Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary diffraction studies of AcrB, an inner-membrane multi-drug efflux protein

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Received 15 May 2002
Accepted 5 August 2002

Resistance of pathogens to antibiotics is often dependent on multi-drug export proteins that reside in the inner membrane of bacteria. This work describes the expression, purification, crystallization and preliminary crystallographic analysis of AcrB of *Escherichia coli*. Together with AcrA and TolC, AcrB forms a proton motive force dependent efflux pump of the resistance–nodulation–cell division (RND) transporter superfamily and is responsible for resistance towards many common antibiotics such as ciprofloxacin and novobiocin. AcrB crystallizes in space group *R*32, with unit-cell parameters $a = b = 143$, $c = 513$ Å; the crystals diffract to 3.0 Å resolution.

1. Introduction

Multi-drug resistance of common pathogens is one of the most serious health threats in the modern industrial world (Felmingham & Washington, 1999). Resistance is often associated with the overproduction of inner-membrane proteins that are capable of extruding antibiotics and in many cases also detergents, dyes and organic solvents. Multiple antibiotic resistant *Escherichia coli* strains have been shown to overproduce two proteins, AcrA and AcrB (Okusu *et al.*, 1996). These proteins are encoded by the *acrA* and *acrB* genes, which form an operon and are under the control of the regulator protein AcrR. AcrB belongs to the RND (resistance–nodulation–cell division) transporter superfamily (Tseng *et al.*, 1999) and, together with AcrA and the outer-membrane component TolC, transports drugs directly into the medium, bypassing the periplasmic space and outer membrane (Fralick, 1996; Tseng *et al.*, 1999; Zgurskaya & Nikaido, 2000*a*,*b*; Koronakis *et al.*, 2000; Nikaido & Zgurskaya, 2001).

The AcrAB–TolC complex is a major contributor to resistance in *E. coli* to drugs and antibiotics such as ciprofloxacin, novobiocin, erythromycin, tetracycline, chloramphenicol and cloxallin (Okusu *et al.*, 1996; Zgurskaya & Nikaido, 2000*a*; Koronakis *et al.*, 2000; Nikaido & Zgurskaya, 2001).

Both AcrA and AcrB have been purified and successfully reconstituted into liposomes (Zgurskaya & Nikaido, 1999). It has been shown that the inner-membrane protein AcrB is a proton motive force dependent efflux protein (H^+/antibiotic and H^+/bile salt antiporter) and that AcrA is involved in the connection between AcrB and TolC, thus spanning the periplasmic space (Zgurskaya & Nikaido, 2000*b*). The efflux of the toxic compounds across two membranes in Gram-negative bacteria results, in combination with the low permeability of the outer membrane, in a synergistically increased resistance towards the drug.

Recently, structural data on AcrA have been published at a resolution of 20 Å (Avila-Sakar *et al.*, 2001). We describe here the successful crystallization and preliminary X-ray diffraction studies of the inner-membrane protein AcrB.

2. Material and methods

2.1. Protein expression and purification

For routine work with recombinant DNA, established protocols were used (Sambrook *et al.*, 1989). For the construction of the $AcrB_{His}$ expression plasmid, the *acrB* gene was amplified from chromosomal *E. coli* DNA using the oligonucleotides acrBfor (5′-GGATCCCATATGCCTAATTTCTTTATCGATC-3′) and acrBrev (5′-AAGCTTCTCGAGATGATGATCGACAGTATGGCTG-3′). In acrBfor, the ATG start codon of *acrB* is part of an *Nde*I restriction site, which is preceded by a *Bam*HI restriction site (six additional nucleotides) to increase restriction efficiency. In acrBrev, an *Xho*I restriction site has been introduced after the last codon encoding an amino acid.

The PCR mixture contained 500 ng genomic *E. coli* DNA, 0.4 μ*M* of each primer, 0.2 m*M* deoxynucleoside triphosphates, 1× buffer for *Pfu* DNA polymerase and 2.5 U *Pfu* DNA polymerase. After an initial denaturation step (2 min at 368 K), 30 cycles consisting of 30 s at 368 K, 20 s at 333 K and 4 min at 341 K were carried out, followed by a terminal elongation step (4 min at 341 K). The PCR mix was

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separated on a 1% agarose gel and the expected 3.15 kbp fragment was isolated with a Qiaquick spin column. After restriction with *Nde*I and *Xho*I, the PCR fragment was again purified with a Qiaquick spin column and ligated with an *Nde*I/*Xho*I-restricted pET24a (Novagen), resulting in pET24acrB$_{His}$.

E. coli C43(DE3) cells (Miroux & Walker, 1996) were transformed with pET24acrB$_{His}$ and a colony on an overnight-incubated LB plate containing 50 µg ml^{-1} kanamycin (Kan^{50}) was picked to inoculate 4 ml LB Kan^{50}. This preculture was grown overnight at 310 K with shaking at 180 rev min^{-1} and used to inoculate 1 l Terrific Broth (Becton, Dickinson and Co.) Kan^{50}. The culture was grown for about 2–3 h to an OD$_{600}$ of 0.6 and the culture was then cooled down on ice for 5–10 min and supplemented with 0.5 m*M* IPTG (final concentration). Growth was continued for 3.5 h at 303 K with shaking at 180 rev min^{-1}. The final OD$_{600}$ was 2.7 and about 7.5 g of wet packed cells were harvested. Cells were resuspended in 20 ml 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl, 2 m*M* $MgCl_2$, 0.2 m*M* diisopropylfluorophosphate and a trace amount of DNase I. Cells were disrupted by passing the suspension twice through a French pressure chamber at 82.7 MPa. The suspension was centrifuged at 277 K for 10 min at 10 000*g* and the supernatant was centrifuged at 277 K for 1 h at 131 000*g*. The membrane pellet was resuspended in 10 ml 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl (to approximately 30 mg ml^{-1} protein) and stored in liquid nitrogen until use.

Membranes (3.6 ml; approximately 110 mg of protein) were solubilized by addition of 4.4 ml buffer *A* [10 m*M* KP_i pH 7.8, 100 m*M* NaCl, 10% glycerol, 10 m*M* imidazole pH 8.0 and 0.05% cyclohexyl-*n*-hexyl-β-D-maltoside (CHM; Calbiochem)] and 2 ml CHM (10% stock solution) and incubated for 1 h at 277 K in the cold room while rotating (on a blood-wheel). The mixture was centrifuged for 1 h at 145 000*g* at 277 K and the supernatant was applied onto an Ni^{2+}–NTA agarose column (1 ml bed volume) pre-equilibrated in buffer *A*. The column was washed with 35 ml buffer *A* and subsequently washed with 25 ml buffer *B* (50 m*M* imidazole pH 7.0, 100 m*M* NaCl, 10% glycerol and 0.05% CHM). AcrB$_{His}$ was eluted in 10 ml buffer *C* (200 m*M* imidazole pH 7.0, 100 m*M* NaCl, 10% glycerol and 0.05% CHM) and concentrated to 0.5 ml using a Ultrafree-4 (BioMax-50K, Millipore) spin column in a Hereaus centrifuge at 277 K at 5300 rev min^{-1}. Concentrated protein was washed with 4.5 ml 10 m*M* Na HEPES pH 7.0 containing 0.05% CHM and again concentrated to a concentration of about 10–20 mg ml^{-1}. Before crystallization setups were carried out, the sample was passed through a 100 nm filter (Anodisc 13, Whatman).

2.2. Crystallization

The hanging-drop vapour-diffusion method was used with Hampton Research screens to identify promising conditions. Grid screens were prepared in order to investigate the effect of different salts (NaCl, LiCl, KCl and Na_2SO_4), pH (varied over pH 6.5–8.5 in 0.5 steps) and precipitant conditions [PEG 4000 over the range 4–12% (in 1% steps) and PEG 400 over the range 6–20% (in 2% steps)]. Protein solution (10–20 mg ml^{-1}) was mixed in a 1:1 ratio with mother liquor (normally 2.5 µl solution plus 2.5 µl mother liquor) and suspended over 0.5 ml reservoir solution. Samples were incubated at 289 K.

2.3. Cryocooling and X-ray diffraction data measurement

Crystals were subjected to cryoprotection using two different cryoprotectants, glycerol (CryoG1 and CryoG2) and PEG 400 (CryoP1 and CryoP2). A single crystal was transferred to a droplet of solution containing, for case (*a*), 0.1 *M* Na HEPES pH 7.5, 0.1 *M* NaCl, 5% PEG 4000, 5% glycerol and 0.05% CHM (CryoG1). This droplet was then suspended over 0.5 ml of 0.1 *M* Na HEPES pH 7.5, 0.1 *M* NaCl, 5% PEG 4000, 30% glycerol and 0.05% CHM (CryoG2). In case (*b*), the crystal was transferred to a droplet of solution containing 0.1 *M* Na HEPES pH 7.5, 0.1 *M* NaCl, 10% PEG 400 and 0.05% CHM (CryoP1). This droplet was suspended over 0.5 ml 0.05 *M* Na HEPES pH 7.5, 0.05 *M* NaCl, 15% PEG 400 and 0.025% CHM (CryoP2 1:1 diluted with H_2O). After overnight incubation at 289 K, the crystals were collected with a loop and in case (*a*) directly frozen into liquid nitrogen or in case (*b*) transferred to a droplet of 0.1 *M* Na HEPES pH 7.5, 0.1 *M* NaCl, 30% PEG 400 and 0.05% CHM (CryoP2). After 1 min incubation, the crystal was frozen in liquid nitrogen.

In-house preliminary X-ray diffraction data were measured using a Schneider (Offenburg, Germany) Cu $K\alpha$ rotating-anode X-ray generator operating at 40 kV and 100 mA and equipped with focusing mirror optics and a MAR Research 345 mm imaging-plate area detector. Data sets from native crystals at 100 K were collected at the DESY synchrotron, Hamburg, EMBL beamlines BW7A, BW7B and X11 using MAR Research (Hamburg, Germany) CCD and image-plate detectors. Data sets were also collected at beamline X06SA of the Swiss Light Source (Villigen, Switzerland) on a MAR Research CCD detector.

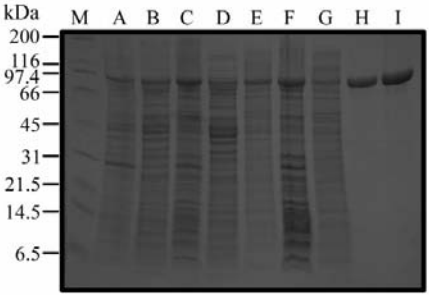

Figure 1
SDS–PAGE analysis of the synthesis and purification of AcrB$_{His}$. 10% SDS–PAGE analysis, gel stained with Coomassie. Lane M: broad-range marker (BioRad, 2 µg); lane A, pellet fraction after French Press and low-spin centrifugation (25 µg); lane B, supernatant fraction after French Press and low-spin centrifugation (25 µg); lane C, *E. coli* C43(DE3)/pET24acrB$_{His}$ membranes (25 µg); lane D, *E. coli* C43(DE3)/pET24acrB$_{His}$ cytoplasm (25 µg); lane E, detergent extract of *E. coli* C43(DE3)/pET24acrB$_{His}$ membranes (25 µg); lane F, extract from *E. coli* C43(DE3)/pET24acrB$_{His}$ membranes (25 µg); lane G, Ni^{2+}–NTA chromatography (10 m*M* imidazole) flow-through fraction (25 µg); lane H, purified and concentrated AcrB$_{His}$ (5 µg); lane I, purified and concentrated AcrB$_{His}$ (10 µg).

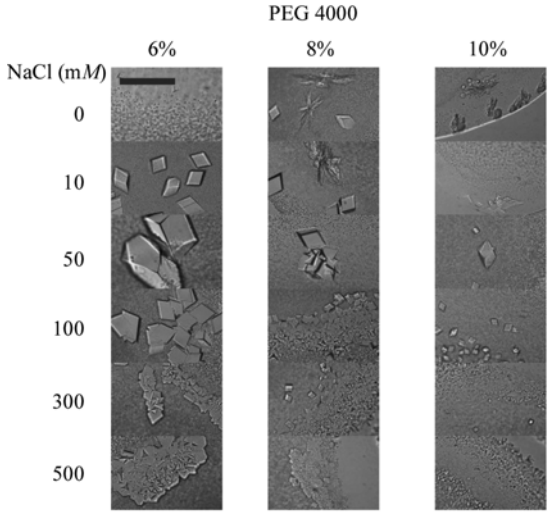

Figure 2
Crystal growth of Ni^{2+}–NTA-purified AcrB$_{His}$. The reservoir solution contained 0.1 *M* Na HEPES pH 7.5, precipitant and salt as indicated. The bar represents about 0.5 mm.

Table 1
Data quality of a native AcrB crystal ($a = b = 143.1$, $c = 513.3$ Å, $\alpha = \beta = 90$, $\gamma = 120°$) collected at beamline BW7A of EMBL/DESY.

Resolution range (Å)	50–3.5	4.0–3.7	3.7–3.5
Unique reflections	26016	4540	3930
Completeness (%)	99.7	99.9	99.9
Multiplicity	4.6	4.6	4.6
$I/\sigma(I)$	9.9	3.7	2.9
R_{meas}† (%)	9.9	43.7	61.5

† Diederichs & Karplus (1997).

3. Results and discussion

$AcrB_{His}$ could be purified in a single step using Ni^{2+}–NTA chromatography. SDS–PAGE analysis revealed the presence of a single band after staining with Coomassie with an electrophoretic mobility of approximately 100 kDa (Fig. 1).

The theoretical molecular weight of $AcrB_{His}$ was calculated to be 114.6 kDa; however, aberrant electrophoretic behaviour is common for membrane proteins (Newman *et al.*, 1981; Pos *et al.*, 1994). After the first crystals were obtained by sitting-drop vapour diffusion from Memfac solution 30 (Hampton Research), crystals of native $AcrB_{His}$ were grown at 293 K from hanging drops containing 2.5 µl of 17 mg ml^{-1} $AcrB_{His}$ and 2.5 µl of reservoir solution containing 0.1 *M* Na HEPES pH 7.5, 6–10% PEG 4000 and 0–500 m*M* NaCl (Fig. 2). Crystals grown in this solution could reach dimensions of up to 700 µm in 4–5 d. However, these larger crystals were not amenable to cryoprotection by immersion into liquid nitrogen and cooling by evaporating nitrogen during data collection. We found that crystals larger than 400 × 400 × 400 µm could not be used for data set collection owing to high mosaicity after freezing. However, smaller crystals diffracted to 3.0 Å.

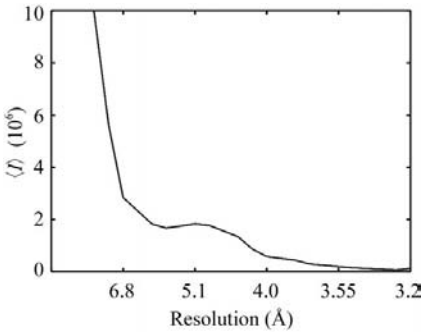

Figure 3
Wilson plot for AcrB.

$AcrB_{His}$ was crystallized in space group *R*32, with unit-cell parameters $a = b = 143$, $c = 513$ Å. The solvent content of the crystals is about 72% if one assumes one molecule per asymmetric unit. Spots were visible to a resolution of 3.0 Å; typical data-collection results are shown in Table 1. The possible molecular packing in space group *R*32 together with the fact that AcrA exists as a trimer (Zgurskaya & Nikaido, 2000*b*) suggests that AcrB might also form a trimeric entity.

Crystals were sensitive to radiation damage, as is apparent from the comparison of images (not shown) taken at the beginning and end of a data set collection, which took about 1.5 to 8 h at SLS and DESY beamlines, respectively. Wilson-plot data are shown in Fig. 3. The intensity of diffraction spots is drastically reduced beyond 6 Å; the mean temperature factor as calculated from the Wilson plot in the 4.0–3.5 range is about 100 Å^2.

A search for suitable heavy-atom derivatives of $AcrB_{His}$ is under way. Preliminary results indicate that MIR phasing is unlikely to succeed owing to high anisomorphism even between native data sets, whereas structure solution by MAD should be possible.

We thank the staff at EMBL/DESY and SLS synchrotron beamlines for support. KMP would like to thank P. Dimroth for his continuous help and support with this work.

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