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Crystallization and preliminary X-ray study of *Vibrio cholerae* uridine phosphorylase in complex with 6-methyluracil

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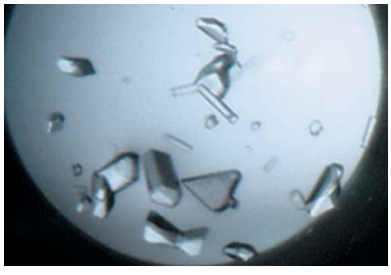

Uridine phosphorylase catalyzes the phosphorolysis of ribonucleosides, with the nitrogenous base and ribose 1-phosphate as products. Additionally, it catalyzes the reverse reaction of the synthesis of ribonucleosides from ribose 1-phosphate and a nitrogenous base. However, the enzyme does not catalyze the synthesis of nucleosides when the substrate is a nitrogenous base substituted at the 6-position, such as 6-methyluracil (6-MU). In order to explain this fact, it is essential to investigate the three-dimensional structure of the complex of 6-MU with uridine phosphorylase. 6-MU is a pharmaceutical agent that improves tissue nutrition and enhances cell regeneration by normalization of nucleotide exchange in humans. 6-MU is used for the treatment of diseases of the gastrointestinal tract, including infectious diseases. Here, procedures to obtain the uridine phosphorylase from the pathogenic bacterium *Vibrio cholerae* (*Vch*UPh), purification of this enzyme, crystallization of the complex of *Vch*UPh with 6-MU, and X-ray data collection and preliminary X-ray analysis of the *Vch*UPh–6-MU complex at atomic resolution are reported.

1. Introduction

Uridine phosphorylase (UPh; EC 2.4.2.3) is a member of the nucleoside phosphorylase enzyme family and plays a significant role in the metabolism of nucleosides in all living organisms. These enzymes contribute to the biosynthesis of nucleotides by producing free purine and pyrimidine bases, and catalyze a reaction that can substitute for expensive *de novo* nucleotide synthesis. Nucleoside phosphorylases are essential in all cells. From this viewpoint, they are very important targets for antibacterial agents. For some time, we have been carrying out investigations of nucleoside phosphorylases, including uridine phosphorylase. Uridine phosphorylase catalyzes the phosphorolysis of ribonucleosides and the reverse reaction of their synthesis. The major physiological substrates of uridine phosphorylase are uridine (Fig. 1*a*) and, for the synthesis reaction of ribonucleosides, uracil (el Kouni *et al.*, 1988; Leer *et al.*, 1977; Molchan *et al.*, 1998; Temmink *et al.*, 2007; Veiko *et al.*, 1996; Watanabe & Uchida, 1995; Woodman *et al.*, 1980).

At present, the most investigated uridine phosphorylases are those from *Escherichia coli* and *Salmonella typhimurium* (Caradoc-Davies *et al.*, 2004; Dontsova *et al.*, 2005; Lashkov *et al.*, 2009, 2010; Morgunova *et al.*, 1995). These enzymes are very similar. Their structures present six identical subunits, each with a molecular mass of 27.5 kDa. Each monomer in the hexamer makes close contacts with an identical hexamer related by a twofold noncrystallographic axis, forming dimers. The hexamer can be described as a trimer of dimers. The active site of uridine phosphorylase is formed by amino acids of the dimer (Caradoc-Davies *et al.*, 2004; Lashkov *et al.*, 2010; Morgunova *et al.*, 1995). The uridine phosphorylase from the pathogenic microorganism *Vibrio cholerae* has about 75% identity to the uridine phosphorylases of *E. coli* and *S. typhimurium*. However, some of the amino acids forming the active site of the enzymes differ (Zolotukhina *et al.*, 2003). Previously, we have investigated the three-dimensional structures of complexes of uridine phosphorylase from *V. cholerae* with phosphate ion (PDB entry 4h1t; I. I. Prokofev, A. A. Lashkov, A. G. Gabdoulkhakov, S. E. Sotnichenko, C. Betzel & A. M. Mikhailov, unpublished work) and with thymidine (PDB entry 4g8j; A. A. Lashkov, A. G. Gabdoulkhakov, I. I. Prokofev, S. E. Sotni-

chenko, C. Betzel & A. M. Mikhailov, unpublished work). In addition, structures of this enzyme in an unliganded state (PDB entry 3o6v; Center for Structural Genomics of Infectious Diseases, unpublished work) and in complex with uracil (PDB entry 3pns; Center for Structural Genomics of Infectious Diseases, unpublished work) have been determined.

The ability of uridine phosphorylase to implement the catalytic reaction using pyrimidine substrates with alkyl or halogen substituents at the 5-position has been demonstrated, and these reactions are reversible (Krajewska & Shugar, 1982; Leer *et al.*, 1977; Temmink *et al.*, 2007; Watanabe & Uchida, 1995; Woodman *et al.*, 1980). However, in the case of 6-alkyl-substituted pyrimidines (for example, 6-methyluracil) the reaction only proceeds in the direction of phosphorolysis of a nucleoside analogue (*P*; Fig. 1*b*), and the synthesis of nucleoside analogues (*S*) does not occur (Krajewska & Shugar, 1982). Krajewska and Shugar assumed that the hydrophobic chemical group attached to the aromatic ring at the 6-position of pyrimidine does not allow the hydrophilic phosphate group of ribose 1-phosphate to move closer to the N1 atom of the heterocycle. To confirm this hypothesis, Krajewska and Shugar initiated investigation of the spatial organization of the complex of uridine phosphorylase from the pathogenic microorganism *V. cholerae* of biotype eltor in serogroup O1 (*Vch*UPh) with 6-methyluracil (6-MU). 6-MU is used to stimulate immunodefence, as an anabolic drug and as an adjuvant in antibiotic therapy (Chadaev & Klimiashvili, 2003; el-On & Weinrauch, 1990; Ganzhii, 2002; Gerasimenko *et al.*, 2002; Petrov *et al.*, 2011; Taran & Shishkina, 1993).

2. Materials and methods

2.1. Cloning, expression and purification

The procedure used to clone the gene for uridine phosphorylase from *V. cholerae* was based on the method described previously by Zolotukhina *et al.* (2003) and Lashkov *et al.* (2012). For amplification of *Vch*UPh, genes were synthesized using two PCR primers. Each primer contains specific restriction sites allowing subsequent cloning into a plasmid. Chromosomal DNA from *V. cholerae* was used as a template for PCR. The PCR was carried out using a TC480 thermal cycler (Perkin Elmer, USA). T7 DNA polymerase (GE Healthcare, Life Sciences, Germany) was used for gene amplification. After amplification, DNA fragments of about 1030 bp were obtained that contained the structural and regulatory regions of the *Vch*UPh gene flanked by recognition sites for the restriction enzymes *Eco*RI and *Bam*HI. After separation of PCR products by agarose gel electrophoresis, the desired DNA fragment was eluted from the gel using a kit supplied by GeneClean (Fermentas, Lithuania). The PCR product was digested with the *Eco*RI and *Bam*HI enzymes and cloned into the multi-copy vector pUC19. The correct sequence of the resulting plasmid pMZ21 containing the *Vch*UPh gene was confirmed by DNA sequencing. The plasmid pMZ21 consisted of one complete open reading frame coding for the *Vch*UPh protein. The protein consisted of 253 amino acids and its predicted molecular mass was 27.5 kDa. Further details are given in Zolotukhina *et al.* (2003).

The plasmid pMZ21 was transformed into *Escherichia coli* strain AM201 which contains a deletion of the uridine phosphorylase gene (Δ*metE-udp*). Analysis of the expression of *Vch*UPh in *E. coli* cells was carried out by electrophoretic separation of the total protein from *E. coli* cells in a polyacrylamide gel under denaturing conditions. For this purpose, overnight cultures of the AM201 strain containing pMZ21 plasmid were grown in rich LB medium supplemented with ampicillin (100 µg ml^{-1}) at 310 K. The cells were collected by centrifugation for 5 min at 5000*g*. The biomass was suspended in a buffer consisting of 62.5 m*M* Tris–HCl pH 6.8, 5% glycerol, 2% *β*-mercaptoethanol (*β*-ME), 0.1% SDS, 0.1% bromophenol blue. The cells were disrupted by boiling for 10 min and samples were analyzed by polyacrylamide gel electrophoresis under denaturing conditions.

The molecular mass of the expressed protein was close to the predicted value of 27.5 kDa calculated from *Vch*UPh gene sequencing.

To obtain pure *Vch*UPh, cultures of *E. coli* strain AM201 containing the pMZ21 plasmid were grown in a thermostatic shaker at 310 K in LB medium overnight. The biomass was collected by centrifugation at 5000*g* for 10 min. The cells were resuspended in a lysis buffer consisting of 50 m*M* Tris–HCl pH 7.5, 1.5 *M* NaCl, 5 m*M* *β*-ME, 0.3 m*M* phenylmethylsulfonyl fluoride and were lysed by sonication. Cellular membranes were removed by centrifugation for 30 min at 10 000*g*. 10% Polymin P pH 6.0 (1/10 of the supernatant volume) was added to the supernatant. This mixture was incubated with stirring overnight at 277 K. The precipitate was collected by centrifugation at 30 min and 14 000*g*, and was solubilized in a buffer consisting of 50 m*M* Tris–HCl pH 7.5, 500 m*M* NaCl, 5 m*M* *β*-ME. Dry ammonium sulfate was added to this solution containing *Vch*UPh to a final concentration of 3 *M* to precipitate the protein,

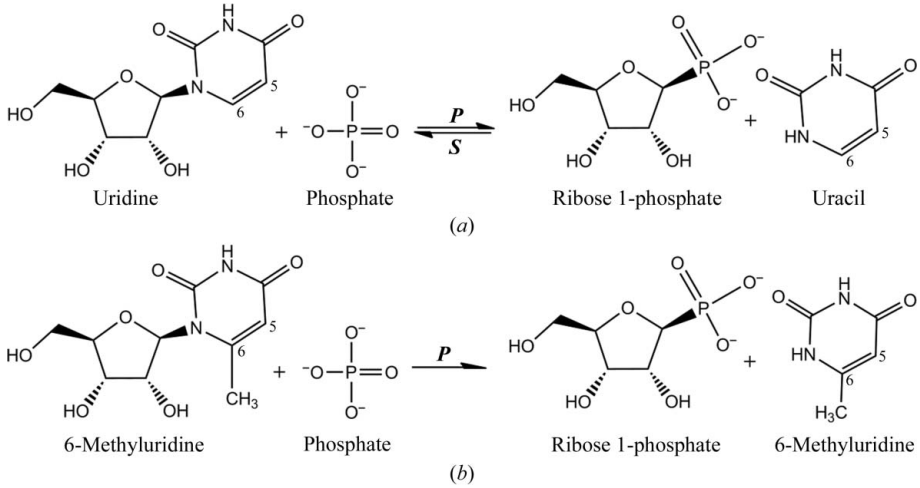

Figure 1
Scheme showing the reaction catalyzed by uridine phosphorylase: (*a*) reversible interconversion of the native substrate uridine to uracil and (*b*) irreversible conversion of the substrate analogue 6-methyluridine to 6-methyluracil. Conventional nomenclature: *P*, phosphorolysis reaction; *S*, nucleoside-synthesis reaction.

and this mixture was incubated at 277 K overnight. The *Vch*UPh precipitate was harvested by centrifugation at 30 min and 14 000*g*. The pellet was then dissolved in a buffer consisting of 50 m*M* Tris–HCl pH 7.5, 2 *M* ammonium sulfate, 5 m*M* β-ME and loaded onto a Butyl Sepharose column. The column was equilibrated with the same buffer. After washing with the equilibration buffer, the protein was eluted at a rate of approximately 0.5 ml min^{-1} at 277 K using a linear gradient of 2–0 *M* ammonium sulfate. The total volume of the gradient was 200 ml and the volume of the fractions was 5 ml. Fractions containing the target protein were pooled. The *Vch*UPh protein was transferred by dialysis into 50 m*M* Tris–HCl pH 7.5, 20 m*M* NaCl, 2 m*M* DTT and applied at a rate of 1 ml min^{-1} onto a Q Sepharose column equilibrated with the same buffer. The column was washed with equilibration buffer, and *Vch*UPh was then eluted using a 20–1000 m*M* NaCl gradient over 200 ml in 50 m*M* Tris–HCl pH 7.5, 2 m*M* DTT. The purified *Vch*UPh was concentrated to 12 mg ml^{-1}. The amount and quality of the *Vch*UPh was monitored by polyacrylamide gel electrophoresis after every purification step.

2.2. Crystallization

Screening to obtain initial crystallization conditions was performed at 295 K by the hanging-drop vapour-diffusion method using the commercially available crystal screening kits The MbClass and MbClass II Suites (Qiagen, Germany). The complex of *Vch*UPh with 6-MU was obtained directly in the drop. Droplets consisting of 2 µl protein solution (12 mg ml^{-1} *Vch*UPh), 2 µl reservoir solution and 1 µl 0.1 *M* 6-MU (Sigma–Aldrich, Germany) were equilibrated against 300 µl reservoir solution in 24-well plates. The most promising conditions were optimized by varying the buffer pH and the amount of precipitant. The final crystallization condition consisted of 15% PEG 4000, 0.1 *M* $MgCl_2$, 0.1 *M* Tris–HCl pH 8.5. Crystals (Fig. 2) of the *Vch*UPh–6-MU complex were grown at room temperature (291 K) in one week. The approximate dimensions of the crystals were 150 × 200 × 30 µm.

2.3. X-ray data collection

For diffraction data collection, a single crystal of the *Vch*UPh–6-MU complex was flash-cooled after soaking in a cryosolution consisting of 40% PEG 400, 0.1 *M* $MgCl_2$, 0.1 *M* Tris–HCl pH 8.5. A data set was collected under cryogenic conditions (100 K) on beamline P13 MX1 at PETRA III (EMBL/DESY, Hamburg, Germany). The wavelength of the radiation was set to 0.827 Å and an oscillation range of 0.1° per frame was used. A Pilatus CCD detector was used to record X-ray diffraction intensities at a crystal-to-detector distance of 247.7 mm. The complex crystals diffracted to 1.17 Å resolution. All data were indexed, merged and processed using the *XDS* package (Kabsch, 2010). The data statistics are summarized in Table 1.

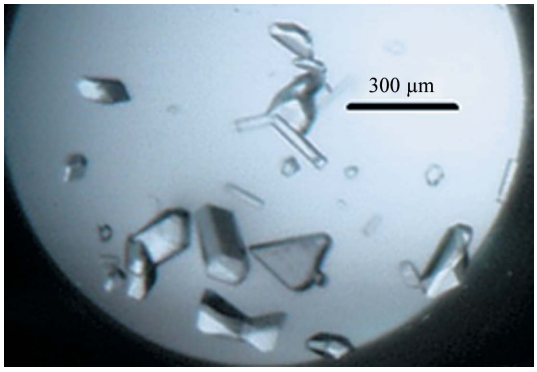

Figure 2
Crystals of uridine phosphorylase from *V. cholerae* in complex with 6-MU.

Table 1
Crystallographic data and statistics of data collection and processing.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 93.01, b = 97.10, c = 93.02, β = 119.99
No. of hexameric molecules per asymmetric unit	1
Molecular weight of hexamer (kDa)	165
No. of amino-acid residues per subunit	253
Wavelength (Å)	0.8266
Radiation source	P13 MX1, PETRA III
Detector type	Pilatus CCD
Crystal-to-detector distance (mm)	247.688
Oscillation range (°)	0.1
No. of frames	1800
Resolution range (Å)	80.56–1.17 (1.20–1.17)
No. of measured reflections	1555812 (62318)
No. of unique reflections	859424 (38791)
Completeness (%)	90.7 (55.3)
$R_{meas}(I)$† (%)	5.1 (27.2)
Wilson plot B factor (Å^2)	12.3
Average $I/\sigma(I)$	11.95 (3.23)
Multiplicity	3.5 (3.2)
Mosaicity (°)	0.07
$CC_{1/2}$‡ (%)	99.9 (89.9)
V_M (Å^3 Da^{-1})	2.27
Solvent content (%)	44.3

† $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$. ‡ $CC_{1/2}$ is the Pearson correlation coefficient calculated between the average intensities of two subsets, each containing a random half of the measurements of each unique reflection (Karplus & Diederichs, 2012).

3. Preliminary X-ray study

Initial phases for the determination and subsequent adjustment of the three-dimensional structure of the complex of *Vch*UPh with 6-MU were obtained by the molecular-replacement method using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP*4 suite (Winn *et al.*, 2011). The previously determined structure of *Vch*UPh in complex with phosphate ion (PDB entry 4ip0; I. I. Prokofev, A. A. Lashkov, A. G. Gabdoulkhakov, C. Betzel & A. M. Mikhailov, unpublished work) was used as a starting model. All ligands, including water molecules, were removed from the starting model before molecular-replacement calculations. The search for a solution was performed using a set of Fourier spectra as a search model at a resolution of 2.5 Å. The program *MOLREP* identified a unique solution characterized by an S_{cor} of 53.8% and an R factor of 43.2%.

The best solution was found for three homodimers of the complex of *Vch*UPh with 6-MU per asymmetric unit. The Matthews coefficient V_M (Matthews, 1968) was calculated to be 2.27 Å^3 Da^{-1}, with a corresponding solvent content of 44.3% (Table 1).

Refinement of the *Vch*UPh–6-MU complex was carried out in *REFMAC* (Murshudov *et al.*, 2011) using a rigid-body procedure. After a few cycles of rigid-body refinement, the R factor and R_{free} decreased to 34.4 and 33.7%, respectively. A subsequent ten cycles of restrained refinement using *REFMAC* reduced the R factor and R_{free} to 22.1 and 26.9%, respectively.

Analysis of a difference electron-density map calculated with ($|F_{obs}| - |F_{calc}|$) coefficients using *Coot* (Emsley & Cowtan, 2004; Emsley *et al.*, 2010) revealed the position of 6-MU in all six active centres of the *Vch*UPh hexamer (Fig. 3). Electron density corresponding to a water molecule was detected in the immediate vicinity of 6-MU (Fig. 3). 6-MU is directly linked by hydrogen bonds to Gln165 and Arg167 in the uracil-binding site of the active centre of

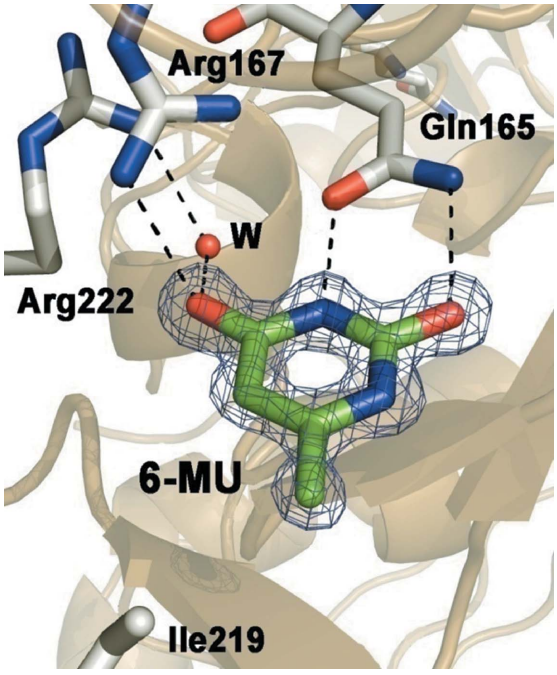

Figure 3
Nucleoside-binding site of *Vch*UPh in complex with 6-MU (prepared using *PyMOL* v.0.99; DeLano, 2002).

the *Vch*UPh molecule and to Arg222 *via* a water molecule (Fig. 3). The position of 6-MU coincides with the position of uracil found in the binding site of uridine phosphorylase from *V. cholerae* (PDB entry 3pns). Detailed information on the structural aspects of the binding of 6-MU will be published in the future.

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References

Caradoc-Davies, T. T., Cutfield, S. M., Lamont, I. L. & Cutfield, J. F. (2004). *J. Mol. Biol.* **337**, 337–354.
Chadaev, A. P. & Klimiashvili, A. D. (2003). *Khirurgiia (Mosk.)*, pp. 54–56.
DeLano, W. L. (2002). *PyMOL*. http://www.pymol.org.
Dontsova, M. V., Gabdoulkhakov, A. G., Molchan, O. K., Lashkov, A. A., Garber, M. B., Mironov, A. S., Zhukhlistova, N. E., Morgunova, E. Y., Voelter, W., Betzel, C., Zhang, Y., Ealick, S. E. & Mikhailov, A. M. (2005). *Acta Cryst.* F**61**, 337–340.
el-On, J. & Weinrauch, L. (1990). *Int. J. Dermatol.* **29**, 232.
Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* D**60**, 2126–2132.
Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* D**66**, 486–501.
Ganzhii, V. V. (2002). *Klin. Khir.*, pp. 18–19.
Gerasimenko, M., Vasil'eva, E. V., Kuvshinov, E. V., Barybin, V. F., Skovorod'ko, S. N., Filatova, E. V. & Lazarenko, N. N. (2002). *Vopr. Kurortol. Fizioter. Lech. Fiz. Kult.*, pp. 12–15.
Kabsch, W. (2010). *Acta Cryst.* D**66**, 125–132.
Karplus, P. A. & Diederichs, K. (2012). *Science*, **336**, 1030–1033.
Kouni, M. H. el, Naguib, F. N., Niedzwicki, J. G., Iltzsch, M. H. & Cha, S. (1988). *J. Biol. Chem.* **263**, 6081–6086.
Krajewska, E. & Shugar, D. (1982). *Biochem. Pharmacol.* **31**, 1097–1102.
Lashkov, A. A., Gabdulkhakov, A. G., Prokofev, I. I., Seregina, T. A., Sotnichenko, S. E., Lyashenko, A. V., Shtil, A. A., Mironov, A. S., Betzel, C. & Mikhailov, A. M. (2012). *Acta Cryst.* F**68**, 1394–1397.
Lashkov, A. A., Zhukhlistova, N. E., Gabdoulkhakov, A. H., Shtil, A. A., Efremov, R. G., Betzel, C. & Mikhailov, A. M. (2010). *Acta Cryst.* D**66**, 51–60.
Lashkov, A. A., Zhukhlistova, N. E., Gabdulkhakov, A. G. & Mikhailov, A. M. (2009). *Crystallogr. Rep.* **54**, 267–278.
Leer, J. C., Hammer-Jespersen, K. & Schwartz, M. (1977). *Eur. J. Biochem.* **75**, 217–224.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Molchan, O. K., Dmitrieva, N. A., Romanova, D. V., Lopes, L. E., Debabov, V. G. & Mironov, A. S. (1998). *Biochemistry*, **63**, 195–199.
Morgunova, E., Mikhailov, A. M., Popov, A. N., Blagova, E. V., Smirnova, E. A., Vainshtein, B. K., Mao, C., Armstrong, S. R., Ealick, S. E., Komissarov, A. A., Linkova, E. V., Burlakova, A. A., Mironov, A. S. & Debabov, V. G. (1995). *FEBS Lett.* **367**, 183–187.
Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* D**67**, 355–367.
Petrov, K. A., Yagodina, L. O., Valeeva, G. R., Lannik, N. I., Nikitashina, A. D., Rizvanov, A. A., Zobov, V. V., Bukharaeva, E. A., Reznik, V. S., Nikolsky, E. E. & Vyskočil, F. (2011). *Br. J. Pharmacol.* **163**, 732–744.
Taran, I. P. & Shishkina, L. N. (1993). *Radiobiologiia*, **33**, 285–290.
Temmink, O. H., de Bruin, M., Turksma, A. W., Cricca, S., Laan, A. C. & Peters, G. J. (2007). *Int. J. Biochem. Cell Biol.* **39**, 565–575.
Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* D**66**, 22–25.
Veiko, V. P., Siprashvili, Z. Z., Ratmanova, K. I., Gul'ko, L. B. & Mironov, A. A. (1996). *Mol. Biol. (Mosk.)*, **30**, 170–176.
Watanabe, S. & Uchida, T. (1995). *Biochem. Biophys. Res. Commun.* **216**, 265–272.
Winn, M. D. *et al.* (2011). *Acta Cryst.* **67**, 235–242.
Woodman, P. W., Sarrif, A. M. & Heidelberger, C. (1980). *Cancer Res.* **40**, 507–511.
Zolotukhina, M., Ovcharova, I., Eremina, S., Errais Lopes, L. & Mironov, A. S. (2003). *Res. Microbiol.* **154**, 510–520.