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Expression, refolding and crystallization of the OpcA invasin from *Neisseria meningitidis*

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OpcA is an integral outer membrane from the Gram-negative pathogen *Neisseria meningitidis* that plays a role in adhesion of meningococci to host cells. The protein was overexpressed in *Escherichia coli* in an insoluble form and a procedure developed for refolding by rapid dilution from denaturant into detergent solution. The refolded material was identical to native OpcA isolated from meningococci, as judged by overall molecular weight, migration on SDS–PAGE and reaction against monoclonal antibodies. Both native and recombinant OpcA crystallized under similar conditions to give an orthorhombic crystal form ($P2_12_12$), with unit-cell parameters a = 96.9, b = 46.3, c = 74.0 Å. Complete data sets of reflections were collected from native and refolded OpcA to 2.0 Å resolution.

1. Introduction

The bacterium *N. meningitidis* is a major cause of meningitis and septicaemia worldwide (Cartwright, 1995). The outer membrane proteins (OMPs) carry out several important functions for the organism, including adhesion to host cells (Poolman *et al.*, 1995). OpcA (formerly Opc) is a member of the class 5 group of OMPs in *N. meningitidis* and has some biochemical features in common with the diverse opacity (Opa) proteins (Achtman *et al.*, 1988). The Opa proteins are predicted to form an eight-stranded β-barrel structure within the outer membrane (Malorny *et al.*, 1998), similar to those adopted by the *E. coli* OmpA and OmpX proteins, the crystal structures of which have recently been determined (Pautsch & Schultz, 1998; Vogt & Schultz, 1999; Pautsch & Schultz, 2000). OpcA does not show any significant sequence homology with the Opa family and has been predicted to adopt a ten-stranded rather than an eight-stranded β-barrel structure (Merker *et al.*, 1997). It therefore appears that OpcA may be a structurally novel member of this expanding group of membrane proteins. OpcA is also a member of a family of related proteins with similar transmembrane regions (Zhu *et al.*, 1999).

There is substantial evidence that OpcA functions as an adhesin, promoting the adhesion of non-encapsulated meningococci to epithelial and endothelial cells (Virji *et al.*, 1992). Work by Virji and coworkers has implicated the vitronectin receptor in mediating binding to OpcA of human endothelial cells (Virji *et al.*, 1994). Heparin sulfate proteoglycans have also been shown to bind OpcA to epithelial cells (de Vries *et al.*, 1998). The predicted two-dimensional structure of OpcA by Merker *et al.* (1997) suggests that a substantial proportion of the protein is exposed on the outer surface of the outer membrane and could therefore be accessible to antibodies and other ligands. There is some evidence for this from an analysis of two-dimensional crystals of OpcA which were obtained by reconstitution into lipid vesicles and showed evidence for features in the protein that protruded above the membrane (Collins *et al.*, 1999). Furthermore, OpcA is known to be highly immunogenic in humans and to induce the production of bactericidal antibodies (Rosenqvist *et al.*, 1993). The determination of the crystal structure of OpcA may therefore provide some clues about the molecular basis for the roles of OpcA as an immunogen and adhesin.

A serious obstacle to the crystallization of any outer membrane protein (OMP) is the isolation of milligram quantities of pure homogeneous material for crystallization trials. This is a particular problem for structural studies on OMPs from bacterial pathogens, where large amounts of native material may be difficult to obtain. There are already examples of refolding protocols for eight-stranded (Pautsch *et al.*, 1999), 12-stranded (Dekker *et al.*, 1995), 16-stranded (Qi *et al.*, 1994; Surrey *et al.*, 1998) and 22-stranded (Buchanan, 1999) β-barrel proteins. Here, we describe a method for the expression, refolding of recombinant OpcA and crystallization from both native and recombinant sources. A comparison of the crystals from both sources shows that their diffraction properties are very similar, thus

establishing that recombinant OpcA is indeed a suitable substitute for structural and functional studies. A previously reported method for refolding OpcA (Musacchio *et al.*, 1997) was less convenient to use for isolation of large quantities of OpcA protein for structural studies. Determination of the three-dimensional structure of OpcA will provide new insights into the mechanism of adhesion to epithelial and endothelial cells.

2. Experimental and results

2.1. Cloning of the opcA gene and expression of the recombinant protein

The DNA encoding the OpcA protein (without its 19-amino-acid leader sequence) was PCR amplified from strain NmA Z3476 (Olyhoek *et al.*, 1991) with PCR primers YLNMC31 (TTC CAT GGA TCC AGC ACA AGA GCT TCA AAC CGC T) and YLNMC32 (GTC ATC TAG ATG ATG ATT TCA AAT CAT CAG AAT TTT A) containing 5′-end *Nco*I/*Bam*HI and 3′-end *Xba*I restriction sites, respectively. The restricted fragment (803 bp) was cloned in pMG1, a pBR322 derivative plasmid which utilizes signals from λ-phage DNA to drive the transcription and translation of inserted foreign DNA in fusion with the sequence coding for the 81 N-terminal amino acids of NS1 (non-structural protein of influenza virus). The NS1 sequence (except the first nine nucleotides) was deleted by a *Bam*HI restriction, resulting in the plasmid pMG MDP OPC. The expressed OpcA protein therefore consists of a fusion of the first three amino acids of NS1 (Met-Asp-Pro), followed by the predicted sequence of the mature OpcA polypeptide after cleavage by the signal peptidase (Ala-Gln-Glu-Leu-Gln-Thr-Ala), giving a total of 255 amino acids (Merker *et al.*, 1997). The OpcA recombinant plasmid was introduced by transformation into an *E. coli* lysogenic AR58 strain by heat shock at 310 K. Expression of OpcA is under the control of the λP_L promoter/O_L operator. The host strain AR58 contains a temperature-sensitive *c*I857 gene in its genome which blocks expression from λP_L at low temperature by binding to O_L. Once the temperature reaches 312 K, *c*I857 is released from O_L and OpcA is expressed. The recombinant *E. coli* strain was cultivated on a 20 l scale (Biolafitte fermenter) in a semi-synthetic medium. The growth phase was performed at 303 K by feeding exponentially increasing amounts of glycerol (Fed-batch mode). When the biomass reached 50 g l^{-1} DCW, the induction was initiated by a shift of temperature to 312.5 K. After 24 h of induction (biomass = 95 g l^{-1} DCW), the culture was harvested by centrifugation and the cell paste was stored at 253 K.

2.2. Refolding and purification

10 g of *E. coli* cell paste was added to 20 ml of 50 m*M* bis-tris propane/HCl pH 7.0 containing a cocktail of protease inhibitors (Boehringer 'Complet' protease inhibitor, using one tablet in 25 ml as recommended by the manufacturer) and allowed to thaw. The cells were lysed by sonication and insoluble material sedimented by centrifugation at 10 000 rev min^{-1} for 15 min at 277 K (Sorval, SS-34). The supernatant was discarded and the pellet resuspended in 100 ml of 50 m*M* bis-tris propane/HCl pH 7.0, 5% LDAO (*N*,*N*-dimethyldodecylamine-*N*-oxide; Fluka) and Boehringer 'Complet' protease inhibitors. The suspension was stirred for 1 h at 277 K. Insoluble material was sedimented by centrifugation at 12 000 rev min^{-1} for 15 min at 277 K (Sorval, SS-34) and the pellet washed twice in 100 ml bis-tris propane/LDAO. The insoluble material in the pellet was resuspended in 50 m*M* bis-tris propane/HCl pH 7.0 plus 6 *M* guanidine–HCl and sonicated for 10 min at 293 K in a sonic water bath, interspersed with vortexing to assist dispersion and solubilization. Residual insoluble material was removed by centrifugation at 13 500 rev min^{-1} for 15 min at 277 K. The supernatant, containing solubilized OpcA, was then added in a dropwise manner to 1 l 50 m*M* bis-tris propane/HCl pH 7.0, 250 m*M* NaCl and 5%(*v*/*v*) LDAO. The resulting solution was dialysed overnight against 9 l of 50 m*M* bis-tris propane/HCl pH 7.0 and 0.1% LDAO. The refolded OpcA was purified on a heparin-affinity column (de Vries *et al.*, 1998), followed by ion-exchange and size-exclusion chromatography as described by Achtman *et al.* (1988). Purified OpcA was stored as a precipitate in 80% ethanol at 253 K. This refolding and purification protocol differs substantially from that described by Musacchio *et al.* (1997); specifically, the OpcA was purified here after refolding from guanidine–HCl rather than before and the conditions for refolding were different. The folded state of the recombinant material was verified by circular dichroism and gave a signal characteristic of a β-sheet protein. The recombinant OpcA also exhibited a change in migration on SDS–PAGE after treatment of the gel sample at 373 K, as reported by Achtman *et al.* (1988) for native OpcA. Finally, the recombinant OpcA also cross-reacted with the human monoclonal antibody LuN*m*03, which reacts with a conformational epitope on the surface of OpcA (Merker *et al.*, 1997). Native OpcA was purified from *N. meningitidis* as described by Achtman *et al.* (1988). The mature native protein (after cleavage of the signal peptide) is predicted to consist of 253 residues, with an approximate molecular weight of 28 000 Da (Merker *et al.*, 1997).

2.3. Crystallization

Crystallization was carried out in hanging or sitting drops as follows. A 100 µl suspension of purified recombinant or native OpcA in 80% ethanol (10 mg ml^{-1} protein concentration) was sedimented by centrifugation at 13 000 rev min^{-1} for 10 min. The supernatant was removed and the pellet dried under vacuum. The OpcA was then solubilized in 100 µl 25 m*M* Tris–HCl pH 7.5 plus 1%(*v*/*v*) *n*-decylpentaoxyethylene ($C_{10}E_5$; Bachem P-1005). The well solution contained 50 m*M* Tris/acetic acid pH 7.5, 150 m*M* zinc acetate, 50 m*M* $ZnCl_2$, 0.5%(*w*/*v*) *n*-heptyl-β-D-glucoside and 20%(*w*/*v*) PEG 4000 (recombinant OpcA) or 10–12%(*w*/*v*) PEG 6000 (native OpcA). The crystallization solution was formed by mixing equal volumes of the OpcA/$C_{10}E_5$ solution with the well solution to give a final volume of 10 µl for hanging drops or 20 µl for sitting drops. Rod or tabular crystals formed within 4–8 d. Dimensions of the crystals varied but were typically up to 300 µm in length, up to 200 µm in the second dimension and up to 100 µm in the third direction. The *n*-heptyl-β-D-glucoside probably functions as a small amphiphile in the crystallization process rather than a deter-

Table 1
Data-collection statistics for crystals of recombinant and native OpcA.

Values in parentheses refer to the outer resolution bin.

	Native	Recombinant
Unit-cell parameters (Å)	96.9, 46.3, 74.0	98.0, 46.3, 74.1
Resolution limits† (Å)	24.0–2.02 (2.08–2.02)	39.0–2.01 (2.06–2.01)
X-ray source	ESRF ID14 EH2, λ = 0.933 Å	ESRF ID14 EH1, λ = 0.934 Å
No. of crystals	1	1
Multiplicity	9.9 (5.9)	6.1 (3.0)
Significance $[\langle I\rangle/\sigma(I)]$	14.9 (4.4)	12.9 (1.7)
Unique reflections	21993	22917
Completeness (%)	98.2 (78.4)	99.2 (89.0)
R_{sym}‡ (%)	10.1 (33.3)	8.7 (40.2)

† Data were processed using *MOSFLM* (Leslie, 1992). ‡ $R_{sym} = (1/N)\sum_{hkl}(1/n)\sum_n(\bar{I} - I)/\bar{I}$.

gent; crystals of OpcA were also obtained without *n*-heptyl-β-D-glucoside, but they were considerably smaller. Prior to data collection, the crystals were transferred into a cryoprotectant containing 100 m*M* Tris/acetic acid pH 7.5, 20%(*w*/*v*) PEG 4000, 200 m*M* zinc acetate, 8%(*v*/*v*) glycerol and 0.5%(*w*/*v*) *n*-heptyl-β-D-glucoside at 298 K for at least 8 h before freezing in liquid nitrogen. In this case, the *n*-heptyl-β-D-glucoside was added to maintain a dynamic equilibrium of detergent between the crystal and the liquor.

Complete data sets of reflections to 2.0 Å were collected from crystals derived from recombinant and native OpcA; the results are summarized in Table 1. The space group of both crystals was assigned as $P2_12_12$ on the basis of the systematic absences along the appropriate *a* and *b* cell axes. The diffraction properties of both crystals were very similar, each having the same space group and essentially the same unit-cell parameters. Assuming one molecule in the asymmetric unit, the Matthews coefficient is 2.97 $Å^3\,Da^{-1}$ (Matthews, 1968) and the calculated solvent content is 52%. The similarity in diffraction properties between the native and recombinant Opc crystals provides good evidence that the procedure described here does indeed refold the recombinant Opc into its native state. Given the inherent practical difficulties in obtaining large quantities of outer membrane proteins for structural analysis from microbial pathogens, this work has shown that recombinant material can be a satisfactory substitute for protein isolated from the original bacterium.

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