The Crystal Structure of Thymidylate Synthase from Pneumocystis carinii Reveals a Fungal Insert Important for Drug Design

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Thymidylate synthase from Pneumocystis carinii (PcTS) is an especially important drug target, since P. carinii is a fungus that causes opportunistic pneumonia infections in immune-compromised patients and is among the leading causes of death of AIDS patients. Thymidylate synthase (TS) is the sole enzyme responsible for the de novo production of deoxythymidine monophosphate and hence is crucial for DNA replication in every organism. Inhibitors selective for P. carinii TS over human TS would be greatly beneficial in combating this disease. The crystal structure of TS from P. carinii bound to its substrate, dUMP, and a cofactor mimic, CB3717, was determined to 2.6 Å resolution. A comparison with other species of TS shows that the volume of the closed PcTS active-site is 20% larger than that of five other TS closed active-sites. A two-residue proline insert that is strictly conserved among all fungal species of TS, and a novel C-terminal closing interaction involving a P. carinii-specific tyrosine residue are primarily responsible for this increase in volume. The structure suggests several options for designing an inhibitor specific to PcTS and avoiding interactions with human TS. Taking advantage of the residue substitutions of P. carinii TS over human TS enables the design of a selective inhibitor. Additionally, the larger volume of the active-site of PcTS is an important advantage for designing de novo inhibitors that will exclude the human TS active-site through steric hindrance.

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Introduction

Thymidylate synthase (TS) is an essential enzyme in the cell-cycle for the production of dTMP, one of the four DNA bases. In an ordered binding reaction (Danenberg & Danenberg, 1979; Fromm, 1967) the substrate, dUMP, followed by the cofactor, 5,10-methylene tetrahydrofolate, or an analog, binds to the active-site cavity of TS, causing the C terminus to fold toward the active-site and the active-site to close (Montfort et al., 1990; Perry et al., 1990). This conformational change sequesters the ligands from the bulk solvent and enables the formation of a covalent bond from the active-site cysteine nucleophile to C6 of dUMP. There is evidence from equilibrium dialysis (Dev et al., 1994), fluorescence spectroscopy (Spencer et al., 1997), differential scanning calorimetry (Chen et al., 1996) and mutagenesis (Maley et al., 1995) studies that TS is a negatively cooperative, or a half-the-sites reactive enzyme. This evidence shows that dUMP and cofactor bind the first active-site with higher affinity than the second active-site.
Because of its essential nature, TS has long been recognized as a target for anticancer drugs (Jackson et al., 1983; Jones et al., 1981; Varney et al., 1992). Previous drug design efforts have focused on either mechanism-based inhibitors, mimicking either the substrate or the cofactor, or novel inhibitors developed through structure-based drug design (Stout et al., 1999; Varney et al., 1992; Webber et al., 1993). TS is also an excellent target for species-selective drug design for antiproliferatives, whereby the structural differences between the enzymes from a pathogenic and human species are exploited. Here, PcTS was crystallized with 10-propargyl-5,8-dideazafolate (CB3717) (Calvert et al., 1986), a potent antifolate inhibitor (IC₅₀ = 90 nM for this species) (Gangjee et al., 1996), in order to determine the interactions of the active-site residues in the closed conformation and assess any potential species-selective drug design opportunities.

We have determined the structure of the ternary complex of PcTS, dUMP and CB3717 to 2.6 Å resolution. One dimer crystallized in the asymmetric unit and exhibits the asymmetry between monomers predicted by the biochemical cofactor binding experiments (Chen et al., 1996; Dev et al., 1994; Spencer et al., 1997). Despite the strong evolutionary conservation of the residues in the active-site, two residues specific to fungi force conserved helices away from the active-site of P. carinii TS. Because of this insertion, the active-site of PcTS is 20% (90 Å³) larger than that of the ternary complexes of Escherichia coli TS (EcTS, PDB ID:2tsc) (Montfort et al., 1990), human TS (P. Sayre, unpublished results), Leishmania major TS (LmTS, coordinates kindly provided by D. Matthews) (Knighton et al., 1994), and Lactobacillus casei TS (LcTS, PDB ID:1lca) (Finer-Moore et al., 1993). A two-residue proline insert specific to fungi force conserved helices away from the active-site, partially accounting for the larger size. In addition, one of the C-terminal closing interactions in PcrTS involves a tyrosine residue that is specific to PcTS (Phe in hTS), reorienting and opening the closed active-site. The positions of active-site residues specific to PcTS with respect to hTS, as well as the increased volume of the active-site of PcrTS as compared to all other closed complexes of TS, are examined with the goal of designing species-selective inhibitors.

Results

Analysis of the structure

A dimer of PcTS (Figure 1) (70 kDa) crystallized in the asymmetric unit of the P2₁2₁2 unit cell. The enzyme is comprised of two identical polypeptides and is an obligate dimer: each active-site uses two arginine residues (Arg153, Arg154; EcTS numbering will follow for reference: 126, 127) contributed across the dimer interface from the opposing monomer. The core of the enzyme comprises an extensive β-sheet (strands i-ν) that forms the dimer interface and resembles the interfaces of other TS species (Finer-Moore et al., 1993; Knighton et al., 1994; Montfort et al., 1990). This dimer interface has a buried accessible surface area of 197.2 Å² (Jones & Thornton, 1995, 1996). In each monomer, helices B, D and J surround active-sites that are separated by 21.6 Å across the dimer interface.

Despite the identity of the polypeptides that comprise the dimer of PcrTS, the structures of the monomers in PcrTS are not equivalent. The crystal contacts for the dimer and its symmetry-related molecules are not in the vicinity of the active-sites, providing evidence that the asymmetry is not induced by crystallization. Monomer A binds the substrate, dUMP, and the cofactor analog, CB3717, and exhibits the “closed” conformation (Fauman et al., 1994; Matthews et al., 1990; Montfort et al., 1990). In this conformation there is a covalent bond from the enzyme to the substrate, a substrate molecule positioned for catalysis, and several hydrophobic bonds with a five dimer interactions between the C terminus and the cofactor and active-site residues. Specifically, in the closed monomer, both Arg26 (Ec:R21) and Asn90 (Ec:W83) form hydrophobic bonds to the carboxyl terminus and five van der Waals interactions between the C terminus and the cofactor and active-site residues. Specifically, in the closed monomer, both Arg26 (Ec:R21) and Asn90 (Ec:W83) form hydrophobic bonds to the carboxyl terminus and five van der Waals interactions between the C terminus and the cofactor and active-site residues. Specifically, in the closed monomer, both Arg26 (Ec:R21) and Asn90 (Ec:W83) form hydrophobic bonds to the carboxyl terminus and five van der Waals interactions between the C terminus and the cofactor and active-site residues.
without a covalent bond. The substrate is misoriented and the C terminus participates in a limited number of interactions with the residues in the active-site. The C-terminal interactions in monomer B include only one hydrogen bond from Arg26 (Ec:R21) to the carboxyl terminus and three van der Waals interactions: Thr27 (Ec:T22) with Ile297 (Ec:I264), His245 (Ec:H212) with Lys294 (Ec:P261) and Leu205 (Ec:L172) with Met293 (Ec:A260).

This asymmetry is supported by biochemical experiments (Chen et al., 1996; Dev et al., 1994; Maley et al., 1995; Spencer et al., 1997) and is analyzed elsewhere (Anderson et al., 1999). Conformational changes that occur when a covalent bond forms in one monomer affect both the dimer interface and active-site residues of the second monomer. These conformational changes prohibit a covalent bond from forming in the second site and shift two key residues: Asn210 (Ec:N177), a residue important for specific hydrogen bonding to the pyrimidine base, and Tyr242 (Ec:Y209), an important hydrogen bond donor to the ribose O3’.

These residues do not form hydrogen bonds to the substrate in monomer B, causing the base to become misoriented and provide an unstable docking surface for the cofactor. This results in an altered Kd value for the cofactor (Finer-Moore et al., 1998).

The effect of this asymmetry, caused by the conformational changes incurred when one monomer binds the antifolate, results in an enzyme that is half-the-sites reactive: binding a ligand in one active-site prohibits ligand binding in the opposite active-site.

There have been two previous structures of ternary eukaryotic TS complexes: L. major DHFR-TS bifunctional enzyme (Knighton et al., 1994) bound to dUMP and CB3717 (in the TS subdomain) and rat TS bound to dUMP and Tomudex (Sotelo-Mundo et al., 1999). Two conserved eukaryotic loops (residues 95-106 and 124-131, shown in red in Figure 1) that have been conserved throughout at least 13 eukaryotic species of TS show the same conformations in all three enzymes. PcTS also contains one loop specific to the fungal family, residues 183-188, but the temperature factors for this region are high and the electron density is disordered, rendering the modeling of this region unreliable.

### Substrate and inhibitor binding

Thymidylate synthase binds the substrate, dUMP, and cofactor, 5,10-methylene tetrahydrofolate, in order to catalyze the reductive methylation of the substrate and produce dTMP and dihydrofolate. Here, monomer A binds the substrate and CB3717 (Figure 2a) the cofactor analog, while monomer B binds only the substrate. This situation is not common, but arises from the fact that the asymmetric dimer formed during the time that the protein and CB3717 incubated together in solution. The heterodimer then formed different lattice contacts between each monomer and other symmetry-related molecules. The substrate is bound (see Figure 2a and (b) and Table 2a) in monomer A in a productive manner typical of the form seen in other structures of TS (Finer-Moore et al., 1993; Knighton et al., 1994; Montfort et al., 1990) but shows a different arrangement of hydrogen bonds to the phosphate moiety. The phosphate moiety of dUMP in PcTS is hydrogen bonded to two arginine residues: Arg199 (Ec:R166) and Arg154’ (Ec:R127’), where Arg154’ represents an arginine residue donated from the opposite monomer. Typically, in EcTS, there are four hydrogen bonds from active-site residues to the phosphate moiety (see Table 2a). In monomer A, N3 and O4 of the pyrimidine base are hydrogen-bonded to the specificity residue, Asn210 (Ec:N177), that discriminates between deoxyuracil and other potential substrates, and Tyr242 (Ec:Y209) and Arg153’ (Ec:R126’) form hydrogen bonds to the O3’ of the ribose. The enzyme in monomer A forms a covalent bond from S’ of the catalytic cysteine residue (Cys173, Ec:C146) to C6 of dUMP. Residues in monomer B show the same hydrogen bonds to the phosphate group and Arg153’ (Ec:R126’) forms a hydrogen bond to O3’ of dUMP. However, in monomer B Asn210 (Ec:N177) does not hydrogen bond to the pyrimidine base, Tyr242 (Ec:Y209) does not hydrogen bond to O3’ and there is no covalent bond from Cys173 (Ec:C146) to C6 of dUMP.

PcTS binds the inhibitor, CB3717 (Figure 2a and (b)), in monomer A in a manner similar to the natural cofactor, 5,10-methylenetetrahydrofolate, in the ternary structure of EcTS withFdUMP (dUMP with a fluorine substitution at the C5 position) (Hyatt et al., 1997). There are seven hydrogen bonds from the enzyme to CB3717: Tyr242 (Ec:Y209) and Ser296 (Ec:A263) bind the exocyclic amino group of the quinazoline, the amide nitrogen atom of Phe58 (Ec:H51) binds the O1 group of the glutamate residue, Asp202 (Ec:D168) binds NH3 of the quinazoline, and two well-ordered water molecules bind both the glutamate oxygen atoms. In addition to these hydrogen bonds, there are several van der Waals interactions: Trp87 (Ec:W80), Met295 (Ec:V262), Phe209 (Ec:F176) and

### Table 1. Refinement statistics (outer shell statistics shown in parentheses)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
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</tr>
<tr>
<td>Unit cell edges (Å)</td>
<td>a = 54.04, b = 66.16, c = 178.76</td>
</tr>
<tr>
<td>Resolution (Å)</td>
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</tr>
<tr>
<td>Observed reflections</td>
<td>57,081</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>20,517</td>
</tr>
<tr>
<td>Completeness (%) (2.76-2.6 Å)</td>
<td>81.2 (63.1)</td>
</tr>
<tr>
<td>Rmerge (50-2.6 Å)</td>
<td>0.127 (0.299)</td>
</tr>
<tr>
<td>Rmerge (50-2.6 Å)</td>
<td>0.216 (0.276)</td>
</tr>
<tr>
<td>Rmerge (50-2.6 Å)</td>
<td>0.293 (0.39)</td>
</tr>
<tr>
<td>Total no. atoms</td>
<td>5897</td>
</tr>
<tr>
<td>No. water molecules</td>
<td>196</td>
</tr>
<tr>
<td>Rmsd bonds (Å)</td>
<td>0.009</td>
</tr>
<tr>
<td>Rmsd angles (deg.)</td>
<td>1.7</td>
</tr>
<tr>
<td>Average B-factor (Å²)</td>
<td>22.2</td>
</tr>
</tbody>
</table>
The uracil base forms close van der Waals interactions with the quinazoline rings, and Phe209 (EcFl76) and Ile86 (EcI79) form interactions with the p-amino benzoic acid (PABA) moiety of CB3717. The electron density corresponding to the active-site residues and ligands is shown in Figure 2(c).

The binding interactions between PcTS and the substrate, dUMP, and cofactor analog, CB3717, are different from those of EcTS bound to dUMP and CB3717, a well-studied TS ternary complex (Montfort et al., 1990; Rutenber & Stroud, 1996; Stout & Stroud, 1996). The closed PcTS active-site is more open than the closed EcTS active-site and the number of hydrogen bonds between the enzyme and dUMP, as well as the longer distances of some of the hydrogen bonds to CB3717, reflect this (see Table 2A and B). With one exception, the residues that form hydrogen bonds in EcTS are structurally conserved in PcTS, but the PcTS residue is too far away (more than 3.2 Å) to form a hydrogen bond. The one exception is Phe58 (His51 in EcTS), which forms a hydrogen bond between its amide nitrogen atom and O1 of the glutamate tail. Phe58 uses a different rotamer than His51 of EcTS, and shifts toward the inhibitor in order to form a hydrogen bond with the glutamate tail. Additionally, in EcTS, there are very few atoms in the quinazoline ring more than 3.6 Å from dUMP, in PcTS this distance is 4.8 Å. The greater number of contacts between EcTS and CB3717 is reflected
in the lower IC\textsubscript{50} value for EcTS versus PcTS for CB3717 (10 nM and 90 nM, respectively).

**Comparison of PcTS to other thymidylate synthase structures**

The structures of all species of TS determined so far are very similar at the core of the enzyme, the β-sheet interface, but vary in the specific arrangement of the active-site residues. The structure of rat TS (Sotelo-Mundo et al., 1999) is notable in that both monomers bind the substrate and antifolate but neither forms a closed complex, making this structure a difficult comparison target. In general, PcTS is very comparable to LmTS, another eukaryotic species, in overall fold and in the positions of the eukaryotic loops. However, the two enzymes differ in several key areas, one of which is the C-terminal closing interaction. Figure 3 shows the C-terminal closing interactions for PcTS, LmTS and EcTS. It is evident that there are several interactions that are different between the prokaryotic and eukaryotic enzymes: Gly204 (Ec:F171) is a residue conserved within eukaryotes and cannot provide the same van der Waals surface as the prokaryotic Phe. Met295 (Ec:V262) and Met293 (Ec:A260) are also conserved within eukaryotes, with the former showing strong van der Waals interactions with the quinazoline portion of the folate molecule. Arg26 (PcTS, Ec:R21) and Arg254 (LmTS) do not form hydrogen bonds to dUMP, whereas the related prokaryotic arginine residue does. Importantly, Arg26 (Ec:R21) in PcTS forms hydrogen bonds to both the carboxyl terminus (Ile297, Ec:I264) and the hydroxyl group of Tyr95 (no corresponding Ec residue). Since Arg26 (Ec:R21) forms a hydrogen bond to Tyr95, it pulls away from the active-site and cannot form a bond with dUMP, as occurs with EcTS. Position 95 is Phe and not Tyr in human and almost all other species of TS, making this interaction specific to PcTS and a limited number of other enzymes, including *S. cerevisiae*. In most other closed ternary complexes of TS, Asn90 (W83 in EcTS, Asn317 in
Table 2. Comparison of interactions

<table>
<thead>
<tr>
<th>Interaction</th>
<th>PcTS</th>
<th>EcTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Comparison of dUMP interactions: PcTS versus EcTS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP1 (PO₄,O)</td>
<td>Arg199 (2.7)</td>
<td>Arg166 (2.5), Ser167 (2.7)</td>
</tr>
<tr>
<td>OP2</td>
<td>Arg154 (2.8)</td>
<td>Arg127 (2.8), Arg166 (2.9)</td>
</tr>
<tr>
<td>OP3</td>
<td></td>
<td>Arg126 (3.4)</td>
</tr>
<tr>
<td>O85 (S O)</td>
<td>Arg153(3.2), Tyr242 (2.6)</td>
<td>Arg21 (3.1)</td>
</tr>
<tr>
<td>O83 (3’ O)</td>
<td>N of Asp202 (2.8)</td>
<td>His207 (2.7), Tyr209 (2.6)</td>
</tr>
<tr>
<td>O2</td>
<td>Asn210 (3.4)</td>
<td>Asp169 (2.9)</td>
</tr>
<tr>
<td>N3</td>
<td>Asn210 (3.0)</td>
<td>Asn177 (2.9)</td>
</tr>
<tr>
<td>O4</td>
<td></td>
<td>Asn177 (3.1)</td>
</tr>
<tr>
<td>B. Comparison of CB3717 interactions: PcTS versus EcTS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄ of quinazoline</td>
<td>Tyr242 (3.3), Ser296 (3.0)</td>
<td>O of Ala263 (2.9), Water 3</td>
</tr>
<tr>
<td>O1</td>
<td>N of Phe58 (2.9), Water 1</td>
<td>Water 2</td>
</tr>
<tr>
<td>N1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH3</td>
<td>Asp202 (3.4)</td>
<td>Asp169 (3.2)</td>
</tr>
<tr>
<td>O4</td>
<td>Water 2</td>
<td>N of Gly173 (3.3)</td>
</tr>
<tr>
<td>vDW (Quinazoline)</td>
<td>Trp 85, Met295, Phe209, Uracil base</td>
<td>Trp 85, Leu195, Trp 82, Uracil base</td>
</tr>
<tr>
<td>vDW (PABA group)</td>
<td>Phe209, Ile86</td>
<td>Leu224, Ile81, Phe228</td>
</tr>
</tbody>
</table>

Structurally identical residues are shown in bold. Hydrogen bond lengths are shown in parentheses following the residue name.

LmTS) hydrogen bonds to the carboxyl terminus, but in PcTS it does not (distance 3.5 Å).

The most striking observation in a comparison of PcTS and other species of the enzyme is that the volume of the closed ternary bound active-site is larger than the volumes of the closed ternary bound sites of E. coli (see above), L. casei (Finer-Moore et al., 1993), human (P. Sayre, unpublished results), and L. major (Knighton et al., 1994). Specifically, in comparison with LmTS, a difference-distance matrix calculation (determination-distance matrix plots were produced using the program DDMP from the Center for Structural Biology at Yale University, New Haven, CT) reveals that there are several regions of the enzymes that differ (see Figure 4(a)). The most significant differences are in the neighborhood of Pro37 (no corresponding Ec residue), Ser38 (Ec:H32) and Pro39 (Ec:Q33) (shown in magenta in Figure 4(b)). These residues belong to a functional insertion that is conserved among the four known sequences of fungi TS, is present in no other family of TS enzymes (see Figure 5), and has a series of structural and functional consequences. The serine residue in PcTS is structurally equivalent to the proline residues found in Cryptococcus neoformans and Candida albicans TS, since the hydroxyl side-chain forms a hydrogen bond to the amide nitrogen atom in the peptide backbone.

The residues form a rigid bulge in the first β-strand (β-strand i) near the dimer interface and affect both the relative positioning of the two monomers and the “second shell” of residues from the active-site. These second shell residues do not interact with the substrate and inhibitor directly, but influence the packing of the residues in the first shell that do interact directly. The β-sheet in TS has a notable and rare right-handed twist and crease in the region of the dimer interface. In all known species of TS, a bulge beginning in β-strand i at position 36 (Ec:G31) propagates through β-strand ii at position Gly237 (Ec:G204) and β-strand iii at Arg199 (Ec:R166). The resulting kink in the β-sheet begins at the protein surface and penetrates 20 Å down to the active-site (Matthews et al., 1989), to position residues that form key hydrogen bonds to the dUMP phosphate moiety and the folate molecule (Matthews et al., 1989; Montfort & Weichsel, 1997). Residues Phe30 and Gly31 of EcTS are intolerant to mutagenesis except for the substitutions Tyr30 and Ser31 (Kim et al., 1992), mutations that render the bacterium temperature-sensitive. The proline insert in the PcTS β-strand i at the beginning of the bulge changes this pattern of interactions and induces a large-scale reorientation of the hydrogen bonding interactions with dUMP and the cofactor (Table 2A and B).

The Pro-Ser-Pro insertion, effectively Pro-Pro-Pro, is located in the middle of the first β-strand, breaking the strand and the hydrogen bonding interactions throughout that entire section of β-sheet. The insertion forces a rearrangement of β-strand ii (residues 239-250, Ec:206-217), a region showing displacements between 1.5 and 2.5 Å from LmTS in a difference-distance matrix plot (Figure 4(a)). β-Strand ii pulls away from the active-site, causing Tyr242 (Ec:Y209), which forms a hydrogen bond to the ribose of dUMP, to reposition. β-Strand ii is also packed against the N terminus of the J helix (residues 202-224, Ec:169-191), forcing it to pull away from the active-site. The J helix contains several residues important for both substrate and cofactor binding (eg. Asp202 (Ec:D169), Phe209 (Ec:F176) and Asn210 (Ec:N177)). The retraction of both β-strand ii and the J helix creates a larger PcTS active-site, concentrated especially in the area immediately adjacent to β-strand ii and the N terminal of the J helix.
Figure 3. C-terminal closing interactions for (a) PcTS, (b) LmTS and (c) EcTS. The five C-terminal residues of each enzyme are shown with a connected backbone, residues forming hydrogen bonds to the C terminus are also displayed.

Importantly, there is a structural rearrangement of the substrate and inhibitor due to the altered hydrogen bonding pattern between PcTS residues and the substrate and cofactor, despite a well-conserved sequence of active-site residues.

Drug design

In order to fight the opportunistic infections caused by \textit{P. carinii} and produce a more selective inhibitor against PcTS than human TS, the struc-
ture of PcTS was assessed for any global differences from hTS and to locate residues specific to PcTS. The structure suggests two pathways for identifying inhibitors selective for PcTS over hTS: first, de novo inhibitor design taking advantage of the size difference between the PcTS active-site and other TS species; and second, exploiting the residues in PcTS that are different from those in hTS.

The larger size of the ternary bound monomer suggests that de novo inhibitors can be developed that will exclude binding to human TS through steric hindrance (Figure 6(a) shows a superposition of the PcTS and LmTS active-sites). To design de novo inhibitors, a database of available chemicals is being scanned for novel inhibitors that target either the open or closed monomers of PcTS (A.A., unpublished results). Since the insert in PcTS is specifically conserved in the fungal TS family and is primarily responsible for the realignment of the active-site and its increased volume, there may be a good chance of designing a fungal-specific inhibitor that does not interact with human TS.

There are only a few residues that differ between PcTS and hTS and that fit the criterion of being within a 5-10 Å shell from the inhibitor (Figure 6(b)); however, it should be possible to...
take advantage of four residues in the active-site that differ between the two proteins. Within a 5 Å shell, Ile84 (Ec:V77) in PcTS is a valine residue in human TS and near the C terminus there are two substitutions, Ser296 (PcTS, Ec:A263) for alanine (hTS) and Ile297 (PcTS, Ec:I264) for valine (hTS). These provide options for the design of an antifolate or a novel inhibitor with more specific interactions with PcTS. The substitution of Ser296 is promising, since a serine to alanine change represents a change of polarity and the possibility of a hydrogen bond with a modified inhibitor. The two isoleucine to valine substitutions represent an opportunity for increased hydrophobic contacts with the larger side-chain. Exploiting hydrophobic contacts has proved successful for the design of the BW1843U89 antifolate inhibitor (Montfort & Weichsel, 1997; Stout & Stroud, 1996; Weichsel & Montfort, 1995). Within the 5-10 Å shell, His85 (Ec:T78) in PcTS is a lysine residue in human TS. A new inhibitor could take advantage of either a ster-

ic conflict that would be present with the lysine residue in hTS but would not be present with the P. carinii TS or the altered geometry of hydrogen bond donors and acceptors presented by the histidine residue.

Discussion

P. carinii is a fungus that infects immune-compromised patients and causes pneumonia infections (PCP). PCP is the most common serious opportunistic infection suffered by AIDS patients; it also affects bone-marrow recipients and donors, cancer chemotherapy patients and the elderly. Trimethoprim-sulfamethoxazole and pentamidine are current therapeutics for PCP but are limited by renal, gastrointestinal and hepatic toxicity. New therapies that specifically target P. carinii, showing reduced toxicity, would be a great advantage.

The co-crystal structure of TS from P. carinii, bound to its substrate, dUMP, and an inhibitor,
CB3717, and solved to 2.6 Å, reveals that PcTS has four *P. carinii*-specific residues that contact CB3717, providing options for the design of a new inhibitor with increased specificity. Importantly, PcTS has a larger active-site in the closed conformation than human, *E. coli*, *L. casei* or *L. major* TS. This difference is largely due to a fungal-specific insert in a β-sheet that, in combination with an adjacent con-

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**Figure 5.** Sequence alignment of three fungal TSs: PcTS, CnTS (TS from *C. neoformans*) and CaTS (TS from *C. albicans*), hTS, rat TS, LmTS and EcTS. The sequence alignment was prepared with CLUSTALW (Thompson et al., 1994) and the NCSA Computational Biology Programs.

**Figure 6.** Drug design opportunities. (a) Stereo view of a superposition of PcTS and LmTS active-site residues showing the difference in active-site volume. PcTS residues (including CB3717) are in blue, LmTS residues are in red. (b) Residues that differ between PcTS and hTS, providing options for the design of an inhibitor with increased specificity for PcTS. The distances of the residues from the nearest atom of CB3717 are in parentheses. A Cα atom trace is shown for perspective.
served fungal-specific proline residue, forms a bulge that reorients the other helices that pack around the active-site. A novel C-terminal closing interaction involving Tyr95, a specific PctS residue, also causes an increase in the size of the active-site. The larger PctS active-site may be crucial to designing PctS-specific inhibitors and may be relevant as well to the design of other fungal-specific TS inhibitors for C. albicans and C. neoformans. These three fungal organisms cause potentially lethal opportunistic infections in immune-compromised patients and could be targeted based on the structural differences between the human and P. carinii TS.

Materials and Methods

Protein purification and enzyme assays

Thymidylate synthase from P. carinii was cloned and expressed from E. coli according to the methods described by Santi et al. (1991), and activity was monitored with a colorimetric assay developed by Wahba & Friedkin (1961).

Crystallizations

Crystallizations were carried out at room temperature using the hanging drop method. Enzyme (100 μM) was incubated with 2 mM CB3717, 2 mM dUMP and 1 mM DTPT for one hour at 4°C. A volume of 3 μl of this mixture was combined with 3 μl of well reservoir (12.5% (v/v) PEG8000, 0.2 M (NH4)2SO4, 1 mM DTPT, 0.05 M Tris (pH 7.5)). Crystals usually appeared within one day and measured 0.5 mm × 0.05 mm × 0.05 mm. The crystals consisted of clusters of thin plates that grew from a single nucleus and were difficult to manipulate both for crystal mounting and data collection.

Cryocrystallography

The crystals were frozen in a cold stream (100 K). Data were collected at Stanford Synchrotron Radiation Laboratory (beamline 7-1) at wavelength 1.08 Å using a Mar image plate detector. Complete data collection was impossible, since the long axis of the crystal (a* in reciprocal space) aligned with the phi axis, leading to a cusp of data that could not be collected given the geometry of the Mar system. However, the data show uniform completeness throughout the entire resolution range.

Structure solution

The data were processed with Denzo and Scalepack (Otwinowski, 1993) and converted to structure factors using the program, Truncate, from the CCP4 suite (Collaborative Computational Project Number 4, 1994). Using a model of thymidylate synthase from C. neoformans (A.A., unpublished results), one copy of the dimer of thymidylate synthase from P. carinii was located in the asymmetric unit using the molecular replacement facilities in XPLOR (Brünger, 1996), resulting in a starting R-factor of 45%. A total of 10% of the reflections were set aside for the Rfree calculation and were never recombined with the reflections used to calculate a working R-factor. The model, after substitution of the C. neoformans residues with P. carinii residues, was refined with several cycles of model building followed by cycles of conjugate gradient and simulated annealing refinement in CNS (Adams et al., 1997; Brünger, 1992; Pannu & Read, 1996). The electron density for the enzyme showed good connectivity in 2Fo − Fc maps and electron density for the ligands became clear after a few cycles of refinement, allowing modeling of two molecules of dUMP and one molecule of CB3717. Non-crystallographic symmetry constraints were initially applied between the two monomers but, since the active-sites appeared to be asymmetric with respect to ligand binding, the constraints were removed early in the refinement process and the residues of each monomer were treated independently. The position of each residue was confirmed with simulated annealing omit map calculations and model fitting in O (Jones et al., 1991). Individual temperature factors were refined once the value of Rfree had reached 32%. Water molecules were included in the model if they had electron density at least three sigma above the average density level and if they showed good hydrogen bonding geometry to a protein donor or acceptor. The refinement of the final model converged with an R_factor of 21.6% and an R_free value of 28.3% and good geometry (rmsd bonds = 0.009 Å, angles = 1.7°) (Table 1). There are no discontinuities in the electron density of the main-chain for any residue, except for the extreme N terminus (residues 1 and 2) and a loop (residues 186-188) in each monomer, and there are no outlying residues in the Ramachandran plot.

Protein Data Bank accession numbers

The coordinates can be accessed from the Protein Data Bank with code 1CI7.

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