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Crystallization and preliminary X-ray analysis of ferric enterobactin receptor FepA, an integral membrane protein from *Escherichia coli*

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Abstract

Diffraction-quality crystals have been obtained of the integral membrane protein ferric enterobactin receptor (FepA) from the outer membrane of *Escherichia coli*. Crystals were grown using the zwitterionic detergent lauryldimethylamine oxide (LDAO), the precipitants polyethylene glycol (PEG) 1000 and sodium chloride, and the additive heptane-1,2,3-triol; they have the symmetry of the orthorhomic space group $C222_1$ with a = 112.2, b = 137.2 and c = 135.4 Å and diffract to 2.5 Å resolution. The crystals were flash-cooled and a preliminary data set was collected at 103 K. The crystals are suitable for three-dimensional structure analysis.

1. Introduction

Gram-negative bacteria such as *Escherichia coli* are protected by an outer membrane composed largely of lipopolysaccharides and phospholipids. Molecules smaller than 600 Da can cross this barrier with the help of either non-substrate-specific porins, or substrate-specific transmembrane channels (Nikaido, 1992). Molecules larger than 600 Da must use a high-affinity energy-dependent transport system; examples are the iron transport systems of *E. coli* and other Gram-negative bacteria (for reviews, see Neilands, 1982; van der Helm, 1998).

E. coli and related enteric bacteria synthesize enterobactin (enterochelin), a tricatecholate siderophore (microbial high-affinity iron carrier), to scavenge ferric ions in the medium (O'Brien & Gibson, 1970; Pollock & Neilands, 1970). The ferric enterobactin complex (Fig. 1) is transported into the cell by a system of transport proteins. Enterobactin is the most powerful ferric ion complexing agent known having a stability constant of 10^{52} (Harris *et al.*, 1979).

Ferric enterobactin is recognized by the receptor protein FepA in the outer membrane of *E. coli* (Cox *et al.*, 1970; Hollifield & Neilands, 1978). Other proteins besides FepA are used for further transport of ferric enterobactin through the inner membrane. These are the periplasmic protein FepB (Elkins & Earhart, 1989; Stephens *et al.*, 1995) and the inner (cytoplasmic) membrane protein, FepC, which has an ATP binding domain, and FepD and FepG (Chenault & Earhart, 1991; Shea & McIntosh, 1991). The *fep* and *ent* genes form a cluster (Ozenberger *et al.*, 1987); the *ent* genes produce enzymes used in the synthesis of enterobactin. Enterobactin and Fep proteins are only produced under iron-deficient conditions (O'Brien & Gibson, 1970; McIntosh & Earhart, 1977). This regulation is achieved by the Fe^{2+} requiring Fur protein (Bagg & Neilands, 1987; de Lorenzo *et al.*, 1988) which binds upstream of the iron-related genes, and prevents tran-

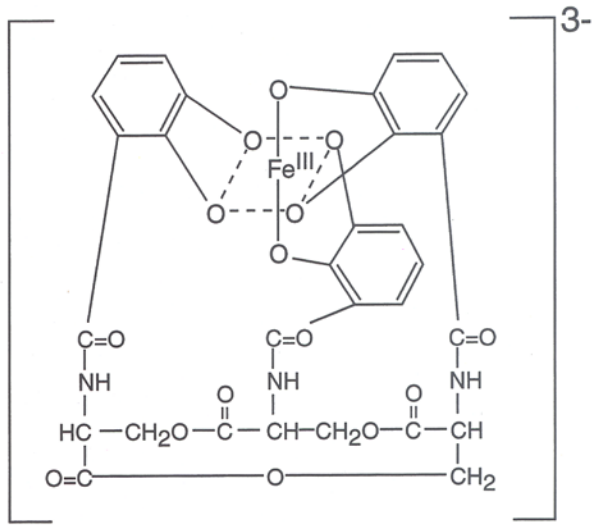

Fig. 1. Structural formula of ferric enterobactin.

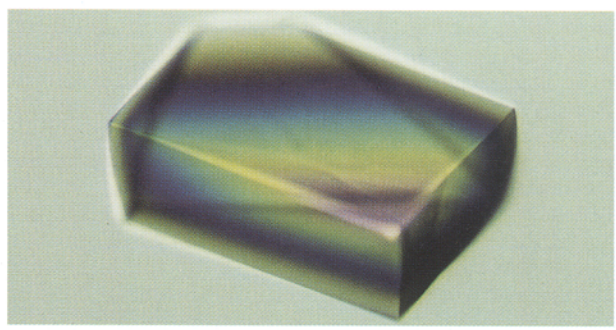

Fig. 2. Crystal of FepA, 80 × 145 × 250 µm, grown from a drop initially containing 0.055% LDAO, 14% PEG 1000, 0.35 *M* NaCl, 1.75% heptane-1,2,3-triol, and 15% glycerol.

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scription of the *fep–ent* cluster of genes when sufficient iron is present in the cell. The iron-receptor outer membrane proteins are also used by colicins and bacteriophages (Hantke & Braun, 1975*a*; Wayne & Neilands, 1975); this aspect has aided the many genetic studies of the iron-uptake systems.

The transport of ferric enterobactin across the outer membrane utilizes an active energy-dependent high-affinity system (Ecker *et al.*, 1986). It is saturable and follows Michaelis–Menten kinetics. The transport is specific because, in addition to ferric enterobactin, only the synthetic analogue FeMECAM is transported with similar kinetic parameters. The number of FepA molecules has been estimated to be 7500 per cell which can be compared to 100 000 porin molecules per cell (Ecker *et al.*, 1986). The iron uptake systems differ from the transport by porins in the fact that they require the TonB protein for energy transduction to the outer membrane (Frost & Rosenberg, 1975; Hantke & Braun, 1975*b*). The outer membrane is without an inherent energy source and it is presumed that conformational changes in TonB aid the transport across this membrane (Klebba *et al.*, 1993; Postle, 1993).

The FepA protein has been sequenced (Lundrigan & Kadner, 1986). It has a molecular weight of 80 009 and consists of 724 amino acids. A model for FepA (Murphy *et al.*, 1990) predicts 29 membrane-spanning regions which are thought to contain predominately β-sheets as has been found in the crystal structures of porins.

An early experiment showed that FepA isolated from the membrane retained its binding activity for ferric enterobactin (Hollifield & Neilands, 1978; Fiss *et al.*, 1982). Once larger amounts of FepA could be produced, these experiments were extended and confirmed (Zhou *et al.*, 1993, 1995). They showed that the binding of detergent solubilized FepA with ferric enterobactin was not only at a single site of the protein, but also that it was saturable and specific, as kinetic uptake experiments had indicated. FepA, once isolated and purified, therefore retains its conformational integrity and a successful crystal structure determination can yield meaningful results in order to study the mechanism of transport. We have obtained diffraction-quality crystals of FepA and a preliminary native data set has been collected from a single flash-cooled crystal. The space-group determination described in this communication does not indicate a trimer formation for FepA, in contrast to porins where this association is consistently observed.

2. Results and discussion

FepA protein was overproduced in a genetically transformed strain of *E. coli* UT5600/pBB2 (Lundrigan & Kadner, 1986; Jalal & van der Helm, 1989). The purification of FepA proved to be difficult due to contamination by a proteolytic product of FepA. This difficulty was eliminated by moving the plasmid to a different host, *E. coli* JM109. The Triton X-100 solubilized protein was purified by a combination of anion-exchange chromatography (HPLC) and chromatofocusing (FPLC) (Zhou *et al.*, 1995). Detergent was exchanged to LDAO† on a 3 ml DEAE Sepharose CL-6B (Pharmacia) anion-exchange column that was equilibrated in 0.1 *M* MOPS (pH 7.4), 0.06% LDAO, and 2 m*M* sodium azide (NaN_3) (buffer *A*). The protein was eluted with buffer *A* containing 0.3 *M* NaCl, washed in a Centricon 30 concentrator (Amicon) with buffer containing 0.025 *M* MOPS (pH 7.0), 0.05% LDAO, 0.1 *M* NaCl, 20% glycerol, and 2 m*M* NaN_3 and concentrated to 15 mg ml^{-1}. Protein concentration was determined by the BCA method (Pierce). The protein was stored at 277 K.

Crystallization conditions were screened using the hanging-drop vapor-diffusion technique (McPherson, 1982). Microcrystals were previously obtained using PEG as the precipitant and β-D-octyl glucoside as the detergent (Jalal & van der Helm, 1989); we therefore screened for better crystals maintaining PEG's of different molecular weights as the precipitants, but changing the detergent.

Diffraction-quality crystals were grown at 294 K using a 3 µl drop initially containing 7.5 mg ml^{-1} of FepA, 14% PEG 1000 (Hampton Research), 0.05 *M* Tricine (pH 8.0), 0.0125 *M* MOPS (pH 7.0), 0.055% LDAO, 0.35 *M* NaCl (Garavito & Rosenbusch, 1986), 1.75% heptane-1,2,3-triol (Sigma high melting point isomer; Michel, 1991), 15% glycerol and 2 m*M* NaN_3. The drop was equilibrated against a 1 ml reservoir containing 28% PEG 1000, 0.1 *M* Tricine (pH 8.0), 0.06% LDAO, 0.35 *M* NaCl, 10% glycerol, and 2 m*M* NaN_3 (Fig. 2). Orthorhombic crystals grew to an average size of 70 × 150 × 230 µm in five to eight weeks.

Oscillation images were recorded on an R-AXIS II image-plate detector using focused Cu $K\alpha$ radiation from a Rigaku RU-300 rotating-anode generator with mirror optics (Molecular Structure Corporation). Because of moderate decay of high-resolution data, the crystals were mounted directly from the mother liquor onto rayon cryoloops (Hampton Research) and flash cooled in liquid propane (Hope, 1990; Teng, 1990). Data were collected at 103 K.

The images were indexed with the program *DENZO* (Otwinowski, 1993) and found to have the symmetry of the orthorhombic space group $C222_1$ with unit-cell dimensions a = 112.2, b = 137.2 and c = 135.4 Å. The space-group assignment was confirmed by statistical analysis of data indexed in a primitive unit cell. The Matthews coefficient, V_m, equals 3.26 Å^3 Da^{-1} for one FepA molecule in the asymmetric unit (Matthews, 1968). This corresponds to a solvent content of 62%.

A single crystal was used to collect a complete native data set to 2.5 Å resolution (Fig. 3). The data were processed and scaled with programs *DENZO* and *SCALEPACK* (Otwinowski, 1993). A total of 376 537 observations were measured and subsequently reduced to 33 895 unique reflections with $R_{\rm merge}$‡ = 0.099. This represents 91.7% of theoretically observable reflections between 100 and 2.5 Å resolution.

Crystals of FepA are suitable for three-dimensional structure analysis. The solution of the structure by the method of multiple isomorphous replacement is currently under way.

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† Abbreviations used: MOPS, 3-(4-morpholino) propane sulfonic acid; TRICINE, [*N*-tris(hydroxymethyl)-methylglycine]; LDAO, lauryldimethylamine oxide; PEG, polyethylene glycol.

‡ $R_{\rm merge} = \sum_{hkl}[\sum_i(|I_{hkl,i} - \langle I_{hkl}\rangle|)]/\sum_{hkl,i}(I_{hkl})$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices, h, k and l, and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection.

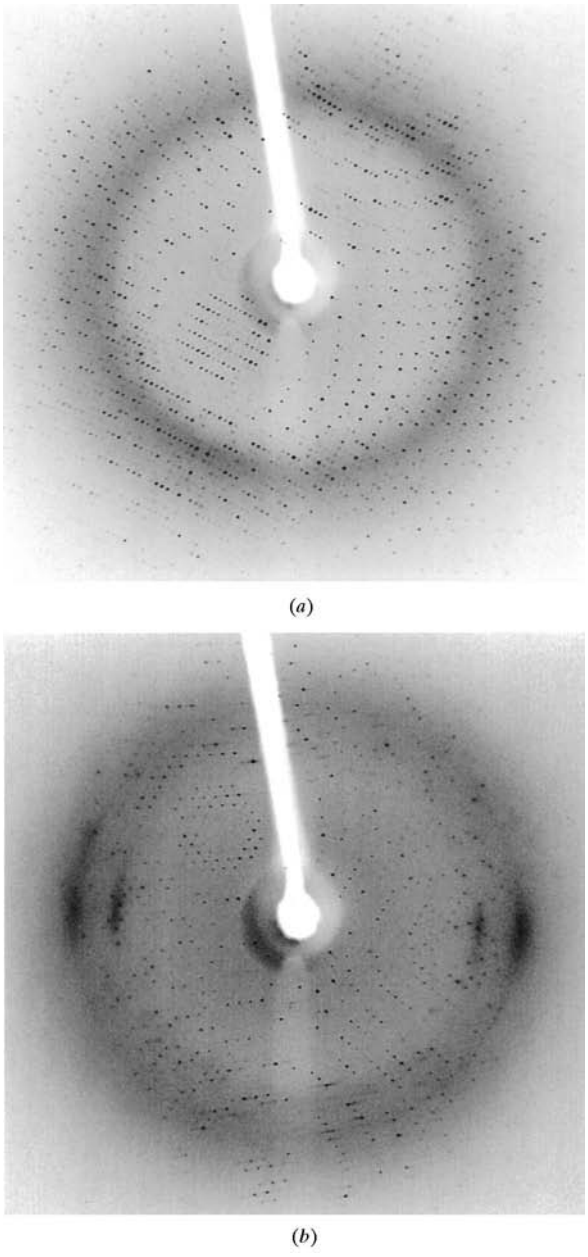

Fig. 3. Diffraction patterns of a flash-cooled (103 K) FepA crystal rotated about the *a* axis. (*a*) The diffraction spots can be observed to the edge of the plate, corresponding to 2.5 Å resolution. (*b*) After 90° rotation from the orientation in (*a*). Diffraction spots can be observed to 2.8 Å resolution, with noticeable anisotropy in this orientation. The exposure time was 30 min for each image, with a crystal-to-film distance of 160 mm and an oscillation of 1.0°.

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