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Purification, crystallization and preliminary X-ray analysis of uridine phosphorylase from *Salmonella typhimurium*

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The structural *udp* gene encoding uridine phosphorylase (UPh) was cloned from the *Salmonella typhimurium* chromosome and overexpressed in *Escherichia coli* cells. *S. typhimurium* UPh (StUPh) was purified to apparent homogeneity and crystallized. The primary structure of StUPh has high homology to the UPh from *E. coli*, but the enzymes differ substantially in substrate specificity and sensitivity to the polarity of the medium. Single crystals of StUPh were grown using hanging-drop vapor diffusion with PEG 8000 as the precipitant. X-ray diffraction data were collected to 2.9 Å resolution. Preliminary analysis of the diffraction data indicated that the crystal belonged to space group $P6_{1(5)}$, with unit-cell parameters $a = 92.3$, $c = 267.5$ Å. The solvent content is 37.7% assuming the presence of one StUPh hexamer per asymmetric unit.

1. Introduction

Uridine phosphorylase (UPh; EC 2.4.2.3) catalyzes the phosphorolytic cleavage of the N-glycosidic bond of uridine to form ribose 1-phosphate and uracil (Leer *et al.*, 1977; Vita *et al.*, 1986). UPhs together with purine nucleoside phosphorylases (PNPs), methylthioadenosine phosphorylases (MTAPs) and methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN) comprise a superfamily with a common monomer fold. UPhs from most microorganisms are hexameric, while PNPs from microorganisms can be either hexameric or trimeric, mammalian PNPs and MTAPs are both trimeric and MTAN is dimeric. Nucleoside phosphorylases are involved in essential biochemical salvage pathways in the cell that provide free purine and pyrimidine bases for subsequent nucleotide biosynthesis, enabling a less costly alternative to *de novo* nucleotide biosynthesis.

Because of the critical role of UPh in maintaining uridine and uracil pools, it has been suggested that UPh inhibitors might be useful as potential chemotherapeutic agents (Niedzwicki *et al.*, 1983). For this reason, we have undertaken a systematic structure–function study of *Salmonella typhimurium* UPh. Inhibitor design will be facilitated by previous functional chracterization of the UPhs from *Escherichia coli*, *S. typhimurium*, *Yersina pseudotuberculosis* and *Vibrio cholerae* (Zolotukhina *et al.*, 2003). In this paper, we report the purification and crystallization of UPh from *S. typhimurium* (StUPh). In addition to structural studies of StUPh, structure determinations of UPh from both *V. cholerae* and *Y. pseudotuberculosis* have been initiated.

2. Materials and methods

2.1. Purification

The *udp* gene from *S. typhimurium* was cloned into pBluescript IISK as described in Molchan *et al.* (1998). *E. coli* strain BL21 (DE3) was transformed with the recombinant plasmid and plated on LB agar with 100 µg ml^{-1} ampicillin. 10 ml LB media containing antibiotics was infected by several colonies and the culture was grown overnight at 310 K. This culture was added to 1 l of LB media containing 100 µg ml^{-1} ampicillin and incubated in a shaker (160 rev min^{-1}) at 310 K until the OD_{590} reached 0.5. At this point, protein production was induced using 0.5 m*M* isopropyl-β-D-thiogalactopyranoside. After 3 h, the cells were harvested by centrifugation (11 000*g*, Beckman JA-10 rotor, 277 K, 10 min). StUPh was purified using a modification of the procedure described by Mikhailov *et al.* (1992). 4 g of wet cells was suspended in 20 ml 100 m*M* Tris–HCl pH 8.0, 1 m*M* β-mercaptoethanol, 1.5 *M* NaCl, 4 m*M* EDTA, 1 m*M* PMSF and broken by sonication. Cellular membranes were removed by centrifugation for 20 min at 14 000*g*. The supernatant was placed into a blender and a solution containing 20 m*M* NaH_2PO_4 pH 7.0, 10% polyimine P (to remove residual nucleic acids), 2 *M* NaCl, 0.5 m*M* β-mercaptoethanol was added slowly under stirring to a final polyimine concentration of approximately 1%. After incubation for 2 h at 277 K, the mixture was

centrifuged for 20 min at 14 000*g*. Precipitated proteins were dissolved in 2 *M* $(NH_4)_2SO_4$, 50 m*M* KH_2PO_4 pH 7.2 and 0.5 m*M* β-mercaptoethanol and loaded onto a butyl-HiTrap column. StUPh was eluted in the same buffer with a linear gradient of ammonium sulfate from 2 to 0 *M*. The fractions, which showed single symmetrical peaks in the UV spectra and were homogeneous by Coomassie blue-stained SDS–PAGE analysis, were pooled and dialyzed into a solution of 50 m*M* Tris–HCl pH 7.5, 20 m*M* NaCl. StUPh was purified by Q-Sepharose HiTrap chromatography. Pure protein was eluted from the column using a linear gradient of NaCl from 20 m*M* to 1 *M*. The fractions were pooled, buffer-exchanged to remove salt, concentrated to 20 mg ml^{-1} and stored for crystallization experiments. This preparation of protein was shown to be homogeneous by native gel electrophoresis in 6% polyacrylamide gel with Tris–borate buffer and Coomassie blue staining (Fig. 1).

2.2. Crystallization

Initial screening for crystallization conditions was performed by hanging-drop vapor-diffusion techniques using commercially available sparse-matrix screens (Jancarik & Kim, 1991) from Hampton Research. All drops were prepared by mixing 2.5 µl protein solution (20 mg ml^{-1} StUPh, 10 m*M* Tris–HCl pH 7.3, 0.4% NaN_3) with 2.5 µl precipitant solution from the crystal screen. The drops were placed on siliconized cover slips and equilibrated against 1.0 ml of the same precipitant solution at 294 K. Microcrystals of StUPh formed in a crystal screen solution containing 0.1 *M* sodium acetate trihydrate pH 4.6 and 8% PEG 4000. In further experiments, PEG 4000 was replaced by 10% PEG 8000 and the ratio of protein solution to precipitant solution in the drops was changed to 3:2. This condition yielded crystals of dimensions 0.10 × 0.05 × 0.04 mm (Fig. 2) that were suitable for diffraction analysis. Before freezing in liquid nitrogen, the crystals were transferred to a solution containing 20% PEG 400 and 100 m*M* sodium acetate trihydrate pH 4.6.

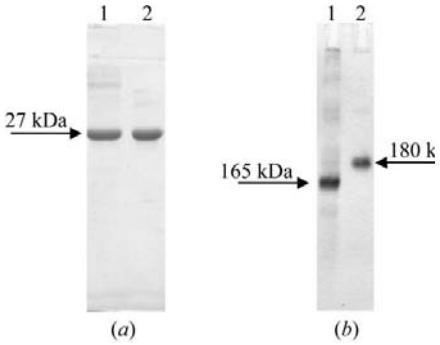

Figure 1
Denaturing and non-denaturing gel electrophoresis for StUPh. (*a*) SDS–gel electrophoresis for EcUPh (lane 1) and StUPh (lane 2). (*b*) Native gel electrophoresis for EcUPh (lane 1) and StUPh (lane 2). The calculated monomer molecular weight is 27 kDa for both enzymes.

2.3. Data collection

The diffraction data were collected on a MAR 345 image-plate system mounted on an Elliott GX-6 rotating-anode generator operating at 35 kV and 35 mA. The generator was equipped with a Kumakhov polycapillary lens (Kumakhov, 2000), which increases the beam intensity at the crystal by more than an order of magnitude. Data were collected at 100 K by the standard oscillation method using a crystal-to-detector distance of 280 mm. Images were collected in 0.5° increments with an exposure time of 20 min per image over a total of 43°. The X-ray diffraction data were processed with the *XDS* package (Kabsch, 2001). Data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

StUPh was produced without affinity tags and was purified to homogeneity by polyimine precipitation followed by hydrophobic interaction and anion-exchange chromatography. Homogeneity was demonstrated by native gel electrophoresis using *E. coli* Uph (EcUPh) as a standard (MW = 165 kDa; Fig. 1). The deduced amino-acid sequence of StUPh contained 253 amino-acid residues (Zolotukhina *et al.*, 2003). From SDS–gel electrophoresis, the monomer is a 27 kDa species (Fig. 1*a*), which is consistent with the calculated molecular weight. The molecular weight of StUPh by native gel electrophoresis was 180 kDa (Fig. 1*b*), suggesting that the native form is probably a hexamer (expected MW = 162 kDa), as is observed for the *E. coli* enzyme (Burling *et al.*, 2003). The different mobility that indicates a molecular weight of approximately 180 kDa results from variations in the amino-acid sequences between StUPh and EcUPh (Zolotukhina *et al.*, 2003).

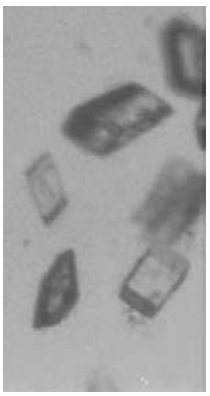

Figure 2
Crystals of StUPh.

Table 1
Crystal data and data-collection statistics for StUPh.

Data in parentheses are for the highest resolution shell.

X-ray source	Elliot GX-6
Wavelength (Å)	1.54
Crystal-to-detector distance (mm)	280
Oscillation range (°)	0.5
Resolution (Å)	20.0–2.9
Space group	$P6_{1(5)}$
Unit-cell parameters (Å)	a = 92.3, c = 267.5
No. observations	75053
No. unique reflections	26462
Multiplicity	2.84 (2.85)
$\langle I\rangle/\langle\sigma(I)\rangle$	6.89 (3.19)
Completeness (%)	93 (90.6)
$R_{\rm merge}$† (%)	15.4 (32.5)
Subunits per asymmetric unit	6 ($V_{\rm M}$ = 2.0 Å^3 Da^{-1})

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$.

The initial crystallization of StUPh was complicated by the small size and high mosaicity of the crystals. After replacing PEG 4000 with PEG 8000 and increasing the concentration from 8 to 10%, crystals suitable for X-ray crystallographic studies were obtained and preliminary data were measured to 2.9 Å resolution using a rotating-anode source (Table 1). Because the space group lacks symmetry elements that can be used to generate the hexamer, the only acceptable crystal packing is one hexamer per asymmetric unit, resulting in a Matthews coefficient of $V_{\rm M}$ = 2.0 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 37.7%. The self-rotation function obtained using *MOLREP* (Vaguine *et al.*, 1999) is consistent with six monomers in the asymmetric unit related by 32 point-group symmetry. Non-crystallographic dyad axes perpendicular to the *c* axis are clearly defined. The non-crystallographic threefold axis is approximately parallel to the *c* axis and is obscured by the large peak corresponding to the sixfold crystallographic symmetry along the *c* axis. Structure determination by molecular replacement using *CNS* (Brünger *et al.*, 1998) and non-crystallographic symmetry averaging is in progress using EcUPh (PDB code 1k3f) as an initial model.

A comparison of the structures of StUPh and EcUPh will explain the differences in their catalytic efficiencies toward phosphorolysis of both natural nucleosides and their analogs. StUPh has higher affinity for all

substrates tested compared with EcUPh. For example, the affinity of StUPh for deoxythymidine is four times higher than that of EcUPh (Molchan *et al.*, 1998). The reason for this is not apparent from amino-acid sequence comparisons and it is hoped that X-ray structural studies of StUPh will provide an explanation.

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