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Purification, crystallization and preliminary crystallographic analysis of the periplasmic binding protein ProX from *Escherichia coli*

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A periplasmic binding protein (ProX) for the compatible solutes glycine betaine and proline betaine from *Escherichia coli* was crystallized using the hanging-drop vapour-diffusion method. Crystals were grown using a protein concentration of 10 mg ml^{-1} and a precipitant of 26–28% PEG 4000 in 50 m*M* PIPES pH 6.2–6.4. Native diffraction data to 1.93 Å resolution have been obtained from crystals at 290 K. The crystals belong to the space group $P2_12_12_1$, with unit-cell parameters a = 48.1, b = 55.0, c = 115.7 Å, and contain one molecule per asymmetric unit.

1. Introduction

Bacteria lack systems for active water transport; therefore, cellular water content is governed by osmosis and is strongly affected by changes in environmental osmolality. Cells maintain turgor by dynamically modulating the pool of osmotically active solutes in their cytoplasm (Booth & Louis, 1999). To limit the loss of water under hyperosmotic conditions, many microorganisms amass specific organic osmolytes, the so-called compatible solutes. These compounds are highly congruous with cellular functions and are accumulated in preference to inorganic ions that are deleterious in high concentrations to the cell. This response to low water activity permits viability and growth over a wide range of osmotic conditions and is found not only in the microbial world but also in plant, animal and even human cells (Burg *et al.*, 1997; Csonka & Hanson, 1991; Kempf & Bremer, 1998; Rhodes & Hanson, 1993).

Compatible solutes are polar highly soluble molecules that usually do not carry a net charge at physiological pH (da Costa *et al.*, 1998). They serve a dual role in osmoregulation. Firstly, because compatible solutes can be accumulated up to molar concentrations, they make a major contribution to the maintenance of turgor and the retention of cell water under conditions of low water activity (Csonka & Hanson, 1991; Kempf & Bremer, 1998; Poolman & Glasker, 1998). Secondly, compatible solutes stabilize proteins and cellular components against denaturation. This protective property is not fully understood, but is generally explained in terms of the preferential exclusion model (Arakawa & Timasheff, 1985; Qu *et al.*, 1998). One of the most effective and ubiquitously used compatible solutes is glycine betaine (*N*,*N*,*N*-trimethylglycine).

Compatible solute accumulation in bacteria may be achieved by uptake from the environment (Kempf & Bremer, 1998; Poolman & Glasker, 1998). One of the most intensively characterized osmoprotectant transporters is the ProU system from *E. coli* and *Salmonella typhimurium*. It is a member of the ATP-binding cassette (ABC) transporter superfamily. The ProU system consists of an inner-membrane-associated ATPase (ProV), the integral membrane protein ProW and the soluble periplasmic substrate-binding protein ProX (Gowrishankar, 1998). Expression of the *proVWX* structural genes is strongly increased under hyperosmotic conditions, thereby allowing increased compatible solute uptake in high osmolaltity environments (Booth & Higgins, 1990; Csonka & Hanson, 1991; Gowrishankar & Manna, 1996; Lucht & Bremer, 1994). ProU was originally identified as an osmoregulated uptake system for the compatible solute proline (Csonka, 1983), but subsequent studies identified it as an efficient transporter for both glycine betaine and proline betaine (*N*,*N*-dimethylproline) (Cairney *et al.*, 1985; Gousbet *et al.*, 1994; Haardt *et al.*, 1995; May *et al.*, 1986). These two compounds are bound with high affinity (K_D = 1 and 5 μ*M*, respectively) and specificity by the substrate-binding protein ProX (Barron *et al.*, 1987; Haardt *et al.*, 1995; Higgins *et al.*, 1987; May *et al.*, 1986). The ProU system also functions in the low-affinity uptake of a wide variety of compatible solutes, most of which

are structurally related to glycine betaine and proline betaine. However, as initially observed for proline (Barron *et al.*, 1987), no binding of any of these compounds to ProX has been observed *in vitro* (Gousbet *et al.*, 1994; Haardt *et al.*, 1995).

Compatible solutes appear to be preferentially excluded from the immediate vicinity of proteins (Arakawa & Timasheff, 1985; Qu *et al.*, 1998). Yet, ligand-binding proteins such as ProX recognize these compounds and bind them with high affinity and specificity. No crystal structure has been reported for any protein with a bound compatible solute. We have therefore crystallized the *E. coli* ProX protein with its ligands glycine betaine and proline betaine and report here a preliminary crystallographic analysis of these receptor proteins in their substrate-bound form.

2. Methods and materials

2.1. Overproduction and purification of ProX

An approximately 3.4 kbp *Sal*I–*Eco*RI restriction fragment containing both the *proW* and *proX* genes from *E. coli* were isolated from the *proU*$^+$ (*proVWX*) plasmid pOS59 and cloned into the low copy number expression vector pPD101 (Dersch *et al.*, 1994) under the control of the bacteriophage T7φ10 promoter, yielding plasmid pSK7. A pre-culture of strain PD141 [λDE3 (pSK7)] was grown in a minimal medium [Na_2HPO_4 (10.5 g l^{-1}), KH_2PO_4 (4.5 g l^{-1}), $(NH_4)_2SO_4$ (1.0 g l^{-1}), Na_3citrate.$2H_2O$ (0.5 g l^{-1}), $MgSO_4.7H_2O$ (0.025 g l^{-1}), 0.4% casamino acids and 0.4% glucose as the carbon source] containing 30 µg ml^{-1} chloramphenicol and was then used to inoculate (dilution 1:8000) a 10 l flask containing 8 l of the same minimal medium with chloramphenicol. This culture was grown overnight under air bubbling until it reached an OD_{578} of approximately 1.7–1.9 and the T7φ10-mediated expression of the *proWX* genes was then induced by the addition of 1 m*M* IPTG. The culture was further grown for 1 h and the cells were then collected by centrifugation and washed once with a solution of 30 m*M* Tris–HCl pH 7.3. Approximately 25–30 g cell paste was obtained from 8 l of bacterial culture; aliquots that corresponded to 1.3 l of the original culture were either processed directly or frozen at 253 K until further use.

Two portions of the cells were washed again with a solution of 30 m*M* Tris–HCl pH 7.3 and were then separately subjected to cold osmotic shock (Neu & Heppel, 1965). The released periplasmic proteins were combined (100 ml) and subjected to FPLC chromatography at a flow rate of 2 ml min^{-1} on DEAE–Sepharose Fast Flow that had been equilibrated with 16 m*M* Tris–HCl pH 8.3 (140 ml chromatography material packed into a XK25/40 column; Pharmacia, Germany). The column was washed with 140 ml equilibration buffer and the ProX protein was eluted with an increasing Tris–HCl pH 8.3 gradient (16–400 m*M*) from the DEAE Sepharose Fast Flow column (Barron *et al.*, 1987). The ProX protein eluted between 150 and 200 m*M* Tris–HCl from the column, as judged by SDS–PAGE. ProX-containing fractions were combined and ammonium sulfate was added to a final concentration of 1.5 *M*. The protein solution was then subjected to hydrophobic interaction chromatography on a phenyl-Sepharose column (70 ml chromatography material packed into a XK26/20 column; Pharmacia, Germany) set at a flow rate of 2 ml min^{-1}. The column was pre-equilibrated with a 10 m*M* Tris–HCl pH 8.3 buffer and washed with equilibration buffer containing 1.5 *M* ammonium sulfate prior to the elution of ProX with a decreasing ammonium sulfate (1.5–0 *M* ammonium sulfate in 10 m*M* Tris–HCl pH 8.3) gradient. The ProX protein eluted from the phenyl-Sepharose column at the end of the ammonium sulfate gradient; it was free from other contaminating proteins, as judged from SDS–polyacrylamide gels stained either with Coomassie brilliant blue or silver nitrate. The purified ProX protein was dialyzed overnight against 5 l of a 10 m*M* Tris–HCl pH 8.3 buffer. The ProX preparations contained between 1 and 2 mg ml^{-1} of protein; approximately 75 mg of purified ProX protein was obtained from 16 l of bacterial cell culture. ProX in complex with glycine betaine was prepared by adding 1 m*M* glycine betaine (Sigma Chemie, Germany) to the growth medium, the column and dialysis buffers. To obtain ProX with proline betaine as the ligand, the protein was first purified without glycine betaine and proline betaine (Extrasynthèse, France) was then added to the substrate-free ProX preparation to a final concentration of 1 m*M* prior to crystallization. For the preparation of ligand-free ProX proteins, casamino acids were omitted from the growth medium of the *proX*-overexpressing cells.

2.2. Protein crystallization

ProX was concentrated to 10 mg ml^{-1} by ultrafiltration and centrifuged at 100 000*g* for 15 min before examination by dynamic light scattering using a DynaPro instrument (Protein Solutions, Charlottesville, VA, USA). Initial screening was carried out by vapour diffusion with Hampton Research Crystal Screen kits I and II (Jancarik & Kim, 1991). Refinement of crystallization conditions was carried out using hanging-drop vapour diffusion. Equal volumes (5 µl) of reservoir and protein solution were mixed and suspended over 250 µl of reservoir. Crystallization took place at 291 K. Diffraction experiments were carried out using a rotating-anode Cu X-ray source (1.54 Å wavelength) and a MAR 345 image-plate system. Data was collected at room temperature to 1.93 Å resolution and was reduced using *XDS* (Kabsch, 1988).

3. Results and discussion

The previously reported purification scheme for the *E. coli* periplasmic ProX protein relied on a strain expressing the *proX* structural gene from a single chromosomal copy under the control of the osmoregulated *proU* promoter (Barron *et al.*, 1987). To increase ProX production, we positioned *proX* under the transcription initiation signals of the bacteriophage T7φ10 promoter in a low copy number vector (Dersch *et al.*, 1994). This prevents the overloading of the *E. coli* secretion machinery with the pro-ProX precursor molecule and allows IPTG-regulated *proX* expression in an *E. coli* strain [PD141 (λDE3)] carrying a chromosomal copy of the T7 RNA polymerase under *lac*PO/*lac*I control. We also modified the ProX purification procedure of Barron *et al.* (1987) to increase the purity of the ProX preparations so that they were suitable for crystallization trials. In brief, periplasmic proteins were released from ProX-overproducing cells by cold osmotic shock (Neu & Heppel, 1965) and these ProX-containing fractions were then subjected to sequential FPLC chromatography steps on DEAE–Sepharose Fast Flow and phenyl-Sepharose columns. In this way, highly pure and soluble ProX protein was obtained that proved to be monodisperse as judged by dynamic light scattering.

Screening experiments gave rise to initial crystallization conditions with various PEGs and ammonium sulfate as precipitants over a wide range of buffer pH values. Optimization experiments yielded three-dimensional crystals suitable for diffraction data collection only from conditions of low ionic strength with PEG 4000 as precipitant and a buffer pH of 6–7. Single crystals of dimen-

Table 1
Crystallographic statistics of ProX data sets with bound glycine betaine (GB) or proline betaine (PB).

All data with $I/\sigma > 0$ are included. Values in parentheses are for the outer shell.

	GB	PB
Resolution (Å)	20.0–1.93 (2.00–1.93)	6.0–2.28 (2.35–2.28)
Reflections	226595 (21511)	55167 (4656)
Unique reflections	24782 (3444)	14196 (1203)
Completeness (%)	99.7 (98.9)	98.5 (98.0)
I/σ	19.4 (5.2)	17.9 (6.7)
$R_{\rm merge}$ (%)	8.8 (26.4)	5.2 (17.3)
$R_{\rm meas}$ (%)	9.9 (19.7)	6.0 (20.0)

sions 1000 × 250 × 250 µm were grown from a reservoir solution of 26–28% PEG 4000 and 50 m*M* PIPIES pH 6.2–6.4. Crystals are first observed after 11 d and required 4–6 weeks to reach full size. No crystals of ProX in the absence of ligand were observed under these conditions.

ProX crystals belong to the space group $P2_12_12_1$ and have unit-cell parameters a = 48.0, b = 55.0, c = 115.7 Å. This gives a solvent content of 46.5% and a $V_{\rm M}$ of 2.32 Å^3 Da^{-1} with one molecule in the asymmetric unit. Diffraction to 1.93 Å was observed using a rotating-anode source. The crystals do not exhibit significant radiation damage at room temperature after more than 24 h X-ray exposure. Data quality is summarized in Table 1.

The favourable influence of compatible solutes on protein stability and folding seems to rely on their effective exclusion from protein surfaces (Arakawa & Timasheff, 1985; Qu *et al.*, 1998). Yet, their acquisition from external sources in both prokaryotic and eukaryotic cells requires highly specific and high-affinity interactions with components of transport systems. Through the crystallographic analysis of the ProX receptor protein in complex with either glycine betaine or proline betaine, we hope to contribute to a firm understanding of those molecular determinants that govern the specific binding of compatible solutes to proteins. In addition, we would like to elucidate how ProX can recognize both a linear (glycine betaine) and a cyclic (proline betaine) substrate with essentially the same binding affinity.

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