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Crystallization and preliminary X-ray diffraction analysis of *Salmonella typhimurium* uridine phosphorylase complexed with 5-fluorouracil

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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. This enzyme also activates pyrimidine-containing drugs, including 5-fluorouracil (5-FU). In order to better understand the mechanism of the enzyme–drug interaction, the complex of *Salmonella typhimurium* UPh with 5-FU was cocrystallized using the hanging-drop vapour-diffusion method at 294 K. X-ray diffraction data were collected to 2.2 Å resolution. Analysis of these data revealed that the crystal belonged to space group *C*2, with unit-cell parameters $a = 158.26$, $b = 93.04$, $c = 149.87$ Å, $\alpha = \gamma = 90$, $\beta = 90.65^\circ$. The solvent content was 45.85% assuming the presence of six hexameric molecules of the complex in the unit cell.

1. Introduction

5-Fluorouracil (5-FU) has been used in chemotherapeutic regimens in patients with gastrointestinal malignancies, including oesophageal, gastric, colon and pancreatic tumours, as well as breast cancer for several decades (Kemeny, 1987; Huang *et al.*, 2007). This drug competes with uracil for interaction with thymidylate synthase. 5-FU interferes with DNA and RNA synthesis, thereby causing cell death. Furthermore, 5-halogen-containing derivatives of uracil are also used as antimicrobial agents. Synergism of 5-FU with antibacterial agents such as ceftriaxone, ceftazidime, cefotiam, piperacillin and netilmicin has been shown (Price *et al.*, 1965; Gieringer *et al.*, 1986; Yamashiro *et al.*, 1986). However, toxicity towards haematopoietic cells, skin, heart and neurons can limit the therapeutic efficacy of 5-FU and structurally similar drugs (Peters & van Groeningen, 1991). Uridine phosphorylase (UPh) catalyzes the phosphorolytic cleavage of the N-glycosidic bond in uridine to produce ribose 1-phosphate and uracil (Leer *et al.*, 1977; Vita *et al.*, 1986). This enzyme activates pyrimidine-based drugs, including 5-FU (Cao *et al.*, 2002; Caradoc-Davies *et al.*, 2004). Therefore, studies of the structure of UPh bound to 5-FU should be instrumental in future modifications aimed at the design of 5-FU derivatives with lower general toxicity and retained antitumour and antimicrobial potencies (Iigo *et al.*, 1990; Temmink *et al.*, 2006; Matsusaka *et al.*, 2007).

2. Protein expression and purification

Salmonella typhimurium UPh (*St*UPh) was expressed in *Escherichia coli* and purified as reported elsewhere (Molchan *et al.*, 1998; Dontsova *et al.*, 2004). The *Escherichia coli* BL21 (DE3) strain was transformed with the recombinant plasmid and grown on solid LB agar for 12 h at 310 K. Protein synthesis was stimulated with 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside. The biomass was sonicated, ammonium sulfate was added to precipitate the proteins and the pellets were dissolved in buffer (pH 7.2) containing 50 m*M* KH_2PO_4 and 0.5 m*M* β-mercaptoethanol. Further steps of *St*UPh purification were performed using chromatography on butyl Sepharose as a first step and Q-Sepharose as the final step. The enzymatic activity was 280 units per milligram of purified protein. The homogeneity of the purified *St*Uph was 96% as determined by nondenaturing gel electrophoresis.

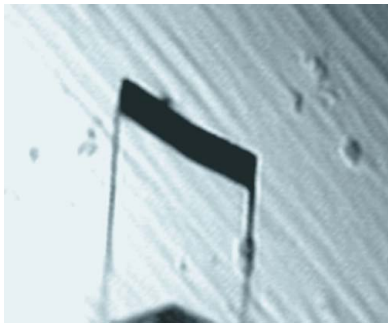

Table 1
Statistics of X-ray data.

Values in parentheses are for the last resolution shell.

Wavelength (Å)	0.918
Temperature (K)	100
Oscillation (°)	0.5
Space group	*C*2
Unit-cell parameters (Å, °)	a = 158.26, b = 93.04, c = 149.87, $\alpha = \gamma = 90$, $\beta = 90.65$
Molecules per unit cell	6
V_M (Å^3 Da^{-1})	2.27
Solvent content (%)	45.85
Resolution limits (Å)	30.0–2.2 (2.25–2.2)
Completeness (%)	90.2 (79.3)
No. of reflections	324446
No. of unique reflections	99573 (7124)
$R_{observed}$ (%)	6.8 (55.6)
$R_{expected}$ (%)	7.0 (55.3)
$\langle I/\sigma(I)\rangle$	12.67 (2.16)
R_{meas}	0.08 (0.66)
R_{merge}	0.017 (0.47)
Redundancy	3.26 (3.24)

3. Crystallization of the *St*Uph–5FU complex

Crystals of the complex of *St*Uph with 5-FU (EBEWE Pharma, Austria) were obtained by cocrystallization (Fig. 1). Crystallization was performed on siliconized glass slides (Hampton Research, USA) in Linbro plates at 294 K using the hanging-drop vapour-diffusion method. The reservoir solution (0.5 ml) consisted of 0.34 ml 0.1 *M* Tris–maleate–NaOH buffer pH 5.5 and 0.16 ml 40%(*w*/*v*) polyethylene glycol 3350. The crystallization drop contained 2 µl *St*Uph solution (11.3 mg ml^{-1}) in 10 m*M* Tris–HCl buffer pH 7.3, 2 µl H_2O, 1.3 µl reservoir solution, 2 µl 100 m*M* 5-FU and 0.3 µl 2-propanol. Crystals of dimensions 0.07 × 0.3 × 0.5 mm were obtained after 1–2 weeks and were used for X-ray diffraction analysis.

4. X-ray analysis

The X-ray data set (Table 1) was collected upon irradiation of *St*UPh–5FU crystals under cryogenic conditions (100 K) on beamline 14.2 at BESSY, Berlin, Germany. The wavelength was 0.9184 Å. A CHESS CCD detector was used with an oscillation range $\Delta\varphi$ of 0.5° and a crystal-to-detector distance of 240 mm. Prior to freezing in liquid nitrogen, the crystals were transferred into cryoprotectant solution containing 100 m*M* Tris–maleate–NaOH buffer pH 5.5, 25%(*w*/*v*) polyethylene glycol 3350 and 20%(*v*/*v*) anhydrous glycerol. All data were processed and scaled using *XDS* (Kabsch, 1988).

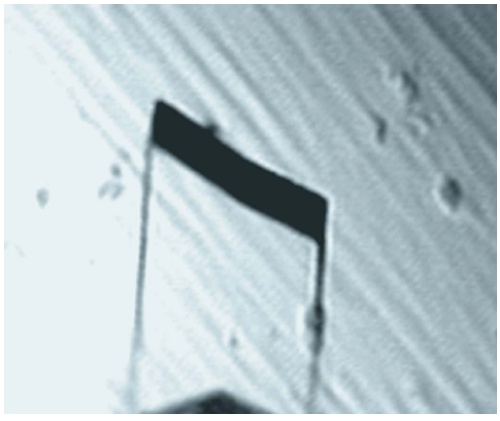

Figure 1
Crystal of *St*UPh complexed with 5-FU.

300 images were obtained during the collection of X-ray diffraction data. All data were indexed, merged and processed using the *XDS* program with XYCORR, INIT, COLSPOT, IDXREF, DEFPIX, XPLAN, INTEGRATE and CORRECT options. For a semi-automatic determination of the space group, the minimal value of the *XDS* 'quality of fit' function was used. The crystals of the *St*UPh–5-FU complex belonged to space group *C*2. Detailed data statistics are presented in Table 1.

The structure was resolved by the molecular-replacement technique using the *Phaser* program with rigid-body refinement option (McCoy, 2007). X-ray diffraction data from 10 to 2.5 Å resolution were used in this step. The X-ray structure of monomer *A* only of ligand-free *St*UPh at 1.76 Å resolution (Timofeev *et al.*, 2007; PDB code 2oxf) was utilized as a search model. Water molecules were removed from the model. Six full homohexamer molecules were found in the unit cell. The Matthews coefficient (Matthews, 1968) was 2.27 Å^3 Da^{-1} and the solvent content was 45.85% (Table 1). Only one solution was evident, with an *R* factor of 37.64% and a correlation coefficient R_{corr} of 76.95%.

To improve the phase model, one macrocycle of simulated annealing using the *phenix.refine* module of *PHENIX* (Adams *et al.*, 2002) was performed in the temperature range 12 000–300 K with 50 K steps and resolution 10–2.2 Å. Before refinement, 5% of the observations were chosen at random and set aside for cross-validation analysis and to monitor the various refinement strategies. Next, σ_A-weighted electron-density maps with coefficients $(2|F_o| - |F_c|)$ and $(|F_o| - |F_c|)$ were obtained using *PHENIX*. Using the $(|F_o| - |F_c|)$ electron-density map in the *Coot* program (Emsley & Cowtan, 2004), we identified 5-FU molecules and water molecules localized in the close vicinity of the 5-FU molecules. After two cycles of restrained refinement in *REFMAC* (Murshudov *et al.*, 1997) and the synthesis of σ_A-weighted $(2|F_o| - |F_c|)$ and $(|F_o| - |F_c|)$ electron-density maps the *R* factor was 26.42% and R_{free} was 29.24%.

One uracil-binding site in *St*Uph complexed with 5-FU and water is shown in Fig. 2. The cartoon representation was generated with *PyMOL* (DeLano, 2008). The drug and water bind to the following atoms of the amino-acid residues in the uracil-binding site:

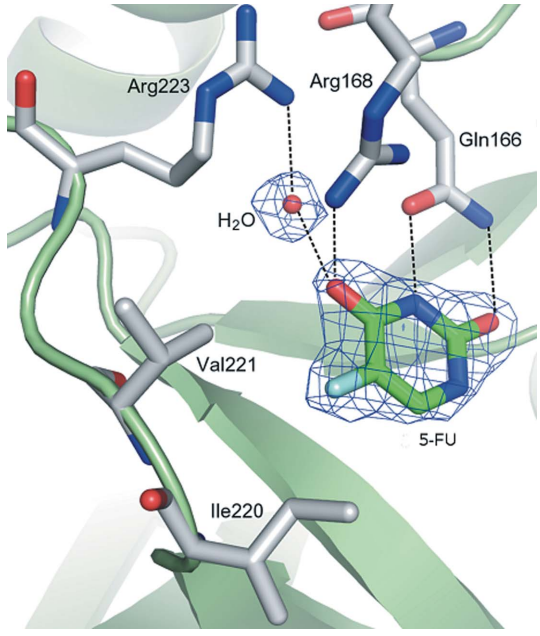

Figure 2
Preliminary structure of the uracil-binding site in the *St*UPh–5-FU complex.

Arg223 NH1–water (2.85 Å), water–5-FU O4 (2.88 Å), Arg168 NH1–5-FU O4 (3.00 Å), Gln166 OE1–5-FU N3 (2.70 Å), Gln166 NE2–5-FU O2 (2.89 Å). Refinement of the structure is currently in progress and will be published elsewhere.

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