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FOR THE RECORD

An internal affinity-tag for purification and crystallization of the siderophore receptor FhuA, integral outer membrane protein from *Escherichia coli* K-12

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Abstract: FhuA (M_r 78,992, 714 amino acids), siderophore receptor for ferrichrome-iron in the outer membrane of *Escherichia coli*, was affinity tagged, rapidly purified, and crystallized. To obtain FhuA in quanties sufficient for crystallization, a hexahistidine tag was genetically inserted into the *fhuA* gene after amino acid 405, which resides in a known surface-exposed loop. Recombinant FhuA405.H_6 was overexpressed in an *E. coli* strain that is devoid of several major porins and using metal-chelate chromatography was purified in large amounts to homogeneity. FhuA crystals were grown using the hanging drop vapor diffusion technique and were suitable for X-ray diffraction analysis. On a rotating anode X-ray source, diffraction was observed to 3.0 Å resolution. The crystals belong to space group $P6_1$ or $P6_5$ with unit cell dimensions of $a = b = 174$ Å, $c = 88$ Å ($\alpha = \beta = 90°$, $\gamma = 120°$).

Keywords: crystallization; FhuA; membrane protein; outer membrane; protein crystals; siderophore; TonB-dependent receptor; X-ray crystallography

Biological membranes serve to partition the cell interior from the external milieu, and they play a critical role in maintaining cell integrity and cell function. The cell envelope of Gram-negative bacteria consists of three distinct layers: the surface-located outer membrane (OM), the periplasm in which the peptidoglycan layer is found, and the cytoplasmic membrane (CM). Bacteria have a variety of transport systems available for the import of essential nutrients through these cell envelope layers (reviewed by Nikaido & Saier, 1992). The OM acts as a permeability barrier for the cell. It also functions as a molecular sieve, excluding deleterious substances and permitting passive diffusion of small solutes through porins into the periplasm (Nikaido, 1996).

Some compounds essential for bacterial growth are found at exceedingly low concentrations; iron is one such nutrient. To scavenge ferric iron from their environment, bacteria synthesize and secrete iron-chelating substances called siderophores of molecular mass 700–1000. Nonspecific porin channels from *Escherichia coli* have an apparent molecular mass exclusion limit of 600 and, therefore, do not function in the import of these complexes. Hence, bacteria have evolved high-affinity iron transport mechanisms, distinct from porin-mediated diffusion, and these mechanisms require proteins in the OM, the periplasm and the CM. OM-localized receptors bind their cognate ferric siderophore with high affinity and in *E. coli*, the OM receptor for ferric hydroxamate uptake is FhuA, 714 amino acids, M_r 78,992 (Coulton et al., 1986). In addition to binding ferrichrome-iron, FhuA also acts as receptor for four bacteriophages (T1, T5, ϕ80, and UC-1), colicin M, and the antibiotics albomycin and microcin 25. Binding of ferrichrome-iron to FhuA induces localized conformational changes (Moeck et al., 1996; Bös et al., 1998). Such structural alterations signal the ligand-bound status of the receptor and, therefore, the requirement for TonB-dependent energy transduction (Moeck et al., 1997). The CM-localized TonB–ExbB–ExbD complex transduces energy from the proton motive force to FhuA (Braun, 1997; reviewed by Moeck & Coulton, 1998). Once transferred to the periplasm, chelated iron is translocated into the cytoplasm through a CM-localized ATP-dependent mechanism (Mademidis et al., 1997).

Crystallographic analyses of nonspecific porins (Weiss et al., 1991; Cowan et al., 1992) and of specific OM channels (Schirmer et al., 1995; Forst et al., 1998) show a conserved β-barrel structure consisting of 16 or 18 amphiphilic transmembrane β-strands. OM receptors are energy-dependent, ligand-specific gated porin channels (Rutz et al., 1992; Jiang et al., 1997) and, therefore, may share this β-barrel structure. One of the OM receptors in *E. coli* is the receptor for ferric enterobactin FepA, a protein that was crystallized by Jalal and van der Helm (1989). FepA was predicted to form a β-barrel containing 29 transmembrane β-strands (Murphy

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Abbreviations: PEG, polyethyleneglycol; C_{10}DAO, dimethyldecylamine-*N*-oxide; C_{12}DAO, dimethyldodecylamine-*N*-oxide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

et al., 1990). Removal of a large centrally located ligand-binding domain converted the protein into an open diffusion pore (Liu et al., 1993). In contrast, the topological organization of FhuA was modeled as 32 transmembrane β-strands (Koebnik & Braun, 1993). This model is further supported by a neural network designed for topology predictions of outer membrane proteins and available at http://strucbio.biologie.uni-konstanz.de/~kay/om_topo_predict.html (Diederichs et al., 1998). FhuA can be induced to form a channel by the electrochemical potential of the CM via the TonB–ExbB–ExbD complex (Postle, 1993) and by the binding of the bacteriophage T5 (Bonhivers et al., 1996; Plaçon et al., 1997). Ligand binding sites on FhuA have been deduced by competitive peptide mapping (Killmann et al., 1995) and by in vivo thiol-labeling of surface-exposed cysteines (Bös & Braun, 1997; Bös et al., 1998). There is a surface-exposed region of FhuA termed the gating loop, excision of which converts the energy-dependent receptor into an aqueous nonspecific diffusion channel (Killmann et al., 1993).

To study structure–function relationships of FhuA, we designed an overexpression and purification protocol, both of which are prerequisites for crystallization trials and ultimately for structural analysis by X-ray crystallography. For proteins possessing an affinity tag of six consecutive histidine residues, the hexahistidine tag (H_6), a highly selective purification strategy, uses metal-chelate matrices (Arnold & Haymore, 1991). We previously identified amino acid 405 of FhuA to be surface exposed by flow cytometry (Moeck et al., 1994, 1995). We reasoned that because this residue is surface exposed, the splicing of an H_6 tag into this position would generate a recombinant FhuA amenable to affinity purification using metal-chelate chromatography. Accordingly, synthetic double stranded oligonucleotides encoding H_6 were spliced into the *fhuA* gene at codon 405. To assess the fidelity of cloning, the recombinant plasmid (pHX405) was confirmed by DNA sequencing across the splice sites. Plasmid pHX405 was transformed into the *E. coli* strain AW740 [Δ*ompF zcb*:Tn*10* Δ*ompC fhuA*31], which lacks the major outer membrane porins OmpC and OmpF. The mutant protein FhuA405.H_6 was strongly expressed and displayed wild-type behavior with respect to ferrichrome-iron transport and its sensitivity to four lytic viruses. Our conclusion is that insertion of an H_6 tag at amino acid 405 does not interfere with the functions of FhuA.

The *E. coli* strain AW740 harboring the plasmid pHX405 was grown as previously described (Moeck et al., 1996). Outer membrane vesicles were isolated by Tris-HCl/lysozyme/EDTA (Hantke, 1981) and treated with 1.0% C_{12}DAO. Solubilized protein extract was dialyzed against 2 L of 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.10% C_{12}DAO, 5 mM imidazole buffer, and loaded onto a Ni^{2+}-NTA agarose (QIAGEN, Ontario, Canada) column coupled to an automated BioLogic high-resolution protein purification system (Bio-Rad Laboratories, Ontario, Canada). No FhuA405.H_6 was observed in the flowthrough, indicating efficient and selective binding of the hexahistidine-tagged protein to metal–chelate resin. A linear gradient of imidazole (5 to 500 mM) was established over five column volumes, and a single symmetrical peak containing purified FhuA405.H_6 eluted at an imidazole concentration of approximately 200 mM. To remove omnipresent lipids from purified FhuA405.H_6, protein-containing fractions were pooled, dialyzed against 1 L of 50 mM ammonium acetate, pH 8.0, 250 mM NaCl, 0.10% C_{12}DAO, 10 mM imidazole buffer, and loaded onto a Ni^{2+}-charged POROS 20 MC (PerSeptive Biosystems, Massachusetts) column. When a linear gradient of imidazole up to 250 mM was applied over five column volumes, the major protein-containing peak eluted at an imidazole concentration of approximately 100 mM. SDS-PAGE analysis of purified FhuA405.H_6 and silver staining of 1,000 ng showed a single band and an apparent absence of lipopolysaccharide. Measurements of dynamic light scattering (Protein Solutions, Virginia) showed purified FhuA405.H_6 preparations to be monodisperse. From 6 L of cell culture harvested at A_{600} = 1.80, the yield of protein was approximately 50 mg.

Detergent exchange of purified protein was performed by chromatography on Q Sepharose Fast Flow (Pharmacia LKB, Uppsala, Sweden), thereby replacing C_{12}DAO with C_{10}DAO. Bound protein was washed extensively with 50 mM ammonium acetate, pH 8.0 and 0.50% C_{10}DAO buffer and then eluted in the same buffer with a gradient of NaCl up to 1 M. Salt was removed by dialysis of pooled fractions against 10 mM ammonium acetate, pH 8.0, 0.50% C_{10}DAO. FhuA405.H_6 was concentrated to 10–20 mg/mL by ultrafiltration (Centricon 30, Amicon, Massachusetts), and dialyzed for three days against 100 mL of 10 mM ammonium acetate, pH 8.0, 0.80% C_{10}DAO.

Initial sparse matrix screening was performed using commercially available screening kits (Hampton Research, California). Using 1.0% C_{10}DAO as primary detergent and protein concentrations in the range of 10–20 mg/mL, small hexagonal crystals formed over a 14-day period. The hanging drop vapor diffusion technique (McPherson, 1982) was used to optimize crystal growth at 18 °C. By mixing 5 μL of protein with an equal volume of 13–16% PEG 6,000, 100 mM sodium cacodylate, pH 6.4, and equilibrated with 1 mL of the reservoir solution, well-diffracting crystals of FhuA405.H_6 were obtained. Crystals grew in seven days to a final size of 750 × 750 × 300 μm.

FhuA405.H_6 crystals, together with a column of mother liquor, were mounted in glass capillaries. X-ray diffraction data were collected at room temperature using a STOE imaging plate detec-

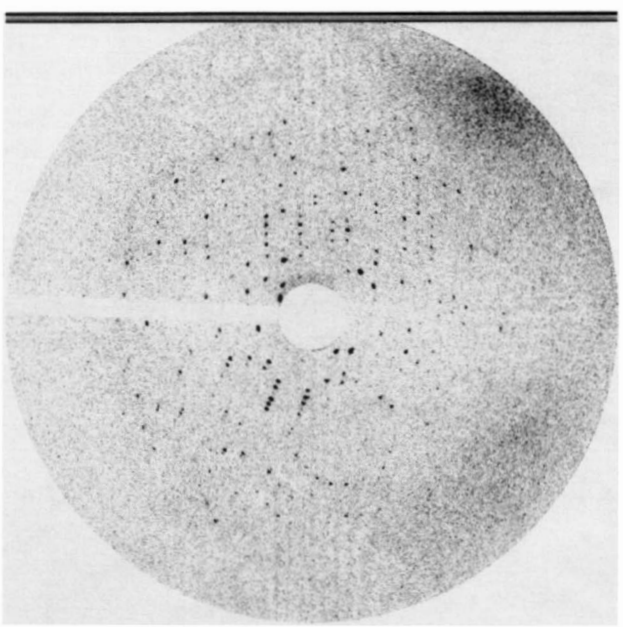

Fig. 1. Still photograph of FhuA crystal on a STOE image plate/rotating anode generator combination. The distance crystal-to-image plate is 159 mm. The detector edge corresponds to 3 Å resolution; outermost reflections are visible at 3.9 Å.

tor. From a rotating anode generator operated at 40 kV and 100 mA, monochromatic CuKα (λ = 1.5418 Å) radiation was used to collect still photographs and native data sets. Reflections could be measured to 3.0 Å (Fig. 1). Reflection intensity did not diminish during data collection, and there was limited crystal damage as the result of exposure to X rays. Data reduction, space group, and unit cell parameters were determined using the DENZO data processing software package (Otwinowski & Minor, 1997). FhuA405.H_6 crystals exhibited the symmetry of the primitive hexagonal lattice. Measured intensities and native Pattersons are consistent with the space groups $P6_1$ or $P6_5$ and refined unit cell parameters of $a = b =$ 173.5 Å, c = 88.1 Å, and $\alpha = \beta = 90°$, $\gamma = 120°$. Given the molecular weight of FhuA405.H_6 (M_r 79,852) and assuming one molecule per asymmetric unit, the Matthews coefficient was calculated to be 4.82 Å^3/Da, with a solvent content of 74.3%. These data are in the range of calculated values for other membrane proteins. Crystal packing arrangements in $P6_1$ or $P6_5$ are compatible with FhuA forming trimers in vivo. We now search for heavy atom derivatives to be used for multiple isomorphous replacement and solution of the crystal structure.

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