Crystal structure of the active form of native human thymidylate synthase in the absence of bound substrates

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Human thymidylate synthase (hTS) provides the sole *de novo* intracellular source of thymidine 5'-monophosphate (dTMP). hTS is required for DNA replication prior to cell division, making it an attractive target for anticancer chemotherapy and drug discovery. hTS binds 2'-deoxyuridine 5'-monophosphate (dUMP) and the folate co-substrate N^5,N^{10} -methylenetetrahydrofolate (meTHF) in a pocket near the catalytic residue Cys195. The catalytic loop, which is composed of amino-acid residues 181–197, can adopt two distinct conformations related by a 180° rotation. In the active conformation Cys195 is close to the active site, while in the inactive conformation it is rotated and Cys195 is too distant from the active site for catalysis. Several hTS structures, either native or engineered, have been solved in the active conformation in complex with ligands or inhibitors and at different salt concentrations. However, apo hTS structures have been solved in an inactive conformation in high-salt and low-salt conditions (PDB entries 1ypv, 4h1i, 4gyh, 3egy and 3ehi). Here, the structure of apo hTS crystallized in the active form with sulfate ions coordinated by the arginine residue that binds dUMP is reported.

1. Introduction

Thymidylate synthase (EC 2.1.1.45) is the only enzyme which catalyzes the formation of thymidine 5'-monophosphate (dTMP) from 2'-deoxyuridine 5'-monophosphate (dUMP) and N^5,N^{10} -methylenetetrahydrofolate (meTHF) by reductive methylation. This mechanism has been extensively studied in both prokaryotes and eukaryotes (Carreras & Santi, 1995; Montfort & Weichsel, 1997). Because its unique activity is crucial to provide the dTMP nucleotide necessary for DNA synthesis, thymidylate synthase is a key target for the development of drugs blocking cell proliferation. Several human thymidylate synthase (hTS) inhibitors have been developed and used in anticancer chemotherapy, such as 5-fluorouracil (Heidelberger *et al.*, 1957) and antifolate drugs such as methotrexate (Allegra *et al.*, 1985), raltitrexed and pemetrexed (Jarmuła, 2010). However, to overcome resistance to and the toxicity of these drugs, new inhibitors of thymidylate synthase need to be found (Garg *et al.*, 2010) based on drug design (Costi *et al.*, 2005), docking (Srivastava *et al.*, 2010) and molecular simulations (Salo-Ahen & Wade, 2011).

Prokaryotic thymidylate synthase has been well studied, and several structures have been solved in different conformations (Finer-Moore *et al.*, 2003; Stroud & Finer-Moore, 2003). The different conformations can be interpreted as changes to accommodate the binding of dUMP and cofactor. Several mechanisms have been proposed. The reaction may either be sequentially ordered with dUMP binding prior to the cofactor meTHF or have a random kinetic mechanism. When

Table 1
hTS production information.

Source organism	<i>Homo sapiens</i>
DNA source	pWHTS-1
Forward primer	GATATACATATGCCTGTGTGCTGGTTC
Reverse primer	TAAAGACTGACTGACAATAAGCTTCGAGCTC
Cloning vector	pET-17xb
Expression vector	pET-17xb
Expression system	<i>E. coli</i> Rosetta2 DE2 pLysS
Complete amino-acid sequence of the construct product	MLVAGSELPRRPLPPAAQERDAEPRPPHGLQYL-GQIQHILRCGVRKDDRTGTLSVFGMQARYS-LRDEFPLLTTRVFWKGVLEELLWFIKGSTNA-KELSSKGVKIWDANGSRDFLDSLGFSTREEGD-LGPVYGFQWRHFGAEYRDMESDYSGQGVQDLQ-RVIDTIKTNPDDRRIIMCAWNPRDLPLMALPP-CHALCQFYVNVNSELSCQLYQRSGMDGLGVFPN-IASYALLTYMIAHITGLKPGDFIHTLGXAHIT

the substrate and cofactor are within a ternary complex, a nucleophilic attack by the thiol of the Cys195 residue occurs at the C6 atom of dUMP (Carreras & Santi, 1995). Human thymidylate synthase (hTS) has a similar structure (average r.m.s.d. on C α atoms of 1.1 Å), which was solved by molecular replacement using the prokaryotic enzyme as a search model (Schiffer *et al.*, 1995). The eukaryotic TS has a specific extension of 28 amino-acid residues in the N-terminal region and two insertions of 12 and eight amino acids at positions 117 and 146 of hTS. The catalytic mechanism of hTS involves a flexible loop (amino-acid residues 181–197) which can adopt two different conformations, involving a 180° twist from the inactive to the active form. In the active form, the loop is flipped out and positions the catalytic Cys195 residue in the active site. The reaction is initiated by the Cys195 residue attacking the 6-position of the pyrimidine base of the nucleotide. However, in the inactive form Cys195 is buried in the subunit interface (Berger *et al.*, 2004).

hTS also exhibits different kinds of regulatory mechanisms. hTS is an RNA-binding protein that is able to bind mRNA, including its own mRNA (Chu *et al.*, 1991), c-Myc (Chu *et al.*, 1995) and p53 (Chu *et al.*, 1999). The binding of apo hTS to its own mRNA inhibits its translation (Liu *et al.*, 2002). hTS is also regulated by monomer–homodimer equilibrium. The homodimeric form is mandatory for catalytic activity (Genovese *et al.*, 2010). Peptides have been designed which block the homodimer in its inactive form (Cardinale *et al.*, 2011). Several structures of hTS have been solved in an inactive form (Schiffer *et al.*, 1995; Phan, Steadman *et al.*, 2001) and in an active form with the substrate dUMP and the drug raltitrexed (Phan, Koli *et al.*, 2001). Several mutants have also been designed that favour the inactive form over the active form (Luo *et al.*, 2011). Other regions, such as amino-acid residues 129–135 and 195–206, corresponding to eukaryotic insertions have also been shown to be flexible. The loop containing amino-acid residues 181–197 can be stabilized in the inactive form by the point mutations M190K or A191K (Lovelace *et al.*, 2009). The inactive form is also stabilized by the phosphorylation of this loop by casein kinase 2 (Luo *et al.*, 2011). The active form can be favoured by the R163K mutation (Gibson *et al.*, 2008). The effect of dUMP binding on the active conformation of the R163K mutant (Gibson *et al.*, 2011)

and that of 1,3-propanediphosphonic acid (PDPA), which is an allosteric inhibitor, have been investigated. hTS complexed with this inhibitor can exist as an asymmetric dimer with only one subunit in the active form (Lovelace *et al.*, 2007). It has been shown that according to the crystallization conditions ionic strength can favour the inactive or the active form. High and low ammonium sulfate salt concentrations favour the inactive form (Lovelace *et al.*, 2005). Here, we present a new crystal form of wild-type apo hTS obtained from a low-salt condition but in the active form. It reveals that hTS is very flexible and can be trapped in the active conformation without the need to engineer the loop. This active form may help in the design of inhibitors which will block the active form and prevent the substrate from entering the active site.

2. Experimental procedures

2.1. Expression and purification

Human thymidylate synthase was produced and purified as described previously (Pedersen-Lane *et al.*, 1997). The *Escherichia coli* Rosetta 2 (DE3) pLysS strain transformed with plasmid pET-17xb expressing full-length hTS with a P2L mutation was grown at 37°C with permanent agitation in an orbital shaker (200 rev min⁻¹) in 2× YT medium (16 g tryptone, 10 g yeast extract, 5 g NaCl per litre of H₂O) supplemented with ampicillin (100 µg ml⁻¹) and chloramphenicol (25 µg ml⁻¹) until the optical density (OD) reached 0.6 at 600 nm. Protein expression was then induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After overnight (16–22 h) induction at 30°C, the cells were centrifuged at 7000g for 25 min at 4°C and the pellet was frozen at –80°C. The cells were resuspended in lysis buffer (buffer A; 500 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA, 20 mM NaH₂PO₄ pH 7.8, 1.0 mM β-mercaptoethanol, 2.0 µg ml⁻¹ DNase I). The cells were lysed by sonication and the cell extract was centrifuged at 9000g for 30 min. Ammonium sulfate was dissolved in the supernatant to a final concentration of 1.5 M. The solution was centrifuged at 9000g for 20 min and the supernatant was retained. Additional ammonium sulfate was dissolved in the supernatant to a final concentration of 2.4 M. After centrifugation at 9000g for 30 min, the pellet was retained and resuspended in a small volume of dialysis buffer (buffer B; 10 mM NaH₂PO₄ pH 7.8, 1.0 mM β-mercaptoethanol, 0.1 mM EDTA). The lysate was transferred into dialysis tubing (10–30 kDa cutoff) and dialysed overnight against dialysis buffer B. The dialysed lysate was loaded onto a DEAE Sepharose Fast-Flow column (GE Healthcare; column volume 50 ml) connected to an ÄKTApurifier (GE Healthcare) and equilibrated with buffer C (10 mM K₂HPO₄ pH 7.8, 1 mM β-mercaptoethanol, 0.1 mM EDTA). After extensive washing of the column with buffer C, elution was carried out with an increasing gradient of buffer D (75 mM K₂HPO₄ pH 7.8, 1 mM β-mercaptoethanol, 0.1 mM EDTA). The fractions that eluted at 10–70% buffer D were analyzed on 12.5% SDS–PAGE. The fractions containing the protein were dialysed against buffer E (1.0 M ammonium

sulfate, 20 mM K_2HPO_4 pH 7.8, 1 mM β -mercaptoethanol, 0.1 mM EDTA) and applied onto a Phenyl Sepharose Fast-Flow column (GE Healthcare; column volume 50 ml) connected to an ÄKTApurifier. After washing the column with buffer *E*, elution was carried out with an increasing gradient from 100% buffer *E* to 100% buffer *F*, where buffer *F* consists of 20 mM K_2HPO_4 pH 7.8, 1 mM β -mercaptoethanol, 0.1 mM EDTA. The fractions containing the protein were pooled and applied onto a Superdex 200 HiLoad 16/60 (GE Healthcare; column volume 120 ml) size-exclusion chromatography column equilibrated with buffer *G* (20 mM K_2HPO_4 pH 7.8, 150 mM NaCl, 1 mM β -mercaptoethanol, 0.1 mM EDTA). The protein was concentrated using Amicon Ultra-15 5K (Millipore) and the purity of the protein was estimated on 12.5% SDS-PAGE. Macromolecule-production information is given in Table 1.

2.2. Crystallization

The native protein was crystallized in the 'low-salt' conditions described previously (Lovelace *et al.*, 2005). Crystals were obtained at 18°C by the hanging-drop vapour-diffusion method from a 1:1 mixture of protein solution (at 10–17 mg ml⁻¹) and reservoir solution consisting of 25–30% PEG 4000, 30 mM ammonium sulfate, 20 mM β -mercaptoethanol, 100 mM Tris-HCl pH 9 (Table 2). Crystals were transferred into reservoir mother liquor containing 10% ethylene glycol before flash-cooling in liquid nitrogen.

2.3. X-ray data collection and processing

X-ray diffraction data were collected on the ID29 beamline at the ESRF, Grenoble, France using a Quantum Q315r

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well VDX plate, Hampton Research
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	10–17
Buffer composition of protein solution	20 mM K_2HPO_4 pH 7.8, 150 mM NaCl, 1 mM β -mercaptoethanol, 0.1 mM EDTA
Composition of reservoir solution	25–30% PEG 4000, 30 mM ammonium sulfate, 100 mM Tris-HCl pH 9, 20 mM β -mercaptoethanol
Volume and ratio of drop	2 μ l, 1:1 ratio
Volume of reservoir (ml)	1

detector (ADSC) and were processed using *XDS* (Kabsch, 2010). The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 91.79$, $b = 106.46$, $c = 101.50$ Å, $\alpha = 90$, $\beta = 108.99$, $\gamma = 90^\circ$ and with four molecules per asymmetric unit. The data-collection statistics are reported in Table 3. Molecular replacement was performed in *PHENIX* (Adams *et al.*, 2010) with *Phaser* (McCoy *et al.*, 2007) using the hTS structure (PDB entry 1ypv; Lovelace *et al.*, 2005) as a template model. Further refinement was performed in *PHENIX* and manual reconstruction was performed with *Coot* (Emsley *et al.*, 2010).

3. Results and discussion

In this work, we present the X-ray structure of native hTS without substrate and inhibitors. The protein was crystallized in the low-salt conditions (Lovelace *et al.*, 2005) that previously yielded an inactive conformation of the molecule. The protein readily crystallized in a different space group ($P2_1$). The $P2_1$ crystal contained four protein molecules in the

Figure 1

The structure of the two dimers of hTS in the asymmetric unit of the $P2_1$ crystal. For comparison, one subunit (in green) is superimposed with the active form of hTS (blue ribbon; PDB entry 1hvy).

Table 3

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Beamline	ID29, ESRF
Wavelength (Å)	0.98
Resolution range (Å)	46.55–2.99 (3.098–2.99)
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 91.79$, $b = 106.46$, $c = 101.50$, $\beta = 108.99$
Total reflections	80957 (4705)
Unique reflections	34338 (2412)
Multiplicity	2.4 (2.0)
Completeness (%)	91.67 (64.96)
Mean $I/\sigma(I)$	14.11 (1.75)
Wilson B factor (Å ²)	68.90
R_{merge}	0.071 (0.56)
R_{meas}	0.09
R_{work}	0.21 (0.33)
R_{free}	0.24 (0.32)
No. of non-H atoms	
Total	9191
Macromolecules	9131
Ligands	60
Water	0
No. of protein residues	1130
R.m.s.d., bonds	0.003
R.m.s.d., angles	0.77
Ramachandran favoured (%)	97
Ramachandran allowed (%)	2.82
Ramachandran outliers (%)	0.18
Clashscore	1.70
Average B factors (Å ²)	
Overall	74.90
Macromolecules	74.80
Ligands	91.40

asymmetric unit assembled as two dimers (Fig. 1). The structure was refined by applying torsion NCS restraints. The four protein molecules therefore have the same structure, with an average pairwise r.m.s.d. of 0.25 Å. The inactive structure (PDB entry 1ypv) was chosen as a search model owing to its crystallization under similar conditions (pH 8.5). However, after reconstruction of the loop containing amino-acid residues 181–197 there was no doubt that the hTS structure was in the active form (Fig. 2) in the four molecules of the asymmetric unit. The first 25 amino-acid residues and the last five amino-acid residues are missing from the structures, but all of the loops, including loop 107–128, are well defined with no chain breaks in the electron density.

Our hTS structure superposes with previously reported apo R163K mutant, bound R163K mutant and $\Delta 7$ –29 deletion mutant ternary-complex active forms (PDB entries 2rd8, 3h9k and 1hzw, respectively) with an r.m.s.d. of less than 0.3 Å (Gibson *et al.*, 2008, 2011; Almog *et al.*, 2001). In the apo R163K mutant of hTS, a phosphate ion mimics the phosphate group of the dUMP substrate (Gibson *et al.*, 2008). In our crystal structure, a sulfate ion is located at exactly the same position as the dUMP phosphate. This ion is at the interface of the dimer, interacting with the Arg50 and Arg215 residues from one subunit and the Arg175 and Arg176 residues from the other subunit (Fig. 3). PDB entry 1hzw is an engineered form lacking the 23 amino-acid residues in the N-terminal region. The N-terminal extension is specific to eukaryotes and may be important for the regulation and stability of hTS. It has been hypothesized that a hydrophobic pocket formed by

Phe137, Gln138, Phe142, Gly143, Trp182 and Leu187 accommodates Val3 in the inactive form (Lovelace *et al.*, 2005). The importance of the Val3 residue has been studied and density corresponding to this residue may appear in crystals of the V3L mutant in the inactive form (Huang *et al.*, 2010). However, the N-terminal region 1–25 has never been observed

Figure 2

The structure of apo hTS is in the active form. (a) Superimposition of apo hTS structures solved in different space groups. Apo hTS in the active form (this work) is represented in cyan with loop 107–128 in green and loop 181–198 in blue. Apo hTS in the inactive form (PDB entry 1ypv) is shown in pink with loop 181–197 in red. Cys195 is shown in ball-and-stick representation. (b) Superimposition of active apo hTS (cyan) on active hTS (ternary complex; PDB entry 1hzw). Ligands are omitted for clarity.

in any crystal in the active form and is also missing in the electron-density map of our structure.

Although the crystallization was performed in low-salt conditions similar to those used by Lovelace *et al.* (2005), we obtained a new crystal of apo hTS. Since loop 107–128, which has been shown to be flexible in the inactive form, and the catalytic loop 181–195 are not involved in crystal packing in space group $P2_1$, some differences may favour the active form in this particular space group. Differences may arise from the hTS construct. Lovelace and coworkers used an N-terminally extended construct of hTS (Phan, Steadman *et al.*, 2001), but here we used the wild-type hTS sequence. The 42-amino-acid N-terminal extension may stabilize the N-terminus. Indeed, Lovelace and coworkers interpreted residual density in a hydrophobic pocket as Val3.

Figure 3
(a) The sulfate ion at the interface of the dimer. The two subunits are coloured green and cyan. The $2F_o - F_c$ electron density at 2σ for the ion is represented in orange. Residues that interact with sulfate are highlighted. (b) The dUMP substrate in the 1hvy structure is represented in the same orientation to illustrate that the phosphate occupies the sulfate position.

Another difference could be the amount of β -mercaptoethanol. In the 1ypv structure the cysteine residues were built as hydroxymethylthiocysteines. All structures are formed of dimers that are related by either crystallographic symmetry or noncrystallographic symmetry. In the 1ypv structure the hTS dimer is related by twofold crystallographic symmetry, but in our structure the dimers are all related by noncrystallographic symmetry. Cys180 is near the twofold axis of the dimer. The Cys180 residues of both monomers are facing each other and are in position to form a disulfide bridge. There is no electron density for this disulfide bridge in our structure, and the two sulfhydryl groups are too distant (3.8 Å in both dimers). In the 1ypv structure the sulfhydryl groups of Cys180 and the symmetry-related residue are 8.7 Å apart. They cannot be closer because of the ethyl modification of the cysteine by β -mercaptoethanol. This modification may change the dimer interface and favour the inactive form. Cys180 has previously been identified as a critical residue by mutagenesis studies (Lin *et al.*, 2003).

Since everything else remained similar, the difference may also be a consequence of the pH. At pH 9 sulfate may make a stronger interaction with the active site. In our structure, a sulfate ion occupies the position of the dUMP phosphate. Other sulfate ions are located on the surface of the protein but do not bridge the subunits. In only one molecule, a sulfate ion occupies the position of the thienyl group of the drug Tomuxed (raltitrexed). The equilibrium between the active and the inactive form of hTS has been extensively studied. It has been shown that hTS exists as a mixture of inactive and active forms in solution. The equilibrium is displaced towards the inactive form by the presence of phosphate or sulfate ions, while the presence of dUMP drives the equilibrium towards the active form (Phan, Steadman *et al.*, 2001). Thus, sulfate ions stabilize the substrate-binding pocket in the active conformation. We show here that both states can be captured in different crystals obtained from similar conditions.

4. Conclusion

We solved the low-resolution structure of hTS in a new crystal form obtained at a low ammonium sulfate concentration. A sulfate ion is in the same position as the phosphate group of dUMP. A sulfate ion is bound to an Arg residue from both subunits of the dimer and seems to stabilize the dimer in the active form. This result suggests that a charge effect may also be important for the development of new drugs.

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References

Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.

- Allegra, C. J., Chabner, B. A., Drake, J. C., Lutz, R., Rodbard, D. & Jolivet, J. (1985). *J. Biol. Chem.* **260**, 9720–9726.
- Almog, R., Waddling, C. A., Maley, F., Maley, G. F. & Van Roey, P. (2001). *Protein Sci.* **10**, 988–996.
- Berger, S. H., Berger, F. G. & Lebioda, L. (2004). *Biochim. Biophys. Acta*, **1696**, 15–22.
- Cardinale, D. *et al.* (2011). *Proc. Natl Acad. Sci. USA*, **108**, E542–E549.
- Carreras, C. W. & Santi, D. V. (1995). *Annu. Rev. Biochem.* **64**, 721–762.
- Chu, E., Copur, S. M., Ju, J., Chen, T., Khleif, S., Voeller, D. M., Mizunuma, N., Patel, M., Maley, G. F., Maley, F. & Allegra, C. J. (1999). *Mol. Cell. Biol.* **19**, 1582–1594.
- Chu, E., Koeller, D. M., Casey, J. L., Drake, J. C., Chabner, B. A., Elwood, P. C., Zinn, S. & Allegra, C. J. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 8977–8981.
- Chu, E., Takechi, T., Jones, K. L., Voeller, D. M., Copur, S. M., Maley, G. F., Maley, F., Segal, S. & Allegra, C. J. (1995). *Mol. Cell. Biol.* **15**, 179–185.
- Costi, M. P., Ferrari, S., Venturelli, A., Calò, S., Tondi, D. & Barlocco, D. (2005). *Curr. Med. Chem.* **12**, 2241–2258.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Finer-Moore, J. S., Santi, D. V. & Stroud, R. M. (2003). *Biochemistry*, **42**, 248–256.
- Garg, D., Henrich, S., Salo-Ahen, O. M., Myllykallio, H., Costi, M. P. & Wade, R. C. (2010). *J. Med. Chem.* **53**, 6539–6549.
- Genovese, F., Ferrari, S., Guaitoli, G., Caselli, M., Costi, M. P. & Ponterini, G. (2010). *Protein Sci.* **19**, 1023–1030.
- Gibson, L. M., Celeste, L. R., Lovelace, L. L. & Lebioda, L. (2011). *Acta Cryst.* **D67**, 60–66.
- Gibson, L. M., Lovelace, L. L. & Lebioda, L. (2008). *Biochemistry*, **47**, 4636–4643.
- Heidelberger, C., Chaudhuri, N. K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Plevin, E. & Scheiner, J. (1957). *Nature (London)*, **179**, 663–666.
- Huang, X., Gibson, L. M., Bell, B. J., Lovelace, L. L., Peña, M. M. O., Berger, F. G., Berger, S. H. & Lebioda, L. (2010). *Biochemistry*, **49**, 2475–2482.
- Jarmuła, A. (2010). *Mini Rev. Med. Chem.* **10**, 1211–1222.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Lin, X., Liu, J., Maley, F. & Chu, E. (2003). *Nucleic Acids Res.* **31**, 4882–4887.
- Liu, J., Schmitz, J. C., Lin, X., Tai, N., Yan, W., Farrell, M., Bailly, M., Chen, T. & Chu, E. (2002). *Biochim. Biophys. Acta*, **1587**, 174–182.
- Lovelace, L. L., Gibson, L. M. & Lebioda, L. (2007). *Biochemistry*, **46**, 2823–2830.
- Lovelace, L. L., Johnson, S. R., Gibson, L. M., Bell, B. J., Berger, S. H. & Lebioda, L. (2009). *Protein Sci.* **18**, 1628–1636.
- Lovelace, L. L., Minor, W. & Lebioda, L. (2005). *Acta Cryst.* **D61**, 622–627.
- Luo, B., Repalli, J., Yousef, A. M., Johnson, S. R., Lebioda, L. & Berger, S. H. (2011). *Protein Sci.* **20**, 87–94.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Montfort, W. R. & Weichsel, A. (1997). *Pharmacol. Ther.* **76**, 29–43.
- Pedersen-Lane, J., Maley, G. F., Chu, E. & Maley, F. (1997). *Protein Expr. Purif.* **10**, 256–262.
- Phan, J., Koli, S., Minor, W., Dunlap, R. B., Berger, S. H. & Lebioda, L. (2001). *Biochemistry*, **40**, 1897–1902.
- Phan, J., Steadman, D. J., Koli, S., Ding, W. C., Minor, W., Dunlap, R. B., Berger, S. H. & Lebioda, L. (2001). *J. Biol. Chem.* **276**, 14170–14177.
- Salo-Ahen, O. M. & Wade, R. C. (2011). *Proteins*, **79**, 2886–2899.
- Schiffer, C. A., Clifton, I. J., Davisson, V. J., Santi, D. V. & Stroud, R. M. (1995). *Biochemistry*, **34**, 16279–16287.
- Srivastava, V., Gupta, S. P., Siddiqi, M. I. & Mishra, B. N. (2010). *Bioinformation*, **4**, 357–365.
- Stroud, R. M. & Finer-Moore, J. S. (2003). *Biochemistry*, **42**, 239–247.