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Structure of *Escherichia coli* OmpF porin from lipidic mesophase

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ABSTRACT

Outer membrane protein F, a major component of the *Escherichia coli* outer membrane, was crystallized for the first time in lipidic mesophase of monoolein in novel space groups, P1 and H32. Due to ease of its purification and crystallization OmpF can be used as a benchmark protein for establishing membrane protein crystallization *in meso*, as a "membrane lyzozyme". The packing of porin trimers in the crystals of space group H32 is similar to natural outer membranes, providing the first high-resolution insight into the close to native packing of OmpF. Surprisingly, interaction between trimers is mediated exclusively by lipids, without direct protein–protein contacts. Multiple ordered lipids are observed and many of them occupy identical positions independently of the space group, identifying preferential interaction sites of lipid acyl chains. Presence of ordered aliphatic chains close to a positively charged area on the porin surface suggests a position for a lipopolysaccharide binding site on the surface of the major *E. coli* porins.

1. Introduction

Gram-negative bacteria are surrounded by two lipid membranes separated by a peptidoglycan layer. The outer membrane (OM) is a protective sieve with a molecular weight cut off of several hundred Daltons that allows the passage of hydrophilic molecules. It prevents uncontrolled penetration of the cell by lipophilic and larger hydrophobic molecules. The OM is asymmetric, with lipopolysaccharides (LPS) forming its outer, and phospholipids and lipoproteins its inner, leaflet (Nikaido, 2003). The major components of the OM, however, are porins, constituting between 60% and 90% of the membrane surface (Jaroslawski et al., 2009; Rosenbusch, 1974). Under typical growth conditions the major porin of the *Escherichia coli* OM is outer membrane protein F (OmpF), present in 10^5 copies per cell and constituting around 7% of the total protein mass of the bacteria (Rosenbusch, 1974).

Because of its high abundance and extreme stability, OmpF was the first membrane protein to be crystallized, producing crystals diffracting to high resolution (Garavito et al., 1983a). It is also one of the first membrane proteins for which a high-resolution crystallographic structure was determined (Cowan et al., 1992). OmpF is a β-barrel protein formed by 16 antiparallel trans-membrane β-strands. Short turns, T1–T8, connect β-strands on the "smooth" periplasmic surface and long loops, L1–L8, on the "rough", extracellular surface.

In vivo OmpF exists in the form of extremely stable trimers (Rosenbusch, 1974) densely packed into two-dimensional hexagonal crystals in the OM (Hoenger et al., 1993). Delipidated OM-peptidoglycan complex also displays the two-dimensional crystalline packing (Rosenbusch, 1974; Steven et al., 1977). The projection structure of similar two-dimensional crystals obtained with synthetic lipids was solved at resolution of 3.5 Å (Sass et al., 1989a), however, their high-resolution three-dimensional structure has not been determined. Neither of the multiple 3D crystal forms of OmpF obtained so far (Cowan et al., 1995; Kefala et al., 2010; Reitz et al., 2009) displayed packing similar to native leaving the details of the porin-porin interactions within OM, important for understanding the integrity of the OM, unknown.

It was again in the laboratory of Rosenbusch that a new approach to the crystallization of membrane proteins was first applied (Landau and Rosenbusch, 1996), in the pursuit of a high-resolution structure of bacteriorhodopsin (bR) (Pebay-Peyroula et al., 1997). It utilized a lipidic cubic phase formed by monoglyceride monoolein (MO) as a matrix from which the membrane proteins crystallized. Whilst general applicability of lipidic cubic phase for crystallizing membrane proteins was demonstrated early (Chiu et al., 2000), initially, the method was successful for bR-like proteins only. It required systematic efforts to understand crystallogenesis in cubic phase and develop tools for the crystallization including robotic automation (Caffrey and Cherezov, 2009) to obtain high-resolution structures of a variety of membrane proteins. These include the β-barrel protein BtuB (Cherezov et al., 2006c), protein complexes as large as light-harvesting II complex

Abbreviations: OM, outer membrane; MO, monoolein; LPS, lipopolysaccharide; bR, bacteriorhodopsin; OmpF, outer membrane protein F; DDM, β-dodecyl maltoside; OG, β-octyl glucoside.

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(Cherezov et al., 2006b) and, recently, GPCR proteins (Cherezov et al., 2007). It was realized that to obtain well diffracting crystals for proteins of higher molecular weight the cubic phase with the characteristic scale of repeating unit around 100 Å has to be swollen (typically using small molecule additives). This creates a less ordered sponge phase with lower curvature (Cherezov et al., 2006b). Both cubic and sponge phase crystallization methods are referred to as *in meso* crystallization.

In spite of its demonstrated success *in meso* crystallization is not a widespread technique, which is due to necessity to manipulate a highly viscous MO and cubic phase, requiring usage of special syringes and due to the optical non-homogeneity of the cubic phase requiring significant practical experience (Caffrey and Cherezov, 2009). Establishing this method in a laboratory would be eased by the existence of a readily obtainable, stable and easily crystallizable model protein.

Here, we show that OmpF satisfies all these requirements. It can be easily crystallized *in meso* and be used as a "membrane lyzozyme" for establishing this crystallization method *de novo*. Unlike bR it is colorless, hence detection of OmpF crystals in optically inhomogeneous lipid mesophase is more representative of other membrane proteins, most of which do not contain chromophore. Novel crystal forms of OmpF were obtained *in meso* with crystal packing similar to the arrangement of porins in the OM, thus providing the first high-resolution evidence for the absence of direct OmpF–OmpF interaction in the bacterial outer membrane.

2. Materials and methods

2.1. Purification of OmpF

OmpF was purified from *E. coli* strain BL21. Cells were grown in 70L fermenter in Luria–Bertani broth at 37 °C to an optical density of ~4.0. One to two liters of cell culture were used to purify OmpF. OM were isolated by modified method of Taylor et al. (1998). Cells suspended in 50 mM bis-Tris pH 6.0, 0.02% PMSF and EDTA free protease inhibitors (Roche Diagnostics) were disrupted using a constant flow cell disruptor (Constant Systems Ltd.). Undisrupted cells were removed by centrifugation (15 min; 9700*g*; 4 °C). Supernatant was centrifuged (90 min; 23,800*g*; 4 °C) and the membrane pellet resuspended in PB buffer (20 mM Tris pH 8.0, 1 mM EDTA). Remains of the inner membranes were solubilized in 1% Triton X-100 at room temperature for 15 min and centrifuged (40 min; 178,000*g*; 4 °C). The resulting pellet was homogenized and stirred in 2% Triton X-100 for 10 min and unsolubilized OM material pelleted as before. This OM pellet was resuspended in PB buffer.

OmpF solubilization and purification was based on the method of Yamashita et al. (2008) with some modifications. OM material was solubilized in 3% β-octyl glucoside (OG, Glycon) for 2 h at room temperature and centrifuged (40 min; 120,000*g*; 4 °C). Supernatant was retained, the pellets extracted again with 3% OG for a further 2 h and clarified by centrifugation as before. Solubilized material was pooled and loaded onto 5 ml Hi Trap Q HP column (Amersham Bioscience) in PB buffer with 0.05% β-dodecyl maltoside (DDM) and washed with 10 column volumes. OmpF was eluted with a 100 ml gradient of 0–100% buffer B (0.8 M NaCl in PB buffer). Eluted protein was diluted with PB buffer, to decrease NaCl concentration to between 50 and 100 mM NaCl, and concentrated in 100 kDa MWCO concentrators Ultracel (Amicon) to a final concentration of 40–50 mg/ml. Protein concentration was measured using absorbance at 280 nm and an extinction coefficient of 54,210 $(\text{cm M})^{-1}$. Detergent concentration in the sample was assayed using an established colorimetric method (Urbani and Warne, 2005).

2.2. Crystallization and crystal freezing

Cubic phase with reconstituted porin was prepared by mixing the protein at a concentration of between 10 and 55 mg/ml (and detergent concentration between 2% and 8.5%) with MO (Nu-Check Prep, USA) 1:1 (v/v) using a mixer made of two 50 µl Hamilton syringes joined tip to tip (Caffrey and Cherezov, 2009). For high throughput screening crystal trials were set up in plastic Laminex plates (Molecular Dimensions) with 100 µm spacers by dispensing 0.1 µl of cubic phase and covering it with 1 µl of precipitant. Optimization and crystal growth for diffraction experiments were performed in MRC Under Oil Crystallization Plates (Swissci) using 0.5 µl of cubic phase overlaid with 3 µl of precipitant solution. The plate was sealed with clear tape.

Initial screening was performed with home made screens based on: salts NaCl, Taximate, $CaCl_2$; swelling additives JeffamineM-600, KSCN, pentaerythritol propoxylate (PPO, Hampton Research) (Cherezov et al., 2006a); and pH in the range 4.5–8.5. For broader screening commercial screens MemGold (Molecular Dimensions), Topaz Opti Mix3 (Fluidigm), JCSG$^+$ and ProComplex (Quiagen) were mixed with swelling additives KSCN (1 M) or PPO (15%). Absorbance of UV and visual light by crystals was measured using a home-built UV microscope (courtesy of G. Schertler et al., MRC LMB).

For diffraction analysis crystals were grown using following precipitant solutions comprising 0.1 M sodium citrate pH 5.6, 0.7 M sodium tartrate, 1 M KSCN (crystal P10); 0.1 M sodium acetate pH 4.6, 2.6–2.7 M $LiNO_3$, 2.0–2.1 M KSCN (crystal P66); and 0.1 M MES pH 6.5, 1.8–2.0 M KSCN (crystal P56).

Crystals were extracted from cubic phase using a cryo-loop, washed in precipitant solution and then transferred to cryo-solution containing 20% glycerol or 30% glucose, with the exception of crystals grown in $LiNO_3$, which did not require additional cryo-protection. Crystals were plunge frozen in liquid nitrogen.

2.3. Data collection and processing

Diffraction data were collected on X06SA (SLS, Switzerland), I02 and I24 (Diamond, UK). Data were processed using the CCP4 suite (1994). Phases were obtained by molecular replacement in PHASER (Mccoy et al., 2007) using an OmpF model with PDB code 2OMF. Model building was done in COOT (Emsley and Cowtan, 2004) and O (Jones and Kjeldgaard, 1997), with refinement in PHENIX (Adams et al., 2002) and REFMAC5 (1994).

3. Results and discussion

3.1. OmpF crystallization

We used OmpF as a stable model protein with the intention of establishing lipidic cubic and, especially, sponge phase crystallization methods in our laboratory. Porin had not been crystallized in cubic phase before so initially we used a homemade crystallization screen. This included the swelling additives needed to obtain a sponge phase in the crystal trials (see Section 2.2). The initial hits were obtained only in the trials with potassium thiocyanide as a swelling additive. These were in the form of barely visible microcrystals that were identified in inhomogeneous and opaque lipidic phase due to their sparkly light scattering properties rather than crystalline shape (Fig. 1B). Under many conditions *in meso*, particularly in high molecular weight polyethylene glycols, crystal-like objects corresponding to crystalline domains of cubic phase often appear (Caffrey and Cherezov, 2009) (Fig. 1A). They do not display optical activity, nor do they absorb UV light. Partially optimized OmpF crystals showed UV absorbance, suggesting that they were