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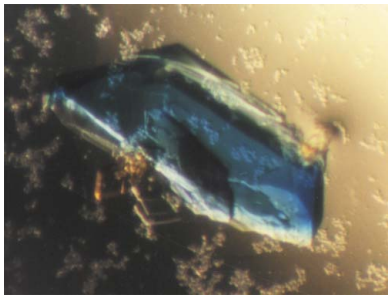

High-resolution diffraction from crystals of a membrane-protein complex: bacterial outer membrane protein OmpC complexed with the antibacterial eukaryotic protein lactoferrin

Crystals of the complex formed between the outer membrane protein OmpC from *Escherichia coli* and the eukaryotic antibacterial protein lactoferrin from *Camelus dromedarius* (camel) have been obtained using a detergent environment. Initial data processing suggests that the crystals belong to the hexagonal space group *P*6, with unit-cell parameters $a = b = 116.3$, $c = 152.4$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. This indicated a Matthews coefficient (V_M) of 3.3 Å^3 Da^{-1}, corresponding to a possible molecular complex involving four molecules of lactoferrin and two porin trimers in the unit cell (4832 amino acids; 533.8 kDa) with 63% solvent content. A complete set of diffraction data was collected to 3 Å resolution at 100 K. Structure determination by molecular replacement is in progress. Structural study of this first surface-exposed membrane-protein complex with an antibacterial protein will provide insights into the mechanism of action of OmpC as well as lactoferrin.

1. Introduction

Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhi* are virulent to humans, causing diseases such as typhoid. *E. coli*, found in the gastrointestinal tract, is an opportunistic human pathogen which is implicated in haemolytic uraemic syndrome (US Department of Health and Human Services; http://www.kidney.niddk.nih.gov/kudiseases). The outer membrane proteins of these bacterial species serve as the major point of nutrient entry into the cell, comprising a distinct class of structurally well characterized β-barrels. *E. coli* OmpC (MW = 38 kDa) belongs to the 16-stranded β-barrel fold (Koebnik *et al.*, 2000) of outer membrane proteins that form trimers with the loops exposed on the outside of the cell membrane. So far, crystals that diffract to high resolution have not been obtained for either *S. typhi* OmpC or *E. coli* OmpC (Arockiasamy & Krishnaswamy, 1999; Kim, 1998). Lactoferrin (Lf) is an iron-binding 76 kDa mammalian glycoprotein found in exocrine secretions and on mucosal surfaces that protects the host against microbial invasion (Oram & Reiter, 1968). Whole cell-binding assays have shown Lf to cause bacteriostasis at a concentration of 1 mg ml^{-1} (Naidu *et al.*, 1993). Lf is a major ingredient in antimicrobial spray recommended by US Food and Drug Administration to fight against *E. coli*. Erdei *et al.* (1994) have reported binding of ^{125}I-labelled lactoferrin to the 38 kDa protein from outer membrane preparations of Gram-negative bacteria. By using porin-deficient *E. coli* K12 isogenic mutants (Sallmann *et al.*, 1999), OmpC and PhoE were found to be specific binding partners of lactoferrin. From solid-phase binding studies, it was shown that lactoferrin binds to purified *E. coli* OmpC trimer with a molar ratio (lactoferrin:OmpC) of 1.9 ± 0.4 and a dissociation constant of 39 ± 18 n*M* (Sallmann *et al.*, 1999). This study also indicated that the interaction involved the N-terminal regions of lactoferrin and the loops of OmpC. Camel lactoferrin (cLf) has been structurally characterized and is homologous to human lactoferrin (hLf; 74% sequence identity). It consists of two homologous N- and C-terminal lobes, each made up of two domains, N-1 (1–90 and 252–320) and N-2 (91–251) and C-1 (345–433 and 596–663) and C-2 (434–595), respectively (Khan *et al.*, 2001). The domains also exhibit the remarkable features of iron binding and iron release. The C-terminal lobe loses iron at pH 6.5, while the N-terminal lobe only loses iron at a pH less than 4.0 (Khan *et al.*, 2001). Mild tryptic digestions of

human and bovine lactoferrin generate 30 kDa N-terminal and 50 kDa C-terminal fragments (Legrand *et al.*, 1984). The purified fragments tend to reassociate through noncovalent reversible interactions.

We report here the crystallization of the complex formed between *E. coli* OmpC and camel lactoferrin and preliminary crystallographic data.

2. Materials and methods

2.1. Protein purification

OmpC was extracted and purified by a modified salt-extraction method (Arockiasamy & Krishnaswamy, 2000). *E. coli* HB101 cells were grown in Luria Broth [1.0%(*w*/*v*) tryptone, 0.5%(*w*/*v*) yeast extract and 1.0%(*w*/*v*) NaCl] for 12 h in an incubator shaker at 310 K and 200 rev min^{-1}. Pelleted cells were sonicated at 80 W using a Labsonic system (Lab-line Instruments, USA). Outer membranes were harvested from lysate by ultracentrifugation at 30 000 rev min^{-1} and 293 K for 90 min. Native OmpC was purified by anion-exchange chromatography using Q-Sepharose to remove bound and unbound lipopolysaccharides. The buffer of the purified sample was exchanged for crystallization buffer using Amicon ultrafiltration devices and concentrated to 21 mg ml^{-1}. The composition of the crystallization buffer was 50 m*M* Tris–HCl pH 7.7, 1%(*w*/*v*) octyl polyoxyethylene and 3 m*M* sodium azide. Purification of apo camel lactoferrin was carried out using ion-exchange and gel-filtration chromatography as reported by Khan *et al.* (2001). The purity of protein samples was determined with SDS–PAGE. Protein aliquots were centrifuged at 11 000 rev min^{-1} for 5 min at 277 K to sediment fibres before use for crystallization.

2.2. Complex preparation

The samples of purified *E. coli* OmpC and camel lactoferrin were mixed in 50 m*M* Tris–HCl buffer pH 7.7 containing 1%(*w*/*v*) octyl polyoxyethylene and 3 m*M* sodium azide and allowed to stabilize overnight at 293 K. This preparation was checked on polyacrylamide gel electrophoresis for complex formation prior to setting up for crystallization.

2.3. Crystallization

Crystals were grown by sitting-drop vapour diffusion at 293 K. A random screen was generated using *CRYSTOOL* (Segelke, 2001). Initial screening was carried out at a protein concentration of 11.7 mg ml^{-1}. A drop containing equal volumes (2 µl each) of protein solution and mother-liquor solution was equilibrated against 100 µl reservoir solution (mother liquor without detergent) using a 96-well plate (Hampton Research). The complex could be crystallized in different forms such as a cluster of needles and thin hexagonal plates within a week. The initial hits were from a variety of buffers (Tris–HCl pH 10.0, sodium phosphate pH 8.0 and HEPES pH 7.5), precipitants (ammonium sulfate, PEG 400, PEG 1000, PEG 1500 and PEG 2000), additives (sodium potassium tartrate, potassium iodide, manganese chloride and ammonium nitrate) and detergents (octyl β-thioglucoside, cymal5, LDAO and octyl hydroxyethyl sulfoxide). The crystals obtained were generally small (maximum size of 50 µm) and clustered as a result of multiple nucleation sites. These conditions were further optimized by variation of precipitant gradient, protein concentration, pH of the buffer, precipitant/additive combination and detergent head groups. For optimization, drops containing equal volumes (5 µl each) of protein solution and mother liquor were equilibrated against 1000 µl reservoir solution (mother liquor without detergent) in Cryschem plates (Hampton Research). Crystals suitable for data collection were obtained from the optimized condition consisting of 200 m*M* Tris–HCl pH 10.0, 13–18%(*w*/*v*) PEG 1500, 150–300 m*M* sodium potassium tartrate, 0.6% β-octyl thioglucoside and 5%(*v*/*v*) ethylene glycol. Crystals of dimensions of up to 2.0 × 0.5 × 0.1 mm that were suitable for diffraction grew in about 8–9 months.

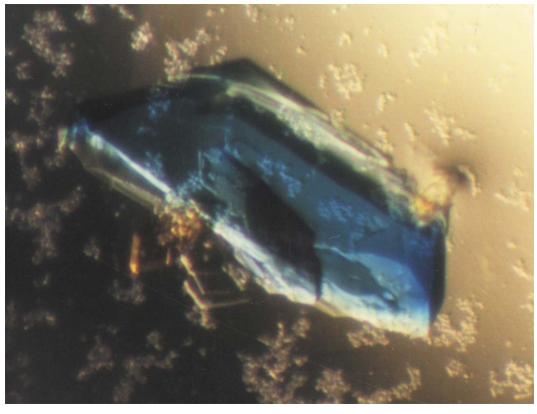

Figure 1
Single crystal of OmpC–lactoferrin complex grown by the sitting-drop vapour-diffusion method that was used for data collection. The crystal has dimensions of 2.0 × 0.5 × 0.1 mm.

2.4. Data collection

The diffraction data were collected on the PX14.2 beamline (Synchrotron Radiation Source, Daresbury, UK; λ = 0.9795 Å) using an ADSC Quantum-4 CCD detector. Prior to data collection, crystals were soaked in mother liquor containing 20%(*v*/*v*) glycerol which served as a cryoprotectant. The crystals were scooped using a cryoloop and were frozen at liquid-nitrogen temperature (100 K) for data collection.

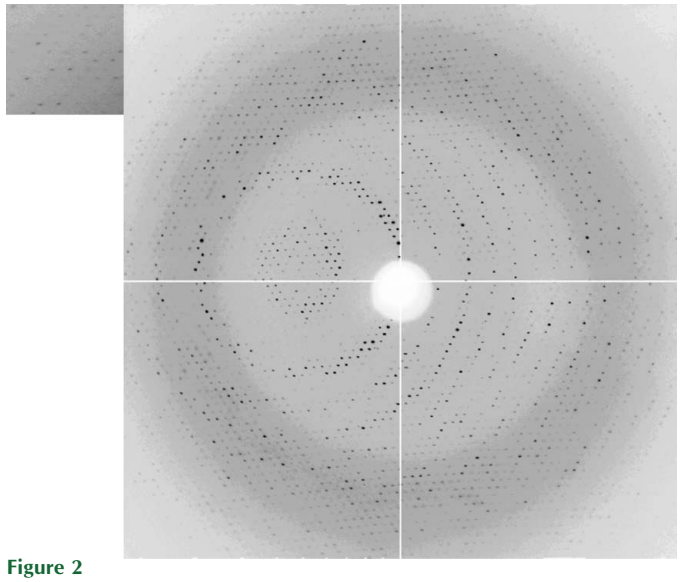

Figure 2
A representative diffraction image from the crystal of the porin–lactoferrin complex. The inset shows weak spots at the corner corresponding to 2.6 Å resolution. There was notable anisotropy and a complete data set was collected to 3 Å resolution.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	SRS, Daresbury, UK
Beamline	PX 14.2
Detector	ADSC Quantum-4 CCD
Wavelength (Å)	0.9795
Space group	*P*6
Unit-cell parameters (Å)	$a = b = 116.3$, $c = 152.4$
Resolution (Å)	50.0–3.0 (3.11–3.0)
Total No. of reflections	307015
Unique reflections	21001
Completeness (%)	89.6 (88.8)
$R_{\rm merge}$	0.081 (0.235)
Average $I/\sigma(I)$	13.0 (9.7)
Redundancy	14.6
Matthews coefficient (Å^3 Da^{-1})	3.3
Solvent content (%)	63
MW per unit cell (kDa)	533.8

3. Results

A single crystal of dimensions 2.0 × 0.5 × 0.1 mm (Fig. 1) was used for data collection. It diffracted to high resolution (Fig. 2). Images were taken with an exposure time of 18 s. The oscillation range for an individual image was 1°. The crystal-to-detector distance was 280 mm. A complete data set was collected to 3 Å resolution. There was notable anisotropy. The data were indexed in the hexagonal space group *P*6, with unit-cell parameters $a = b = 116.3$, $c = 152.4$ Å. Each frame was processed using *DENZO* (Otwinowski & Minor, 1997) and scaled using *SCALEPACK* (Otwinowski & Minor, 1997) (Table 1). Preliminary molecular-replacement analysis in *P*6 with the model of osmoporin from *Klebsiella pneumoniae* (PDB code 1osm), which has 79% sequence identity with *E. coli* OmpC, and packing considerations suggested that the trimer axis of the porin coincides with the crystallographic threefold along the *c* axis at (1/3, 2/3) in the *ab* plane, leading to the presence of two trimers of porin in the unit cell. Packing consideration and initial estimates suggest a possible molecular complex of four molecules of lactoferrin with two trimers of porin in the unit cell. The complex corresponds to a Matthews coefficient ($V_{\rm M}$) of 3.3 Å^3 Da^{-1}, corresponding to 63% solvent content.

4. Discussion

Various approaches have been used to increase the resolution limits of membrane-protein structures by increasing the polar surface through co-crystallization with antibodies (Hunte & Michel, 2002), by deletion of loops and by introducing point mutations (Pautsch *et al.*, 1999). This is the first report on a bacterial outer membrane protein diffracting to higher resolution by co-crystallization with a soluble protein having antibacterial properties (Byrne & Iwata, 2002). Earlier crystallographic studies on *S. typhi* OmpC and *E. coli* OmpC have reported diffraction patterns which were limited to 7 and 4 Å resolution, respectively (Arockiasamy & Krishnaswamy, 1999; Kim, 1998). The lack of high-resolution diffraction has been attributed to the presence of bound lipopolysaccharides. However, the low-resolution diffraction analyses of *S. typhi* OmpC together with structure-based sequence alignment of *S. typhi* OmpC and *E. coli* OmpC imply that the longer loops involved in packing interactions might be a reason for crystallographic disorder. Therefore, in the present approach, we have minimized the conformational flexibility of loops by immobilizing them with an interacting partner, lactoferrin. A complex with strong affinity is formed between OmpC (from *E. coli*/*S. typhi)* and lactoferrin (from camel/human) even under denaturing conditions on SDS–PAGE. The complex is stable in the presence of SDS and is recognized by anti-porin monoclonal antibodies. Although various antibacterial mechanisms for lactoferrin such as iron chelation leading to iron limitation for bacterial growth, binding to lipopolysaccharides and leakage through the outer membrane have been suggested, the exact nature is not yet known. The preliminary structure determination by molecular replacement indicates that the structural characterization of this membrane-protein complex may reveal significant biological insights into the antibacterial action of lactoferrin. This knowledge is expected to help in the rational design of effective mimetic drugs.

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