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Crystal structures of thymidylate synthase from nematodes, *Trichinella spiralis* and *Caenorhabditis elegans*, as a potential template for species-specific drug design

Abstract: Crystal structures were solved of the binary complexes *Trichinella spiralis* and *Caenorhabditis elegans* thymidylate synthases with deoxyuridine monophosphate (dUMP), with crystals obtained by the vapor diffusion method in hanging drops. For the *T. spiralis* thymidylate synthase-dUMP complex, the diffraction data were collected at the BESSY Synchrotron to 1.9 Å resolution. The crystal belongs to the space group P1 with two dimers in the asymmetric unit (ASU). For the *C. elegans* TS-dUMP complex crystal, the diffraction data were collected at the BESSY Synchrotron to 2.48 Å resolution, and the crystal belongs to the space group P 32 2 1, with two monomers (one dimer) in the ASU. Structural comparisons were made of both structures and each of them with the corresponding mouse thymidylate synthase complex.

Keywords: 3D structure; nematode; thymidylate synthase.

Enzymes: thymidylate synthase (EC 2.1.1.45).

PDB reference: *Trichinella spiralis* thymidylate synthase-dUMP complex, 4G9U; *Caenorhabditis elegans* thymidylate synthase-dUMP complex, 4IRR.

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Introduction

Thymidylate synthase (TS; EC 2.1.1.45) is an important target in chemotherapy, catalyzing the conversion of deoxyuridine monophosphate (dUMP) and $N^{5,10}$-methylenetetrahydrofolate (mTHF) to deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF) [1]. Enzyme levels in two species of nematodes, parasitic *Trichinella spiralis* (causing a serious disease, trichinellosis) and free-living *Caenorhabditis elegans* (considered a model for parasitic nematodes), have been shown to remain high throughout the life cycle of each parasite, and the latter concerned also developmentally arrested, non-growing larvae, *T. spiralis* muscle, and *C. elegans* dauer larvae [2–4]. Thus, the enzyme might constitute a potential target for nematode-selective chemotherapy.

As TS protein, and particularly its active center, belongs to the most conservative, inhibitors designed as substrate/cofactor analogs are beyond hope as candidates for species-selective inhibitors of the pathogen versus the mammalian enzyme. A promising way of solving such problems is virtual selection of an inhibitor, based on comparison of the 3D structures of pathogen and mammalian enzyme proteins, aimed at non-conservative protein fragments differing between enzymes from both groups [5]. To make such an approach possible, crystal structures were solved of *T. spiralis* and *C. elegans* binary TS-dUMP complexes and structural comparisons were made with the corresponding mouse TS-dUMP complex [6]. Unfortunately, a similar comparison with the human enzyme has been so far impossible, as an analogous structure of the non-mutant human TS-dUMP complex is not available in the Protein Data Bank. However, in view of high similarity (94.9%) between mouse and human TS protein sequences, with 88.8% of these sequences being identical, indicated by the sequence alignment with FASTA [7], the conclusions derived upon comparison with the mouse enzyme should hold true for the human enzyme. Further comparisons with ternary human and mouse complexes are planned but these are beyond the scope of the present paper.

Materials and methods

Crystallization and data collection

Each *T. spiralis* and *C. elegans* TS recombinant protein, overexpressed and purified as previously described [3, 8], was dialyzed against 5 mM Tris HCl buffer, pH 7.5, containing 5 mM DTT and then concentrated using an Amicon Centricon centrifugal filter. Crystals were grown by the vapor diffusion method in hanging drops at room temperature (*T. spiralis* TS) or at 4°C (*C. elegans* TS). With the *T. spiralis* enzyme, 3.5 µL of the protein 20 mg/mL solution, containing 10 mM dUMP, and 2 µL of the well solution were mixed and allowed to equilibrate with 0.5 mL of the well solution, containing 0.1 M Tris HCl, pH 7.9; 0.08 M $MgCl_2$ and 18.5% (w/v) PEG 4000. With the *C. elegans* enzyme, 2.5 µL of the protein 23 mg/mL solution, containing 9.5 mM dUMP, and 2.5 µL of the well solution were mixed and allowed to equilibrate with 0.5 mL of the well solution, containing 0.1 M Bis Tris pH 7.2, 0.2 M sodium acetate, 15% PEG 3350.

X-Ray diffraction data were collected from a single flash-frozen crystal at the BESSY Synchrotron using an X-ray wavelength of 0.918 Å.

Data processing: structure determination and refinement

Data were processed with the use of DENZO and SCALEPACK [9]. The structure was determined by molecular replacement carried out with Phaser from the CCP4 package [10], using the mouse TS ternary complex with N^6-hydoxy-dCMP and DHF as the search model. The correctness of the structure was evaluated using Sfcheck and Procheck from the CCP4 suite. Some X-ray data and model refinement parameters are presented in Table 1.

Results and discussion

The structure model of the parasitic nematode *T. spiralis* TS complexed with dUMP consists of two dimers (dimer AB shown in Figure 1). It comprises the following amino acid residues: 17–300/chain A, 17–304/chain B, 18–303/chain C, and 18–299/chain D. The N- and C-termini, being not ordered, are not visible in the electron density map. In each of the four active centers a clear electron density corresponding to the dUMP molecule is present. The

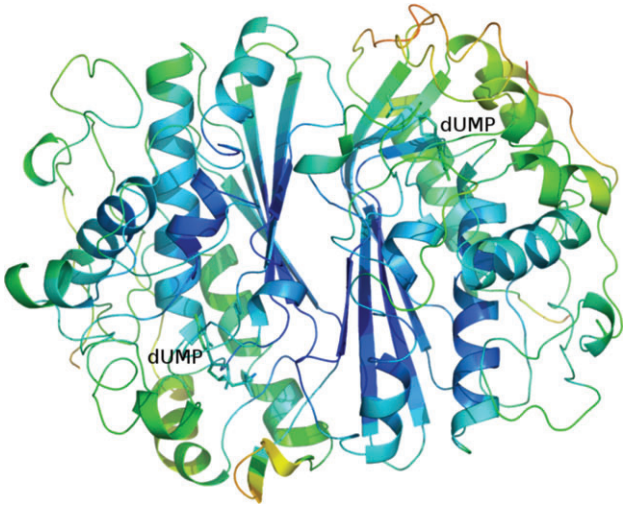

Figure 1 Dimer AB of *Trichinella spiralis* TS-dUMP structure model. Coloring is according to B-factor values. Both active centers contain the substrate molecule.

Table 1 Data collection and refinement statistics for structures of *Trichinella spiralis, Caenorhabditis elegans*, and mouse TS-dUMP binary complexes.

Crystal and refinement parameters	*T. spiralis* TS-dUMP	*C. elegans* TS-dUMP	Mouse TS-dUMP [6]
Lattice type	Triclinic	Monoclinic	Monoclinic
Space group	P1	P 32 2 1	C 1 2 1
Unit cell parameters	A=51.695 Å	a=135.09 Å	a=160.35 Å
	b=65.914 Å	b=135.09 Å	b=88.54 Å
	c=96.511 Å	c=155.77 Å	c=136.76 Å
	α=85.305°	α=90.00°	α=90.00°
	β=85.327°	β=90.00°	β=95.99°
	γ=67.117°	γ=120.00°	γ=90.00°
Resolution range, Å	19.96–1.90	29.2–2.48	19.95–1.70
Number of unique reflections	89,350	58,033	207,806
Redundancy	5	6.4	6.4
$<I/\sigma(I)>$	6.1	8.1	16.9
Number of reflections used in refinement	89,301	55,056	205,349
R factor, %	16.5	22.4	23.6
R_{free} factor, %	22.0	26.3	29.2
RMS bond, Å	0.022	0.0117	0.022
RMS angle, °	1.834	1.5183	1.956

distance between dUMP C6 and catalytic Cys S atoms is in each subunit longer than 3 Å, pointing to the lack of covalent bond. The model reveals a high degree of similarity to the mouse structure (model of mTS-dUMP complex; PDB ID: 4E5O). The Cα root mean square deviation (RMSD) for *T. spiralis* TS-dUMP/chain $A_{(22Glu-299Pro)}$ versus mouse TS-dUMP/chain $A_{(23Gly-299Pro)}$ amounts to 0.798 Å (the sequence identity for this range being 67.3%). Of interest is that *T. spiralis* catalytic Cys189 appears capable of adopting two alternative conformations (Figures 2 and 3). Also the next residue, His190, appears to acquire two conformations: the minor one resembles the His conformation in all determined models of mouse enzyme (and also in human TS model of the complex with dUMP and Tomudex; PDB ID: 1I00) and the dominant one leans towards the Tyr224 OH group. The primary His190 conformation in *T. spiralis* TS is similar to that of the corresponding His conformation in each *C. elegans* and *Escherichia coli* TS (cf. PDB ID: 1BID). Instead of mouse TS Leu192 and

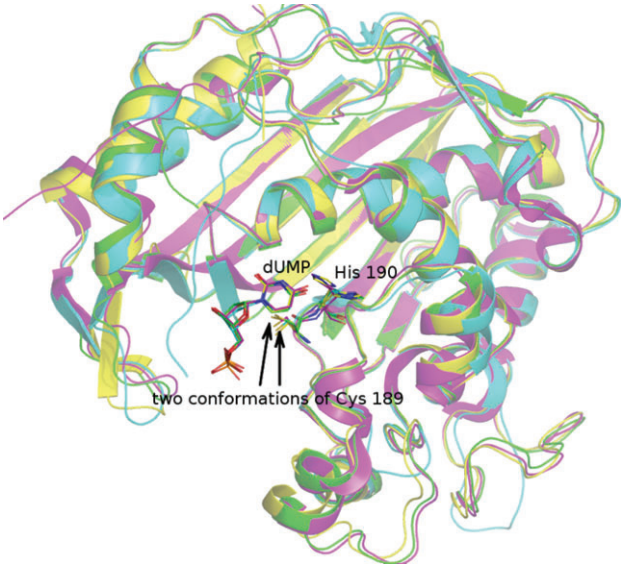

Figure 2 Superimposition of monomers A of *Trichinella spiralis* (yellow), *Caenorhabditis elegans* (green), mouse (pink), and *Escherichia coli* (cyan) TSs, depicting the substrate molecule, and catalytic Cys and His (*T. spiralis*/mouse TS His190) residues. For each subunit of the parasitic nematode, TS model His190 can adopt two alternative conformations; whereas the dominant conformation is similar to that of the corresponding *C. elegans* TS and *E. coli* TS His residue, the minor one resembles His residue in mouse TS. The image also shows two conformations of the *T. spiralis* TS catalytic Cys.

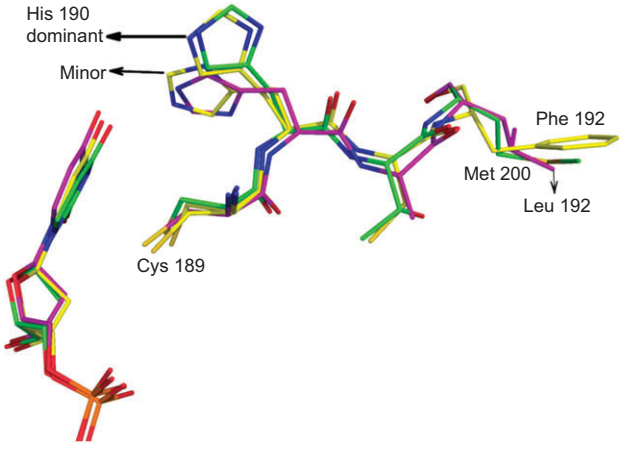

Figure 3 Substrate molecule, catalytic Cys189, and the next fragment of three residues of *Trichinella spiralis* TS (chain A/yellow), compared with *Caenorhabditis elegans* TS (green) and mouse TS (pink). The mouse TS Leu192 corresponds to Phe192 residue in *T. spiralis* TS and Met200 residue in *C. elegans* TS.

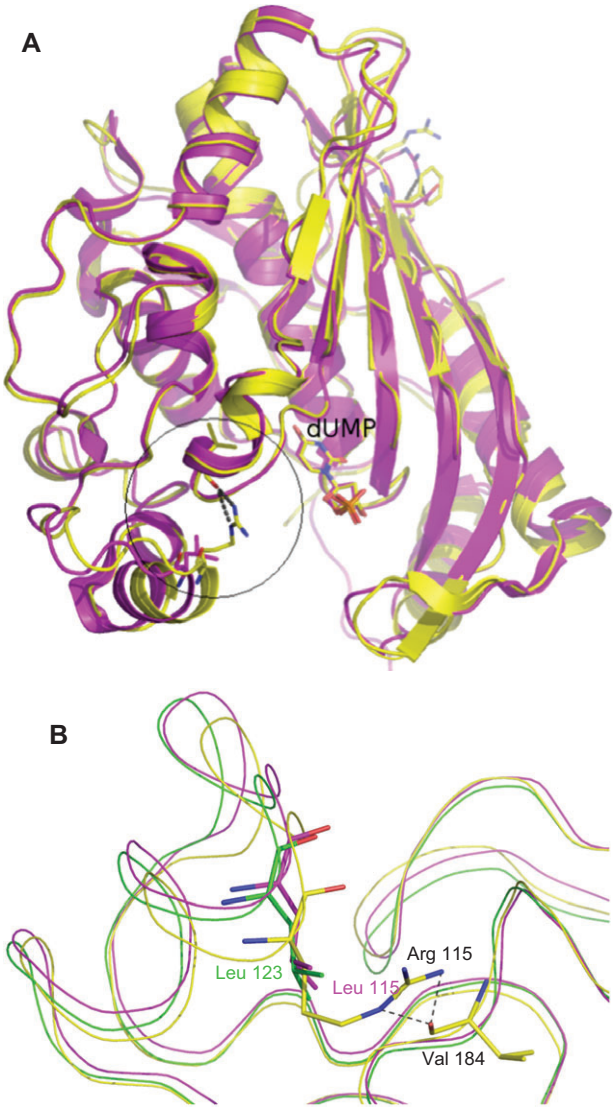

Figure 4 *Trichinella spiralis* TS (yellow) Arg115, equivalent of mouse TS (pink) Leu115 and *Caenorhabditis elegans* (green) Leu123, hydrogen bonded to Val184. Panel (B) shows the circled fragment of (A) in magnification.

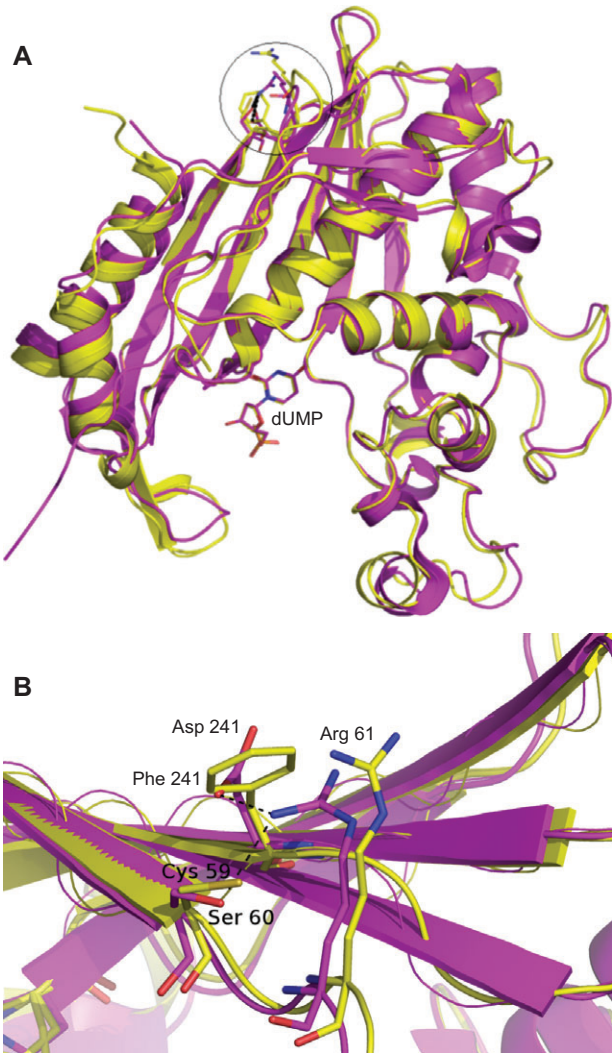

Figure 5 *Trichinella spiralis* TS (yellow) Cys59 and Phe241 are in the case of mouse and human (structure 1I00) replaced by Ser and Asp residues, correspondingly. This change involves adjacent arginine residue conformation modification. Panel (B) shows the circled fragment of (A) in magnification.

human TS Leu198, the enzyme molecules of *T. spiralis* and *E. coli* contain Phe residue (e.g., *T. spiralis* Phe192), and *C. elegans* TS contains Met (Met200) residue (Figure 3). Comparing TS active sites, *T. spiralis* Met206 is replaced by leucine residue in both *C. elegans* and mouse TS (cf. PDB 1BID and 1I00).

Interestingly, the overall similarity between the *C. elegans* and mouse enzyme structures appears higher than that between *T. spiralis* and mouse TSs. The Cα RMSD for *C. elegans* TS-dUMP/chain $A_{(30Asp\text{-}307Pro)}$ versus mouseTS-dUMP/chain $A_{(23Gly\text{-}299Pro)}$ amounts to 0.582 Å. In spite of a high degree of similarity, superimposing the mouse over *T. spiralis* enzyme structures shows approximately 40 significant differences with regard to the physicochemical character of amino acid residues or the distance between them. In most cases, amino acid substitution results in rupture of the hydrogen bonding network and is compensated by water molecules. Outside the active site, a substitution of *T. spiralis* Arg115 to leucine residue in mouse (Leu115) and *C. elegans* (Leu123) TS leads to the loss of two structurally essential hydrogen bonds, connecting with a residue (e.g., *T. spiralis* Val184) from a different part of the amino acid chain (Figure 4). Another interesting structural distinction concerns the presence of Cys59 and Phe241 in the parasitic nematode TS instead of Ser60 and Asp241 in mouse TS (Figure 5). Together with altered conformation of the adjacent arginine residue (*T. spiralis* Arg61), the two sets of residues form distinctly different local protein surfaces.

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