Crystallization and preliminary X-ray analysis of the ATP-binding domain of the ABC transporter haemolysin B from *Escherichia coli*

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Haemolysin B (HlyB) is a transmembrane protein which belongs to the superfamily of ABC transporters. *In vivo*, it mediates the non-classical translocation of the 107 kDa toxin HlyA across both membranes of *Escherichia coli* together with haemolysin D and the outer membrane protein TolC. The cytosolic ATP-binding domain of HlyB has been overexpressed and purified as an N-terminal His-tag fusion protein. Here, the crystallization of the ATPase domain of HlyB in the presence of ATP is described. A native data set has been obtained at a resolution of 2.8 Å. Crystals belong to the primitive tetragonal space group $P4_x2_12$, where x is very likely to be 1 or 3, with unit-cell parameters $a = b = 104.6$, $c = 125.8$ Å, $\alpha = \beta = \gamma = 90°$.

1. Introduction

Haemolysin B (HlyB) is part of a type I secretion machinery (Buchanan, 2001) which transports the toxin haemolysin A in a one-step process across both membranes of *E. coli* (Blight & Holland, 1994). This translocation machinery is composed of HlyB, located in the inner membrane of *E. coli*, with a cytoplasmic ATPase HlyD, anchored in the inner membrane and spanning the periplasm, and the outer membrane protein TolC. HlyD is thought to act as a bridge between HlyB and TolC, thereby facilitating direct extrusion into the medium (Thanabalu *et al.*, 1998).

HlyB belongs to the superfamily of ABC (ATP-binding cassette) proteins (Higgins, 1992; Holland & Blight, 1999) which contain a highly conserved ATP-binding motif. These proteins are ubiquitous ATP-dependent transmembrane pumps or ion channels. Although highly diverse with respect to their individual transport substrates, all members of this family are thought to share a common four-domain architecture. This consists of two TMDs (transmembrane domains) and two NBDs (nucleotide-binding domains) or ABC domains. The ABC domain contains the Walker A and B motifs as well as the signature motif of ABC transporters, which is located between the Walker A and B motifs. Well known human members of this family are MDR1 (multidrug-resistance protein 1), which confers resistance to many anticancer drugs, TAP (transporter associated with antigen processing), which mediates the transport of antigenic peptides from the cytosol into the lumen of the ER for loading onto class I MHC molecules, and CFTR (cystic fibrosis transmembrane conductance regulator). Mutations in CFTR lead to the most common deadly inherited disease among Caucasians, cystic fibrosis (Schmitt & Tampé, 2000).

Despite the importance of ABC proteins, structural information is limited and only the crystal structures of HisP (Hung *et al.*, 1998), the ABC subunit of the histidine permease, MalK (Diederichs *et al.*, 2000), the ABC subunit of the maltose transporter, MJ1276 (Karpowich *et al.*, 2001) and MJ0796 (Yuan *et al.*, 2001) and the ABC domain of human TAP1 (Gaudet & Wiley, 2001) have been solved. In addition, the three-dimensional structure of MsbA has been successfully solved at 4.5 Å resolution (Chang & Roth, 2001). MsbA is involved in lipid A transport export from the cytoplasm. However, the N-terminal part of the NBD, including the Walker A motif, is not visible in the electron density. Here, we describe the crystallization and preliminary X-ray analysis of the ABC-domain of HlyB, an ABC transporter devoted to export.

2. Materials and methods

2.1. Protein purification and crystallization

The ABC domain of HlyB (amino acids 467–707) was overexpressed and purified as an N-terminal His-tagged fusion protein by metal-affinity chromatography as described elsewhere (Benabdelhak *et al.*, 2002). It displayed basal ATPase activity with a K_m value of 3.5 mM and a v_{max} value of 0.3 µmol min^{-1} mg^{-1}. In order to obtain highly purified and homogenous protein, an additional size-exclusion chromatography step was introduced. Protein fractions corresponding to the ABC domain of HlyB were pooled after the affinity chromatography, concentrated and immediately applied to a Superdex S200 column (16/60 prep grade, Pharmacia, Frei-

burg, Germany). The column was equilibrated in either 10 mM Tris–HCl pH 8.0, 100 mM KCl or 10 mM sodium phosphate pH 8.0, 100 mM KCl. Fractions corresponding to monomeric HlyB ABC domain were analysed by SDS–PAGE, pooled if their purity was greater than 99% (as judged from SDS–PAGE) and concentrated immediately to the desired concentration (10–20 mg ml^{-1}) using an Ultrafree concentrator unit with a molecular-weight cutoff of 10 kDa (Millipore). Protein concentration was determined by absorption at 280 nm using the theoretically calculated extinction coefficient of 15 400 M^{-1} cm^{-1}. All crystallization trials were performed at 277 K using the hanging-drop vapour-diffusion technique by mixing equal volumes of protein and reservoir solutions. All trials were conducted in the presence of 50 mM ATP (final concentration), which was incubated with the protein solution for at least 30 min at 277 K prior to crystallization.

2.2. Cryocooling

Various cryoprotecting agents were analysed for potential use. The agents tested included glycerol [10–30%(v/v)], ethylene glycol [5–20%(v/v)], MPD [10–30%(v/v)], PEG 400 [10–20(v/v)], sorbitol [5–15%(w/v)], xylitol [(5–15%(w/v)], paratone N (Hampton Research) and Panjelly oil (Jena Bioscience). The crystals were transferred into stabilization buffer (150 mM ADA pH 6.2, 25% PEG 8000, 80 mM ammonium sulfate complemented with the appropriate amounts of cryoprotectant) either directly in a step-wise fashion (3–5% steps of cryoprotectant with varying times between the individual transfer steps) or by dialysis and were then flash-frozen in liquid nitrogen. Based on the mosaicity and diffraction limit of the frozen crystals compared with crystals mounted in a glass capillary, the only suitable cryoprotectant was ethylene glycol [10–20%(v/v)].

2.3. Data collection

Initial X-ray experiments were performed in-house using a Rigaku X-ray generator with Cu $K\alpha$ radiation and an R-AXIS IV image plate. Owing to the low resolution, the best diffracting crystals were analysed at either BW6 (DESY, Hamburg, Germany) or ID14-1 (ESRF, Grenoble, France). All data were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997).

Table 1
Summary of crystallization conditions and data statistics for the three crystal forms of the ATP-binding domain of HlyB.

Crystallization trials were performed by mixing protein and reservoir solution in a 1:1 ratio at 277 K. Crystals appeared within 2 d (crystal form *C*) to three weeks (crystal form *A*) and grew within three weeks to their final dimensions ($300 \times 100 \times 50$ µm for crystal form *A*, $300 \times 50 \times 30$ µm for crystal form *B* and $500 \times 150 \times 80$ µm for crystal form *C*). Resolution limits are based on $I/\sigma(I) > 3$ statistics. Values in parentheses refer to data in the highest resolution shell (4.3–4.2 Å for crystal form *B* and 2.9–2.8 Å for crystal form *C*). During data refinement spots were omitted when below 3σ.

	Form *A*	Form *B*	Form *C*
Crystallization conditions	10 mg ml^{-1} HlyB ABC domain in 10 mM Tris–HCl pH 8.0, 100 mM KCl† mixed with 100 mM MES pH 6.0, 10% PEG 6000	15 mg ml^{-1} HlyB ABC domain in 10 mM phosphate buffer pH 8.0, 100 mM KCl† mixed with 200 mM sodium citrate pH 5.8, 12% PEG 6000	10 mg ml^{-1} HlyB ABC domain in 10 mM phosphate buffer pH 8.0, 100 mM KCl† mixed with 150 mM diamino acetic acid pH 6.2, 16% PEG 8000, 300 mM (NH$_4$)$_2$SO$_4$
Wavelength (Å)	1.54	1.005	0.934
Resolution (Å)	<15	20–4.2	20–2.8
Space group	Not determined	$P4_x2_12$	$P4_x2_12$
Unit-cell parameters (Å)	Not determined	$a = 64.3$, $b = 64.3$, $c = 124.4$	$a = 104.6$, $b = 104.6$, $c = 125.8$
Mosaicity (°)	Not determined	2.6	1.4
Observations		20215	562378
Unique observations		8121	17574
Completeness (%)		89 (85.5)	100 (99.8)
$I/\sigma(I)$		4.4 (3.3)	32.9 (4.8)
R_{sym}‡ (%)		10.2 (15.4)	4.3 (24.7)

† HlyB ABC domain was pre-incubated with ATP (final concentration 50 mM) for at least 30 min at 277 K. ‡ $R_{sym} = \sum(|I_{hkl} - \langle I_{hkl}\rangle|)/\sum I_{hkl}$.

3. Results and discussion

An initial PEG 6000 screen revealed small crystals of the ABC domain at 100 mM MES pH 6.0, 10% PEG 6000 together with precipitated protein. Optimization of the crystallization conditions resulted in crystal form *A*, with average dimensions $300 \times 50 \times 30$ µm. In-house X-ray experiments at 277 K showed that these crystals diffracted below 15 Å (Table 1). During the course of optimization, the protein was isolated in 10 mM phosphate buffer pH 8.0, 100 mM KCl instead of 10 mM Tris pH 8.0, 100 mM KCl to increase the stability. In addition, the influence of different PEGs ranging from a molecular weight of 400 to 20 000 as well as the nature and concentration of the buffer of the reservoir solution was investigated. While the nature of the PEG had virtually no influence, the nature and the concentration of the buffer was very important. The best diffracting crystals were obtained from citrate buffer. At low concentrations of citrate (120 mM citrate or lower) cubic shaped crystals (crystal form *A*) were obtained. However, increasing concentra-
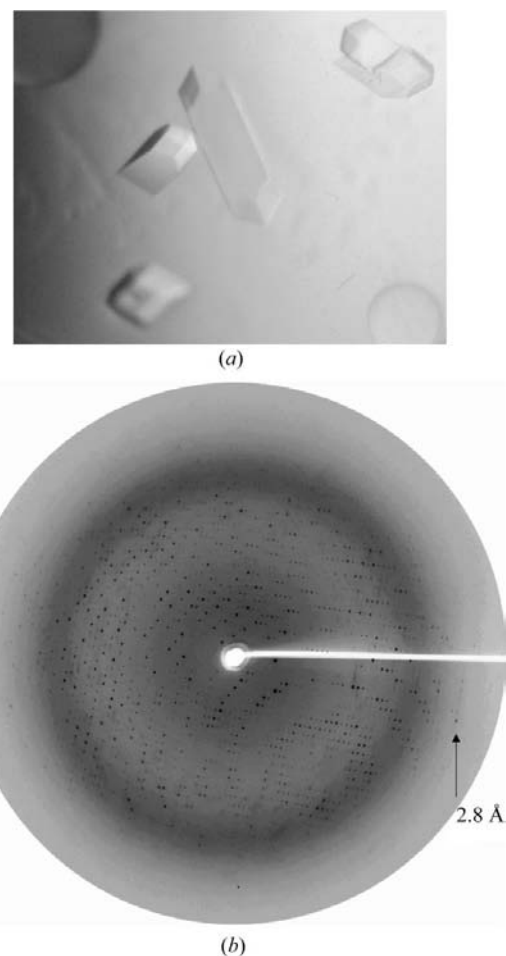
Figure 1
(*a*) Crystal form *C* of the ATP-binding domain of HlyB (for crystallization conditions and further details, see Table 1). (*b*) Diffraction image of crystal form *C* obtained at ESRF beamline ID 14-1 (further details are given in Table 1).

tions of citrate resulted in the appearance of pyramid-shaped crystals. These crystals diffracted in-house to approximately 6 Å. After careful optimization of the freezing conditions, crystal form *B* could be flash-frozen in liquid nitrogen by soaking the crystals for 30 s in 250 m*M* citrate pH 5.8, 25% PEG 8000, 20% ethylene glycol. Native reflection intensities were measured at BW-6 (DESY, Hamburg) using a wavelength of 1.005 Å and an oscillation range of 0.5°. The results are summarized in Table 1. To further improve crystal quality, various additives and salts were investigated. Finally, crystal form *C* (Fig. 1*a* and Table 1) was obtained in the absence and presence of 1 m*M* EDTA. Crystals diffracted in-house to 4.2 Å. A native data set was obtained at ID14-1 (ESRF, Grenoble) from a single crystal flash-frozen in liquid nitrogen after a 30 s soak in a solution containing 150 m*M* ADA pH 6.2, 25% PEG 8000, 80 m*M* ammonium sulfate and 17% ethylene glycol (Table 1). The native data set contained 562 378 reflections, which reduced to 17 574 unique reflections. The completeness of the data set was 100%, with an R_{sym} of 4.3% in the resolution shell 20–2.8 Å (24.7% in the last resolution shell). Crystals belong to the space group $P4_x2_12$ (most likely $x = 1$ or 3), with unit-cell parameters $a = b = 104.6$, $c = 125.8$ Å. Currently, we are working on the improvement of crystal quality and phase determination by either molecular replacement or experimental methods.

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