibodies used to detect PorA and RmpM were MN23G2.38 (P. van der Ley, National Institute of Public Health and the Environment, Bilthoven, The Netherlands) and MN2D6D [24], respectively. The antibodies, which recognize the denatured forms of the proteins, were used at a dilution of 1/4000. MN23G2.38 recognized both subtypes of PorA.

For quantification of protein bands, the gels were scanned with a densitometer (Molecular Dynamics) and analysed using the programme Image Quant. When the intensity of the denatured monomeric form of the protein detected in the lane with the sample incubated at room temperature is designated '*a*' and that at 100°C as '*c*', the folding efficiencies can be expressed as $(c-a)/c \times 100\%$.

2.5. Immunoprecipitation

PorA P1.6 was incubated for 3 h at room temperature with the monoclonal antibody MN19D6.13, which recognizes a conformational epitope (P. van der Ley, National Institute of Public Health and the Environment, Bilthoven, The Netherlands), under agitation. Protein A-Sepharose CL-4B (2.5

mg per immunoprecipitation; Pharmacia Biochemicals) was added, and the mixture was incubated for 1 h at room temperature. The Sepharose beads with the bound immunocomplexes were collected by centrifugation (13 000×*g*, 1 min), and the pellet was washed twice with 50 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.1 mM EDTA, 0.1% BSA, 2.5 mM SB12 and finally with 10 mM Tris-HCl pH 8.0, 2.5 mM SB12. To dissociate the antigen from the beads, the pellet was incubated for 4 min at room temperature in 200 mM glycine-HCl pH 4.0, 2.5 mM SB12. After centrifugation (13 000×*g*, 1 min), the supernatant was collected and neutralized with 1 M Tris-HCl pH 8.0.

2.6. Protein sequencing

Protein sequencing was performed by repetitive Edman degradation with a Protein Sequencer, model 476A (Perkin-Elmer).

2.7. Isolation of outer membrane complexes

Outer membrane complexes of *N. meningitidis* were isolated as described [25] with some modifications. In short, after harvesting the cells of 200 ml cultures, the pellet was resuspended in 10 ml of 0.2 M LiCl, 0.1 M EDTA pH 7.0 and incubated for 2 h at 45°C. Intact cells were removed by centrifugation (30 min, 12 000×*g*, SM-24 rotor, Sorvall). The outer membrane complexes were collected by ultracentrifugation (1 h, 61 000×*g*, 50Ti rotor, Beckman) and resuspended in 200 μl of 62.5 mM Tris-HCl pH 6.8.

2.8. Liposome swelling assay

In vitro folded and purified porins were extracted from gels with 1% OPOE in 20 mM Tris-HCl pH 7.5 [20]. Liposome swelling assays were performed as described [26,27]. In short, liposomes were prepared from 4 μmol 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; Avanti) and 1 μmol egg phosphatidyl-DL-glycerol (EPG; Avanti). In the proteoliposomes, 17% (w/v) dextran T40 (Pharmacia) in 5 mM Tris pH 7.5 was included. The isotonic concentration was determined by diluting the proteoliposomes into different concentrations of raffinose (BDH) in 5 mM Tris-HCl pH 7.5. The tested solutes (all in 5 mM Tris-HCl pH 7.5) for diffusion into the proteoliposomes were arabinose (Sigma), glucose (Merck), *N*-acetylglucosamine (Sigma) and maltose (Serva). The permeability (p) of porins was calculated from the initial rates of swelling monitored at 500 nm and normalized for the initial absorbance (A_i) by the formula: $p = (1/A_i)^2 \times \Delta OD/\Delta t$ [27]. At least three independent measurements were performed. The swelling rates obtained with arabinose were set at 100%, and those obtained with the other sugars were related to this value.

2.9. Planar bilayer measurements

Black lipid membranes were formed from 1% solutions of dioleoylphosphatidylglycerol (DOPG) in *n*-decane at room temperature. For comparison, some experiments were performed with asymmetric membranes, prepared according to the Montal Mueller technique [28], from lipopolysaccharide (LPS) of the deep-rough mutant *E. coli* strain F515 on one side and a phospholipid mixture consisting of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin in the molar ratio of 81:17:2 on the other side of the membrane as described previously [29]. Since the acyl chains of the LPS have to be in the fluid state for the formation of asymmetric membranes, these measurements were performed at 37°C. The aqueous subphase was buffered with 5 mM HEPES pH 7.0. In the case of the asymmetric membranes, 5 mM $MgCl_2$ was added for stabilization of the membranes. Porins were added at a final concentration of 500 ng/ml to the *trans* side. The *trans* side corresponds to the phospholipid side in the case of asymmetric membranes. A transmembrane potential of 20 mV was applied, which was negative at the *cis* side, while the *trans* side was grounded. The single-channel conductance of the pores was determined from the stepwise conductance increments after porin addition. For each value, at least 100 porin insertions measured in at least three different experiments were evaluated. For the comparison of the ion permeability of the porins under different ion concentrations, the size parameter was calculated as the quotient of the single-channel conductance Λ and the specific conductance σ of the subphase. Voltage gating was measured by applying a voltage ramp across the black lipid membrane. The

critical closing potential was defined as the potential at which closing of the pores starts to occur.

3. Results

3.1. Expression system

To produce large quantities of the PorA proteins of subtypes P1.6 and P1.7,16, we cloned the parts of the *porA* genes encoding the mature domains of the proteins (i.e., without the signal sequences) behind the T7 promoter, resulting in plasmids pCJ40 (*porA* P1.6) and pCJ41 (*porA* P1.7,16). In the plasmids, the codon for the first amino acid residue of the mature protein was situated directly downstream from the ATG start codon. Furthermore, to facilitate radiolabelling of the proteins in future studies, two codons for leucines were replaced by codons for methionine residues. In strain BL21(DE3), the recombinant genes were expressed upon addition of IPTG (shown for PorA P1.6 in Fig. 1, lane 2) and the proteins accumulated in cytoplasmic inclusion bodies. These inclusion bodies were separated from the majority of proteins in the cell lysate by centrifugation (Fig. 1, lanes 3 and 4). After dissolving the inclusion bodies in 8 M urea and a final ultracentrifugation step to remove residual membrane fragments, the proteins in the supernatant were about 90% pure as estimated from gel (Fig. 1, lane 5). The total yield of PorA protein was approx. 200 mg from 1 l cultures and did not contain detectable amounts of LPS (results not shown).

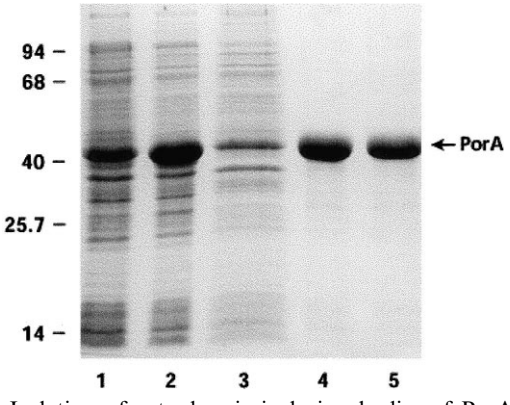

Fig. 1. Isolation of cytoplasmic inclusion bodies of PorA P1.6. Whole cells of *E. coli* BL21(DE3) containing pCJ40, before (lane 1) and after 3 h induction with IPTG (lane 2). Cells were disrupted by sonication, the inclusion bodies were collected by centrifugation (lane 4) and the supernatant (lane 3) contained almost all other bacterial proteins. The inclusion bodies were dissolved in 8 M urea and a last ultracentrifugation step was performed. Lane 5 contains the isolated PorA P1.6 (indicated by an arrow). Samples were incubated at 100°C for 10 min in sample buffer prior to SDS-PAGE. The proteins were stained with Coomassie. Positions of molecular weight standard proteins are indicated at the left (in kDa).

N-Terminal amino acid sequencing of the purified PorA P1.6 revealed the sequence MDVSMY. Apparently, the first methionine was not removed in vivo, resulting in an N-terminal extension of the native mature sequence with one amino acid residue. Such an extension was not expected to interfere with folding, since various studies with porins have indicated that even much longer N-terminal extensions do not prevent folding in vivo and in vitro [30–32]. Furthermore, the sequence confirmed the substitution of methionine for leucine at the fourth position of the mature sequence.

3.2. Folding and purification of PorA P1.6 and P1.7,16

Previously, it has been shown that urea-denatured neisserial porins (PorB from *N. meningitidis* and pIb from *Neisseria gonorrhoeae*) could attain a native-like trimeric structure by adding Zwittergent 3-14 and subsequently removing the urea on a molecular sieve column [9]. In addition, it was shown that Zwittergent 3-14 was a suitable detergent for refolding SDS-denatured PorA produced in *Bacillus subtilis* into a native-like conformation [10]. Based on these findings, we developed a straightforward system for the in vitro folding of the urea-denatured PorA P1.6 and PorA P1.7,16. The denatured protein stocks in urea were quickly diluted into an appropriate buffer containing a zwitterionic detergent at a concentration of 10 times the critical micelle concentration (CMC). Protein folding was analysed by SDS-PAGE, since folded monomers, dimers and trimers can be distinguished from denatured protein because of their different migration behaviour. Folded monomers display a higher electrophoretic mobility than the denatured monomers, probably because of their compact shape [33]. Oligomeric forms of the proteins with apparent molecular weights of approx. 2 and 3 times that of the folded monomer, were considered

to represent dimers and trimers, respectively. We started to determine the folding conditions for PorA P1.6. Zwittergent 3-14 and Zwittergent 3-12 (SB12) showed similar folding efficiencies (not shown). For large scale folding and purification, we chose, for economical reasons, the use of SB12 (0.5%, w/v). We tested various buffers of pH 7.0. Folding was slightly less efficient in HEPES and 3-morpholinopropanesulphonic acid (MOPS) buffer (70–75% folding) than in phosphate buffer or Tris buffer (85% folding) (results not shown). In Tris buffer, similar folding efficiencies were obtained in the pH range of 7–10, while lower folding efficiencies were obtained at a pH below 7 (not shown). Surprisingly, the folding conditions for PorA P1.7,16, which is highly homologous to PorA P1.6 (91% identity), turned out to be quite different. This protein required high alkaline conditions for folding, since it folded much more efficiently in an ethanolamine buffer pH 10.8 or a glycine buffer pH 10.4 (70% folding) than in various other buffers at pH 7.0 (results not shown). The subsequent purification step determined finally the folding conditions used: 20 mM sodium phosphate pH 7.0, 0.5% SB12 and 20 mM ethanolamine pH 10.8, 0.5% SB12 for PorA P1.6 and PorA P1.7,16, respectively. Folding reactions were routinely incubated overnight at room temperature, although the folding was essentially complete already after 30 min (data not shown).

Folded PorA P1.6 and P1.7,16 were purified on SP-Sepharose HP and Q-Sepharose HP ion-exchange columns, respectively. Prior to loading on the columns, small aggregates formed by PorA P1.6 (about 20%) were removed by centrifugation. Each PorA subtype eluted in two separate peaks from the column; the most pure fractions from the second peak were pooled. The total yield of the 99% pure folded porins was approx. 20 mg/l of cell culture, i.e. 10% of the amount of protein present in the inclusion bodies. The final preparations of folded and purified PorA P1.6 and P1.7,16 proteins are shown in Fig. 2, lane 5 and 14, respectively. The presence of two trimeric forms in both cases was remarkable. The PorA P1.7,16 preparation initially showed only the lower migrating species, and the second band was only formed after several months of storage at 4°C. In the case of PorA P1.6, both bands were directly visible after the purification. Furthermore, putative dimers and folded monomers (indicated as monomer* in Fig. 2) were detected in the case of PorA P1.6. The four forms of PorA P1.6, i.e. two trimeric, a dimeric and a folded monomeric form, turned out to be in an equilibrium: when one form was isolated from gel, and subsequently analysed once more by

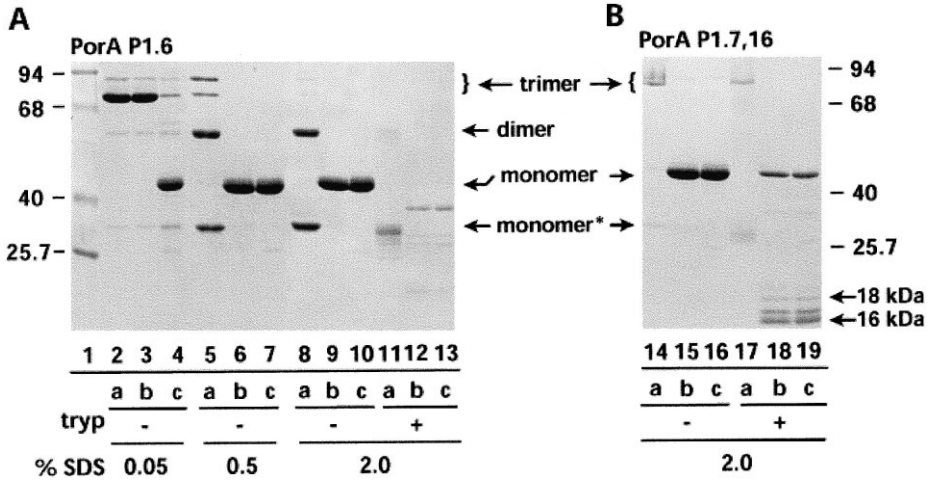

Fig. 2. SDS-PAGE of in vitro folded and purified PorA P1.6 (A) and PorA P1.7,16 (B). Trypsin treatment was performed when indicated (tryp+). The samples were dissolved in sample buffer with either 0.05, 0.5 or 2% SDS as indicated, and subsequently incubated at room temperature (a), 56°C (b) or 100°C (c). Lane 1 contains the molecular weight standard proteins, whose molecular weights are indicated at the left (kDa). The positions of trimer, dimer, monomer and folded monomer (monomer*) are indicated, as well as the tryptic fragments of 16 and 18 kDa.

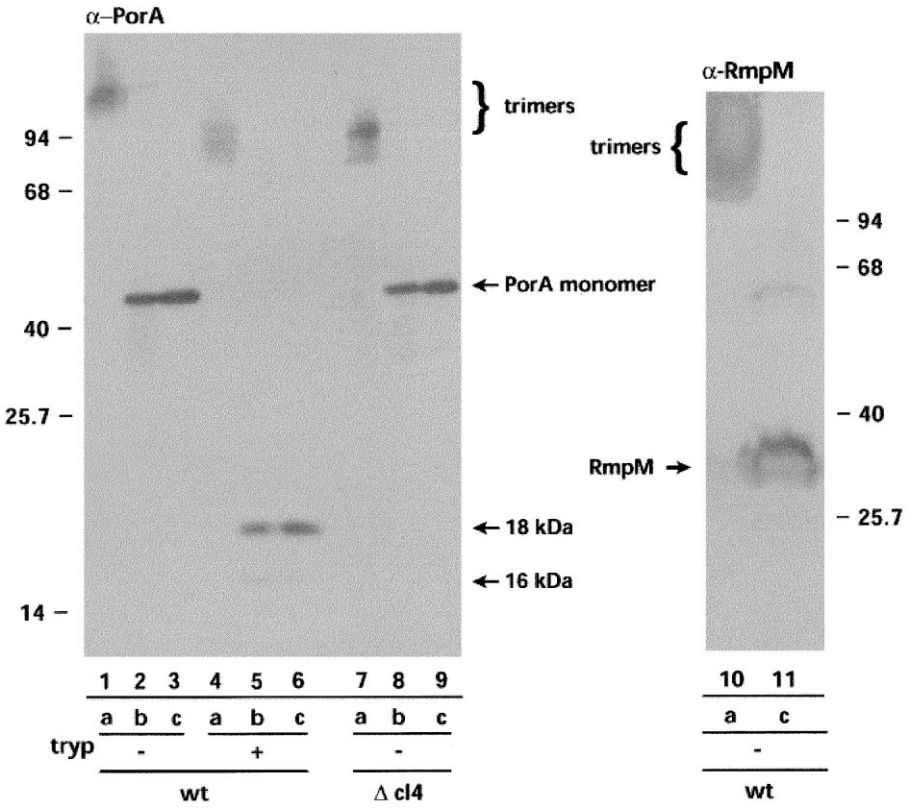

Fig. 3. Western immunoblot of whole cells of the meningococcal strain H44/76 (wt) and its *rmpM* mutant derivative (Δcl4) incubated with MN23G2.38 (α-PorA) or MN2D6D (α-RmpM) antibodies as indicated. Trypsin treatment was performed when indicated (tryp+). The samples were incubated at room temperature (a), 56°C (b) or 100°C (c) in the presence of 2% SDS. The positions of molecular weight standard proteins are indicated (kDa). The trimeric complexes, the PorA and RmpM monomer and the tryptic fragments of 16 and 18 kDa are indicated.

SDS-PAGE, all four different forms were detected again in approximately the same ratio. Furthermore, all four forms could be immunoprecipitated with antibody MN19D6.13 directed against a conformational epitope (data not shown), consistent with the notion that all these forms represent native-like forms of the protein.

3.3. Sensitivity of folded PorA to SDS and trypsin

To determine whether the in vitro folded porins had attained the native configuration, their SDS and trypsin sensitivity were assessed. SDS-PAGE revealed two trimeric, a dimeric and a folded monomeric form of PorA P1.6, when an unheated sample with 0.5% SDS in the sample buffer was loaded (Fig. 2, lane 5). With 2% SDS in the sample buffer, the ratio of the trimeric forms over the dimeric and folded monomeric ones was lower (Fig. 2, lane 8), whereas almost all PorA P1.6 was in the trimeric forms when the SDS concentration was 0.05% (Fig. 2, lane 2). It is conceivable that the protein consisted solely in a trimeric conformation in the absence of any SDS. The oligomeric conformation of PorA P1.7,16 was much less dependent on the SDS concentration in the sample buffer, since only a minor amount of folded monomer was visible at higher SDS concentrations (Fig. 2, lane 14). Both PorA subtypes denatured when samples were heated to 56°C prior to SDS-PAGE (Fig. 2, lanes 6, 9 and 15), unless the SDS concentration was very low (lane 3). The SDS sensitivity of PorA produced in its native environment was examined as well. Proteins from whole cells of *N. meningitidis* were separated by SDS-PAGE, and analysed by Western blotting (Fig. 3). When solubilized at room temperature in 2% SDS, the in vivo formed trimers of PorA from strain H44/76 (lane 1) and M990 (results not shown)

did not dissociate into dimeric and folded monomeric forms as did in vitro folded PorA P1.6. Apparently, in vitro folded PorA P1.6 is not as stable as the protein isolated from its native environment. Like the in vitro folded PorAs, the in vivo folded PorAs denatured when the samples were heated to 56°C prior to electrophoresis (Fig. 3, lane 2).

E. coli porins are known to be highly resistant to trypsin and hence, trypsin resistance can be used as a facile parameter to assess correct folding [34]. We investigated the trypsin resistance of the two in vitro folded neisserial porins. After trypsin digestion, folded forms of PorA P1.6 and P1.7,16 were still detectable showing that a majority of the proteins were not extensively degraded (Fig. 2, lanes 11 and 17). However, when the samples were denatured prior to electrophoresis, a defined breakdown pattern was observed (Fig. 2, lanes 12, 13, 18 and 19). Apparently, only a few digestion sites are accessible for trypsin, and the tryptic fragments generated remain non-covalently associated, unless heated in SDS-containing sample buffer. Also PorA produced in meningococci was only digested to a limited extent by trypsin. When the samples were solubilized at room temperature, trimers were still visible on Western blot (Fig. 3, lane 4), whereas breakdown products of 18 and 16 kDa were recognized by the monoclonal antibody MN23G2.38 upon denaturation of the sample (lanes 5 and 6). When in vitro folded PorA of the corresponding subtype P1.7,16 was treated with trypsin, fragments of 18 and 16 kDa were also observed in addition to breakdown products which probably do not contain the epitope for the monoclonal antibody used (Fig. 2, lanes 18 and 19). Taken together, according to the trypsin experiments, the in vitro folded porins seemed to be correctly folded.

3.4. RmpM association to trimeric porins and stabilization of the oligomeric state

Interestingly, the apparent molecular weight of the trimeric form of PorA produced in meningococci decreased significantly upon trypsin treatment (Fig. 3, compare lanes 1 and 4), and was, after trypsin digestion, more in correspondence to the apparent molecular weight of the in vitro folded PorA trimers (Fig. 2, lanes 2 and 14) than the undigested trimers were. This result suggested that a trypsin-sensitive, proteinaceous component is associated to the PorA trimers produced in meningococci. Since porin PI of *N. gonorrhoeae* (the homologue of PorB of *N. meningitidis*) has been reported to be associated with PIII [35,36], we hypothesized that the PorA trimers might be firmly associated with RmpM (class 4 protein), which is the meningococcal homologue of PIII [37]. To test this possibility, the electrophoretic mobility of PorA trimers produced in an *rmpM* mutant derivative of H44/76 was investigated. Indeed, these trimers migrated considerably faster than PorA trimers produced in the wild-type strain (Fig. 3, compare lanes 1 and 7). Furthermore, Western blot experiments demonstrated that RmpM migrated in the unheated samples (Fig. 3, lane 10) with the same mobility as the trimeric forms of PorA (Fig. 3, lane 1). Heating of the samples for 10 min at 100°C prior to electrophoresis (lane c) resulted in dissociation of the complexes of PorA trimers and the class 4 protein. These results demonstrate that the RmpM outer membrane protein is tightly associated with the PorA trimers in the meningococci. Similarly, the RmpM protein turned out to be tightly associated with the trimers of the meningococcal PorB protein (results not shown).

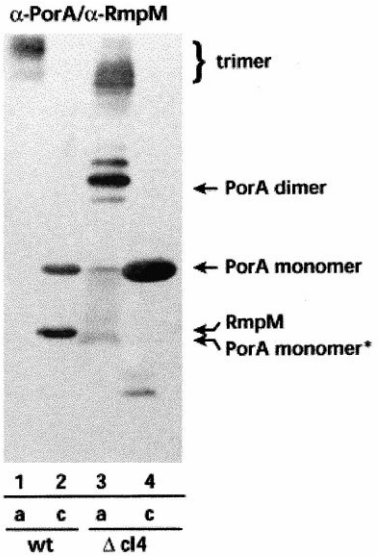

Fig. 4. Western immunoblot of isolated outer membrane complexes of the meningococcal strain H44/76 (wt) and its *rmpM* mutant derivative (Δcl4) incubated with MN23G2.38 (α-PorA) and MN2D6D (α-RmpM). The samples were incubated at room temperature (a) or 100°C (c) in the presence of 2% SDS. Trimers, dimers, monomers and folded monomers (monomer*) of PorA and the denatured RmpM are indicated.

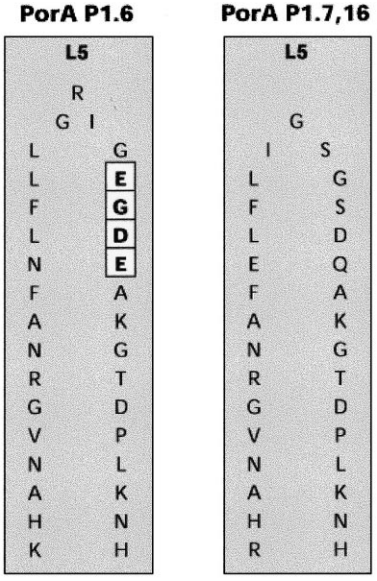

Fig. 5. Cartoon of the surface-exposed loops 5 of PorA P1.6 and P1.7,16, according to the proposed topology model [5]. The cluster of negatively charged residues in PorA P1.6 is indicated.

When outer membrane complexes of *N. meningitidis* were isolated and analysed by SDS-PAGE, it turned out that the neisserial porins were less stable in the absence of RmpM. Immunodetection revealed that both PorA (Fig. 4) and PorB (results not shown) porins were partly dissociated into dimers and folded monomers in the case of the *rmpM* mutant strain (lane 3). Apparently, the RmpM protein has a stabilizing function on the oligomeric state of the porins.

3.5. PorA pore functioning

Liposome swelling assays were performed to determine whether the in vitro folded PorA proteins formed functional pores. Both proteins showed pore formation, and the swelling rates depended on the sugar size (Table 1). No significant difference was observed between the two subtypes or between the two electrophoretically separable trimeric forms of P1.6 (Fig. 2, lane 5). As a control, the assay was performed without adding porins to the liposomes. No swelling was observed with any of the sugars, showing that the detergent alone was not able to form channels in the liposomes.

To study the pore characteristics of the folded PorA proteins in further detail, planar lipid bilayer experiments were performed. PorA P1.6 was studied most extensively in symmetrical DOPG membranes (Table 2). The average single-channel conductance Λ in 1.0 M KCl was 2.8 nS resulting in a size parameter of 0.22 nm (Table 2). The size parameters of PorA P1.7,16, either folded in vitro or isolated from meningococcal outer membranes of *porB* mutant strain CE2002, were 0.19 nm and 0.22 nm, respectively, in this subphase.

Surprisingly, no linear decrease in the conductance of PorA P1.6 was observed at lower KCl concentrations, which is reflected in the size parameters (Table 2). While the size parameter of PorA P1.7,16 remained constant when decreasing the salt concentration, the size parameter of PorA P1.6 increased (Table 2) implying an important difference in the functional characteristics of the two subtypes.

To investigate the influence of the lipid matrix on the pore size, some measurements were performed in asymmetric membranes consisting of deep rough *E. coli* LPS on one side and a mixture of phospholipids on the other side (Table 2, measurements indicated with superscript e). To stabilize the asymmetric membranes, 5 mM $MgCl_2$ was added to the subphase. While rather similar size parameters were measured for PorA P1.7,16 in both types of membranes, the size parameter for PorA P1.6 was significantly smaller at low salt concentrations and comparable to the value observed at 1.0 M KCl. This difference was, however, not due to the different type of membrane used, but to the presence of $MgCl_2$, since the addition of 5 mM $MgCl_2$ to the symmetric DOPG mem-

Table 1
Relative swelling rates of liposomes containing PorA P1.6 or PorA P1.7,16

Sugar	Fw (Da)	Swelling rate (%)[a]	
		PorA P1.6[b]	PorA P1.7,16
Arabinose	150	100	100
Glucose	180	69 ± 12	66 ± 9
N-Acetylglucosamine	221	33 ± 9	23 ± 16
Maltose	342	5 ± 3	6 ± 5

[a]The swelling rates obtained with arabinose were set at 100%, and those obtained with the other sugars were related to this value.

[b]Both trimeric species of PorA P1.6 (as detected on gel, Fig. 1, lane 5) were extracted from gel and studied separately. Since the pore properties of the two forms were indistinguishable in this assay (data not shown), their values were averaged.

branes also reduced the size parameters to lower values (Table 2).

Single-channel experiments for PorA P1.6 were also performed with salts other than KCl to gain information on the selectivity of the porin (Table 2). Replacement of the chloride ion by the less mobile acetate ion hardly affected the single-channel conductance and the size parameter when salt concentrations with similar specific conductances in the subphase are compared (i.e. 1.0 M KAc and 0.5 M KCl, Table 2). However, replacement of K^+ by the less mobile cations Li^+, Na^+ or Mg^{2+} resulted in a strong decrease in the size parameter (Table 2), indicating a preference of PorA P1.6 for cations. No significant difference was observed when K^+ was replaced by the equally mobile ion NH_4^+.

The ion selectivity of PorA P1.6 was more accurately determined in zero current membrane potential measurements after applying a 3.7-fold KCl concentration gradient (*cis* side 0.1 M; *trans* side 0.37 M). The *cis* side became positive with a potential of about 18 mV, again indicating the preferential movement of cations through the pores. The ratio of the cation permeability over the anion permeability (p_K/p_{Cl}) was calculated, using the Goldman-Hodgkin-Katz equation according to Benz et al. [38]. When the ionic activity coefficients (0.77 for 0.1 M KCl and 0.672 for 0.37 M KCl) were included, a permeability ratio of 4.6 was calculated.

Porin channels tend to close when a high transmembrane potential is applied (see [39] for a recent review). The critical closing potential is characteristic for the porin species studied. Voltage gating of PorA P1.6 (in the symmetric as well as asymmetric membranes) and PorA P1.7,16 (only determined once in the asymmetric membranes) was observed at poten-

Table 2
Single channel conductance as measured in symmetrical DOPG membranes, unless otherwise indicated, the specific conductivity of the subphase and the calculated size parameter for PorA P1.6 and P1.7,16

PorA P1.6 in vitro[a]			
Subphase	Single channel conductance (Λ) (nS)[b]	Specific conductance (σ) at 20°C (S/m)[c]	Size parameter (Λ/σ) (nm)
1.0 M KCl	2.80 ± 0.52	11.9	0.22 ± 0.04
0.5 M KCl	1.71 ± 0.22	5.62	0.30 ± 0.04
0.2 M KCl	0.88 ± 0.14	2.27	0.37 ± 0.06
0.1 M KCl	0.63 ± 0.17	1.12	0.54 ± 0.15
0.1 M KCl[e]	0.31 ± 0.04	1.2	0.18 ± 0.02
0.1 M KCl+5 mM $MgCl_2$	0.24 ± 0.05	1.4	0.17 ± 0.03
1.0 M KAc	1.79 ± 0.16	6.0	0.28 ± 0.02
1.0 M LiCl	0.67 ± 0.01	6.9	0.09 ± 0.01
1.0 M NaCl	0.73 ± 0.11	8.36	0.08 ± 0.01
1.0 M $MgCl_2$	0.73 ± 0.09	10.7	0.07 ± 0.01
1.0 M NH_4Cl	2.34 ± 0.18	11.06	0.20 ± 0.02
PorA P1.7,16 in vitro[a]			
1.0 M KCl	1.92 ± 0.17	9.6	0.19 ± 0.02
0.5 M KCl	1.17 ± 0.15	5.12	0.22 ± 0.03
0.2 M KCl	0.52 ± 0.10	2.25	0.22 ± 0.04
PorA P1.7,16 in vivo[d]			
1.0 M KCl	2.16 ± 0.19	9.6	0.22 ± 0.02
0.5 M KCl	1.23 ± 0.17	4.8	0.23 ± 0.03
0.5 M KCl[e]	1.35 ± 0.04	5.28	0.19 ± 0.06
0.2 M KCl	0.55 ± 0.11	2.25	0.23 ± 0.05
0.1 M KCl[e]	0.33 ± 0.10	1.24	0.19 ± 0.06

[a]Porins isolated, folded and purified as described in Section 2.
[b]At least 100 opening events in at least three independent experiments were measured.
[c]The specific conductance was determined with a conductivity meter.
[d]Porin isolated after SDS-PAGE from outer membrane complexes of *N. meningitidis* strain CE2002 as described in Section 2.
[e]Asymmetric membranes with 5 mM $MgCl_2$.

tials higher than ±80 mV (results not shown). The closures of single porins were observed in three discrete steps, each one third of the single-channel conductance, consistent with a trimeric unit of the PorA protein.

4. Discussion

Although PorA P1.6 and PorA P1.7,16 are for 91% identical at the amino acid sequence level, the in vitro folding of the two urea-denatured porins required different conditions. PorA P1.6 could be folded efficiently at neutral pH, whereas PorA P1.7,16 needed high alkaline conditions for efficient folding. Considering the calculated p*I* values of the proteins (i.e., 6.8 for P1.6 and 9.06 for P1.7,16, PC Gene, IntelliGenetics, Switzerland), it appears that a pH above the p*I* is needed for efficient folding in vitro. Such a requirement could explain the broad pH range in which PorA P1.6 could be folded efficiently (pH 7–10). Refolding and insertion of the OmpA protein of *E. coli* into dimyristoylphosphatidylcholine (DMPC) vesicles also required a pH above the p*I* of the protein [40]. Similarly, the OmpF protein of *E. coli* inserted optimally at a pH of 6.5, while the p*I* of OmpF is 4.5 [41]. In both cases, optimal refolding and insertion was observed when the proteins were soluble in water. Probably, this also accounts for the PorA proteins of *N. meningitidis*.

To verify whether the in vitro folded porins are structurally identical to their in vivo assembled counterparts, several biochemical and biophysical characteristics were investigated. Some novel characteristics of the porins were revealed.

4.1. Characteristics of the PorA protein

The trimeric form of the in vitro folded PorA P1.6 was more sensitive to SDS than the in vivo assembled protein, since it dissociated into dimers and folded monomers at high SDS concentrations. This SDS sensitivity may be explained by the absence in vitro of stabilizing components, such as RmpM, LPS and/or divalent cations, which are present in vivo. Alternatively, or in addition, the recombinant PorA P1.6 may be hampered in its stability by the two methionine for leucine substitutions at position 4 and 369 of the mature sequence, respectively, or, more likely, by the N-terminal extension of a methionine, which had to be added to initiate translation of the mRNA for the recombinant protein in *E. coli*. In this respect, it should be noted that a short N-terminal His tag has been reported to weaken the subunit interactions in the porin PhoE of *E. coli* [30]. Furthermore, the N- and C-termini of the porins from *Rhodobacter capsulatus* and *Rhodopseudomonas blastica* are forming intersubunit salt bridges [42,43]. If similar salt bridges are present in the meningococcal porins, it is conceivable that their formation is hindered by the additional N-terminal methionine. In PorA P1.7,16, the effect of the N-terminal extension may be less detrimental, due to additional subunit interactions. Similar differences in SDS susceptibility have been reported in the case of the PorB class 2 and class 3 proteins, the latter being highly sensitive to SDS resulting in dissociation and partial unfolding of the trimeric form [44], while the former is more resistant to SDS [6].

Remarkable was the observation of two trimeric bands on gel of PorA P1.6 and, in time, also of PorA P1.7,16. They probably reflect two conformational states. Whether the two trimeric forms are also present in vivo is difficult to assess, since lipo-oligosaccharides remain associated to the porins produced in vivo, resulting in a smear on SDS-PAGE.

The meningococcal PorA porin is, in vivo and in vitro, more prone to trypsin digestion and heat denaturation than the porins of *E. coli*. This difference might be explained by the different niches in which the bacteria live. *N. meningitidis* does not face the extremes of bile salts and proteases in the intestine in which *E. coli* lives. Also a fraction of the PorB class 2 porin of *N. meningitidis* was sensitive to trypsin [6], while PorB class 3 protein was resistant [44].

4.2. RmpM association to PorA

The PorA and PorB trimers produced in vivo appeared to be firmly associated to RmpM. This observation might explain the severely reduced expression of the RmpM protein that was observed in a mutant strain lacking the PorB porin [3]. Possibly, the association of RmpM to porin is essential for its outer membrane localization and stability. In this

respect, it should be noted that RmpM is homologous to the *E. coli* OmpA [37], but it lacks the N-terminal β-barrel domain, which anchors OmpA in the outer membrane [45,46]. Hence, the association of RmpM to porins is probably essential for its anchoring to the outer membrane. Both OmpA and RmpM contain a peptidoglycan-binding motif in the C-terminal domain [47,48]. Hence, these proteins might function to anchor the outer membrane to the peptidoglycan. Furthermore, RmpM appeared to stabilize the oligomeric state of the porins PorA and PorB.

4.3. PorA pore functioning

Both the liposome swelling assay and the black lipid membrane experiments showed comparable results for each of the two PorA subtypes, indicating that the two subtypes have a similar pore size. Furthermore, in the swelling assay, the different trimeric forms of PorA P1.6, detected by SDS-PAGE, were functionally similar. Moreover, in vitro folded and in vivo assembled PorA P1.7,16 yielded similar size parameters, consistent with the assumption that the in vitro folded form has a native configuration. Apparently, the RmpM protein, which is associated to the in vivo assembled PorA, did not significantly influence the pore characteristics. Previously, an average single-channel conductance in 1 M KCl of 1.8 nS for PorA P1.7,16 isolated from *Neisseria* has been reported [3]. Calculating with the theoretical specific subphase conductance of 10.3 S/m [49], this results in a size parameter of 0.17 nm, which is close to the values observed in this report.

Surprisingly, the conductance of PorA P1.6 was not proportional to the salt concentration in the aqueous phase. A similar non-linearity was reported for the cell wall porin of the Gram-positive bacterium *Nocardia farcinica* [50]. In that case, it was argued that negative point charges at the channel mouth might be responsible for this behaviour. Furthermore, introduction of negative charges by succinylation of amino groups resulted in a non-linear relation between the single-channel conductance and the salt concentration in the case of the porin of *R. capsulatus* [51]. Moreover, Omp34 of *Acidovorax delafieldii* [52] and Omp32 of *Comamonas acidovorans* [53] also displayed a relatively high conductance at low salt concentrations. Mathes and Engelhardt [53] argued that a surplus of charges, not completely shielded at low salt concentrations, may attract ions, resulting in an enhanced conductance. A similar mechanism might explain the non-linearity observed with the meningococcal PorA P1.6. The amino acid sequences of the PorA subtypes P1.6 and P1.7,16 are for 91% identical, but a suspicious cluster of three negatively charged residues is present in the putative external loop 5 of subtype P1.6 and lacking in subtype P1.7,16 (Fig. 5). This cluster of residues might be responsible for the enhanced movement of cations at low KCl concentrations. Consequently, these negatively charged residues have to be located at a critical position for the passage of ions. Since magnesium ions are expected to neutralize the negative charges in this cluster, the observation that the addition of 5 mM $MgCl_2$ to the aqueous subphase decreased the size parameter to values observed at higher salt concentrations is in agreement with our proposal. An alternative explanation for the non-linearity of the single-channel conductance of PorA P1.6 with the salt concentration could be that the structure of the porin changes with ionic strength of the aqueous subphase. However, this explanation does not account for the observed effect of the magnesium ions, as described above.

The degree of cation selectivity measured for the in vitro folded PorA P1.6 is consistent with that of PorA P1.7,16 as reported previously [3], but much lower than that of PorA/C1 (24:1 Na^+ over Cl^-) reported recently [54]. In the latter study, PorA/C1 was isolated from a derivative of strain H44/76, from which also PorA P1.7,16 of our work originates, but the sequence presented deviates considerably from the published sequence [55], most notably by the presence of five instead of one negatively charged residues in loop 5. This difference might explain the higher degree of cation selectivity of PorA/C1. The selectivity of the PorB porin is less clear since both cation selectivity [56] and anion selectivity [1,2] of this porin have been reported. Whereas strain differences may account for this discrepancy, it seems most likely that *N. meningitidis* contains in general a cation-selective pore (PorA) as well as an anion-selective pore (PorB). Moreover, PorB is thought to be the equivalent of porin PI from *N. gonorrhoeae*,

which is studied extensively and has been shown to be anion-selective [1,57].

In our studies, PorA showed voltage gating and closed at voltages above ±80 mV. In contrast, in vivo assembled PorA P1.7,16 was even not observed to close when the applied voltage was increased up to 100 mV [3]. The PorB protein of *N. meningitidis* seems to be more sensitive to a membrane potential. Closing potentials of 40–50 mV [56] and even of 15 mV [2] have been reported. For the PI porin of *N. gonorrhoeae*, closing potentials of 80 mV [57] and even 120 mV [58] have been reported. These differences might be due to different purification and reconstitution procedures [59,60]. In addition, the mutations introduced in the recombinant PorA used in our study might decrease the stability of the porins, which might result in a lower closing potential [61].

In conclusion, we succeeded in obtaining high yields of functional, native-like PorA porins of two subtypes, namely PorA P1.6 and PorA P1.7,16. Previously, the porin of *Rps. blastica* could be refolded in vitro after production in inclusion bodies, in which case the recombinant porin was shown to be identical at the atomic level to the native porin [62]. Immunization studies with both subtypes of in vitro folded and purified PorA proteins have demonstrated that bactericidal antibodies can be elicited [63]. Furthermore, the purified proteins will be used in crystallization studies to solve the 3D structure of the proteins.

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