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Purification and crystallization of ferric enterobactin receptor protein, FepA, from the outer membranes of *Escherichia coli* UT5600/pBB2

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The ferric enterobactin receptor protein, FepA, was isolated and purified from the outer membranes of a genetically transformed strain of *Escherichia coli* (UT5600/pBB2) using anion-exchange chromatography, chromatofocusing and gel filtration. The purified protein was found to crystallize from 25 mM sodium phosphate buffer in the presence of 0.8% β-D-octylglucoside under a range of conditions. The protein formed mostly small rods and needle-shaped crystals in the hanging drop method.

Ferric enterobactin protein; Purification; Crystallization; Outer membrane protein; (*E. coli*)

1. INTRODUCTION

Membrane receptor proteins perform the specialized functions of recognizing, binding and subsequent processing of smaller molecules involved in transport activities. An example of such a system is the ferric enterobactin receptor protein (FepA) localized in the outer membranes of *E. coli* [1–3]. Enterobactin is a tricatecholate type siderophore, which is biosynthesized inside the bacterial cells and then excreted to scavenge ferric ion. The siderophore forms a very stable hexacoordinate octahedral trianionic ferric complex with a *Δ-cis* coordination geometry [4,5]. The FepA receptor protein recognizes and binds enterobactin only in its ferric complex form and allows it to enter into the cell in order to deliver the essential metal ion. Biosynthesis of both enterobactin and FepA receptor protein is repressed when a sufficient level of iron is present in the growth medium.

The chemical and physical mechanisms of recognition and binding as well as other important functions of membrane receptor proteins are not well understood. A clear knowledge of the structures of both the bound molecule and its receptor protein is essential for an explanation of these phenomena. Since X-ray crystallography offers the best way to determine three-dimensional structures of complex molecules, attempts have been made to purify and crystallize the ferric enterobactin receptor protein with a view to determining its structure.

Isolation and purification of the ferric enterobactin receptor protein, FepA from *E. coli* BN3040 (using an iron-deficient Tris medium to express the protein), has been described by Neilands and co-workers [1,2]. They also showed that the isolated FepA protein retained an ability to bind ferric enterobactin. Crystallization trials, aimed at determining the protein structure by X-ray diffraction, usually requires large quantities of

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Abbreviations: Tris, trihydroxymethylaminomethane; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PEG, polyethylene glycol

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protein of the highest purity grade. Initial attempts to purify sufficient quantities of FepA from *E. coli* BN3040 proved to be an exhaustive and laborious project.

Lundrigan and Kadner [6] made the genetically transformed strain of *E. coli* UT5600/pBB2, during their DNA sequencing studies of the *fepA* gene. This strain has been found, however, to be a suitable source of FepA. The strain grows on LB broth to an A_{600} as high as 2.5 and overproduces FepA since it contains a high copy number plasmid. This communication describes the procedure used for the purification and crystallization of FepA from the outer membrane of this bacterium.

2. MATERIALS AND METHODS

2.1. *Organism source and growth conditions*

E. coli UT5600/pBB2: *leu, proC, TrpE, rpsL, Δ(ompT-fepA)/Amp^r, fepA$^+$* was received from Dr M. Lundrigan of the University of Virginia, School of Medicine, Charlottesville, VA. The bacteria were maintained on agar slants made of LB medium [7] containing 40 mg/l of ampicillin and 25 mg/l of streptomycin. To produce FepA, cells were grown in TY medium [8] with the above antibiotics at room temperature. Cultures, at their late log phase, were rapidly cooled by adding crushed ice and harvested by centrifugation.

2.2. *Extraction and solubilization of FepA*

The freeze-dried cells (5 g) suspended in buffer A (10 mM Tris-HCl, 10 mM benzamidine, pH 8.0) (700 ml) were sonicated in small batches in an ice-salt bath. The suspension was centrifuged for 10 min at 8000 × *g* and the pooled supernatants were recentrifuged at 200000 × *g* for 1 h in a Beckman L8-80 ultracentrifuge. The membrane pellet was suspended in 40 ml buffer B (buffer A with 2% Triton X-100, pH 8.0), shaken for 30 min at room temperature and centrifuged for 1 h at 200000 × *g*. Extraction of the pellet with buffer B was repeated once again. The pellet was then extracted three times with buffer C (buffer B with 5 mM EDTA, pH 8.0) and two additional times with buffer D (buffer C with 1% β-D octylglucoside). The five supernatants containing the FepA protein (from the extractions with buffers C and D) were pooled and dialysed against 3 l of buffer E (50 mM Tris-HCl, 10 mM benzamidine, 2% Triton X-100, 5 mM EDTA, pH 7.5).

2.3. *Purification of FepA*

Dialysed protein solution was loaded onto an anion-exchange column (Whatman DE-52, 43 cm × 1.5 cm) and washed with 250 ml of buffer E. Proteins were eluted with a 0–0.2 M NaCl gradient in buffer E using a Kratos Spectroflow 430 gradient former. Fractions containing FepA were pooled, dialysed and rechromatographed in a second anion-exchange column of the same composition and size. The FepA band was dialysed against buffer F (25 mM imidazole, 5 mM EDTA, 3 mM NaN_3, 5 mM benzamidine, 1% Triton X-100, pH 6.9). To prepare the chromatofocusing column, 20 ml of the gel (PBE 94, Pharmacia) was washed (3 changes) with buffer F and deaerated. A column (25 cm × 0.7 cm) was packed with the gel (7 ml) and topped with preswollen Sephadex G10 beads (2 cm). 20 bed vols of buffer F was passed through the column to stabilize pH at 6.9. FepA sample was loaded onto the column with a flow rate of 0.07 ml/min. After washing the column with 4 bed vols of buffer F, the pH gradient was started with buffer G (10% polybuffer 74, 5 mM EDTA, 3 mM NaN_3, 1 mM benzamidine, 1% Triton X-100, pH 3.9). Fractions containing FepA were dialysed and chromatographed on a DE-52 column (11 cm × 0.7 cm) with buffer H (10 mM Na-phosphate, 3 mM NaN_3, 5 mM benzamidine, 1% β-D-octylglucoside, pH 7.5). After washing the column (10 bed vols), the protein was eluted with buffer H containing 0.2 M NaCl. The purified FepA was finally subjected to gel filtration through Sephadex G100 (1 cm × 23 cm) with buffer I (25 mM Na-phosphate, 3 mM NaN_3, 5 mM benzamidine, 1% β-D-octylglucoside, pH 7.5).

2.4. *Electrophoresis and thin-layer chromatography*

Identification of FepA was primarily based on the migration of the protein as the 81 kDa band in the SDS-PAGE system [9] described by Neilands and co-workers [1,2]. A sample of FepA purified from *E. coli* BN3040 had the same mobility. The band was not observed, when the medium, in which cells were grown, contained sufficient iron. Thin-layer chromatography of the protein samples (to check phospholipid contamination and detergent composition) was performed on 0.25 mm Silica gel KGF plates (Whatman). Visualization agents included (i) iodine vapour, for detergents, (ii) 0.2% ninhydrin in ethanol followed by heating at 100°C for 5 min, for proteins, and (iii) Molybdenum blue (Sigma), for phospholipid. The solvents used were (i) water saturated 2-butanone and (ii) chloroform/methanol/ammonia, 65:35:5. Protein concentration was determined by the UV absorption method [10], after removal of Triton X-100 and benzamidine from the sample. Concentration of protein solutions in Triton X-100 free buffers was carried out on Centriprep 30 and Centricon 30 concentrators (Amicon).

2.5. *Crystallization of FepA*

Preliminary crystallization experiments of FepA were carried out using the hanging drop method. A number of variables were used: pH 6.0, 6.6, 6.9 and 7.5; precipitant types, PEG 2000, 3000 and 4000; precipitant concentration in the protein solution, 4%, 8%, 12% and 16%; precipitant concentration in the reservoir well, 16% and 20%. The buffer used for crystallization was 25 mM Na-phosphate, 3 mM NaN_3 and 0.8% β-D-octylglucoside. The concentration of FepA in the hanging drop was 16 μg/5 μl.

3. RESULTS AND DISCUSSION

This report outlines the total extraction of FepA from the outer membranes of *E. coli* UT5600/pBB2 and the purification and initial crystallization of the protein. The extraction pro-

cedure was primarily based on selective solubilization of inner and outer membrane proteins with Triton X-100 in the absence and the presence of EDTA [1,11]. However, Triton X-100 and EDTA could not solubilize all of the FepA even after three extractions (fig.1). A mixture of two detergents (Triton X-100 and β-D-octylglucoside) in the presence of EDTA was found to completely solubilize FepA from the outer membrane pellet. The initial solubilization of the inner membrane proteins with buffer B was essential in order to solubilize the outer membrane proteins during later steps.

Separation of most of the major contaminant proteins from the main band of FepA was achieved in the first DE-52 column (fig.2). The minor contaminant proteins were removed in the second ion-exchange column, after which FepA appeared pure from the Coomassie blue staining. However, the protein was still contaminated with membrane phospholipids. These are known to interfere seriously with protein crystallization [12]. Chromatofocusing with polybuffer exchanger (PBE94) on a pH gradient of 6.9–3.9 removed the phospholipids and FepA eluted out as a sharp band at pH 5.5. Chromatofocusing was also used by Garavito and Rosenbusch [12] to remove the phospholipids from porin, another outer membrane protein of *E. coli*. Finaly the buffer and the detergent were changed in a DE-52 column followed by gel filtration of the protein in Sephadex G100. Thin-layer chromatography showed that the purified protein solution was free from phospholipids and Triton X-100. The final yield of the purified FepA from 5 g of freeze-dried cells was approx. 10 mg.

The molecular mass of FepA was predicted recently to be 79908 Da on the basis of the DNA sequence of the *fepA* gene [6]. FepA moved in SDS-PAGE as the usual 81 kDa band when heated for 5 min with the treatment buffer (125 mM Tris-HCl, 4% SDS, 20% (v/v) glycerol, 10% 2-mercaptoethanol and 2% bromophenol blue) [1] prior to electrophoresis. When this heating step was omitted, FepA moved as a 62 kDa band protein instead of 81 kDa (fig.3). Therefore, FepA most probably exists as a monomer and retains its tertiary structure when it moves to the 62 kDa band position.

PEG 2000, 3000 and 4000 were found to be suitable crystallizing agents for FepA. Crystalliza-

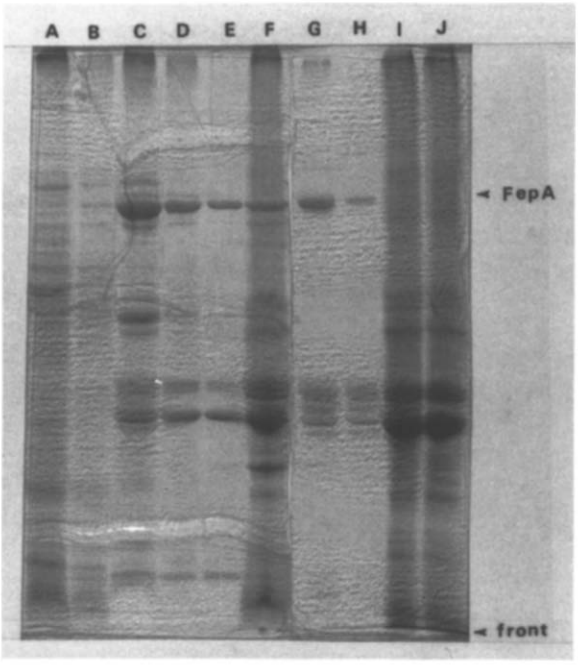

Fig.1. SDS-PAGE gel showing total extraction of FepA. The two initial extractions with buffer B (lanes A,B) dissolved inner membrane proteins. After three extractions (lanes C,D,E) with buffer C, a considerable quantity of FepA still remained in the pellet (lane F). Two more extractions of the pellet with buffer D dissolved FepA almost quantitatively (lanes G,H), while the bulk of the porins remained in the pellet (lanes I,J).

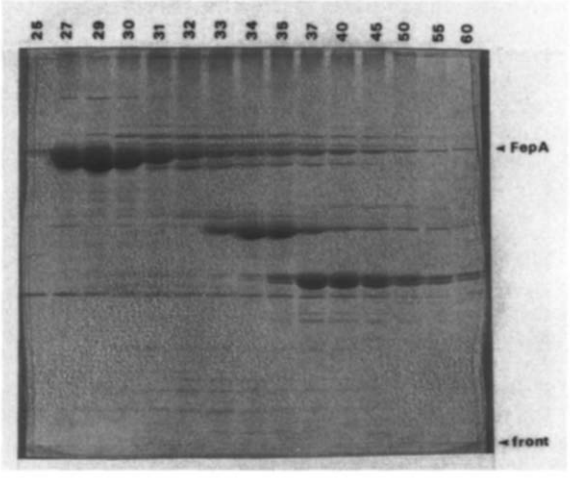

Fig.2. SDS-PAGE gel showing the results of the first DE-52 column. Lane numbers correspond to the fractions (2 ml each) eluted from the column.

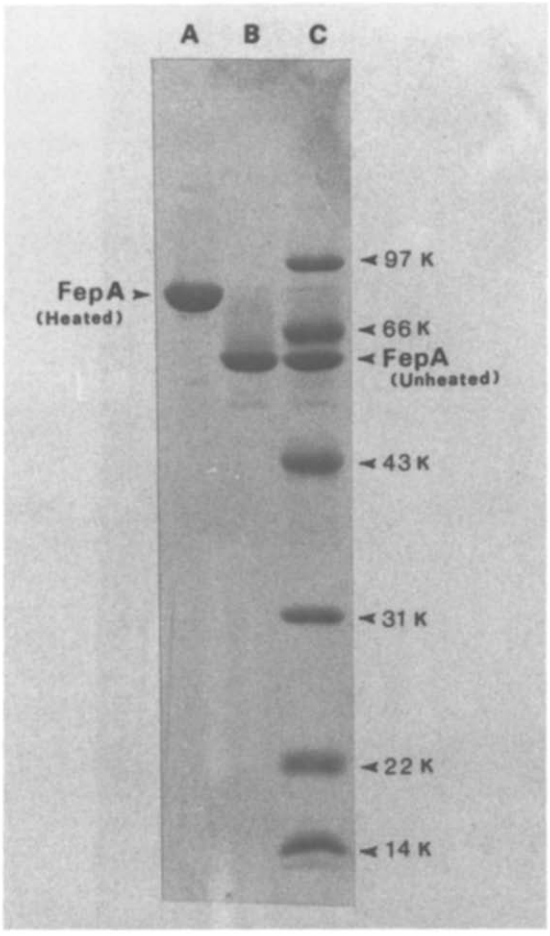

Fig.3. Lane A, FepA heated with treatment buffer; lane B, heating omitted but the treatment buffer present; lane C, protein markers (Biorad): phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa). FepA sample was added after the heat treatment of the protein standards.

tion occurred within a month, when hanging drops of the protein solution containing 12–16% of PEG were equilibrated against the buffer containing 20% PEG at pH 6.0–6.9. Better crystals were observed at lower pH values. Although most of these crystals in these initial crystallization trials were minute needles, some small, rod-shaped, single crystals were also observed (fig.4). After six months some of the rods have grown to 0.3 mm in length. Further systematic attempts employing better crystallization methods (e.g. microdialysis) are

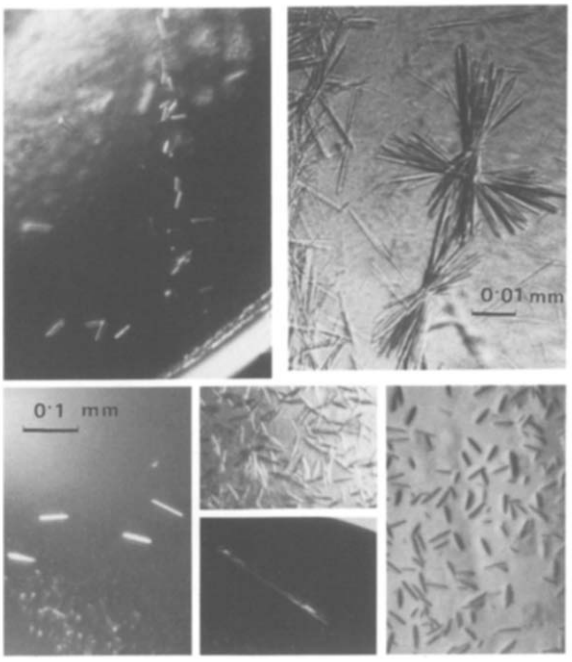

Fig.4. Various types of FepA crystals observed in the initial crystallization trials (magnification in the upper right side picture is greater than the others).

in progress to yield crystals suitable for single crystal X-ray diffraction studies.

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