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Crystallization and initial X-ray diffraction of BtuB, the integral membrane cobalamin transporter of *Escherichia coli*

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BtuB, the cobalamin transporter from *Escherichia coli*, has been overexpressed, purified and crystallized. The purified protein was solubilized in *n*-octyl tetraoxyethylene (C_8E_4) and was crystallized using sitting-drop vapor diffusion with PEG 3350 and magnesium acetate as precipitants (pH 6.5). Two crystal forms have been obtained. Crystal type I belongs to space group $P3_121$, with unit-cell parameters $a = b = 81.6$, $c = 210.0$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Crystal type II belongs to space group $P3_121$, with unit-cell parameters $a = b = 81.6$, $c = 226.0$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Each crystal form contains a monomer in the asymmetric unit. Diffraction for crystal type I extends to 2.0 Å and diffraction for crystal type II extends to 2.7 Å. Both crystal forms are suitable for structure determination.

1. Introduction

The outer membrane (OM) of *Escherichia coli* forms a semipermeable barrier to the extracellular environment. It allows the passage of solutes that are hydrophilic and smaller than 600 Da in molecular weight. This property prevents the maintenance of a chemiosmotic gradient across the OM. Two classes of transport proteins, passive diffusion porins and TonB-dependent active transporters, exist in the OM. Porins are non-specific (*i.e.* OmpF) or specific (*i.e.* LamB) β-barrel proteins that allow diffusion of small molecules such as ions or small sugars into the periplasmic space (Shultz, 1996). TonB-dependent active transporters bind scarce nutrients including ferric siderophores, hemin and cobalamins with high affinity and transport them in an energy-dependent step requiring the TonB protein (Postle, 1999). TonB is associated with the cytoplasmic membrane and couples the proton motive force to outer-membrane receptors (Cadieux & Kadner, 1999; Kadner, 1990). BtuB is the 66 kDa cobalamin transporter from *E. coli* (Heller & Kadner, 1985). BtuB binds cobalamins with high affinity: a K_d of $\sim$0.3–3 n*M* for cyanocobalamin (vitamin B_{12}) has been reported (Bradbeer *et al.*, 1986). Cells containing BtuB and the TonB complex accumulate cyanocobalamin in the periplasm to 1000-fold excess over the external media (Bradbeer, 1993). Energy coupling to active transport across the outer membrane is not well understood. We have undertaken structural studies of BtuB in its apo and substrate-bound forms. In this paper, we describe the overexpression, purification, crystallization and initial X-ray diffraction studies of BtuB.

2. Results and discussion

2.1. Protein expression and purification

The *btuB* gene (including its native signal sequence) was subcloned from pAG1 (Gudmundsdottir *et al.*, 1988) by standard PCR methods into a pET22b expression vector (Invitrogen) for overexpression in BL21 (DE3) plysS (Invitrogen). For protein expression, cells were grown at 307 K in minimal 'A' media (Sambrook *et al.*, 1989) containing ampicillin (100 µg ml^{-1}) and chloramphenicol (30 µg ml^{-1}). BtuB expression was induced by addition of 1 m*M* IPTG at a cell density of $OD_{600} \simeq 0.25$. Cells were grown for 4 h post-induction to a final $OD_{600} \simeq 1.0$ and harvested by centrifugation. Cell pellets were resuspended in lysis buffer [20 m*M* HEPES pH 7.2, 1 m*M* EDTA, 5 µ*M* Pefabloc (Pentapharm), 0.5 m*M* tris-(2-carboxyethyl)phosphine hydrochloride (TCEP; Molecular Probes), 100 m*M* magnesium chloride and 10 ng ml^{-1} deoxyribonuclease I (Sigma)], followed by French pressure cell disruption (Spectronic Unicam). Centrifugation (12 000*g* for 20 min) was performed on cell lysate to pellet unbroken cells. Centrifugation (100 000*g* for 2 h) of the supernatant yielded a pellet composed of the inner and outer membranes (total membranes). Selective solubilization of the inner membrane from the total membranes was achieved by resuspension of the pellet in HEPES pH 7.2, 25 m*M* *n*-lauroyl sarcosine and 2 m*M* EDTA and incubation on a rocker platform for 45 min at 295 K. Centrifugation (100 000*g* for 1 h) of the total membrane fraction yields soluble inner membranes. The outer membrane was resuspended in 50 m*M* bis-tris pH 6.9, 2 m*M* EDTA. Concentrated

n-octyl tetraoxyethylene (C_8E_4; Anatrace) was added to 150 m*M* concentration followed by gentle stirring for 2.5 h at 295 K. Centrifugation (100 000*g* for 1 h) was then performed to pellet any residual insoluble material. SDS–PAGE analysis of the supernatant revealed that BtuB is efficiently solubilized and is expressed as the second most abundant protein in the outer membrane (Fig. 1*a*, lanes 1 and 2). Two anion-exchange chromatography steps yield homogeneous BtuB. In the first step, solubilized outer membrane was loaded onto a Q-Sepharose fast flow (Pharmacia) 15 × 1 cm column (Kontes). The column was washed with 60 ml buffer *A* (30 m*M* bis–tris pH 6.8, 20 m*M* C_8E_4, 0.5 m*M* TCEP). Protein fractions eluted in a 100 ml gradient of buffer *A* and buffer *A* plus 0.9 *M* LiCl (starting LiCl concentration 150 m*M*; final LiCl concentration 750 m*M*; 1 ml min^{-1} flow rate). This first chromatography step leaves small amounts of impurities composed mainly of the outer-membrane porins OmpF and OmpC. BtuB-containing fractions were pooled and the salt concentration was decreased tenfold by dilution into buffer *B* (20 m*M* Tris pH 8.0, 20 m*M* C_8E_4, 0.5 m*M* TCEP). The sample volume was reduced to less than 50 ml in a stirred-cell concentrator (Amicon/Millipore) using a 25 kDa molecular-weight cutoff (MWCO) membrane. In the second step of purification, the sample was loaded onto a 2 ml Q2 high-resolution column (BioRad). The column was washed with 20 ml buffer *B* containing 200 m*M* LiCl, which removes the contaminating porins. BtuB was eluted in a 20 ml gradient of buffer *B* and buffer *B* plus 0.9 *M* LiCl (starting LiCl concentration 200 m*M*; final LiCl 600 m*M* LiCl; 1 ml min^{-1} flow rate). Fractions were pooled and desalted as above. SDS–PAGE analysis showed the homogeneity of purified BtuB (Fig. 1*a*, lane 3). Silver staining showed that lipopolysaccharide copurifies with BtuB (Fig. 1*b*). The lower molecular-weight band in Fig. 1(*b*) was identified as lipopolysaccharide by separate experiments utilizing a modified version (Austin *et al.*, 1990) of the method of Hitchcock & Brown (1983). Briefly, proteinase K added to protein–lipopolysaccharide mixtures will digest the protein component of the mixture while leaving the lipopolysaccharide component unaffected. Silver-stained gels of BtuB treated with proteinase K in this manner yielded a single band at the position of the lower band in Fig. 1(*b*), confirming that the lower band is lipopolysaccharide (data not shown). The solubility of purified BtuB is very sensitive to ionic strength and BtuB-containing fractions precipitate unless these fractions are immediately diluted to reduce the LiCl concentration to <50 m*M*. A CDM 210 conductivity meter (Radiometer Copenhagen) was used to monitor the LiCl concentration of protein fractions. The yield of purified BtuB was approximately 1 mg per litre of bacterial culture. Purified BtuB was concentrated by Centricon ultrafiltration (MWCO 25 kDa, Millipore) to 12 mg ml^{-1} (based upon a calculated extinction coefficient of 136 690 M^{-1} cm^{-1}). BtuB was dialyzed against buffer *B* for 5 d in a 500 µl Dispodialyzer (MWCO 25 kDa, Spectrapor) with one buffer exchange.

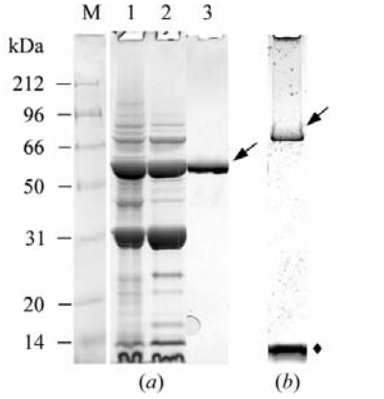

Figure 1
SDS–PAGE gel of BtuB expression and purification. (*a*) Lane M, molecular-weight markers; lane 1, outer membrane prior to solubilization; lane 2, C_8E_4-solubilized membranes (arrow indicates BtuB); lane 3, homogeneous BtuB after second chromatography step. (*b*) Silver-stained gel of purified BtuB. Lipopolysaccharide (indicated with a diamond) copurifies with BtuB (indicated with an arrow) through two anion-exchange steps. Gel (*b*) was optimized for visualization of lipolpoysaccharides and proteins migrate differently to gel (*a*).

2.2. Crystallization

Purified BtuB (12 mg ml^{-1} in 20 m*M* Tris pH 8.0, 20 m*M* C_8E_4, 0.5 m*M* TCEP) was set up in several detergent-specific crystallization screens (Snook & Wiener, 2001; Song & Gouaux, 1997). Crystallization experiments were set up by mixing 1.5 µl of BtuB and 1.5 µl of reservoir buffer in a Cryschem sitting-drop vapor-diffusion tray (Hampton Research) with 300 µl total reservoir buffer, followed by incubation at 293 K. Optimization of crystallization conditions yielded the best crystals from the Song and Gouaux screen condition No. 25 (200 m*M* magnesium acetate, 10% PEG 3350, 50 m*M* cacodylate pH 6.6 and 20 m*M* C_8E_4). The final crystallization condition consisted of BtuB (12 mg ml^{-1}) mixed in a 1:1 ratio with reservoir solution (200–400 m*M* magnesium acetate, 4–7% PEG 3350, 50 m*M* cacodylate pH 6.6 and 20 m*M* C_8E_4). BtuB crystals are typically 100–200 µm in the longest dimension and have a cubic or diamond-like morphology (Fig. 2*a*). The crystals are visible after 2–3 d and achieve their maximum dimensions in 2–4 weeks. Preliminary data from substrate-binding experiments, performed by equilibrium dialysis of purified BtuB (in calcium-containing buffer) in the presence of varying concentrations of ^{57}Co-cyanocobalamin, yielded an initial estimate of $K_d \simeq 1$ n*M* (data not shown). Thus, we proceeded to produce crystals of the BtuB–cyanocobalamin (vitamin B_{12}) complex. Cyanocobalamin was soaked into BtuB crystals by transfer of the crystals into a soaking buffer (150 m*M* calcium chloride, 18% PEG 3350, 25 m*M* bis-tris pH 6.6 and 10 m*M* C_8E_4) containing an approximately tenfold molar excess of cyanocobalamin over BtuB (Fig. 2*b*). We screened soaking buffers both with and without calcium, as a role for calcium in high-affinity cyanocobalamin binding has been reported (Bradbeer *et al.*, 1986). Crystals became more intensely colored in the calcium-containing soaking solution compared with the calcium-free solution. Calcium was soaked into BtuB by repeating the above method without the addition of cyanocobalamin to the soaking buffer. X-ray data were collected from both

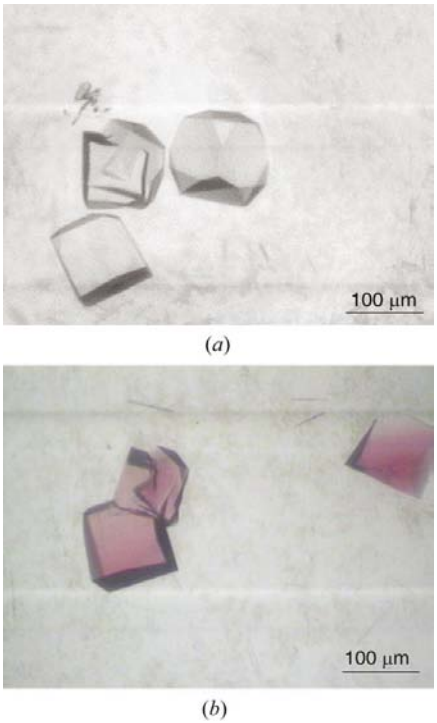

Figure 2
Crystals of BtuB. (*a*) Typical BtuB crystals obtained in this study. The crystals measure 140 µm in the longest dimension. (*b*) BtuB crystals from (*a*) after soaking for 24 h in cyanocobalamin-containing soaking buffer.

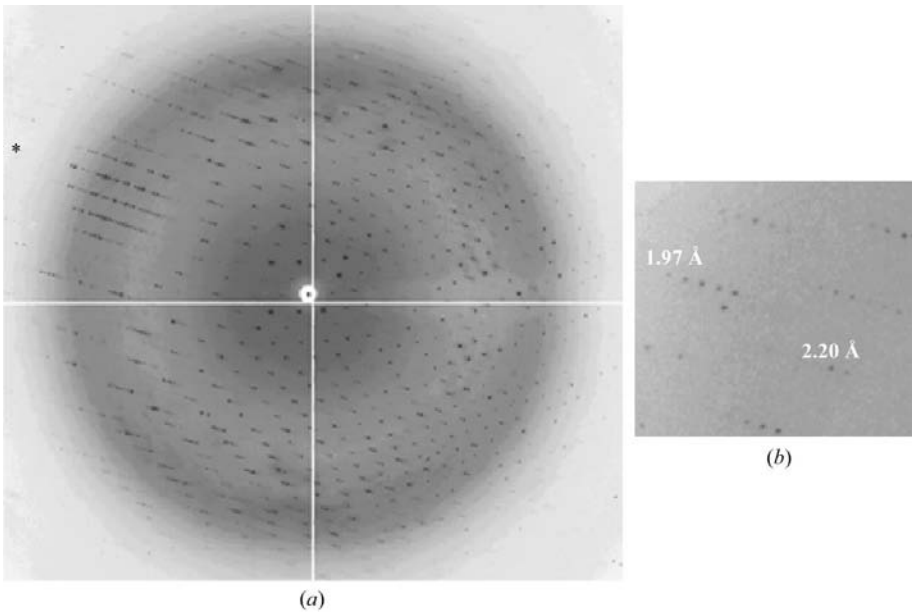

Figure 3
(*a*) Diffraction from a type I BtuB crystal recorded at CHESS F1 (0.5° oscillation, 20 s exposure, λ = 0.900 Å). The region marked with an asterisk is shown magnified in (*b*). (*b*) Diffraction extending beyond 2.0 Å resolution.

types of soaked crystals following ∼24 h incubation in the respective soaking buffers.

2.3. Data collection and analysis

For data collection, crystals were transferred into cryobuffer (150 m*M* magnesium acetate or calcium chloride, 18% PEG 3350, 25 m*M* bis-tris pH 6.6 and 10 m*M* C_8E_4) for 30–60 s followed by loop mounting and cryocooling (either by plunging into a nitrogen bath or insertion into a nitrogen stream). Diffraction data were processed with *HKL*2000 (Otwinowski & Minor, 1997); diffraction collected in-house extended to 3.7 Å. The crystals belong to the trigonal space group $P3_121$, with unit-cell parameters $a = b = 81.6$, $c = 210.0$ Å (type I) or 226.0 Å (type II). The calculated solvent content based upon a monomer of BtuB in the asymmetric unit is 59.9% (V_M = 3.0 Å^3 Da^{-1}) for type I crystals and 61.4% (V_M = 3.2 Å^3 Da^{-1}) for type II crystals (Matthews, 1968). BtuB crystals diffracted to 2.0 Å for type I crystals and to 2.7 Å for type II crystals at the CHESS F1 beamline (Fig. 3). Statistics for data sets collected from type I and type II crystals are shown in Table 1.

Table 1
Crystallographic data statistics.

Values in parentheses are for the highest resolution shell.

Crystal form	Type I	Type II
Unit-cell parameters (Å, °)	$a = b = 81.6$, $c = 210.0$, $\alpha = \beta = 90$, $\gamma = 120$	$a = b = 81.6$, $c = 226.0$, $\alpha = \beta = 90$, $\gamma = 120$
Beamline	CHESS F1	APS SBC 19-ID
Wavelength (Å)	0.9000	0.9787
Resolution range	25.0–2.0 (2.18–2.00)	20.0–2.7 (2.78–2.70)
Unique reflections	55620	24020
Redundancy	8.9	6.0
Completeness (%)	99.6 (97.8)	96.4 (89.3)
R_{sym} (%)	6.5 (41.2)	9.1 (56.0)

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