Multi-targeted Antifolates Aimed at Avoiding Drug Resistance Form Covalent Closed Inhibitory Complexes with Human and *Escherichia coli* Thymidylate Synthases

Peter H. Sayre¹, Janet S. Finer-Moore¹, Timothy A. Fritz¹
Donna Biermann¹, Susan B. Gates², Warren C. MacKellar²
Vinod F. Patel² and Robert M. Stroud¹*

¹Department of Biochemistry and Biophysics, University of California, San Francisco
Box 0448, San Francisco CA 94143-0448, USA
²Lilly Research Laboratories
Eli Lilly and Company
Indianapolis, IN 46285, USA

Crystal structures of four pyrrolo(2,3-d)pyrimidine-based antifolate compounds, developed as inhibitors of thymidylate synthase (TS) in a strategy to circumvent drug-resistance, have been determined in complexes with their *in vivo* target, human thymidylate synthase, and with the structurally best-characterized *Escherichia coli* enzyme, to resolutions of 2.2-3.0 Å. The 2.9 Å crystal structure of a complex of human TS with one of the inhibitors, the multi-targeted antifolate LY231514, demonstrates that this compound induces a “closed” enzyme conformation and leads to formation of a covalent bond between enzyme and substrate. This structure is one of the first liganded human TS structures, and its solution was aided by mutation to facilitate crystallization. Structures of three other pyrrolo(2,3-d)pyrimidine-based antifolates in complex with *Escherichia coli* TS confirm the orientation of this class of inhibitors in the active site. Specific interactions between the polyglutamyl moiety and a positively charged groove on the enzyme surface explain the marked increase in affinity of the pyrrolo(2,3-d)pyrimidine inhibitors once they are polyglutamylated, as mediated in *vivo* by the cellular enzyme folyl polyglutamate synthetase.

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*Corresponding author

Keywords: antifolate; dTMP synthesis; drug resistance; X-ray crystal structure; cancer

Introduction

Cytotoxic compounds directed at intracellular targets remain the mainstays of anti-cancer therapy. The substrate-based antimetabolite prodrug 5-fluorouracil directed in part at thymidylate synthase (TS) is first-line therapy for high-risk and metastatic colon cancer, and is part of adjuvant regimens aimed at reducing the risk of breast cancer recurrence. However, the efficacy of antimetabolites such as 5-fluorouracil (5-FU) directed at TS is limited by action in other pathways, toxicity toward non-tumor cells, and by several mechanisms of drug resistance in tumor cells. For example, gene amplification and consequent increased intracellular expression of TS are associated with decreased sensitivity to the active metabolite of 5-FU, 5-fluoro-2′-deoxyuridine 5′-monophosphate (5-FdUMP). Similarly, structural mutations in the TS gene itself that mediate drug resistance have been observed in cultured cell lines² or in chemically mutagenized cells selected for

Abbreviations used: TS, thymidylate synthase; hTS, human TS; EcTS, *Escherichia coli* TS; dUMP, 2′-deoxyuridine 5′-monophosphate; 5-FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; 5-FU, 5-fluorouracil; 5-FdUR, 5-fluoro-2′-deoxyuridine; meTHF, 5,10-methylenerethrofolate; FPGS, folylpolyglutamate synthetase; DHFR, dihydrofolate reductase; GARFT, glycaminide ribonucleotide formyltransferase; PABA, p-aminobenzoic acid.

E-mail address of the corresponding author: Stroud@msg.ucsf.edu

Present addresses: P.H. Sayre, Valentis, Inc., 863A Mitten Road, Burlingame, CA 94010; V. F. Patel, Kinetix Pharmaceuticals Inc., 220 Boston Ave., Medford, MA 02155, USA.
the ability to grow in the presence of TS-inhibitory antifolates.\textsuperscript{3}

One strategy to overcome drug resistance of cancer cells that is based on overexpression or structural mutation of the gene for a single target uses a combination of chemotherapeutic agents directed at several intracellular targets, as demonstrated, for example, in the first effective combination therapy for leukemia. A cell might overcome inhibition of a single enzyme but may not accumulate sufficient changes to become resistant to a combination of compounds that inhibit several enzymes. This approach, however, carries with it the risk of additive or synergistic toxicities from the several drugs. An alternative is to use a single agent aimed at multiple intracellular targets within a single metabolic pathway: a multi-targeted inhibitor might be associated with decreased development of resistance and a more limited toxicity profile.

Resistance may be reflected in increased excretion, or in diminished uptake of the compound that leads to inadequate intracellular drug accumulation. Overexpression of multidrug-resistance genes that encode efflux pumps can lead to rapid drug excretion and consequently reduced exposure of the target protein to the compound. Charged compounds are less likely to be effluxed from inside the cell, and the intracellular addition of glutamate residues to folates is a normal mechanism exploited by cells to increase intracellular retention of folates.\textsuperscript{4} In this reaction, three to five glutamate groups are added enzymatically to the \(p\)-aminobenzoic acid (PABA) moiety of reduced intracellular folates by the enzyme folylpolyglutamate synthetase (FPGS). Likewise, when antifolates are recognized by FPGS, the addition of multiple negatively charged carboxyl groups to such folate-based inhibitors results in a markedly increased intracellular half-life and cytotoxicity.\textsuperscript{5} Polyglutamylation also directly increases binding affinity for TS and hence has two desirable effects on inhibitory efficacy. However FPGS then provides another avenue for drug resistance by being a site for mutations that abrogate glutamylation of the antifolate.

A novel class of antifolates has been developed that addresses both of these mechanisms of cellular resistance, first by inhibiting multiple enzymes in the folate pathway and in the TS cycle, and second by presenting itself as a substrate for polyglutamylation by FPGS. LY231514 (Figure 1) is one of a family of antifolate compounds that contain a 6-5-fused pyrrolo(2,3-d)pyrimidine nucleus instead of the 6-6-fused pteridine or quinazoline ring found in many other mechanism-based antifolates.\textsuperscript{6} Unlike other antifolates that inhibit TS, such as raltitrexed\textsuperscript{5} (also known as Tomudex or ZD1694), CB3752 or BW1843U89,\textsuperscript{7} this compound is an effective inhibitor of several enzymes in the folate pathway, including glycaminide ribonucleotide formyltransferase (GARFT) and dihydrofolate reductase (DHFR). LY231514 is also among the better substrates for FPGS (\(k_m = 1.6 \text{ } \mu\text{M}\)). In addition, its pentaglutamylated form has a 100-fold lower \(K_i\) (1.3 \text{ } \mu\text{M}) for TS relative to its non-polyglutamylated form (\(K_i = 109 \text{ } \mu\text{M}\)). Inhibition of GARFT by the compound is similarly sensitive to polyglutamylation (\(K_i = 9,300 \text{ } \mu\text{M}\) for the monoglutamate compared to 65 \text{ } \mu\text{M} for the pentaglutamate). In contrast, binding affinity for DHFR is insensitive to the addition of glutamate groups.\textsuperscript{8} LY231514 is cytotoxic for tumor cells in culture\textsuperscript{10} and has been shown to be tolerable and to lead to responses in initial phase I evaluation against advanced malignancies.\textsuperscript{11,12} Phase II clinical trials in metastatic colon cancer suggest that response rates are 15-20\%.

To understand the chemical and structural basis of inhibitor binding of this novel class of antifolates, we determined the structure of TS in complex with four members of this drug family (Figure 1). Our initial aim was to develop an efficient system for solving X-ray crystallographic structures of human TS in complex with ligands including

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\end{center}

\textbf{Figure 1.} The 5,6-pyrrolo-pyrimidine antifolate TS inhibitors LY231514, polyglu-LY231514, LY347170 and LY338529, with their \(K_i\) values for human TS.
LY231514 and its analogues. We previously reported the structure of unliganded human TS. However, crystallization of liganded complexes of human TS had proven intractable, perhaps because of the surface charge characteristics of human TS, or because of a 27 residue amino-terminal segment that is loosely ordered in the unliganded structure. We attempted to circumvent these problems by a mutational strategy aimed at variously modifying surface charge and removing amino-terminal residues.

One mutated form of human TS retained full activity, could be crystallized, and gave diffraction at 2.8 Å resolution. These crystals allowed determination of the active site configuration and the orientation of the inhibitors within the active site. The crystal structure shows that binding of a member of the pyrrolo(2,3-d)pyrimidine family of inhibitors to human TS induces the catalytically activated “closed” active site, and induces formation of a covalent bond between the cysteine nucleophile and C6 of dUMP.

To extend the findings with the human enzyme, we took advantage of the crystallographically more tractable Escherichia coli enzyme, which can be crystallized reliably in liganded complexes that diffract to higher resolution than human TS. In this case, numerous well-refined structures of fully liganded EcTSs allow comparisons that reveal critical details about mechanisms of inhibition.

Results

Human TS with antifolate and substrate

The specific activity of wild-type recombinant human TS expressed in E. coli was 0.4-0.6 μmol/minute per mg. Extensive attempts to crystallize wild-type human TS, dUMP and various antifolates yielded small crystals (<50 μm) that diffracted to only 5.0 Å. To obtain better-ordered crystals of human TS in complex with ligands, a series of enzymes modified by site-directed mutation were designed and created. The strategy was that charged residues deduced by homology with other TS structures to be on the enzyme surface were modified to residues of opposite charge. Cysteine residues that might be prone to oxidation and thereby interfere with proper folding were mutated to serine. We deleted the 29 amino residue amino-terminal extension in human TS (del29) compared with bacterial T5s, since this region is disordered in crystals of unliganded human TS and in ternary complexes of rat TS. Specific activity measurements showed that mutants R46E, P2L, C1805, C1995 and multiple mutants C1805/C1995, R46E/C1995 and R46E/C1805/C1995 had specific activities in the range 0.2-0.4 μmol/minute per mg. Mutant proteins del29 and del29/C1805 that lack the amino-terminal extension were inactive. To understand the details of interaction of the pyrrolo(2,3-d)pyrimidine inhibitor with the human enzyme, active mutants were complexed with inhibitor and substrate dUMP, and the complexes subjected to crystallization trials. Human TS mutant R46E in complex with LY231514 and dUMP crystallized in 7-21 days. This mutant has been reported to crystallize at high frequency in crystallization screens. Diffraction intensities to 2.8 Å were collected from crystals at −170 °C, and to 3.0 Å resolution at 20 °C. Structures of these complexes were solved by molecular replacement in a triclinic and monoclinic form, respectively. The results presented here are for the structure refined to 2.9 Å resolution.

In attempts to understand why R46E assisted crystallization, we examined the crystal packing arrangement around R46E, which is at a crystal packing interface; however, it is 7 Å from the nearest contact, which is a glutamate residue that is ion-paired with a lysine residue of the same molecule. Thus, it could be that R46E removed a potentially bad interaction, or that the charge in this area is in some way a better match for this inhibitor complex.

The overall fold

Human TS, like other TSs, is an obligate homodimer in which each monomer is composed of three layers of α-helices lying adjacent to a β-sheet that forms the dimer interface. In triclinic and monoclinic crystal forms, the asymmetric unit includes two dimers related by non-crystallographic symmetry. Within each dimer in the triclinic cell, the monomers are related by approximate 2-fold symmetry and have nearly identical structures. The two dimers within the asymmetric unit are very similar: the rmsd distance (rmsd) between the dimers after they have been overlapped is 0.19 Å for Cα atoms and 0.24 Å for all atoms.

The overall fold closely matches that of bacterial and eukaryotic species of TS; compared to EcTS (PDB accession code 2kce), the rmsd of 264 equivalent Cα atoms is 1.85 Å; compared to 280 equivalent Cα atoms in the rat structure, the rmsd is 1.08 Å. There are two insertions in human TS relative to EcTS (residues h117-h128 and residues h146-h153), both visible in the electron density, that are distant from the active site and make no contact with it. These insertions pack together and comprise part of the small domain of the enzyme. The small domain of TS is structurally conserved among eukaryotic species but structurally diverse in non-eukaryotic TSs. Despite its variable structure, the small domain contains two conserved residues, Trp h109 and Asn h112 (Trp in bacterial TSs) that contact the cofactor in closed conformations of TS ternary complexes. These two residues in the human TS ternary complex overlay the equivalent residues Trp Ec80 and Trp Ec83 in EcTS-dUMP-raltitrexed when the two structures are aligned.
Closed conformation

In the ternary complex with LY231514, human TS adopts a closed conformation, similar to that adopted by EcTS in ternary complexes with dUMP and antifolate inhibitors. This represents a dramatic, and functional conformational change from that seen in the unliganded, or in the dUMP bound enzyme, that occurs on binding cofactor or other antifolate inhibitors. In ternary complexes of EcTS with the antifolates CB3717 or raltitrexed the C-terminal residues are close to the active site and make specific contacts with folate or antifolate ligands. In contrast, rat TS solved in complex with dUMP and raltitrexed does not adopt this closed conformation, with corresponding differences in binding of the antifolate. In the 2.6 Å rat structure, no density was visible for the C-terminal six residues, suggesting that they were disordered in the crystal.

The positions of the corresponding residues in the human structure were assessed by calculating simulated annealing omit maps after removing residues Lys h308-Val h313. Well-resolved density for main-chain atoms for these six C-terminal residues (Figure 2) demonstrates specific contacts between some of them and the LY231514 fused ring system. For example, the carbonyl group of Ala h312 makes a hydrogen bond to the exocyclic amino group at LY231514 C2. In the rat TS-dUMP-raltitrexed complex, a similar interaction of the inhibitor with the C-terminal residues is impossible, since the exocyclic amino group is replaced by a methyl substituent in raltitrexed, and this could contribute to the open conformation of the complex. A recent crystal structure of human TS in a ternary complex with raltitrexed and dUMP shows that the enzyme is in a closed conformation and the C terminus is well ordered even though it does not hydrogen bond with the ligands. That complex has a crystal form similar to the triclinic crystal form of hTS-dUMP-LY231514 we report, suggesting that crystal packing or crystallization conditions influence the protein conformations of TS in ternary complex crystal structures.

In addition to the hydrogen bond between Ala h312 O and the exocyclic amino group, there is a hydrophobic contact between Met h311 and the pyrrolo(2,3-d)pyrimidine ring system in the antifolate LY231514. Side-chain density for C-terminal residues Lys h308 and Glu h310 is not well defined. These side-chains point toward solvent and are more mobile than the side-chains from Met h309 and Met h311 that make contacts with ligand or the protein interior.

The closed conformation of the human TS ternary complex is reinforced by a water-mediated hydrogen bond from the δ²-NH of Asn h112 to either Ala h312 O (as shown in Figure 3) or to one of the carboxyl oxygen atoms of the C-terminal residue Val h313 across the active-site cavity (Figure 3). The uncertainty concerns the position of the interposed water molecule Wat330. This hydrogen bond stabilizes the position of the C terminus and helps to close the active site from solvent. Similarly, in EcTS ternary complex structures, one of the carboxyl oxygen atoms of the C-terminal residue Ile Ec264 serves as a hydrogen bond acceptor from the ε¹-N in Trp Ec83 across the front of the active site.

Consistent with the closed conformation, the active-site cavity in liganded human TS is smaller than in open structures. The distance between Cα atoms of the catalytic Cys h195 and Tyr h258 on the opposite side of the active site is 15.5 Å, while

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**Figure 2.** Divergent-eyes stereo plot of 2σ density (orange cage around the residues) from a simulated annealing omit map for carboxy-terminal residues in the hTS complex with dUMP and LY231514. The Fo - Fc density map was calculated after omitting all atoms within a 3 Å cushion around residues h308-h313 and refining the protein by simulated annealing in CNS. Ligands are shown with black stick bonds. The positions of the carboxy-terminal residues in the open conformation of TS, taken from the overlapped crystal structure of the *E. coli* TS-dUMP binary complex (PDB accession code 1BID), is shown with brick-red ball-and-stick bonds. The Figure was prepared using BOBSCRIPT.
the corresponding distance in closed ternary complexes of antifolates with EcTS (Cys Ec146 to Tyr Ec209) is 15 Å. The structure of the rat enzyme in complex with raltitrexed shows a larger active site, in which the corresponding distance (Cys r189 to Tyr r252) is 16.5 Å. Difference-distance matrices that compare the rat complex to hTS-dUMP-LY231514 or hTS-dUMP-raltitrexed (PDB accession code 1HVY) show shifts of protein segments toward the active site in the hTS complexes relative to the rat TS complex. The same shifts indicated in the matrices characterize the conformational change seen between apo and fully-ligated EcTS. These shifts are more exaggerated for one protomer than for the second, indicating that the rat structure is an asymmetric dimer, more open in one protomer than in the other. A difference-distance matrix comparing hTS-dUMP-LY231514 with hTS-dUMP-raltitrexed shows the latter complex has not closed as completely, implying that the specific hydrogen bond interactions between the C terminus and inhibitor stabilize the most compact closed state of the enzyme.

**Comparison to the human apo-TS structure**

The current structure for hTS includes a covalently bound dUMP ligand, a bound antifolate inhibitor and 286 of the 313 residues in the human enzyme. No density is apparent for N-terminal residues h1-h27, suggesting that they are disordered in the crystal. A similar finding was reported for rat TS and for the hTS ternary complex with raltitrexed. Our previously reported structure of unliganded human TS included 240 residues. As in the current structure, the 27 amino-terminal residues were disordered. In addition, the eukaryotic insertions at residues h117-h128 and h146-h153, a nearby loop at h95-h110 and residues h141-h145 were not ordered in apo-hTS. These regions are apparent in the density for the presently reported ternary complex structure. The C-terminal three residues, h310-h313, of the unliganded structure protrude more deeply into the empty active-site cavity than they do in the ternary complex, where the cavity is occupied by LY231514.

The most striking difference between liganded and apo-enzyme structures lies in the active-site loop, residues h177-h201. In the unliganded human TS structure, this loop is twisted inside out, by nearly 180° around an axis through the Cα atoms of residues h188 and h200, which leaves the γ-S nucleophile of the active-site Cys h195 pointing away from a potential interaction with C6 of dUMP, suggesting that there is a mechanism associated with chaperoning the ligands into the active site (Figure 4). In the ternary complex, the h177-h201 loop is in a configuration typical of other TS structures. This configuration is required for nucleophilic attack, and the covalent bond formation observed here between Cys h195 γ-S and C6 of dUMP. After alignment of the unliganded and ternary complex structures, the overall rmsd between all Cα atoms present in both crystal structures is 2.52 Å; when the Cα atoms in the distinctly twisted loop are omitted from the comparison, however, the overall similarity is very high, with the rmsd for Cα atoms = 0.98 Å.

**dUMP binding and covalent inhibition**

The orientation of dUMP in the active site is similar to that found in the ternary complex of, for example, EcTS-dUMP-raltitrexed. Simulated annealing refined omit difference electron density maps generated after exclusion of dUMP and inhibitor atoms reveal the orientation and conformation of both ligands (Figure 5). The dUMP pyrimidine ring binds in an anti orientation and the ribose has the typical C2-exo conformation. Figure 6 demonstrates several conserved hydrogen bonds that are maintained with side-chains in the active site. Asn h226 δ-NH2 donates a hydrogen atom to dUMP O4; Asn h226 δ-O serves as a hydrogen acceptor from dUMP N3. Ribose O3’ makes hydrogen bonds to His h256 and Tyr h258. In contrast to the hTS complex with dUMP and raltitrexed, His h196 does not contact dUMP O4. Rather, it adopts the conventional conformation seen in ternary complexes of other TS species, where it participates in a water-mediated hydro-
gen-bonding network to dUMP. However, all of the water molecules in this network could not be identified conclusively in our structure because of limited resolution. Clearly, the His h196 conformation is sensitive to the solvent structure in the active site, which is in turn influenced by the structure of the inhibitor.

The phosphate group of dUMP is coordinated by side-chains of four arginine residues: Arg h50 and Arg h215 (corresponding to Ec21 and Ec166) from one monomer, and Arg h175' and Arg h176' (corresponding to Ec126' and Ec127') from the other monomer. Ser h216 (Ec167) γ-O also shares a hydrogen atom with O2P of the phosphate group. Refined omit difference electron density after exclusion of dUMP and γ-S of the active-site Cys h195 clearly reveals density for a covalent bond between Cys h195 γ-S and C6 of dUMP (Figure 5). Thus inhibition by LY231514 results from formation of a non-productive covalent complex that is unable to proceed, in the absence of the methylene group of 6mTHF, from C-5 activation to methyl group exchange.

Inhibitor binding

LY231514 is anchored in the active site by aromatic stacking between the fused pyrrolo(2,3-d)pyrimidine ring system and the dUMP pyrimidine ring. In addition to the hydrogen bond between the donor exocyclic amino group N10H of LY231514 to Ala h312 O, there is another between the donor N3H of LY231514 and the δ-O of Asp h218. (Figure 7(a)). Analogous interactions are seen in ternary complexes with EcTS and CB3717. However, raltitrexed does not have an amino group at N10, therefore in the complex of raltitrexed with rat TS (Figure 7(b)) or human TS (Figure 7(c)), the hydrogen bond interactions with this group are lost.

A conserved water molecule (Wat330) donates a hydrogen bond to the acceptor N1 in the pyrrolo(2,3-d)pyrimidine ring, and donates another to either the Ala h312O or to Val h313 δ-O. Wat330 is surrounded in a tetrahedral arrangement by these two acceptors, and two hydrogen bond donors (Arg h50 η²-NH, and Asn h112 δ-NH), enabling the water molecule to completely satisfy its hydrogen bonding potential (Figures 3 and 7(a)). This water molecule is conserved in EcTS ternary complexes where it donates hydrogen bonds to N1 of the pterin (or quinazoline ring) of the folate (or anti-folate), and to Ala h312 O, the penultimate carbonyl group in the protein, and accepts a hydrogen bond from the Arg Ec21 η²-NH, analogous to the human complex.
An additional specific hydrogen bond is observed between the donor N7H of LY231514 and δ1′-O of Asn h112. This interaction is not possible in bacterial TSs in which asparagine is replaced by Trp at that position; a suitable hydrogen acceptor is not present with pyrrolo(2,3-d)pyrimidines in complex with EcTS (Figure 7(d), and see the text below), in which the corresponding residue is Trp Ec83.

The PABA ring of LY231514 is surrounded by a collar of hydrophobic side-chains: Ile h108, Leu h221, Phe h225 and Met h311 (Figure 8). This hydrophobic environment provides several favorable interactions important for tight binding of folate-based inhibitors. The charged glutamic acid tail occupies a shallow, positively charged groove on the enzyme surface, as defined by the polyglutamylated folates.22 The Glu carboxyl group is 4.3 Å from highly conserved Lys h77 ζ-N, and may form water-mediated hydrogen bonds to this group, as in other TS species. In the cell, LY231514 is polyglutamylated and can make additional charge-charge interactions with the tail-binding groove, as seen in the structure of EcTS with polygl-YL231514 described below.

**Mutation interpretation**

**Mutants selected for antifolate resistance.** Mutants selected in vivo under ethyl methanesulfonate and Thymitaq pressure led to modifications in the loop encompassing residues h47-h52 that contains the phosphate-binding Arg h509 (Figure 9). Mutants D49G and G52S generated resistance to both the folate inhibitor Thymitaq and the substrate analog 5-fluoro-2-deoxyuridine (5-FdUR). Random sequence mutagenesis of the Arg h50-loop followed by selection for growth in TS-deficient E. coli in the presence of 5-FdUR generated several other 5-FdUR-resistant variants.23 Substitutions at K h47, D h48, D h49, T h51 and G h52 were often present in these drug-resistant mutants. The four phosphate-binding arginine residues are known to provide significant binding energy for the substrate, but mutations of these residues frequently have $k_{cat}$ values similar to that of wild-type TS, since the arginine residues do not have a direct role in catalysis and since ligand binding is not rate-determining in TS.24,25 Besides binding to the dUMP phosphate moiety, Arg h50 provides key hydrogen bond interactions that stabilize the closed conformation of TS required for tight binding of folate analogs. The Arg h50-loop becomes progressively better ordered as first substrate, then cofactor binds. The wild-type residues at the substitution sites in the 5-FdUR-resistant mutants all have clear roles in stabilizing the conformation of the Arg h50-loop in the closed ternary complex of human TS with LY231514. For example, Thr h51 γ-O hydrogen bonds to both Thr h53 γ-O and Asp h49 δ1′-O, but does not make these interactions in the open rat TS ternary complex. K h47 ζ-N and D h48 δ1′-O are linked to the loop containing phosphate-binding arginine residues Arg h175 and Arg h176 from the other protomer; this loop shifts towards the active site during ternary complex for-
Figure 7. Diagrams showing interactions of (a) LY231514 with human TS. (b) The interactions seen in the rat TS structure with raltitrexed that no longer has an equivalent of N10 on the inhibitor, and results in a more open structure. (c) Interactions seen in the human TS-raltitrexed structure. (d) Interactions seen in the complex of polyglutamyl-LY231514 with EcTS. In (d) the inhibitor is cut off at the polyglutamate tail. Interactions of the polyglutamate tail with EcTS are detailed in Figure 10(a). The Figure was prepared using LIGPLOT.53

mation. Finally, G h52 adopts a conformation in the LY231514 complex, but not the open rat TS structure, that is disfavored for any residue besides glycine (φ, ψ angles of 92° and −19°, respectively). Thus, mutations to h47-h52 could confer resistance by destabilizing closed ternary complexes of TS
Figure 8. The folate-binding hydrophobic neck of human TS is depicted. The PABA ring is surrounded by a collar of hydrophobic residues. Site-directed mutagenesis of H h108, L h221 and F h225 produced drug-resistant variants. The location of the collar in the dimer and a close-up view of its interactions with LY231514 are shown. The hydrophobic side-chains in the collar are plotted as dark green stick bonds in the former view, and rendered as dark green (carbon) or yellow (sulfur) space-filling atoms in the second. In both views, the inhibitor atoms are drawn using the same color scheme as that in Figure 3. The Figure was prepared using MOLSCRIPT.51,52

relative to loosely bound open structures without abrogating activity.

Site-directed mutagenesis of highly conserved residues H h108, L h221 and F h225 forming the hydrophobic collar around the PABA ring (Figure 8) also generated molecules resistant to Thymitaq, raltitrexed, 5-FdUR or BW1843U89. Such mutations modify the hydrophobic environment surrounding the PABA ring and could have a large impact on binding affinity and binding rate for both antifolates and cofactor, but, since the hydrophobic collar is distant from the active site and not directly involved in the chemical steps of the enzyme reaction, these mutations would not necessarily lead to large decreases in kcat.

Mutations of F h225 and I h108 are the only two residues that make direct hydrophobic contacts with the PABA ring, while L h221 is in hydrophobic contact with the glutamate (Figures 7(a) and 8). Together, the structure and the drug-resistance data suggest that hydrophobic interactions with the PABA ring contribute more towards cofactor or antifolate affinity than hydrophobic interactions with the glutamyl moiety.

Active-site residues associated with 5-FdUR resistance. Landis et al.27,28 randomized residues h197-h199 and h204 (EcTS homologous residues are Ec148-Ec150 and Ec155) and selected for mutant enzymes that retained near wild-type TS activity but acquired resistance to the inhibitory effects of 5-FdUR. In such mutants, Ala h197 was changed to larger hydrophobic residues (Leu, Met

Figure 9. The Arg-50 loop of human TS showing its location in the dimer and a close-up of its interactions with dUMP. Atoms are color-coded as in Figure 3. Mutants that lead to resistance to TS inhibition cluster in this loop. The Figure was prepared using MOLSCRIPT.51,52
or Val), Cys h199 was modified to Leu, Ile, Met or Phe and Val h204 was changed to a polar or charged residue. These concurrent changes may give rise to a tighter active site that will not accommodate the fluoro-dUMP derivative. Alternatively, Cys h199 abuts Tyr h133 in the human TS ternary complex structure reported here. Tyr h133, in turn, hydrogen bonds to His h196 ε²-N, stabilizing a conformation of this residue commonly seen in closed ternary complexes of several TS species, but not in dUMP-bound or unliganded TS. Substitutions at Cys h199 may force a shift in the Tyr h133 side-chain, interfering with its role in orienting the environmentally important His h199. In EcTS, the corresponding histidine residue, His h147, has a clear role in forming the covalent ternary complex intermediate during the enzyme reaction, even though several mutants at this site are active.²⁹

The P303L hTS mutant was recently isolated from a 5-FdUR-resistant cell line.³⁰ Pro h303 is in the long C-terminal tail of hTS. In the hTS closed ternary complex with LY231514 the side-chain of Pro h303 is in hydrophobic contact with Arg h78 β-C (3.8 Å away) whereas in the open rat TS structure, the Pro side-chain is not in contact with other residues. Thus, mutation of Pro h303 to a bulkier group such as Leu may sterically disfavor the closed ternary complex conformation of the enzyme.

dUMP binding residues. Ser h216 is important for ligand binding and contributes to catalysis.³¹ Of 16 amino acids substituted into position h216, only threonine with its hydroxyl group yields an active enzyme.³¹ The hydroxyl group of Ser h216 makes a hydrogen bond to an oxygen atom of the dUMP phosphate group. However, the direct role of Ser h216 in binding phosphate does not, in itself, explain the essential nature of this residue, since phosphate-binding arginine residues, which contribute at least two hydrogen bonds each to the phosphate oxygen atoms, can be mutated without loss of function.³² The side-chain of Ser h216 is buried completely in the closed conformation seen in the human TS ternary complex. It is 3 Å from the dUMP ribose ring, 3.2 Å from His h256 ε²-C (or ε²-N depending on the rotamer of H h256) and 3.8 Å from the guanidinium group of Arg h216. Thus, bulky substituents at this site would greatly disfavor ternary complex formation.

Site-directed mutagenesis of Gln h214 produced variants that affect the rate at which hTS undergoes interconversion from an unliganded or binary open complex to a closed ternary complex.³³ The human TS ternary complex structure shows that, as in other species of TS, the highly conserved Gln h214 (Gln Ec165) participates in a hydrogen bonding network that stabilizes the binding of substrate dUMP via interactions with the backbone carbonyl group of Ser h216 (Ser Ec167) and with neighboring side-chains Asn h226 (Asn Ec177), and His h250 (Trp Ec201). Asn h226 in this network encodes substrate specificity by making hydrogen bonds to N3 and O4 of dUMP that optimize the orientation of the pyrimidine ring prior to cofactor binding.³³ By fixing the orientation of the carboxamide group of Asn h226, Gln h214 contributes indirectly to dUMP orientation, an important factor in the rate of ternary complex formation.³⁴

High-resolution structures of EcTS with pyrrolo(2,3-d)pyrimidine antifolates

Crystals with pyrrolo(2,3-d)pyrimidine antifolates

In crystallization trials with human TS, diffraction-quality crystals could not be obtained with the polyglu-LY231514 or with two other members of the pyrrolo(2,3-d)pyrimidine series of compounds, LY341770 (containing a unique five-membered nitrogen-containing ring in the tail) and LY38529 (with a single valyl group instead of a glutamyl group attached to the PABA moiety) (Figure 1). Ternary complexes of the readily crystallizable EcTS were therefore made with dUMP and the antifolates polyglu-LY231514, LY341770, or LY38529. Crystals of these ternary complexes contained a TS homodimer in the asymmetric unit, formed in the space group P6₁ characteristic of most EcTS ternary complexes, and diffracted to 2.2, 2.5 and 2.7 Å, respectively. The structures were solved utilizing initial phases calculated from the EcTS-dUMP-raltitrexed structure (PDB accession code 2kce) with ligands and solvent removed, and refined to an R-factor of 19.8% (R_free = 24.1%) for EcTS-dUMP-polyglu-LY231514, R-factor = 18.1% (R_free = 21.9%) for EcTS-dUMP-LY341770 and R-factor = 22.0% (R_free = 24.9%) for EcTS-dUMP-LY38529.

dUMP binding at the active site

Simulated annealing refined omit difference electron density maps, generated after exclusion of dUMP and inhibitor atoms, reveal the orientation and conformation of both ligands. The overall orientation of substrate dUMP in all six active sites and its hydrogen bonding interactions with the enzyme are similar to those seen in complexes of EcTS with dUMP and the inhibitors CB3717, raltitrexed or BW1843U89. Refined omit difference electron density after exclusion of dUMP and the γ-S of Cys Ec146 shows unambiguous density for a covalent bond between Cys Ec146 γ-S and C6 of dUMP in each active site. This confirms the finding in the human structure that TS inhibition by polyglu-LY231514 results from formation of a non-productive covalent complex unable to proceed from C5 activation on dUMP to methyl group exchange.

Inhibitor binding at the active site

In common with other TS anti-folates, planar ring stacking interactions orient the inhibitors in each structure over the pyrimidine ring of dUMP.
The fused ring system is approximately centered over the dUMP pyrimidine. When the complexes are superimposed on EcTS ternary complexes with dUMP and CB3717 or raltitrexed, the pyrrolo(2,3-d)pyrimidine and quinazoline ring systems overlap closely, as do the FABA ring moiety in each class of inhibitors. As in meTHF and CB3717, but in contrast to other antifolate TS inhibitors in current clinical use or development (raltitrexed and BW1843U89), the present series of compounds includes an exocyclic amino group at the 2-position of the pyrrolo(2,3-d)pyrimidine nucleus. The amino nitrogen atom is hydrogen bonded to Ala Ec263 O (Figure 7(d)). In addition, a conserved water molecule, seen in most other ternary complexes of TS, mediates hydrogen bonds between the amino group, protein backbone, and δ-O of invariant residue Asp Ec169. Asp Ec169 δ-O is a proton acceptor from N3; this conserved interaction, seen in ternary complexes with both cofactor and with antifolate in several TS species, appears to be critical for forming covalent ternary complexes in TS.35 A second conserved water molecule donates hydrogen bonds to N1 and Ala Ec263 O, and accepts a hydrogen bond from Arg Ec21 η2-N. This water molecule forms analogous hydrogen bonds in EcTS-dUMP-raltitrexed,36 TS-dUMP-CB371718 and TS-FdUMP-meTHF.36

The five-membered pyrrolo group in this series is unique among anti-TS folate compounds. The pyrrolo group does not make specific hydrogen bonding contacts with EcTS side-chains. However, it participates in hydrophobic interactions similar to those made with the cofactor or with quinazoline ring-based antifolates. There are edge-to-face interactions at distances of 3.7 and 3.2 Å with Trp Ec80 and Trp Ec83, respectively, and N7 approaches δ-C of the conserved hydrophobic residue Leu Ec143 at a distance of 3.4 Å.

In summary, the interactions made by the pyrrolo(2,3-d)pyrimidine ring system with EcTS closely parallel those made by the pterin and quinazoline ring systems of meTHF and CB3717. They are the same as the interactions seen in the human TS-dUMP-LY231514 structure, with two important exceptions (Figure 7). In human TS, Met h311 provides a hydrophobic interaction with the ring system that cannot be made in EcTS, where this residue is valine. Second, in the human structure Asn h112 is a hydrogen bond acceptor from N7 of the pyrrolo ring. The corresponding residue in EcTS, Trp Ec83, cannot form this hydrogen bond, but packs against the ring, contributing to hydrophobic interactions with the inhibitor.

The negatively charged carboxylate groups of polyglu-LY231514 increase affinity through specific electrostatic interactions

Antifolates with a glutamate residue are typically converted to polyglutamylated forms by polyglu poly-γ-glutamate synthetase. In contrast to most TS-inhibitor complexes solved to date, polyglu-LY231514 includes four physiologically relevant carboxyl groups, one from each of four glutamyl residues, thus it closely mimics the inhibitor’s intracellular characteristics. As in EcTS-dUMP-polyglu-CB3717,22 the polyglutamyl tail binds in a positively charged shallow groove on the protein surface. The binding site is plastic: specific interactions are mainly electrostatic and characterized by hydrogen bonds that are either water-mediated, or involve flexible side-chains of Arg or Lys. In spite of this flexibility, the binding modes for the polyglutamyl groups of polyglu-CB3717 and polyglu-LY231514 are very similar, with an rmsd in position of 0.79 Å. The higher resolution of the polyglu-LY231514 structure compared to the polyglu-CB3717 complex structure allows a more precise characterization of the polyglutamate-protein interactions (Figure 10(a)).

A water-mediated hydrogen-bonding network coordinates the carboxyl oxygen atoms of the first glutamyl group (O28 and O29 in Figure 10) with ζ-N of conserved Lys Ec48 and with the main-chain carboxyl atoms of Leu Ec172 and Ile Ec258. The same water-mediated network between the first glutamate residue and the protein is seen in TS complexes with monoglutamylated inhibitors (e.g. Figure 10(b)). The side-chain of the first Glu makes no direct interactions with the protein. The second carboxyl group is directed outward from the active site toward solvent and makes no specific contact with the enzyme. The last two carboxyl groups are in the vicinity of positively charged side-chains that line a shallow surface groove bordering the enzyme’s deep active site. In particular, a carboxyl oxygen atom of the third glutamyl group (O46 in Figure 10(a)), which is well localized in refined omit difference electron density, makes hydrogen bonds of good geometry with the highly conserved Arg Ec53 and with His 51 ζ-N in one of the active sites of the protein. The most distal carboxyl group is less well resolved in difference electron density and does not contact specific residues.

Discussion

A closed, liganded human complex

This is one of the first crystallographic structures of liganded human TS, solved after many years of diligently attempted crystallization. The crystallization itself was achieved by mutagenesis experiments involving many sites, aimed at altering surface properties of the enzyme. The structure demonstrates that the potent antifolate inhibitor LY231514 forms a covalent, closed ternary complex with hTS and the substrate dUMP. In contrast to the structure of the hTS apo-enzyme, the h177-h201 loop bearing the active-site cysteine residue adopts an untwisted conformation that allows a productive nucleophile attack by the γ-S of the catalytic cysteine residue on C6 of dUMP. Ligand binding is required for the change
from the non-productive apo conformation to the productively folded conformation.

Binding of the 6,5-fused pyrrolo[2,3-d]pyrimidine ring to human TS demonstrates a unique feature compared to other antifolates. The side-chain of Asn h112 donates a hydrogen bond to N7 of LY231514. The corresponding EcTS residue Trp Ec83 would not make a similar interaction. A hydrogen bond acceptor is not present in raltrexed or other antifolates.

**Drug-resistant mutations in human TS**

With the aim of creating molecules resistant to TS inhibitors, various mutations were generated and assessed under pressure for TS to become resistant. The hTS structures allow the direct observation of the binding mode of a folate inhibitor to the active site and enable an analysis of the kinetic basis of resistance incurred by the human TS mutants.

The drug-resistant mutants are distant from the site of dUMP methylation and have no direct role in catalysis but have clear structural roles in preferentially stabilizing the closed ternary complex conformation of the enzyme. Drug-resistant TSs that retain activity could be transduced into normal hematopoietic stem cells and protect the cells against cytotoxic agents that target TS, so reducing the toxic effects of these anti-cancer drugs. This can be an important contribution to protecting stem cells from the effects of cytotoxic anti-cancer drugs. These mutations cluster in two regions around the active site: the Arg50 loop and the hydrophobic neck that surrounds the PABA ring (Figures 8 and 9).

**Polyglutamyl interactions**

In crystallization experiments with wild-type or mutant human TS, crystals could be made only with LY231514, and not with its polyglutamylated counterpart. However, LY231514, like many antifolates, is a substrate for FPGS, which leads to formation of an intracellular polyglutamylated species with distinctly higher affinity for TS and greater intracellular retention. Clearly the surface properties of a ligand-protein complex determine the crystal contacts, as evidenced also by the crystallization of R46E but not wild-type human TS.

Polyglu-LY231514 formed ternary complex crystals with EcTS and dUMP and the crystal structure of the EcTS complex elucidated interactions between the glutamyl groups and a shallow groove on the enzyme surface. These interactions demonstrate why the polyglutamylated form binds with dramatically increased affinity. Four additional hydrogen bonds are created with the cofactor carboxyl groups; three of them are salt-bridges between charged groups. At least within
buried active sites, hydrogen bonds between charged groups can be estimated to be ~2-3 kcal higher than between uncharged groups.25,38

Conclusion

The ternary complex structure of human TS with LY231514 shows formation of a closed covalent inhibitory complex. Complexes with EcTS provide detail of the interactions of the polyglutamate residues with the enzyme surface. This human TS structure instructs in the design of antifolate drugs based on this novel “multitargeting inhibitor” complex, and it enables the development of species-specific antifolates.

Materials and Methods

Human TS

Protein preparation

Human TS was expressed in E. coli from plasmid pGCHTS-TAA.39 Site-directed mutagenesis was carried out with a double-stranded method40 using the Chamelon kit (Stratagene, CA).

Transformed bacteria (E. coli DH5x) were grown overnight and pellets were sonicated with four 30 second bursts and 90 second rests in lysis buffer (20 mM Tris (pH 7.4), 1 mM EDTA, 2 mM DTT, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 400 nM PMSF). Lysates were cleared by centrifugation at 30,000 g for 30 minutes at 4°C. Cleared lysates were made 30 mM in KCl and applied to FF Sepharose (Pharmacia). Protein was eluted with a KCl gradient from 30 mM to 600 mM and human TS-containing fractions were identified by monitoring TS activity in each fraction. Fractions with TS activity were pooled. (NH₄)₂SO₄ was added to a final concentration of 0.75 M. The pool was applied to a phenyl-Sepharose column. Protein was eluted with a gradient of 0.375 M to 0.0 M (NH₄)₂SO₄. Fractions containing TS activity were pooled and applied to a Cibacron Blue (Pharmacia) column and eluted with a KCl gradient (530 mM to 800 mM KCl). Fractions with the highest specific activity were pooled and dialyzed against 20 mM Tris (pH 7.4), 1 mM EDTA, 2 mM DTT. Glycerol was added to 10% (v/v) and the protein stored at -80°C.

Activity was measured by monitoring folate turnover spectrophotometrically.41 Activity is reported in µmol substrate turned over per minute per mg of protein.

Crystallization of human TS mutants with ligands

Crystallization trials by the hanging drop method were carried out for wild-type TS and all of the active mutants. These consisted of sparse matrix and focused screens followed by fine grid optimization. Ternary complexes were formed with protein (5-10 mg/ml), dUMP and LY231514, polyglu-LY231514, LY338529 or LY341770. A single mutant, human TS R46E, yielded diffraction quality crystals in 30% (w/v) PEG-monomethyl ether 5000 in 0.1 M sodium cacodylate (pH 6.5), 0.2 M ammonium sulfate, 0.5 M KCl.

Crystallography

Room temperature diffraction. In total 99, and 96 1°, 20 minute frames were collected from each of two crystals, at room temperature on an image plate detector (Raxis II) using monochromated CuKα radiation from a Rigaku rotating anode generator operating at 50 kV and 300 mA. Data reduction was carried out using Denzo from the HKL suite of programs.42 The space group was C2 with a = 138.1 Å, b = 98.1 Å, c = 112.0 Å, β = 110.7° and with two dimers in the asymmetric unit. Scaling and merging diffraction intensities with I > 1.0 σ from the two crystals using Scalepack from HKL.42 yielded an 84% (3.0 Å) complete data set with Rsym = 14% and I/σI = 5.8.

Cryocrystallographic diffraction. In total, 180 and 42 1°, 20 minute frames were collected at -170°C for each of two crystals respectively, on an image plate detector (Raxis IV) using CuKα radiation from a Rigaku rotating anode generator operating at 50 kV and 300 mA. The space group was P1 with a = 68.1 Å, b = 69.6 Å, c = 73.9 Å, α = 71.7°, β = 85.7°, γ = 75.1° and with two dimers in the asymmetric unit. The solvent content for the triclinic unit cell was 54%. Scaling and merging of all data (no σ cutoff) from the two isomorphous crystals using Scalepack yielded an 88% complete data set to 2.8 Å with Rsym = 13.8% and I/σI = 7.4.

Structure solution and refinement

Human TS crystal form A (monoclinic). The structure of the complex in the monoclinic crystal form was determined by molecular replacement from 15 to 4 Å in CNS.43 The TS dimer of the bifunctional DHFR-TS enzyme from Leishmania major 16 with ligands and solvent molecules removed was used as the search model. Two redundant solutions (peaks in the cross-rotation function at 0.179 and 0.174; mean = 0.035; standard deviation = 0.036) related by the 2-fold symmetry of the dimer were obtained. The search model was placed in one of those orientations and a translation search performed. A clear peak allowed placement of one dimer in the unit cell, resulting in a protein content of 23%. With this dimer fixed, and a second dimer rotated according to one of the 2-fold related peaks from the cross-rotation function, a second translation search was performed. A clear peak in the translation function specified the position of a second dimer, resulting in a protein content of 46%. The two dimers were non-crystallographically related by a translation only. After refinement treating each monomer as a rigid body, the crystallographic R-factor was 42.0% (Rfree = 42.6%) for data between 50 and 3 Å, with bulk solvent correction applied.

Strict non-crystallographic symmetry was applied during refinement in CNS.43 Simulated annealing using torsion angle dynamics was performed. Density maps at this stage clearly showed the presence of dUMP and LY231514 ligands in the active site and they were therefore added to the model. Their inclusion lowered the crystallographic R-factor as well as Rfree by 0.9%. Non-crystallographic symmetry matrices were refined after a nearly complete model was built. The positions of ligands were checked in each active site separately by calculating simulated annealing omit maps,45 omitting successively the four pairs of ligands. The final model includes residues 28-313 of hTS, dUMP and LY231514. The R-factor was 26.8% (Rfree = 31.1%) for all data between 38 and 3.0 Å.
**Table 1. Data statistics with outer shell statistics given in parentheses**

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**Human TS crystal form B (triclinic).** We used a partially refined structural model for the human TS-dUMP-LY231514 dimer from the monoclinic crystal (R-factor = 28.8 %, Rfree = 33.3 %) as a search model for molecular replacement in the triclinic crystal form. A rotation search in AMoRe gave two pairs of peaks (values of rotation function 39.2 and 40.6 with mean = 0 and standard deviation 2.70). These two rotation solutions were used as the starting point for a translation search in AMoRe. With one dimer fixed, a single translation solution emerged for the second dimer (value of the translation function = 17.5 with mean = 0 and standard deviation = 1.41; R-factor = 46.6 for data from 20 to 3.5 Å). The asymmetric unit thus comprises two dimers that are non-crystallographically related by a translation and a rotation. Refinement in X-PLOR treating each monomer as a rigid body gave an R-factor = 42.4 % (Rfree = 43.2 %) for data from 7.0 to 3.0 Å. Strict non-crystallographic symmetry was applied among the four monomers in the asymmetric unit. Hand rebuilding was carried out in O by positional and grouped B-factor refinement in X-PLOR and CNS. A bulk solvent correction was applied in X-PLOR. Non-crystallographic constraints were replaced with restraints and individual isotropic B-factors were refined with restraints in the final stages. The final model includes residues 28-313 of human TS, dUMP and LY231514, 48 water molecules and one phosphate group. The crystallographic R-factor was 23.6 % (Rfree = 26.4 %) for all data between 23 and 2.9 Å.

**E. coli TS**

**Expression, crystallization and data collection**

EcTS was expressed from the thyA gene in E. coli strain 2913 and stored as an ammonium sulfate precipitate at −20 °C. Protein was dialyzed against 20 mM KPO₄ (pH 8.0), 3.8 mM DTT, 0.05 mM EDTA for crystallization. Ternary complex crystals were grown by hanging drop crystallization of TS with the substrate dUMP and polyglu-LY231514, LY341770 or LY338529. A 5.0 g/l drop of protein solution (20 mM KPO₄ (pH 8.0), 1.5 mg/mL TS, 1.9 mM LY231514, 5.8 mM dUMP, 3.8 mM MgCl₂, 3.8 mM DTT, 0.05 mM EDTA, 1.25 M (NH₄)₂SO₄) was equilibrated at 23 °C against an excess of precipitant solution (2.5 M (NH₄)₂SO₄, 20 mM KPO₄ pH 8.0). Crystals with hexagonal rod morphology grew in two to three days. Diffraction intensities were collected at room temperature using a Raxis II area detector system with CuKα X-rays from a Rigaku rotating anode detector operating at 50 kV and 300 mA. Intensities were integrated, scaled and reduced with the programs Denzo and Scalepack from the HKL suite.

**Structure determination and refinement**

Structures were determined by isomorphous molecular replacement using the structure of the EcTS-dUMP-ralitrexed ternary complex (PDB accession code 2kce), which crystallizes in the same crystal form, but omitting

**Table 2. Refinement statistics with outer bin statistics in parentheses**

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<th>HTS A</th>
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<th>EcTS</th>
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the ligands and water molecules. Rigid body least-squares minimization of position using X-PLOR gave R-factors of 28-29% using all data between 7.0 Å and 2.5 Å. Difference electron density maps calculated based on the protein alone displayed clear density for the folate inhibitors in both structures. Well-defined density was also apparent for dUMP. The ligands were placed into the difference electron density using O.18 Ligand density was clearly defined and well-ordered in one of the dimer's two active sites, while ligands were more flexibly defined in the second. Structures were subjected to alternating cycles of rebuilding, and positional and B-factor refinement using X-PLOR or CNS.43,47 Water molecules were chosen at difference density peaks based on favorable hydrogen-bonding geometry. Data collection and refinement statistics are summarized in Tables 1 and 2.

Structure comparisons and analysis

As TS binds ligands, concerted shifts of secondary structural elements toward the active site are accompanied by a marked migration of the four C-terminal residues that allow for ordered binding of the substrate and cofactor, and for product release.18 In addition, substrate binding to the human apo-enzyme may involve more dramatic rearrangements.15 Therefore, a comparison with the apo-form of the enzyme illustrates common and unique features of folate binding to human TS. To further analyze the basis for binding specificity of the pyrrolo[2,3-d]pyrimidine-based inhibitors, and the potential for improvement in these properties, we compared our structures with two other closed, fully liganded complexes, EcTS-dUMP-ralitrexed20 and hTS-dUMP-ralitrexed,21 and with a more open ternary complex, rat TS-dUMP-ralitrexed.14 Conformational differences were identified using difference distance matrices produced with the DDMP program from the Center for Structural Biology at Yale University, New Haven, CT. Structures were superimposed by overlapping their structural cores (the largest set of contiguous residues that showed no structural differences between the two structures) as identified using our NEWDOME program, by Eric Fauman. LSQMAN from the DEJAVU suite of programs was used to superimpose the structural cores.

Protein Data Bank accession numbers

Coordinates and structure factor amplitudes have been deposited in the RCSB Protein Data Bank with the following accession codes: hTS-dUMP-LY231514 (P1 space group), 1JU6; hTS-dUMP-LY231514 (C2 space group), 1JUJ; EcTS-dUMP-LY341770, 1JTP; EcTS-dUMP-polyglu-LY231514, 1JTJ, EcTS-dUMP-LY38529, 1JUT.

Acknowledgments

This work was supported by NIH grant CA 63081 (R.M.S.) and Eli Lilly, Inc. P.H.S. is the recipient of a Jane Coffin Childs Fellowship, and T.A.F. is supported by a postdoctoral fellowship from NIH (AI 10279). We thank Dr Richard Schevitz for comments on the manuscript.

References


*Edited by I. A. Wilson*

(Received 18 June 2001; received in revised form 4 September 2001; accepted 5 September 2001)