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Asymmetric pore occupancy in crystal structure of OmpF porin from *Salmonella typhi*

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ABSTRACT

OmpF is a major general diffusion porin of *Salmonella typhi*, a Gram-negative bacterium, which is an obligatory human pathogen causing typhoid. The structure of *S. typhi* Ty21a OmpF (PDB Id: 3NSG) determined at 2.8 Å resolution by X-ray crystallography shows a 16-stranded β-barrel with three β-barrel monomers associated to form a trimer. The packing observed in *S. typhi* Ty21a rfOmpF crystals has not been observed earlier in other porin structures. The variations seen in the loop regions provide a starting point for using the *S. typhi* OmpF for structure-based multi-valent vaccine design. Along one side of the *S. typhi* Ty21a OmpF pore there exists a staircase arrangement of basic residues (20R, 60R, 62K, 65R, 77R, 130R and 16K), which also contribute, to the electrostatic potential in the pore. This structure suggests the presence of asymmetric electrostatics in the porin oligomer. Moreover, antibiotic translocation, permeability and reduced uptake in the case of mutants can be understood based on the structure paving the way for designing new antibiotics.

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1. Introduction

Outer membrane protein OmpF is a member of non-specific general diffusion porin family of Gram-negative bacteria, such as *Salmonella* and *Escherichia coli* (Nakae, 1976; Nikaido, 1994) whose main function is to facilitate the transport of hydrophilic solutes with molecular mass up to 600 Da across the outer membrane (Jap and Walian, 1990; Nikaido, 1993). *Salmonella typhi* is an obligatory human pathogen that causes typhoid which continues to be a major health problem in developing countries (Crump et al., 2004). Among the newer generation vaccines against typhoid, *S. typhi* Ty21a vaccine and Vi polysaccharide have proven to be safe (DeRoeck et al., 2007; Ochiai et al., 2007). The *S. typhi* Ty21a vaccine is orally administered live attenuated vaccine licensed for use in persons 2 years of age or older but requires 3–4 immunisations to induce long-term (at least 6–7 years) protective immunity in two thirds of the immunised individuals (Levine et al., 1999). Interestingly, it has been found that highly immunogenic live oral *Salmonella* vaccine would ideally be suited as a carrier of genes that express protective antigens cloned from other antigens (Aggarwal et al., 1990; Formal et al., 1981; Wu et al., 1989) and such hybrid recombinant *Salmonella* vaccines are expected to invoke protective immunity against both the carrier strains as well as the foreign antigens (Fraillery et al., 2007; Hone et al., 1992). In this context, outer membrane proteins (OMPs) of *Salmonella* have been shown to elicit a protective immunity (Isibasi et al., 1988; Udhayakumar and Muthukkaruppan, 1987). It has also been shown that the *Salmonella* antiOmpF and antiOmpC antibodies reached maximum bactericidal titres during the secondary response, antiOmpF antibodies being less immunogenic than antiOmpC antibodies (Secundino et al., 2006). *S. typhi* porins (OmpC) has been shown to display heterologous epitopes on the cell surface (Puente et al., 1995) which can be exploited as vaccine candidate carrying antigens of other disease causing organisms in their loops, making it possible for a double protective therapy.

In addition to their immunological properties as potent surface antigens, porins also act as entry port for various antibiotics (Nikaido, 2003). The bacteria uses either one of the following mechanism to develop antibiotic resistance using porins: loss/reduction of porins, by expression of other porins not involved in antibiotic translocation and by expression of porins with mutations in the key residues involved in the uptake of antibiotics (Delcour, 2009; Pages et al., 2008). Much of the biophysical and mechanistic studies in determining the pathway of antibiotic translocation through porins have focussed on *E. coli* OmpF, as its structural and functional properties are well understood (Cowan et al., 1992; Danelon et al., 2006). The influx of antibiotics through porins is not just a passive diffusion but involves interactions with key residues in the porin channel and it has been shown in *E. coli* OmpF that any mutations in these key residues alter the pore properties in terms of diffusion of antibiotics (Bredin et al., 2002; Hajjar

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et al., 2010b). Hence the crystal structure of porins from different bacterial sources are pre-requisite to understand the specific atomic details, electrostatic pore potential and favourable channel properties involved in antibiotic translocation. Also, structure of porins from pathogenic species like *Salmonella* will help in designing specific vaccines and improved antibiotics therapy. However, structure determination of membrane proteins is still a bottleneck due to difficulties in producing large amounts of protein and crystallisation. Refolding of porins from inclusion bodies (IBs) and their structure determination was successful in the case of *Rhodopseudomonas blastica* porin (Schmid et al., 1996) and OpCA from *Neisseria meningitidis* (Prince et al., 2001) where the refolded proteins showed structural similarity to their native structures. OmpF from *E. coli* had been overexpressed and refolded in the presence of detergents (Miedema et al., 2004; Visudtiphole et al., 2005). However, this is the first report of crystallisation and structure determination of *in vitro* refolded OmpF from a human pathogen.

Here we report the crystal structure of OmpF from *S. typhi* Ty21a at 2.8 Å resolution (PDB: 3NSG).

2. Materials and methods

2.1. Overexpression, purification and crystallisation

Genomic DNA was isolated from the vaccine strain of *S. typhi* Ty21a (Germanier and Furer, 1975). Primers for *ompF* gene were designed for the mature *S. typhi* OmpF (SwissProt Accession: Q56113). PCR amplified product was cloned into *NdeI* and *BamHI* sites of pET20b (Novagen). *E. coli* GJ1158 (Bhandari and Gowrishankar, 1997), a salt-inducible overexpression host, was transformed with ompF/pET20b. Protein was expressed into cytoplasmic inclusion bodies (IBs). Purification, solubilisation and refolding to native trimeric form and further purification of trimers were carried out with slight modifications from the procedures used for *S. typhi* OmpC (Kumar and Krishnaswamy, 2005).

GJ1158/*pompF* was grown overnight at 37 °C in LBON (Luria–Bertani Omitted NaCl) medium containing 50 μg ml^{-1} ampicillin. 1% of this culture was inoculated into 1 L of LBON supplemented with ampicillin and grown in a shaker at 37 °C till OD_{600} 0.6. The culture was induced by addition of 0.3 M NaCl and grown further for 5 h before harvesting at 7000 rpm for 13 min at 4 °C. The cell pellet was washed twice with saline. Cells were resuspended in 50 mM Tris pH 7.5 and disrupted by sonication (Vibra cell 300 W, SONICS). The crude inclusion bodies (IBs) were pelleted by centrifugation at 7000 rpm for 7 min at 20 °C (rotor RPR-12-2, Hitachi high speed centrifuge). The membrane fractions from the inclusion body pellet were removed by washing twice with TTN buffer (50 mM Tris pH 7.5, 0.1 M NaCl and 2% Triton X-100) and twice with TN buffer (50 mM Tris pH 7.5, 0.1 M NaCl).

IBs were solubilised with 50 mM Tris pH 7.5, 0.1 M NaCl and 8 M urea for 5 h at 37 °C with moderate shaking. Undissolved inclusion bodies were removed by centrifugation at 13,000 rpm for 45 min at 25 °C. The solubilised and unfolded OmpF was passed through 0.45 μm filter to remove particulate matter.

Unfolded OmpF was added into 10-fold volume of the refolding buffer containing 50 mM Tris pH 7.5, 0.1 M NaCl, 10% (v/v) Glycerol and 0.2% *n*-dodecyl-N, N-dimethylamine-N-oxide (LDAO from Anatrace) by drop dilution with gentle stirring at a flow rate of 25 ml/h at 25 °C. The refolded protein was left undisturbed for overnight with stirring. The refolded solution turned light turbid after overnight incubation and was centrifuged at 12,000 rpm for 45 min at 25 °C to remove aggregates and particulate matter. The refolded protein in the clear supernatant was loaded onto the Q-Sepharose FF anion-exchange column at a flow rate of 1 ml min^{-1}. The column was washed with five column volumes of the refolding buffer. The bound protein was eluted with 0.1 M NaCl and 1 M NaCl gradient. Fractions containing pure protein were concentrated using 50 kDa MWCO ultrafiltration device (Centricon-50, Millipore). By a series of dilutions and concentration steps, the buffer was exchanged to reduce the NaCl concentration to 0.1 M. Further purification of *S. typhi* rfOmpF was done using Superdex-200 size exclusion chromatographic column (Supplementary Fig. SF1). The column was pre-equilibrated with a filtered and degassed refolding buffer. The peak fractions corresponding to trimer were pooled and concentrated.

In order to produce SeMet substituted *S. typhi* OmpF *E. coli* B834(DE3)/pLys (Novagen) cells were transformed with ompF/pET20b. The cells were grown in M9 minimal media supplemented with 0.5% glucose and 50 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol. Then the cells were induced with 100 μM IPTG at 0.6 OD_{600} and allowed to grow further for expression at 37 °C supplemented with 50 mg L^{-1} of amino acid mix (without methionine and cysteine) and 50 mg L^{-1} of selenomethionine. Harvesting the cells, obtaining IBs, solubilisation, refolding and purification of SeMet substituted *S. typhi* OmpF were done using the same method used for native protein preparation except that all buffers additionally contained 5 mM DTT, in order to prevent oxidation of SeMet.

Crystallisation was performed by vapour diffusion (hanging drops) with different drop ratios. Initial crystals were obtained with 10 mg/ml protein. The best crystals of Se-Met *S. typhi* rfOmpF were grown from 0.1 M trisodium citrate pH 5.4, 0.25 M potassium sodium tartrate tetrahydrate and 2 M ammonium sulphate at a protein concentration of 30 mg ml^{-1}. Crystals were taken using a cryoloop and were flash frozen at liquid nitrogen. Prior to data collection, all crystals were soaked in mother liquor containing 20% or 25% v/v glycerol serving as a cryoprotectant. Out of other cryoprotectants tried (Ethylene glycol, MPD, PEG 400) glycerol acted as better cryoprotectant. There was no difference when using either 20% or 25% Glycerol.

2.2. Crystallography

The selenomethionine (SeMet) substituted *S. typhi* Ty21a rfOmpF crystals, obtained by hanging drop, diffracted to 2.8 Å resolution. The space group is $P2_12_12_1$ with one trimer in the asymmetric unit (V_M 4.02 and solvent content 69.5%). The diffraction data and refinement statistics are given in Table 1. The MRSAD trimer solution had 21 selenium (Se) sites, as there are seven methionines in a monomer, indicating the complete incorporation of SeMet during expression of the protein. The side chain of 253Q is not visible in all three monomers. Apart from the water molecules, 17 LDAO detergent molecules, 80 sulphate ions, 38 glycerol, six tris, four citrate anions and two tartaric acid molecules were assigned (Supplementary Fig. SF2 A–G). There were two PCR induced substitutions, W212R and D285G identified in the recombinant *S. typhi* OmpF. These residues are located in the strand β11 (212R) and loop L7 (285G).

Diffraction data for SeMet labelled *S. typhi* OmpF were collected on a CCD detector (MARMOSAIC225) using 0.979 Å X-rays at the beamline BM-14 of ESRF, Grenoble, France. A total of 858 frames were collected with 5 s exposure and 0.3° oscillation per frame. The detector to crystal distance was 279.94 mm. Data collection and refinement statistics are given in Table 1. Data were indexed, integrated and scaled using HKL-2000 package (Otwinowski and Minor, 1997). Initial attempts to find a molecular replacement solution with *E. coli* native OmpF structure (PDB ID: 2OMF) were not successful. A model was generated from the sequence of the *S. typhi* OmpF using CHAINSAW (Stein, 2008) of CCP4 package and 2OMF as a template. Structure solution for *S. typhi* OmpF was obtained using this model by MRSAD (Schuermann and Tanner, 2003) from PHENIX suite (Adams et al., 2002). Manual

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