Crystal structure of a deletion mutant of human thymidylate synthase $\Delta(7-29)$ and its ternary complex with Tomudex and dUMP

RAMI ALMOG, CHRISTOPHER A. WADDLING,¹ FRANK MALEY, GLADYS F. MALEY, AND PATRICK VAN ROEY

Division of Molecular Medicine, Wadsworth Center, Albany, New York 12201, USA

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Abstract

The crystal structures of a deletion mutant of human thymidylate synthase (TS) and its ternary complex with dUMP and Tomudex have been determined at 2.0 Å and 2.5 Å resolution, respectively. The mutant TS, which lacks 23 residues near the amino terminus, is as active as the wild-type enzyme. The ternary complex is observed in the open conformation, similar to that of the free enzyme and to that of the ternary complex of rat TS with the same ligands. This is in contrast to *Escherichia coli* TS, where the ternary complex with Tomudex and dUMP is observed in the closed conformation. While the ligands interact with each other in identical fashion regardless of the enzyme conformation, they are displaced by about 1.0 Å away from the catalytic cysteine in the open conformation. As a result, the covalent bond between the catalytic cysteine sulfhydryl and the base of dUMP, which is the first step in the reaction mechanism of TS and is observed in all ternary complexes of the *E. coli* enzyme, is not formed. This displacement results from differences in the interactions between Tomudex and the protein that are caused by differences in the environment of the glutamyl tail of the Tomudex molecule. Despite the absence of the closed conformation, Tomudex inhibits human TS ten-fold more strongly than *E. coli* TS. These results suggest that formation of a covalent bond between the catalytic cysteine and the substrate dUMP is not required for effective inhibition of human TS by cofactor analogs and could have implications for drug design by eliminating this as a condition for lead compounds.

Keywords: Thymidylate synthase; Tomudex; dUMP; crystal structure; ternary complex

Thymidylate synthase (TS) (EC 2.1.1.45) converts dUMP to dTMP and is required for DNA synthesis in most organisms (Carreras and Santi 1995). Consequently, TS has been studied extensively as a drug target (Stout et al. 1999). A well-defined structure of human TS can be a very useful tool for

the development of novel drugs for cancer therapy or as TS inhibitors that are selective for pathogenic organisms.

The first step in the TS reaction is a nucleophilic reaction at the C6 position of dUMP with the cysteine sulfhydryl. This is followed by a unique mechanism of reductive methylation that involves the transfer of a hydroxymethyl group from 5,10-methylenetetrahydrofolate (5,10-CH₂H₄PteGlu) to the 5-position of dUMP, accompanied by a reducing hydrogen from the 6-position of its tetrahydropteridine ring (Lorenson et al. 1967). The resulting products, dTMP and H₂PteGlu, are released, in that order, from the enzyme following reduction of an exocyclic-methylene intermediate associated with the 5-position of dUMP (Carreras and Santi 1995). How the reducing hydrogen is actually transferred to the intermediate and how the hydrogen at the 5-position of

Reprint requests to: Patrick Van Roey, Wadsworth Center, P.O. Box 509, Albany, NY 12201-0509; e-mail: vanroey@wadsworth.org; fax: (518) 474 7992.

¹Present address: Department of Biochemistry and Biophysics, University of California/San Francisco, San Francisco, CA 94143.

Abbreviations: ecTS, *Escherichia coli* thymidylate synthase; hTS, human thymidylate synthase; mhTS, $\Delta(7-29)$ human thymidylate synthase; PEG, polyethylene glycol; r.m.s.d., root-mean-square-deviation; rTS, rat thymidylate synthase.

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dUMP is released to the surrounding water has been the subject of much speculation. Irrefutable proof for this step has not been presented to date, although recent sulphydryl trapping experiments have demonstrated the existence of this putative intermediate (Barrett et al. 1998).

Many TS substrate and cofactor analogs have been studied as potential anticancer drugs (Stout et al. 1999). Among the cofactor analogs, Tomudex (Fig. 1) is one of the most extensively evaluated compounds and has been approved for cancer treatment in Europe.

The three-dimensional structure of TS has been reported for several different organisms, including *Lactobacillus casei* (Finer-Moore et al. 1993), *Escherichia coli* (ecTS) (Matthews et al. 1990; Montfort et al. 1990), *Bacillus subtilis* (Stout et al. 1998; Fox et al. 1999), and *Pneumocystis carinii* (Anderson et al. 2000) as well as rat (rTS) (Sotelo-Mundo et al. 1999) and human (hTS) (Schiffer et al. 1995). ecTS has become the standard for structural studies because it appears to be most suitable for crystallographic analysis, readily yielding high-quality crystals and high-resolution structures in both the presence and absence of ligands (Stout et al. 1999). In contrast, mammalian TS complexes have been difficult to crystallize, with the only known structures consisting of two crystal forms of the ternary complex of rTS (Sotelo-Mundo et al. 1999) with dUMP and Tomudex, with the best crystal form diffracting to 2.7 Å resolution. The only report to date for the human enzyme consists of a 3.0 Å resolution structure of uncomplexed hTS (Schiffer et al. 1995), but the coordinates for this structure are not available from the Protein Data Bank. During mutagenesis experiments aimed at generating hTS protein suitable for high-resolution crystallographic studies, we inadvertently encountered a deletion mutant, Δ7–29 hTS (mhTS). The deletion occurs near the amino terminus, in an area of the protein not required for activity or for homodimer association. Therefore, it is not surprising that the activity of mhTS

is comparable to that of wild-type hTS (Pedersen-Lane et al. 1997). This protein crystallized well, and the crystals diffract to 2.0 Å resolution. In this article, we present the structure of mhTS apoenzyme and its ternary complex with dUMP and Tomudex.

Results and Discussion

Structure determination

Crystals of mhTS were grown by hanging-drop vapor-diffusion methods using polyethylene glycol (PEG) 3350, buffered with potassium phosphate (pH 7.4) as the precipitating agent. The mhTS crystals belong to space group P3₁21 with cell parameters $a, b = 90.2$ Å, $c = 143.3$ Å and contain one homodimer in the asymmetric unit. The crystals of free mhTS diffract to 2.3 Å resolution using a rotating anode source and to 2.0 Å resolution at a synchrotron source (beamline X12C, NSLS). The structure was determined by molecular replacement methods, using the structure of rTS as the model. The ternary complex was prepared by diffusing dUMP and Tomudex into crystals of mhTS. Crystals of the ternary complex diffract to 2.5 Å resolution on a rotating anode source. The final models of free and complexed mhTS include residues 5 to 6 and 30 to 307, and residues 6 and 30 to 306, respectively. Electron density is missing for residues 1 to 4 and 308 to 313, suggesting that these residues are disordered. Both active sites of the free mhTS dimer contain a phosphate ion, recruited from the purification and crystallization buffer, that occupies the site of the phosphate of dUMP. Data collection and refinement statistics are listed in Table 1. Figure 2 shows the final (2F_o–F_c)–electron density map for the ligands. The atomic coordinates and structure factors have been submitted to the Protein Data Bank, RCSB (entries 1HZW and 1I00).

Fig. 1. Schematic diagram of the chemical structure of the TS cofactor analog Tomudex (ZD1694).

Table 1. Crystallographic data

	Free mhTS	Ternary complex
Resolution (Å)	30.0 – 2.0	30.0 – 2.5
Completeness (%) ^a	94.7/87.2	92.8/87.9
Redundancy	4.1	5.6
I/σ (% > 2)	74.2/41.7	80.8/48.5
R _{mer}	0.044/0.232	0.083/0.490
R _{crys}	0.205	0.207
R _{free}	0.238	0.261
r.m.s.d. bonds (Å)	0.005	0.007
angles (°)	1.4	1.35
dihedrals (°)	23.1	22.6
Average B		
protein (Å ²)	26.0	46.1
ligands (Å ²)	38.5	62.6
water (Å ²)	31.2	39.6
Most favored phi/psi (%)	90.0	88.2

^a Full data set/highest resolution shell.

Fig. 2. Stereodescription of the final ($2F_o - F_c$)-map for the ligands bound to monomer A of the mhTS homodimer. The electron density map is contoured at the 1.25 σ -level. Figure prepared using the program SETOR (Evans 1993).

Overall structure of mhTS

The structure of mhTS offers two major advantages over the previously reported structure of hTS: much higher resolution (2.0 Å compared to 3.0 Å), and the presence of a homodimer in the asymmetric unit, which can be important for studies of inhibitor interaction since TS appears to be a half-of-the-sites activity enzyme under physiological conditions (Maley et al. 1995; Anderson et al. 1999). As expected, the overall fold of mhTS is very similar to that of all other known TSs and nearly identical to that of rTS. The topology of a TS monomer consists of an α/β -fold containing 7 α -helices and 10 β -strands, arranged in three layers: a six-stranded mixed β -sheet, a long α -helix across the sheet flanked by two shorter helices, and a mixed layer containing the remaining four helices and two antiparallel two-stranded β -sheets. The large β -sheets from the monomers stack against each other to form the dimer interface. The dimer contains two active sites, one within each monomer, except that two arginine residues from the second monomer (Arg175 and Arg176 in hTS) participate in the binding of the phosphate group in the first monomer.

Monomers 1 and 2 are related by pseudo-twofold symmetry and are essentially indistinguishable in geometry, with an r.m.s.d. for all α -carbon atoms of 0.35 Å. The active site areas of TSs are formed by a set of 17 core residues (mhTS sequence numbers), Arg50, Phe80, Gly83, Asn112, Tyr135, Cys195, His196, Arg215, Ser216, Leu221, Gly222, Phe225, Asn226, His256, Tyr258, and Arg175 and Arg176 from the second monomer. While most of these residues are conserved, differences are observed in specific residues

among the set; most notably, residues 80, 83 and 112 differ between bacterial and mammalian TSs. Superposition of the core residues from monomers 1 and 2 of free mhTS indicates that the geometries of the active site areas are very similar, with an r.m.s.d. of 0.27 Å (0.82 Å) for the α -carbon (side chain) atoms.

The structure of free mhTS is very similar to that of rTS (Sotelo-Mundo et al. 1999), although the latter was reported as a ternary complex with dUMP and Tomudex. The r.m.s.d. for all α -carbon atoms of monomer A of free mhTS with monomer A of complexed rTS is only 0.61 Å. In the structure of rTS, residues 1–20 and 301–307 are disordered, as are the 27 amino-terminal residues in the previous report regarding hTS (Schiffer et al. 1995). Therefore, one can assume that the $\Delta(7-29)$ mutation in mhTS results in shortening of a section of the molecule that is highly flexible. The absence of a long disordered N-terminus may have contributed to the increased suitability for crystallization of mhTS compared to full-length hTS. Glu30 is the first residue that makes intramolecular contacts, in the form of hydrogen bonds to Thr75 and Thr76. This interaction is conserved among all known TSs and has been shown to be essential for enzymatic activity in *E. coli* and *L. casei* TS (Carraras and Santi 1995). This is identical to the first N-terminal contact seen for rTS, further confirming that the deletion mutation does not affect the molecular conformation. A similar relationship has been reported for ecTS, but in this instance the glutamic acid residue is replaced by N-carbamoyl methionine (Fauman et al. 1994).

Other than a longer amino-terminus, the main sequence differences between mhTS (or rTS) and ecTS, are insertions in two surface loops of mhTS, residues 115–126 and 145–152. The relatively weak electron density and high thermal parameters for these loops in the mhTS structure indicate that they are highly flexible, a finding that was also obtained in the rTS structure. Despite the insertions and differences the geometries of some surface loops, the overall structure of mhTS is very similar to that of uncomplexed ecTS (Perry et al. 1990), with an r.m.s.d. of 0.9 Å for all α -carbon atoms.

A loop in the active site area of hTS involving residues 180 to 204 was reported by Schiffer et al. (1995) to adopt an unusual conformation that buries the catalytic cysteine, Cys195. A conformational change in this loop was implicated in a mechanism to facilitate substrate docking. However, in the current structure this loop adopts the conformation seen in all other TS structures, with the side chain of Cys195 extending into the active site cavity and in position to interact with C6 of dUMP. It is possible that the difference in the conformations of this loop can be attributed to the presence of a phosphate anion in the mhTS active site. This anion mimics the binding of the dUMP phosphate group and could stabilize a conformation that more closely resembles the substrate-bound conformation. However, the fact that Schiffer et al. (1995) report the presence of electron

density for a large anion thought to be a sulfate anion recruited from the crystallization buffer, at the same site, reduces the likelihood of an effect of the anion on the conformation of this loop.

Structure of the mhTS-Tomudex-dUMP ternary complex

The overall structure of mhTS in its ternary complex is almost identical to that of the free enzyme, with an r.m.s.d. for all α -carbon atoms of 0.46 Å. The conformations of the core residues (Fig. 3) do not change significantly upon dUMP binding, with r.m.s.d.'s of 0.44 Å (1.1 Å) and 0.29 Å (1.2 Å) for the superposition of the α -carbon (side chain) atoms of monomers 1 and 2, respectively. The ternary complex with dUMP and Tomudex, like the same complex of rTS, is in the "open" conformation and does not adopt the "closed" conformation observed for the Tomudex-dUMP complex of ecTS (Rutenber and Stroud 1996). However, the interactions of the ligands, through aromatic ring stacking with average quinazoline-pyrimidine ring separation of about 4.2 Å, are identical in the ecTS, rTS and mhTS complexes. In addition, no difference is observed in the active-site conformations of the two monomers of mhTS, with an r.m.s.d. for the core residues of 0.36 Å (0.68 Å). However, the average thermal parameters of the ligands in monomers 1 and 2 are 57.0 Å² and 68.3 Å², respectively, compared to average thermal parameters of the protein molecules of 44.3 Å² and 47.9 Å². This suggests that neither active site is fully

occupied and that the occupancy of the active site of monomer 2 is somewhat lower than in monomer 1. It is not likely that this is caused by differences in the accessibilities of the two active sites due to crystal packing obstructions, because the crystal was soaked in the ligand-containing solution for three days, but it does hint at a subtle difference in the active sites of the two subunits. These data are consistent with the inability to demonstrate complete saturation of both subunits of wild-type ecTS with FdUMP and CH₂H₄-folate by nondenaturing gel electrophoresis (Maley et al. 1995) and are consistent with the proposed half-of-the-sites activity of this enzyme. Similarly, the crystal structure of *Pneumocystis carinii* TS in complex with dUMP and the cofactor analog 10-propargyl-5,8-dideazafolate (CB3717) revealed an asymmetric dimer with dUMP present in both active sites but only one CB3717 molecule (Anderson et al. 1999), providing evidence that this enzyme is also a half-of-the-sites activity enzyme.

The main differences in the active sites of free and complexed mhTS are in the conformations of three side chains (Arg50, Trp109 and Asn112) and a tightening of the cavity in the complex by about 1.0 Å at Leu221. Arg50 adopts very different conformations in the complex compared to the free enzyme, and its hydrogen bonding distance to the substrate phosphate group is longer than that to the free phosphate. Trp109 appears to be disordered in the complex, showing two conformations with nearly equal occupancy, whereas it is observed in only one conformation in the free enzyme. The additional conformation seen in the complex involves a rotation of the indole ring by about 55° that brings the aromatic ring within van der Waals distance of

Fig. 3. Stereodiagram showing the active site of mhTS in the ternary complex with dUMP and Tomudex. The ligands are shown as ball-and-stick models with open bonds with dUMP at the *top* and Tomudex at the *bottom* of the view. The residues that interact with the protein are shown in solid black lines, with hydrogen bonding contacts with the ligands indicated by dashed lines. Figures 3, 4 and 5 prepared with the program MOLSCRIPT (Kraulis 1991).

the quinazoline ring of Tomudex and is similar to the conformation seen in the rTS ternary complex. This dual conformation of the Trp109 side chain, in addition to the relatively high thermal parameters of the Tomudex molecule, 62.7 Å² in monomer 1 and 75.0 Å² in monomer 2, suggests that the cofactor analog in the mhTS complex is not fully occupied. The change in the conformation of Asn112 does not result in the formation of a protein-ligand contact but appears to be related to a change in the solvent structure, because a water molecule that is in hydrogen-bonding contact with Oδ1 of Asn112 in the free enzyme is absent in the complex.

Small changes in the backbone conformation within the segment spanning residues 217 to 223 result in a movement of about 1.0 Å of the side chain of Leu221 to within van der Waals distance from the thiophene ring of Tomudex. Leu221 and Ile108 sandwich the ring, and the distances between the α-carbons of Leu221 and Ile108 are 11.3 Å and 12.0 Å in complexed and free mhTS, respectively. Ligand binding also induces a shift, by as much as 1.1 Å, towards the ligands in residues 258 to 271. A similar shift has been reported previously for ecTS (Rutenber and Stroud 1996) and rTS (Sotelo-Mundo et al. 1999).

The active site cavity is highly hydrophobic, and many contacts between the protein and the Tomudex molecule involve van der Waals interactions; the side chains of Ile108 and Phe225 are in van der Waals contact with the thiophene ring, Trp109 and Leu221 interact with the quinazoline ring, and Phe80 makes stacking interactions with the glutamyl side chain. The only hydrogen bonding contacts are between Asp218 and nitrogen N3 of the quinazoline ring (2.50 Å), the nitrogen atom of Gly222 with O4 of the quinazoline ring (2.92 Å) and a water-bridged hydrogen bonding contact between the nitrogen of Phe80 and O2 of the glutamyl tail (O2-water 3.19 Å, water-N 2.69 Å). In contrast, the substrate interacts primarily through hydrogen bonding contacts, including contacts of the phosphate group with Arg50 (3.26 Å), Arg215 (2.82 Å), and Ser216 (2.87 Å), and with Arg175 (2.65 Å) and Arg176 (2.68 Å) from the other monomer. In addition, all three potential hydrogen bond donors or acceptors of the pyrimidine base are in contact with the protein: O2 with the nitrogen atom of Asp218 (2.83 Å), N3

with the side chain Oδ1 of Asn226 (2.93 Å), and O4 with Nδ2 of Asn226 (2.80 Å) and Nε2 of His196 (2.67 Å). The O3'-hydroxyl group of the ribose ring interacts with His256 (Nε2, 2.64 Å).

The Tomudex and dUMP molecules displace water molecules that are bound by amide nitrogens or carbonyl oxygens of residues Ile108, Trp109, Ala111, Asn112, and Tyr135 in the free enzyme, but these are not replaced by hydrogen bonding interactions. However, water molecules interacting with the amide nitrogen of Asn218, Oδ1 of Asn226, and Nε2 of His256 are replaced by O2, O3', and N3 of dUMP, respectively. A water molecule bridging O4 of the pyrimidine and the carboxylate of Glu87, which is thought to be catalytically important (Sage et al. 1996), is clearly observed ($B = 31 \text{ Å}^2$), although it does not make a strong hydrogen bond with the substrate (distance of 3.1 Å). Interestingly, this water molecule was not observed in the rTS structure but was reported to be present in the ecTS-dUMP-Tomodex complex.

Comparison with the ecTS-dUMP-Tomodex ternary complex

The ternary complex of mhTS with dUMP and Tomudex, like that of rTS (Sotelo-Mundo et al. 1999), is observed in an open conformation which closely resembles that of the free enzyme. In contrast, the same ternary complex of ecTS is observed in the closed conformation (Rutenber and Stroud 1996) (Fig. 4, Fig. 5A). The closed conformation is distinguished from the open conformation by the presence of a covalent bond between C6 of dUMP and the catalytic cysteine residue (Cys195 in hTS), by an ordered carboxy terminus that encloses the active site cavity, and by a narrower binding site in which the distance between residues that flank the substrate (Cys195 and Tyr258 in mhTS) is reduced by about 1.0 Å.

In the mhTS structure, the ligands interact with each other through base stacking, as observed for all other ternary TS complexes, but they are shifted away from the catalytic cysteine residue by about 1.0 Å compared to their position in ecTS. As a result, the covalent bond between the Cys195

Fig. 4. Stereodiagram showing the superposition of a monomer of the ternary complex of mhTS (open line) with that of ecTS (solid line). The main differences between mhTS and ecTS are two insertions in loops away from the active site (upper left corner). The closed conformation (ecTS) is distinguishable from the open conformation (mhTS) by small inward shifts of the loops surrounding the ligands and by the ordered C-terminus that lies over the active site.

Fig. 5. Stereodiagrams showing the superposition of the dUMP-Tomudex ternary complexes of mhTS (open line) and ecTS (solid line). (A) Overall view of the location of the ligands in the active site. There is a shift of about 1.0 Å away from the catalytic cysteine residue (top of the figure), shown with its covalent bond to C6 of the pyrimidine ring in the ecTS structure, in the locations of the dUMP and Tomudex molecules. (B) Detailed view of the differences in the interactions of Tomudex with mhTS and ecTS. The largest differences are seen in the lower left corner where amino acid substitutions, Phe80 replacing His51 and Gly83 replacing Ser54, result in a very different environment for the glutamyl tail.

sulphydryl and C6 of dUMP, which is observed in the dUMP-Tomudex ternary complex of ecTS as well as in other ternary complexes of the bacterial TSs, is not formed (Fig. 5A). Formation of this bond constitutes the initial step of the reaction mechanism of TS. While the side chains of the cysteine in mhTS and ecTS are in very similar conformations, the shift in the position of dUMP results in a distance of about 3.4 Å between the C6 of the pyrimidine base, and S γ of Cys195 prevents the nucleophilic addition at C6.

Since there is no difference in the amino acid composition of most TSs in the dUMP binding site, it would appear that the difference between the locations of dUMP relative to the cysteine residue in ecTS and mhTS must result from

a range of small but significant differences in the interaction of the protein with the Tomudex molecule. These differences include amino acid substitutions as well as conformational changes in the main chains and side chains (Fig. 5A,B). The most important amino acid substitutions are the replacement of ecTS residues His51, Ser54, and Trp83 by mhTS residues Phe80, Gly83, and Asn112. Substitution of Trp83 by the much smaller Asn112 results in the inability of the residue at this location in the polypeptide to interact with the quinazoline ring of Tomudex. Similarly, the replacement of Ser54 by Gly83 and His51 by Phe80 results in losses of two hydrogen bonds between the protein and the glutamyl tail of the cofactor. The latter loss is somewhat

compensated for in mhTS by a rearrangement in that area of the binding site. The helix containing residues 80 and 83 shifts about 1.0 Å closer to Tomudex, allowing Phe80 to stack against the glutamyl tail. This movement of Phe80 also has an effect on the conformation of Phe225, which, although conserved between ecTS and mhTS, is in a different conformation and interacts with the thiophene ring of Tomudex in mhTS but not in ecTS. However, the environment of the glutamyl tail of Tomudex is much more hydrophobic in mhTS than it is in ecTS.

The final major difference between the ecTS and mhTS ternary complexes is the conformation of the carboxy terminus, which is ordered and actively participates in enclosing the active site cavity in ecTS. In mhTS, well-defined electron density is seen for Pro305, which is in the same location as the conserved Pro256 in ecTS, but is absent for the final seven residues (307 to 313). In ecTS, the carboxy terminus interacts with two residues that are important for substrate and cofactor binding. The first interaction is a hydrogen bond between the terminal carboxyl oxygen and Arg21 (ecTS), which is one of the residues that binds to the phosphate group of dUMP. In the mhTS ternary complex, the corresponding residue, Arg50, is not well ordered (average side chain thermal parameters 69 Å²) and is involved in only one contact, a weak (3.5 Å) hydrogen bond with the phosphate group. Disorder of the carboxy terminus in mhTS appears to reduce the restraint on the conformation of Arg50 and weakens the binding of the phosphate group. On the other hand, the shift in the location of dUMP, in tandem with Tomudex, weakens its interaction with Arg50 but allows O4 of the base of dUMP to form an additional hydrogen bond with His196. The second interaction in ecTS involves a hydrophobic contact between the side chains of the carboxy-terminal residue Ile264 and the active site residue Trp83 (ecTS), accompanied by a water-bridged hydrogen bond between a carboxy-terminal oxygen and Nε1 of the Trp83 side chain. EcTS Trp83 is replaced by Asn112 in mhTS, which could maintain the hydrogen bonding contact with the carboxy terminus in the closed form of the enzyme. However, the absence of the hydrophobic interaction and the smaller residue in hTS at this location may affect cofactor binding and require further examination.

The comparison of the structures of the ternary complexes of mhTS, rTS and ecTS with Tomudex and dUMP reveals that the mammalian enzymes bind these ligands in a location that differs by about 1.0 Å from that of the *E. coli* enzyme. This shift prevents the mammalian enzymes from adopting the closed conformation and from proceeding through the first step of the reaction. It is important to note that this is true regardless of how the complexes were prepared. The ternary complex of mhTS was prepared by soaking the ligands in preformed crystals, while the rTS complex was prepared by cocrystallization. Therefore, it is unlikely that the observed open conformations of the ternary com-

plexes of both enzymes result from crystal packing interactions. A competitive inhibition assay, comparing the 50% inhibition of ecTS and mhTS, indicates that Tomudex binds more strongly to the human enzyme than to the bacterial enzyme ($K_i = 4.6 \times 10^{-7}$ M vs. 4.6×10^{-6} M). This is consistent with the observation that the interactions of Tomudex with the enzyme are the dominant force in determining the position of the ligands in the active site and that the differences in the Tomudex binding result from a number of subtle differences in the amino acid compositions between bacterial and mammalian enzymes as well as associated changes in the side-chain conformations of surrounding residues. These results suggest that cofactor analogs can be very good inhibitors of TS, without inducing the closed conformation. However, it is very likely that other cofactor analogs do induce the transition to the closed form or even different conformations, because diffusion of other folyl-derived cofactor analogs such as CB3717 and 1843U89 into mhTS crystals under identical conditions as those used for the preparation of the Tomudex complex shatters the crystals. Regardless, the results presented here suggest that ecTS may not be an optimal model for drug design based on folate-based TS inhibitors since bacterial and mammalian TSs can interact differently with cofactor analogs.

Materials and methods

Materials

Tomudex was a gift from Astra-Zeneca (Cheshire, England). dUMP, and PEG were obtained from Sigma (St. Louis, MO), and (R,S)CH₂H₄-folate from Schircks Labs (Jona, Switzerland). All other chemicals were at least reagent grade as obtained from commercial sources without further purification.

Methods

During the course of preparing the P2L mutation of hTS, as described for the wild-type enzyme in an earlier publication (Pedersen-Lane et al. 1997), we found that one of the colonies isolated yielded an expressed protein that migrated somewhat faster than the wild-type TS on SDS-PAGE. DNA sequence analysis of the corresponding DNA fragment encoded in pET17xb-hTS showed that it contained not only the desired P2L mutation but also a deletion corresponding to amino acids 7–29. The resulting protein was expressed in a manner described earlier (Pedersen-Lane et al. 1997) except that TX61, a thy^rkanamycin-resistant mutant of BL21 (DE3) was transformed with pET17xb-hTS (Δ7–29, P2L) and employed as the expression system. The transformed TX61 cells were grown in an enriched medium at 37°C containing 50 μg/mL of kanamycin and 100 μg/mL of carbenicillin. When the cells reached an A₆₀₀ of 0.6 they were induced with 0.4 mM IPTG and allowed to grow for another 18 h. The induced mutant TS was isolated and purified to homogeneity as described for the wild-type enzyme. The k_{cat} of the mutant TS was determined to be about 1.67 sec⁻¹, which is comparable to that of the wild-type enzyme (1.80 sec⁻¹).

Crystallization

Pellets of ammonium sulfate precipitated mhTS were dissolved in 0.02 M potassium phosphate (pH 7.2) and 2 mM dithiothreitol (DTT). The protein solution was dialyzed in a Micro Collodion Bag (Sartorius Corp, Edgewood, NJ) against the phosphate buffer for over 24 h. Crystals of mhTS were obtained by hanging drop vapor diffusion methods at 10°C and 22°C. The mhTS protein concentration determination was based on 1.21 A₂₈₀/mg of TS, calculated from the mhTS amino acid composition using the method of Gill and von Hippel (1989). The diffraction data was collected on a single crystal (0.3 × 0.25 × 0.1 mm) that was obtained in a drop formed by pipetting 3 µL 5 mg/mL of mhTS protein and 2.3 µL of well solution (32% (w/v) PEG 3350, 0.1 M potassium phosphate (pH 7.4), and 10 mM DTT). The drop was equilibrated over 800 µL of the well solution for 2 weeks at 22°C. A day before data collection, 5 µL cryoprotectant solution (20% (w/v) PEG 400 – 20% (w/v) PEG 3350 in 0.1 M potassium phosphate (pH 7.4)) was added to the drop and the crystal was flash-cooled in the cold nitrogen gas stream (100 K).

Crystals of the ternary complex, mhTS-Tomudex-dUMP, were obtained by diffusing the cofactor analog and substrate into mhTS crystals grown at 22°C in a drop formed by pipetting 1.5 µL of 15 mg/mL of mhTS and 2.5 µL of well solution (30% w/v) PEG 3350, 0.08 M potassium phosphate (pH 7.1), and 10 mM DTT). The drop containing the crystal was mixed with 8 µL of ligand solution (6.2 mM Tomudex, 6.2 mM dUMP, 21% (w/v) PEG 400, 21% (w/v) PEG 3350, and 10 mM DTT in 0.1 M potassium phosphate (pH 7.1)) and incubated over 800 µL well solution (21% (w/v) PEG 400, 21% (w/v) PEG 3350, and 10 mM DTT in 0.1 M potassium phosphate (pH 7.1)) for 3 days. Data were collected from a flashcooled crystal.

Data collection and structure determination

Diffraction data for the free mhTS were measured at beamline X12c at the National Synchrotron Light Source, Brookhaven National Laboratory with a Brandeis B1 CCD detector. The data were processed to 2.0 Å resolution with Denzo and Scalepack (Otwinowski and Minor 1997). The crystals belong to space group P3₁21 space group with *a*, *b* = 90.2 Å and *c* = 143.3 Å, and have one TS dimer in the asymmetric unit with a solvent content of about 45% (*V*_M = 2.5 Da/Å³). The structure was determined by molecular replacement methods, using the structure of rTS-Tomudex-dUMP (with ligands removed) as the model. There is 93% sequence identity between mhTS and rTS. The CCP4 (CCP4, 1994) version of the program AMORE (Navaza 1994) was used to position the search model. The search in P3₁21 gave a solution with a correlation coefficient of 63.4 % and an R-value of 36.7%, while that in P3₂21 produced a significantly lower correlation coefficient (25.9%) and a higher R-value (49.7%). The model was refined against the diffraction data using CNS, version 0.9a (Brunger et al. 1998), and model building was performed using O (Jones et al. 1991). The first cycle of simulated annealing refinement resulted in an R of 0.29 and an R_{free} (10% of the data) of 0.36. The final model consists of residues 5–6 and 30–307 for monomer 1, 5–6 and 30–306 for monomer 2, one phosphate anion per monomer and 288 water molecules. The WATERPICK and WATERDELETE routines of CNS were used to locate water molecules. The final data collection and refinement statistics are listed in Table 1. Structure comparisons and stereochemical quality were calculated using the CCP4 version of LSQKAB program, O, and PROCHECK (Laskowski et al. 1993).

Diffraction data for the mhTS-Tomudex-dUMP complex were measured on a Rigaku Raxis-IV image-plate detector with a Rigaku RU200 rotating-anode generator and MSC/Yale mirror optics. The data were processed to 2.5 Å resolution with Denzo and Scalepack (Otwinowski and Minor 1997). For the initial refinement of the structure of the mhTS-Tomudex-dUMP complex, the structure of apo-mhTS was subjected to rigid-body and energy minimization refinement in CNS at 2.5 Å resolution with resulting R- and R_{free}-values of 0.285 and 0.296. The difference electronic density maps of the refined model showed clear electron density for dUMP and Tomudex. Several rounds of minimization in CNS and rebuilding in O produced the final model for protein residues 6 and 30–306, the ligands, and 110 water molecules.

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Note added in proof

After acceptance of this manuscript we became aware of a publication of the crystal structure of a ternary complex of hTS with Tomudex and dUMP in which the ligands were cocrystallized with the protein and which showed the protein to be in the closed conformation (Phan et al. 2001). Together, the three crystal structures of the ternary complexes of mammalian TSs with Tomudex and dUMP (rTS cocrystallized, hTS cocrystallized and mhTS, backsoaked) indicate that there are two different stable binding modes for this ternary complex. This appears to be unique to complexes involving Tomudex as the cofactor analog because soaking mhTS with other cofactor analogs tends to destroy the crystals, presumably due to conformational changes in the protein.

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