Structural characterization of the fusobacterial non-specific porin FomA suggests a 14-stranded topology, unlike the classical porins

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Native and recombinant FomA proteins were extracted by detergent from the cell envelopes of *Fusobacterium nucleatum* and *Escherichia coli*, and purified to near homogeneity by chromatography. Circular dichroism analysis revealed that the FomA protein consists predominantly of β -sheets, in line with the previously proposed 16-stranded β -barrel topology model. Results obtained by trypsin treatment of intact cells and cell envelopes of *F. nucleatum*, and from limited proteolysis of purified FomA protein, indicated that the N-terminal part of the FomA protein is not an integral part of the β -barrel, but forms a periplasmic domain. Based on these results a new topology model is proposed for the FomA protein, where the C-terminal part forms a 14-stranded β -barrel separate from the periplasmic N-terminal domain.

Keywords: *Fusobacterium nucleatum*, porins, outer-membrane proteins, circular dichroism

INTRODUCTION

Porins from several Gram-negative bacteria have been studied to variable extents over the last two decades. However, the majority of studies have been performed on porins from proteobacterial species. These studies have shown that porins are outer-membrane proteins (OMPs) that allow non-specific diffusion of solutes usually smaller than 650 Da (Nakae, 1976). The structures of several proteobacterial porins have been resolved, revealing that they exist as trimers, where each monomer forms a separate channel across the outer membrane (OM) (Cowan *et al.*, 1992; Dutzler *et al.*, 1999; Hirsch *et al.*, 1997; Kreusch & Schulz, 1994; Weiss & Schulz, 1992; Zeth *et al.*, 2000). Three of the four proteobacterial subgroups now have at least one solved porin structure, and despite substantial sequence variability, the structures show remarkably similar features. The monomer consists of 16 transmembrane antiparallel β -strands that hydrogen bond to form a β -barrel. The β -

strands are connected by turns or short loops at the periplasmic side, and longer loops exposed to the outside. In general, hydrophobic residues face the lipids and the trimer interface, whereas hydrophilic residues face the channel, thus allowing the channel to be water-filled. Due to the stability of the β -barrel structure, most β -barrel proteins, including the porins, are resistant to denaturation by SDS at lower temperatures (Heller, 1978; Koebnik *et al.*, 2000). This property is termed heat modifiability, since the compactly folded native proteins migrate differently in SDS-PAGE compared to the denatured proteins. Also common to all porins with resolved structure is that the third surface-exposed loop actually folds back into the pore lumen, where it constricts the pore and plays a major role in determining the permeability properties of the porin (Cowan, 1993).

The Gram-negative anaerobe *Fusobacterium nucleatum* is an oral bacterium that exists as a part of the normal oral microflora (Bolstad *et al.*, 1996). It has a pathogenic potential and is implicated in periodontal diseases as well as being the most common periodontal bacterium in clinical infections of other body sites (Moore & Moore, 1994). The major OMP of *F. nucleatum*, FomA, has been shown to function as a non-specific porin in lipid bilayer membranes (Kleivdal *et al.*, 1995), and to function as a porin *in vivo* when expressed recombin-

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Abbreviations: CD, circular dichroism; FomA, fusobacterial outer-membrane protein A; OBG, octyl β -glucopyranoside; OM, outer membrane; OMP, outer-membrane protein.

antly in *Escherichia coli* (Kleivdal *et al.*, 1999). A topology model, suggesting that the FomA protein possesses the general topology of the non-specific porins, has been proposed on the basis of structural principles derived for OMPs of *E. coli* (Bolstad *et al.*, 1994). This model was largely confirmed by the demonstration of the surface exposure of loops L3 to L7 by epitope insertion mutagenesis (Puntervoll *et al.*, 2000), together with the fact that deletions in loops L1 to L7 did not impede the pore function (Kleivdal *et al.*, 2001). In addition, deletions made in loop L6 led to a marked increase in the uptake of antibiotics through the FomA porin (Kleivdal *et al.*, 2001), suggesting that this loop may function analogously to the pore constriction loop L3 of the structured non-specific porins of *E. coli* and *Rhodobacter capsulatus* (Cowan, 1993). Furthermore, a cluster of arginines that contributes to the constriction of the pore has been identified, similar to the cluster observed in the matrix porin OmpF of *E. coli*, but positioned closer to the C terminus (Kleivdal *et al.*, 1999). We recently proposed that these structural deviations from the well-known porins may imply that FomA belongs to a separate subgroup of non-specific porins, reflecting the evolutionary distance between the fusobacteria and the proteobacteria (Kleivdal *et al.*, 2001), but further structural information is clearly needed to establish whether this actually is the case. In the present study, we present the first physicochemical evidence that FomA is a β -barrel, and examine the role of the proposed loop L1. The results presented here indicate that the proposed loop L1 is located in the periplasm, and that the FomA protein is a 14-stranded β -barrel.

METHODS

Bacterial strains and growth conditions. *Fusobacterium nucleatum* ATCC 10953 was used as a source for wild-type FomA, and was grown anaerobically at 37 °C in a liquid Bacto-tryptone/yeast extract/ascorbic acid/glucose/0.5 % NaCl medium (Bolstad *et al.*, 1991).

Escherichia coli strain DH5 α (Hanahan, 1983) was used for cloning purposes. The porin-deficient *E. coli* K-12 strain CE1224 (Tomassen *et al.*, 1983) was used to express recombinant FomA protein as previously described (Puntervoll *et al.*, 2000). The *E. coli* strains AK101 and DH5 α *mutS* were used with the double-primer method for site-directed mutagenesis (Ohmori, 1994). *E. coli* was grown aerobically at 37 °C in LB medium, or, to impose phosphate limitation, in a low-phosphate medium described by Levinthal *et al.* (1962). For large-scale growth under low-phosphate conditions, *E. coli* was grown in 200 ml LB medium overnight; the cells were harvested and washed twice in a low-phosphate medium and used to inoculate a 1 l low-phosphate medium culture, which was incubated overnight. The antibiotics chloramphenicol (35 μ g ml⁻¹), ampicillin (50 μ g ml⁻¹) and tetracycline (10 μ g ml⁻¹) were added for selective purposes when necessary.

Plasmids and constructs. The plasmid pHB14 (Jensen *et al.*, 1996) contains the part of the *fomA* gene encoding the mature part of the protein, immediately downstream of the *phoE* promoter and signal sequence encoding part of *phoE*, and was

used to express the FomA protein recombinantly in *E. coli* in a functional manner (Jensen *et al.*, 1996). Plasmid pHB14E2C was constructed by site-directed mutagenesis using the double-primer method (Ohmori, 1994). The pTZ19U_{rrh}-derived phagemid pHKU_{rrh} containing the chimeric *phoE/fomA* gene from pHB14 and a defective *ori* region (Kleivdal *et al.*, 1999) was used to produce single-stranded DNA (Sambrook *et al.*, 1989). The E2C (5'-GGTGCAGGCATAACACATGCAGCC-TGTACAGATGC-3') and *ori* repair (5'-GGGAAGCGTGG-CGCTTTCTCATAG-3') primers were used, and the site-directed mutagenesis was performed as described by Kleivdal *et al.* (1999). The pHB14E2C plasmid sequence was confirmed by using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Isolation of FomA from the outer membrane by detergent extraction. The procedure described below was used to isolate FomA protein from the outer membrane of both *F. nucleatum* and *E. coli*. Bacterial cells from 1 l of the desired culture were harvested in the late exponential phase of growth by centrifugation, washed twice with 100 ml 50 mM Tris/HCl (pH 7.7), resuspended in 25 ml of the same buffer, and disrupted by passage through a French pressure cell three times at 900 p.s.i. (6.2 MPa). The samples were collected in pre-cooled tubes, and intact cells were removed by a low-speed centrifugation at 1000 g for 20 min at 4 °C. All succeeding centrifugations were performed at 20000 g for 45 min. The cell envelopes were isolated by centrifugation at 4 °C. The cell envelopes of *F. nucleatum* were resuspended in 20 ml 2 % Triton X-100, 10 mM MgCl₂, 10 mM HEPES (pH 7.4), sonicated for 30 min in a water bath sonicator, incubated for 45 min at room temperature, and subjected to centrifugation at 4 °C. The cell envelopes of *E. coli* were resuspended in 20 ml 0.05 % CHAPS, 50 mM Tris/HCl, 20 mM EDTA (pH 8.0), incubated at room temperature for 10 min on a rotating wheel, and subjected to centrifugation at 14 °C. The supernatant was discarded, and the pellet was resuspended in 10 ml 1 % octyl β -glucopyranoside (OBG), 50 mM Tris/HCl, 20 mM EDTA (pH 8.0), and incubated with vigorous shaking (250 r.p.m.) at 37 °C for 30 min. The residual cell envelope material was pelleted by centrifugation at 24 °C, and the supernatant containing the extracted proteins was collected and stored at 4 °C.

Purification of FomA by chromatography. Anion-exchange chromatography was used as the first step in the purification of FomA. A 20 ml Q-Sepharose column (1.5 cm diameter) was equilibrated with 20 mM Tris/HCl buffer (pH 8.0), and the flow-through of protein was monitored by measuring A₂₈₀. When the applied sample contained a detergent other than OBG the column was washed with 20 ml 1 % OBG, 10 mM Tris/HCl (pH 8.0) to exchange the detergent. The protein was eluted from the Q-Sepharose column by using a LiCl gradient. A total volume of 50 ml was used, and the gradient ranged from 0 to 300 mM LiCl with fixed concentrations of 1 % OBG and 10 mM Tris/HCl (pH 8.0). Fractions of 1.5 ml were collected, A₂₈₀ was measured, and fractions of interest were analysed by SDS-PAGE.

Further purification of the FomA protein was achieved by applying size-exclusion chromatography. Fractions enriched in FomA were pooled, and the volume was reduced to less than 2 ml by using a concentration cell (Amicon) with a cut-off filter of 30 kDa. This sample was applied to a Sephacryl 200 column pre-equilibrated with 1 % OBG, 10 mM Tris/HCl (pH 8.0), 200 mM LiCl, and size-exclusion chromatography was conducted in the same buffer at a constant flow of 0.5 ml min⁻¹. Fractions of 1.0 ml were collected, A₂₈₀ was

measured, and fractions of interest were analysed by SDS-PAGE.

SDS-PAGE and Western blotting. SDS-PAGE was performed as described by Lugtenberg *et al.* (1975), and the samples were incubated in sample buffer at room temperature or 95 °C for 5 min prior to electrophoresis. The SDS-PA gels were either stained with Coomassie brilliant blue R250 (Sigma) or subjected to Western blot analysis as described by Towbin *et al.* (1979). Anti-FomA antiserum ϕ 239 (Bakken *et al.*, 1989b) and horseradish-peroxidase-coupled goat anti-rabbit antibody (Bio-Rad) were used as primary and secondary antibodies, respectively, and the immunoblots were developed with 4-chloro-1-naphthol (Bio-Rad).

Analysis of secondary structure content by circular dichroism. Secondary structure content of the FomA protein was evaluated by circular dichroism (CD) spectroscopy using a Jasco J-810 spectropolarimeter equipped with a Jasco PTC-423S Peltier element for temperature control. Prior to analysis, the samples were dialysed against a 1% OBG, 20 mM Tris/HCl (pH 8.0) buffer to reduce the LiCl concentration to less than 5 mM. The CD spectra were recorded at 25 °C with samples containing 0.1–0.3 mg protein ml⁻¹ by four consecutive scans at 0.5 nm wavelength intervals, and the mean spectra were stored. The temperature-dependent stability of secondary structure was monitored by heating the samples at a programmed rate of 1 °C min⁻¹.

Trypsin treatment of intact cells and cell envelopes containing FomA, and of purified FomA. Trypsin treatment of intact cells and cell envelopes was in essence performed as described by Merck *et al.* (1997). In short, cells from an overnight culture were pelleted and resuspended in a buffer containing 0.1 M Tris/HCl (pH 8.0), 0.25 M sucrose and 10 mM MgCl₂, and the samples were incubated on ice for 60 min with 0.1 mg trypsin ml⁻¹. The trypsin reaction was stopped by adding soybean trypsin inhibitor (Sigma) to a final concentration of 500 µg ml⁻¹, and the samples were subsequently washed twice with the above-described buffer containing trypsin inhibitor. Cell envelopes to be trypsin treated were isolated by passage through a French pressure cell and subsequent centrifugation as described by Jensen *et al.* (1996), and subjected to trypsin digestion as described for intact cells. Purified FomA protein was subjected to trypsin digestion by adding trypsin to final concentrations of 10, 1 or 0.1 µg ml⁻¹ and incubating on ice for 60 min; the reaction was stopped as described above. All trypsin-treated samples were immediately analysed by SDS-PAGE and Western blotting to avoid unwanted potential activity of (residual) trypsin.

N-terminal amino acid sequence determination. Samples containing the FomA protein were subjected to SDS-PAGE, and the Coomassie-stained FomA bands were excised. The protein was extracted from the gel by incubation in 70% formic acid at room temperature overnight (Tsugita *et al.*, 1987), and submitted to the protein sequencing facilities at the University of Oslo for N-terminal sequencing.

Single-channel conductance experiments of FomA reconstituted in lipid bilayer membranes. FomA protein was prepared for single-channel conductance experiments by extracting the protein from OM with 1% OBG, followed by SDS-PAGE. Samples were incubated with sample buffer at room temperature prior to SDS-PAGE, and run in parallel in all lanes of a 15-lane SDS-PA gel. The outer lanes were stained with Coomassie blue to guide the excision of non-stained native FomA proteins from the gel, and the protein was eluted with 0.5% OBG in 20 mM Tris/HCl (pH 8.0). The reconstituted

of pore-forming activity in artificial membranes was performed by using black lipid membranes as described previously (Kleivdal *et al.*, 1995).

RESULTS

Purification of FomA from the cell envelopes of *F. nucleatum* and *E. coli*

For structural analysis, the FomA protein was purified to near homogeneity from both cell envelopes of *F. nucleatum* ATCC 10953 (FomA₁₀₉₅₃) and the OM of *E. coli* CE1224 cells expressing FomA (rFomA₁₀₉₅₃). Purification was performed by ion-exchange and size-exclusion chromatography as described in Methods. In addition, a truncated 37 kDa FomA protein (FomA_{10953TRU}) was purified in the same manner, after treating OM fractions of *F. nucleatum* ATCC 10953 with trypsin as described in Methods. N-terminal sequencing yielded mixed signals and demonstrated that the FomA_{10953TRU} protein had either D27 or E29 as a new N-terminus, giving a theoretical molecular mass of approximately 37 kDa. All the purified FomA proteins were heat-modifiable, since samples prepared at room temperature prior to SDS-PAGE migrated with a higher mobility than boiled samples (Fig. 1). This strongly suggests that the non-boiled purified FomA proteins, including the truncated FomA_{10953TRU}, migrate as folded monomers in SDS-PAGE. These observations are in line with previous results obtained by our group (Bakken *et al.*, 1989a; Jensen *et al.*, 1996).

Fig. 1. SDS-PAGE analysis of the purified FomA proteins used in this study. FomA₁₀₉₅₃, FomA purified from the cell envelope of *F. nucleatum* ATCC 10953; FomA_{10953TRU}, a truncated 37 kDa FomA protein purified from trypsin-treated cell envelopes of *F. nucleatum* ATCC 10953. rFomA₁₀₉₅₃, FomA purified from the cell envelope of *E. coli* CE1224. Samples were either boiled for 5 min (+) or incubated at room temperature (–) prior to SDS-PAGE.

Fig. 2. Evaluation of secondary structure content of the FomA protein by CD analysis. CD analysis of the FomA₁₀₉₅₃, FomA_{10953TRU} and rFomA₁₀₉₅₃ proteins was done in a 20 mM Tris/HCl buffer (pH 8.0) containing 1% OBG. The CD spectra were recorded at 25 °C (left-hand panels, full lines), and the lowest mean residue ellipticity (θ) value was determined (214 nm). Subsequently, thermal denaturation of the protein was monitored by increasing the temperature from 25 °C to 100 °C at a rate of 1 °C min⁻¹, using 214 nm as fixed wavelength (right-hand panels). After heating the sample at 100 °C, the temperature was immediately lowered to 25 °C, and another CD spectrum was recorded (left-hand panels, dotted lines).

Secondary structure content analysis by circular dichroism

The CD analysis was performed on the FomA₁₀₉₅₃, FomA_{10953TRU} and rFomA₁₀₉₅₃ proteins, as described in Methods. The CD spectra of the FomA proteins (Fig. 2, left panels), obtained at 25 °C (full lines), suggest that the FomA protein is composed predominantly of β -pleated sheet structures, as the topology model suggests. Furthermore, the similarity between the spectra of FomA₁₀₉₅₃ and FomA_{10953TRU} strongly indicates that the truncated FomA_{10953TRU} protein possesses essentially the same secondary structure content as the native FomA₁₀₉₅₃ protein. The spectrum obtained with the rFomA₁₀₉₅₃ protein differed from the other two spectra (Fig. 2), suggesting that the solubilized rFomA₁₀₉₅₃ protein has a somewhat different secondary structure content. The FomA proteins were subjected to a thermal denaturation analysis by monitoring at 214 nm the disruption of secondary structure (Fig. 2, right panels). The thermal melting profiles of FomA₁₀₉₅₃ and rFomA₁₀₉₅₃ show cooperative unfolding transitions at 92 °C. However, FomA_{10953TRU} does not seem to unfold cooperatively. The rFomA₁₀₉₅₃ protein shows an additional transition step at 68 °C, possibly reflecting two (or more) different conformational populations. CD spectra were also recorded for the samples after heating to 100 °C and immediate subsequent cooling to 25 °C (Fig. 2, left panels, dotted lines). The CD spectra of the heat-treated FomA₁₀₉₅₃ and rFomA₁₀₉₅₃ proteins suggest partial refolding but also significant loss of material, possibly due to aggregation. Unlike the FomA₁₀₉₅₃ and

rFomA₁₀₉₅₃ proteins, the truncated FomA_{10953TRU} protein does not seem to aggregate upon heating, thus displaying similar secondary structure content after cooling to 25 °C.

Assessment of the topology model of FomA by trypsin treatment of intact cells and cell envelopes, and purified FomA proteins

The FomA protein has been shown to be highly resistant to trypsin digestion when situated in the outer membrane of both *F. nucleatum* and *E. coli* (Bakken *et al.*, 1989a; Jensen *et al.*, 1996), despite having several R and K residues distributed evenly in the entire primary structure. It was however reported that a small N-terminal segment was cleaved off when intact cells or cell envelopes of *F. nucleatum* were treated with trypsin, and that E29 became the new N-terminal amino acid, indicating surface exposure of the postulated loop L1 (Bakken *et al.*, 1989a). However, when these experiments were repeated using fresh cells and the conditions described in Methods, the FomA protein appeared to be completely resistant to trypsin (Fig. 3a, lanes 1–4), in the same manner as when present in the OM of intact *E. coli* cells (Jensen *et al.*, 1996). In contrast, when cell envelopes were treated with trypsin, a small fragment was cleaved off (Fig. 3a, compare lanes 5 and 8), indicating that the residues R26 and R28 were accessible to trypsin and a periplasmic location of these residues. If the N-terminus of the FomA protein was an integral part of the β -barrel of the FomA protein, as the proposed topology model suggests (Bolstad *et al.*, 1994), one

Fig. 3. Trypsin accessibility of FomA protein in intact cells, in cell walls and in solution. (a) Western blot analysis of FomA in whole cells and cell walls. Samples were treated with trypsin as described in Methods where indicated, and either boiled for 5 min (+) or incubated at room temperature (–) prior to SDS-PAGE. (b) Western blot analysis of FomA_{Fev1} protein isolated from the OM of *F. nucleatum* Fev1 (lower blot), and rFomA₁₀₉₅₃ isolated from the OM of *E. coli* CE1224 (upper blot), following treatment with trypsin. Three trypsin concentrations were used (0.1, 1 and 10 μg ml⁻¹), and the experiment was performed as described in Methods. All samples were applied to SDS-PA gels in duplicate: one was boiled (+) in sample buffer, whereas the other was incubated at room temperature (–). Molecular mass standards are given in kDa to the left.

would expect that at least the β -strand part of the N-terminus would remain as part of the folded β -barrel, when migrating as a folded monomer in SDS-PAGE. This does not seem to be the case, since the folded monomer migrates faster after trypsin digestion than the undigested one (Fig. 3a, compare lanes 6 and 7). Furthermore, N-terminal sequencing of the truncated folded monomer FomA_{10953TRU} isolated by excision from an SDS-PA gel (corresponding to the one shown in Fig. 1, lane 3), revealed that the original N-terminus was not present, and that the new N-terminus started from either D27 or E29. Since the trimers of the FomA₁₀₉₅₃ protein cannot be observed in SDS-PAGE, we also

performed this analysis on the FomA protein from *F. nucleatum* T18 (FomA_{T18}) (not shown), which displays stable trimers (apparent molecular mass ~70 kDa) in SDS-PAGE (Haake & Wang, 1997). The FomA_{T18} trimer was not disrupted by the trypsin treatment, since the migration in SDS-PAGE only increased slightly compared to full-length trimers, and the truncated FomA_{T18} protein had new N-termini identical to those observed with FomA_{10953TRU}.

Several porins are completely resistant to tryptic hydrolysis also when solubilized (Minetti *et al.*, 1998; Rosenbusch, 1974). Since the 37 kDa C-terminal fragment of the FomA protein was completely trypsin resistant when situated in the OM (Bakken *et al.*, 1989a; Jensen *et al.*, 1996), trypsin digestion experiments were applied to the purified FomA proteins to assess the tertiary structure. Since the FomA protein from *F. nucleatum* ATCC 10953 only displays weak trimeric bands on SDS-PAGE (Fig. 1, lane 1), the FomA protein from *F. nucleatum* Fev1 (FomA_{Fev1}), which has stable trimers in SDS-PAGE (Kleivdal *et al.*, 1995), was purified and included in this analysis. The initial analysis using 10 μg trypsin ml⁻¹ revealed extensive digestion, indicating that several trypsin sites, in addition to R26/R28, were susceptible in the FomA proteins in solution. Fig. 3(b) shows a more detailed analysis that was performed with the rFomA₁₀₉₅₃ and FomA_{Fev1} proteins. Three different trypsin concentrations were used, 0.1, 1 and 10 μg ml⁻¹, and the samples were applied to an SDS-PA gel in duplicate; one sample was incubated at room temperature and the other was boiled prior to electrophoresis. It is evident that both populations of OM-extracted FomA protein were much more susceptible to trypsin when in solution than when present in the membrane, since the protein is degraded to fragments smaller than the 37 kDa OM-protected fragment (Fig. 3b, lanes 6–8). Furthermore, the unheated samples show that trypsin digestion apparently leads neither to a dissociation of the trimers of the FomA_{Fev1} protein, nor to the disintegration of the β -barrel structure of the folded monomers (Fig. 3b, lanes 3–5). This is in line with the very stable structure revealed by the CD experiments. Finally, trypsin digestion leads to a shift in mobility of both trimers and folded monomer (Fig. 3b, compare lanes 2 and 3), suggesting that the N-terminus is not an integral part of the β -barrel.

In order to pursue the hypothesis that the N-terminus is not part of the β -barrel, the 37 kDa truncated FomA_{10953TRU} was incorporated into artificial bilayer membranes, as described in Methods. Fig. 4 shows that FomA_{10953TRU} indeed functions as a pore, and a mean single-channel conductance of 1.22 nS was found, similar to that observed with full-length FomA₁₀₉₅₃ (1.17 nS) (Kleivdal *et al.*, 1995).

Role of the N-terminus of FomA in trimer stability

The strong sequence conservation of the N-terminus among the different FomA proteins suggests an important structural and/or functional role (Bolstad *et al.*,

Fig. 4. Analysis of single-channel events due to incorporation of FomA_{10953TRU} into artificial bilayer membranes.

Fig. 5. Western blot analysis of the rFomA_{10953E2C} protein: trimer dissociation and denaturation *in vitro*. The rFomA_{10953E2C} protein extracted from the OM of *E. coli* CE1224 was incubated at different temperatures in the OBG buffer with and without urea for 20 min, as indicated. The samples were subsequently incubated in a non-reducing sample buffer at room temperature for 5 min prior to SDS-PAGE. The analysis allowed the observation of intact trimers (T) (lanes 1–4 and 6–9), folded dimers (D) and monomers (M) (lanes 8 and 9), denatured dimers (D*) and monomers (M*) (lanes 4, 5 and 9). Molecular mass standards are given in kDa to the left.

1994). In a parallel work, an rFomA₁₀₉₅₃ mutant carrying an E2C substitution was constructed (named rFomA_{10953E2C}), in an attempt to identify the trimeric interface of the FomA protein (L. J. Bruseth, unpublished). This E2C modification was found to have a trimer-stabilizing effect when the protein was recombinantly expressed in *E. coli*, leading to trimers in a non-reducing SDS-PAGE analysis (Fig. 5, e.g. lane 1), unlike the rFomA₁₀₉₅₃ protein (e.g. Fig. 1, lane 5). The observed trimer-stabilizing effect is the result of the cysteine of one monomer of the rFomA_{10953E2C} protein forming a disulphide bond with the cysteine of one of the neighbouring monomers within the trimer, since no trimers were visible after reduction of the disulphide bond (not shown). This disulphide bond apparently stabilizes the entire trimeric structure, implying the direct involvement of the N-terminus in trimer stability. In order to analyse this E2C mutant more closely, the

rFomA_{10953E2C} protein was isolated from the OM of *E. coli* CE1224 cells as described in Methods, and solubilized rFomA_{10953E2C} was subjected to a denaturation analysis (Fig. 5). This analysis shows that the rFomA_{10953E2C} protein is highly resistant to denaturation by temperature, and that, in the absence of a reducing agent, the protein denatures into a monomer and a covalently linked dimer, when heated at 100 °C for 20 min prior to SDS-PAGE. The effects of exposure to various temperatures were followed both in 0.5% OBG, 10 mM Tris/HCl (pH 8.0) solution (Fig. 5, lanes 6–9) and in the same solution also containing 4 M urea (Fig. 5, lanes 1–5). The results clearly show that when the rFomA_{10953E2C} protein was heated to 90 °C in the presence of urea, a portion of the protein denatured completely into the 40 kDa monomer and the 80 kDa dimer. But, in the absence of urea, several additional protein bands can be observed on the SDS-PAGE gel, including bands corresponding to folded monomers (38 kDa) and folded dimers (54 kDa, 62 kDa). This demonstrates that the trimers of the FomA protein can disintegrate into folded monomers (and in the case of the E2C mutant, folded dimers), and that the monomers and dimers are less stable than the intact trimer *in vitro* (Fig. 5, compare lanes 4 and 9).

DISCUSSION

The CD spectra strongly suggest that the FomA protein is rich in β -sheet structure and are similar to those obtained with other porins from *E. coli* (Rosenbusch, 1974) and from *Neisseria meningitidis* (Minetti *et al.*, 1997, 1998). Furthermore, the thermal melting profiles suggest that the FomA protein possesses a β -barrel architecture typical for OMPs (Schulz, 2000), since the cooperative unfolding transition occurs at high temperatures (>80 °C), reflecting the very stable three-dimensional structures of these proteins. The evidence for a β -barrel architecture is in line with the published topology model for the FomA protein suggesting that the FomA protein is a 16-stranded β -barrel (Bolstad *et al.*, 1994, 1995). The apparent lower content of secondary structure observed with the rFomA₁₀₉₅₃ protein in combination with its melting profile suggests that at least a fraction of this protein has a different conformation in solution than the FomA₁₀₉₅₃ protein. This may indicate either that a population of the rFomA₁₀₉₅₃ protein expressed in *E. coli* CE1224 does not attain the native conformation, or that a change in conformation is induced as a consequence of the detergent extraction. Since the rFomA₁₀₉₅₃ protein in the OM possesses other characteristics indicating proper folding, such as heat characterifiability and trypsin resistance, and the isolated protein has a mean single-channel conductance very similar to that of the FomA₁₀₉₅₃ protein (Jensen *et al.*, 1996), the latter explanation is favoured. Trypsin analysis of the purified FomA proteins strongly suggests that both the quaternary and tertiary structures are intact in solution, in spite of increased sensitivity to trypsin compared to the FomA proteins present in the OM, since neither folded monomers nor trimers are

(completely) disrupted by proteolysis (Fig. 3b, lanes 3–5).

The trypsin analysis of the FomA protein present in the OM and in solution also provided new structural information. In contrast to earlier published work (Bakken *et al.*, 1989a), no R or K residues seem to be accessible to trypsin on the surface of intact cells. Instead, the results presented here suggest that the trypsin site reported to be exposed on the cell surface is in fact exposed to the periplasm, since it is fully accessible to trypsin only when cell envelopes are treated with trypsin. Thus residues 1–28 do not seem to be part of the β -barrel, since the N-terminal fragment containing these residues apparently dissociates from the rest of the protein, as evident from the increased mobility in SDS-PAGE and the fact that none of these residues can be found by N-terminal sequencing. Furthermore, the first putative membrane-spanning segment of the topology model of the FomA protein contains several alternating proline residues, and hence this segment cannot exist as a classical β -strand (Bolstad *et al.*, 1994). The 28 aa N-terminal fragment also seems to dissociate from the FomA protein when subjected to trypsin treatment in solution, since there is a shift in the mobility of both the folded trimers of FomA_{Fev1} and the folded monomers of rFomA₁₀₉₅₃ in SDS-PAGE (Fig. 3b, compare lanes 2 and 4). Finally, the fact that several additional trypsin sites were cleaved in solubilized FomA protein (Fig. 3b, lanes 6 and 7), with no apparent effect on the migration of the folded barrel (Fig. 3b, lanes 3 and 4), together with the fact that FomA_{10953TRU} functions as a pore (Fig. 4) with properties similar to those of the FomA₁₀₉₅₃ protein, strongly supports the hypothesis that the N-terminus is not part of the β -barrel.

Earlier work has demonstrated that it is possible to replace residues 8 to 23 with the residues RS without the loss of pore function (Kleivdal *et al.*, 2001), suggesting that this segment is not critical for maintaining the β -barrel structure of the FomA protein. In support of this is also the fact that the 37 kDa trypsin fragment of the FomA (FomA_{10953TRU}) protein functioned as an intact pore in artificial bilayer membranes (Fig. 4), with a mean single-channel conductance similar to that of the full-length FomA₁₀₉₅₃ protein. On the other hand, the results obtained with the E2C FomA mutant protein suggest that the N-terminus plays a role in stabilizing the quaternary structure, since the rFomA_{10953E2C} protein displays more stable trimers than the wild-type FomA₁₀₉₅₃ protein under non-reducing conditions, due to a disulphide bond between two monomers within the trimer.

In the light of the results presented here, we propose an alternative topology model where the C-terminal part of the protein forms a 14-stranded β -barrel (Fig. 6). Although the results do not directly implicate the second putative β -strand of the original model, the constriction rules for transmembrane β -barrels formulated by Schultz (2000) imply an even number of β -strands, thus leading us to propose that the first two strands and loop L1 of

Fig. 6. The 14-stranded topology model of the FomA protein of *F. nucleatum* ATCC 10953. The top of the model shows the putative surface-exposed loops and the central part represents the presumed transmembrane segments. Amino acid residues, shown in one-letter code, are indicated by boxes when they are supposed to form β -strands (shaded box if proposed to face the lipids or the subunit interface) and circles for turns (T), loops (L) and the putative periplasmic N-terminal domain. Amino acid residues referred to in the text are numbered, and the arrows indicate the sites that were accessible to trypsin when cell envelopes containing FomA were treated. The GenBank accession number for the FomA₁₀₉₅₃ sequence is X72583.

the original model are located in the periplasm. The 14-stranded β -barrel topology model not only conforms to the results presented here, but is also in accordance with previously published results for the FomA porin (Kleivdal *et al.*, 1999, 2001; Puntervoll *et al.*, 2000). The insertion of a foreign epitope after E19 did not result in the surface exposure of this epitope, in contrast to similar insertions in loops L2 to L6 (named according to the current topology model – Fig. 6) (Puntervoll *et al.*, 2000), in line with a periplasmic location of this part of the protein. Furthermore, neither the insertion of a foreign epitope after E19 (Puntervoll *et al.*, 2000), nor the deletion of residues 8–23 (Kleivdal *et al.*, 2001), severely affected the function of the FomA porin, in line with the N-terminal part not being an integral part of the β -barrel. Recently, the projection structure of the *E. coli* OmpG porin was published at 6 Å (0.6 nm) resolution (Behlau *et al.*, 2001), and it was suggested that this porin is a 14-stranded β -barrel. Compared to the classical non-specific proteobacterial porins, the OmpG porin is also atypical in that it does not seem to form trimers, in contrast to the FomA porin (Kleivdal *et al.*,

1995). In conclusion, the FomA porin, along with the OmpG porin from *E. coli*, seems to deviate from the common understanding that non-specific porins are 16-stranded β -barrels. In order to assess the accuracy of this novel 14-stranded topology model, obtaining the crystal structure of FomA would be of utmost value, and this is our current goal. The purification procedures presented here form a solid base for obtaining large amounts of pure protein for crystallization.

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REFERENCES

- Bakken, V., Aaro, S. & Jensen, H. B. (1989a). Purification and partial characterization of a major outer-membrane protein of *Fusobacterium nucleatum*. *J Gen Microbiol* **135**, 3253–3262.
- Bakken, V., Aaro, S., Hofstad, T. & Vasstrand, E. N. (1989b). Outer membrane proteins as major antigens of *Fusobacterium nucleatum*. *FEMS Microbiol Immunol* **1**, 473–483.
- Behlau, M., Mills, D. J., Quader, H., Kuhlbrandt, W. & Vonck, J. (2001). Projection structure of the monomeric porin OmpG at 6 Å resolution. *J Mol Biol* **305**, 71–77.
- Bolstad, A. I., Skaug, N. & Jensen, H. B. (1991). Use of synthetic oligonucleotide DNA probes for the identification of different strains of *Fusobacterium nucleatum*. *J Periodontol Res* **26**, 519–526.
- Bolstad, A. I., Tommassen, J. & Jensen, H. B. (1994). Sequence variability of the 40-kDa outer membrane proteins of *Fusobacterium nucleatum* strains and a model for the topology of the proteins. *Mol Gen Genet* **244**, 104–110.
- Bolstad, A. I., Høgh, B. T. & Jensen, H. B. (1995). Molecular characterization of a 40-kDa outer membrane protein, FomA, of *Fusobacterium periodonticum* and comparison with *Fusobacterium nucleatum*. *Oral Microbiol Immunol* **10**, 257–264.
- Bolstad, A. I., Jensen, H. B. & Bakken, V. (1996). Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. *Clin Microbiol Rev* **9**, 55–71.
- Cowan, S. W. (1993). Bacterial porins: lessons from three high-resolution structures. *Curr Opin Struct Biol* **3**, 501–507.
- Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N. & Rosenbusch, J. P. (1992). Crystal structures explain functional properties of two *E. coli* porins. *Nature* **358**, 727–733.
- Dutzler, R., Rummel, G., Alberti, S., Hernandez-Alles, S., Phale, P., Rosenbusch, J., Benedi, V. & Schirmer, T. (1999). Crystal structure and functional characterization of OmpK36, the osmoporin of *Klebsiella pneumoniae*. *Struct Fold Des* **7**, 425–434.
- Haake, S. K. & Wang, X. (1997). Cloning and expression of FomA, the major outer-membrane protein gene from *Fusobacterium nucleatum* T18. *Arch Oral Biol* **42**, 19–24.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Heller, K. B. (1978). Apparent molecular weights of a heat-modifiable protein from the outer membrane of *Escherichia coli* in gels with different acrylamide concentrations. *J Bacteriol* **134**, 1181–1183.
- Hirsch, A., Breed, J., Saxena, K., Richter, O. M., Ludwig, B., Diederichs, K. & Welte, W. (1997). The structure of porin from *Paracoccus denitrificans* at 3.1 Å resolution. *FEBS Lett* **404**, 208–210.
- Jensen, H. B., Skeidsvoll, J., Fjellbirkeland, A., Høgh, B., Puntervoll, P., Kleivdal, H. & Tommassen, J. (1996). Cloning of the *fomA* gene, encoding the major outer membrane porin of *Fusobacterium nucleatum* ATCC10953. *Microb Pathog* **21**, 331–342.
- Kleivdal, H., Benz, R. & Jensen, H. B. (1995). The *Fusobacterium nucleatum* major outer-membrane protein (FomA) forms trimeric, water-filled channels in lipid bilayer membranes. *Eur J Biochem* **233**, 310–316.
- Kleivdal, H., Benz, R., Tommassen, J. & Jensen, H. B. (1999). Identification of positively charged residues of FomA porin of *Fusobacterium nucleatum* which are important for pore function. *Eur J Biochem* **260**, 818–824.
- Kleivdal, H., Puntervoll, P. & Jensen, H. B. (2001). Topological investigations of the FomA porin from *Fusobacterium nucleatum* and identification of the constriction loop L6. *Microbiology* **147**, 1059–1067.
- Koebnik, R., Locher, K. P. & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* **37**, 239–253.
- Kreusch, A. & Schulz, G. E. (1994). Refined structure of the porin from *Rhodospseudomonas blastica*. Comparison with the porin from *Rhodobacter capsulatus*. *J Mol Biol* **243**, 891–905.
- Levinthal, C., Signer, E. R. & Fetherolf, K. (1962). Reactivation and hybridization of reduced alkaline phosphatase. *Proc Natl Acad Sci USA* **48**, 1230–1237.
- Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P. & van Alphen, L. (1975). Electrophoretic resolution of the “major outer membrane protein” of *Escherichia coli* K12 into four bands. *FEBS Lett* **58**, 254–258.
- Merck, K. B., de Cock, H., Verheij, H. M. & Tommassen, J. (1997). Topology of the outer membrane phospholipase A of *Salmonella typhimurium*. *J Bacteriol* **179**, 3443–3450.
- Minetti, C., Tai, J. Y., Blake, M. S., Pullen, J. K., Liang, S. M. & Remeta, D. P. (1997). Structural and functional characterization of a recombinant PorB class 2 protein from *Neisseria meningitidis*. Conformational stability and porin activity. *J Biol Chem* **272**, 10710–10720.
- Minetti, C. A., Blake, M. S. & Remeta, D. P. (1998). Characterization of the structure, function, and conformational stability of PorB class 3 protein from *Neisseria meningitidis*. A porin with unusual physicochemical properties. *J Biol Chem* **273**, 25329–25338.
- Moore, W. E. & Moore, L. V. (1994). The bacteria of periodontal diseases. *Periodontol* **2000** **5**, 66–77.
- Nakae, T. (1976). Outer membrane of *Salmonella*. Isolation of protein complex that produces transmembrane channels. *J Biol Chem* **251**, 2176–2178.
- Ohmori, H. (1994). A new method for strand discrimination in sequence-directed mutagenesis. *Nucleic Acids Res* **22**, 884–885.
- Puntervoll, P., Kleivdal, H., Dahl, K. O., Bitter, W., Tommassen, J. & Jensen, H. B. (2000). The *Fusobacterium nucleatum* porin FomA possesses the general topology of the non-specific porins. *Microbiology* **146**, 1437–1445.

- Rosenbusch, J. P. (1974).** Characterization of the major envelope protein from *Escherichia coli*. *J Biol Chem* **248**, 8019–8029.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schulz, G. E. (2000).** Beta-barrel membrane proteins. *Curr Opin Struct Biol* **10**, 443–447.
- Tomassen, J., van Tol, H. & Lugtenberg, B. (1983).** The ultimate localization of an outer membrane protein of *Escherichia coli* K-12 is not determined by the signal sequence. *EMBO J* **2**, 1275–1279.
- Towbin, H., Staehelin, T. & Gordon, J. (1979).** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.
- Tsugita, A., Ataka, T. & Uchida, T. (1987).** Approaches for submicrosequencing. *J Protein Chem* **6**, 121–130.
- Weiss, M. S. & Schulz, G. E. (1992).** Structure of porin refined at 1.8 Å resolution. *J Mol Biol* **227**, 493–509.
- Zeth, K., Diederichs, K., Welte, W. & Engelhardt, H. (2000).** Crystal structure of Omp32, the anion-selective porin from *Comamonas acidovorans*, in complex with a periplasmic peptide at 2.1 Å resolution. *Structure Fold Des* **8**, 981–992.
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