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## Pairwise Specificity and Sequential Binding in Enzyme Catalysis: Thymidylate Synthase<sup>†</sup>

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**ABSTRACT:** The structures of thymidylate synthase (TS) from *Escherichia coli*, in ternary complexes with substrate and an analogue of the cofactor, are the basis of a stereochemical model for a key reaction intermediate in the catalyzed reaction. This model is used to compare the reaction chemistry and chirality of the transferred methyl group with structures of the components, to identify those residues that participate, and to propose a stereochemical mechanism for catalysis by TS. Effects of chemical modification of specific amino acid residues and site-directed mutations of residues are correlated with structure and effects on enzyme mechanism. The ordered binding sequence of substrate deoxyuridine monophosphate and methylene-tetrahydrofolate can be understood from the structure, where each forms a large part of the binding site for the other. The catalytic site serves to orient the reactants, which are sequestered along with many water molecules within a cavernous active center. Conformational changes during the reaction could involve nearby residues in ways that are not obvious in this complex.

Thymidylate synthase (TS)<sup>1</sup> catalyzes methyl transfer from the cofactor 5,10-methylenetetrahydrofolate ( $\text{CH}_2\text{-H}_4\text{folate}$ ) to 2'-deoxyuridine 5'-monophosphate (dUMP) to give 2'-deoxythymidine 5'-monophosphate (dTDP) and dihydrofolate ( $\text{H}_2\text{folate}$ ). The cofactor  $\text{CH}_2\text{-H}_4\text{folate}$  is normally polyglutamylated by up to seven glutamyl residues. Here we relate what is known of the mechanism of this multistep reaction to the high-resolution structures of TS that we solved first for the *Lactobacillus casei* enzyme (Hardy et al., 1987) and later for complexes formed between the *Escherichia coli* enzyme, substrate, and cofactor analogues (Perry et al., 1990; Montfort et al., 1990). These are three of six highly refined TS structures solved in our laboratory. In the *E. coli* pair there are no disordered residues; thus, we have very high confidence in the total correctness of the structures.

A proposed chemical mechanism, consistent with the protein structure for TS, is shown in Figure 1 (Pogolotti & Santi, 1977; Heidelberger et al., 1983). The cofactor  $\text{CH}_2\text{-H}_4\text{folate}$  plays a dual role. First, it is a one-carbon donor, and then it is a reductant of the transferred methylene at different steps in the reaction (Humphries & Greenberg, 1958; Friedkin, 1959). It is regenerated stereospecifically by dihydrofolate reductase and serine hydroxymethyltransferase in the thymidylate synthase cycle. Because of the increased needs for thymine in dividing cells, TS, with its essential role in DNA synthesis, is a target for anticancer drug design.

A major factor in understanding catalysis by TS has been the discovery of analogues of reaction intermediates. The stable, reversible ternary complex of TS,  $\text{CH}_2\text{-H}_4\text{folate}$ , and the 5-fluoro derivative of the substrate dUMP (FdUMP) has

been particularly useful (Santi & McHenry, 1972). This complex is an analogue of the steady-state intermediate II of Figure 1, differing only by substitution of a fluorine for a hydrogen atom at C-5 of dUMP (Santi & Danenberg, 1984; Moore et al., 1986). C-6 of FdUMP is covalently bound to Cys-198 (Pogolotti et al., 1976; Bellisario et al., 1979). Cys-198 lies in a wide cavity on the protein surface, surrounded by conserved hydrogen-bonding residues. Reaction of this thiol with C-6 of dUMP activates C-5 for condensation with cofactor.

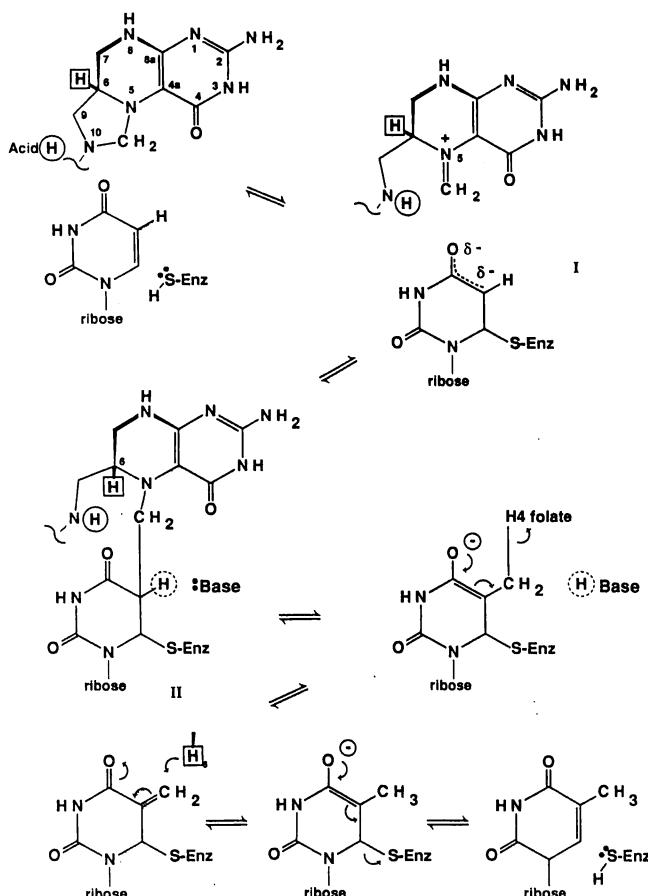
The stable TS-FdUMP- $\text{CH}_2\text{-H}_4\text{folate}$  complex provides a means of determining kinetics of the first half of the reaction in detail since the mechanism of formation of this analogue complex is thought to be very similar to the mechanism of formation of II (Santi et al., 1987). FdUMP and monoglutamated folate bind in an ordered, sequential manner (Lorenson et al., 1967; Danenberg & Danenberg, 1978). TS is an obligate dimer, with components of each monomer contributing to each of two active sites. Some evidence suggests that the two active sites in TS, which are identical with one another in all four structures we determined for unliganded TS species, also react sequentially (Danenberg & Danenberg, 1979). This result implies an asymmetry during the binding and during reaction of substrates with enzyme.

The stereochemistry of the stable ternary TS-FdUMP- $\text{CH}_2\text{-H}_4\text{folate}$  complex has been characterized in great detail. The stability of the covalently bound cofactor (Pogolotti et al., 1976) and the sensitivity of the C-9-N-10 bond of the cofactor to cleavage by specific chemical reagents (Pellino &

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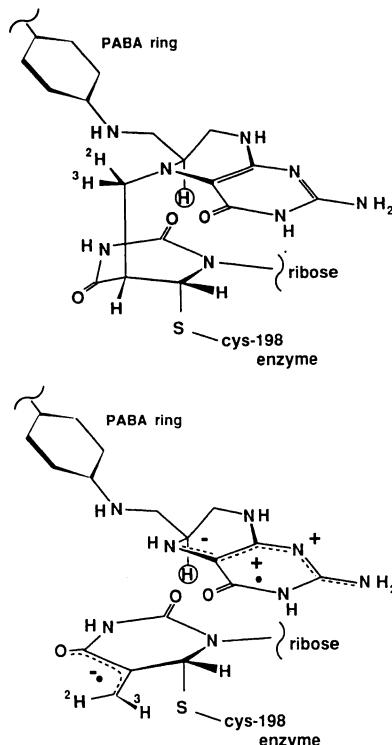
<sup>1</sup> Abbreviations: TS, thymidylate synthase; dUMP, 2'-deoxyuridine 5'-monophosphate;  $\text{CH}_2\text{-H}_4\text{folate}$ , 5,10-methylenetetrahydrofolate; dTDP, 2'-deoxythymidine 5'-monophosphate;  $\text{H}_2\text{folate}$ , dihydrofolate; CB3717, 10-propargyl-5,8-dideazafolate; FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate.



**FIGURE 1:** Proposed mechanism for methyl transfer to dUMP by TS, which also shows the numbering scheme for  $\text{CH}_2\text{-H}_4\text{folate}$ . Formation of a covalent bond between the catalytic thiol of Cys-198 and C-5 of dUMP activates C-5 of dUMP for condensation with the one-carbon unit of  $\text{CH}_2\text{-H}_4\text{folate}$ . Presumably, an activated form of the cofactor, such as the iminium ion  $5\text{-CH}_2=\text{H}_4\text{folate}$ , is the electrophilic species in this condensation reaction (Kallen & Jencks, 1966; Benkovic & Bullard, 1973). An acidic group on the enzyme was proposed to catalyze opening of the imidazolidine ring of  $\text{CH}_2\text{-H}_4\text{folate}$  by protonation at N-10 (Benkovic, 1980; Fife & Pellino, 1981). A basic group on the enzyme was postulated to abstract a proton from C-5 of dUMP. However, no such acidic or basic groups are identified in the structure. Elimination and hydride transfer from C-6 of the cofactor then generate dTMP and dihydrofolate ( $\text{H}_2\text{folate}$ ). The mechanism for the formal transfer of a hydride may occur in two steps: transfer of an electron followed by transfer of  $\text{H}^+$  (Sleicher & Benkovic, 1984), as suggested in Figures 2 and 4.

Danenberg, 1985) suggest that the methyl group being transferred to dUMP remains attached to N-5 of the cofactor (rather than N-10) in the ternary complex. The methylene bridge and enzyme sulfhydryl are trans-equatorial after unfolding (James et al., 1976; Byrd et al., 1978) but are in a trans-diaxial conformation in the folded protein (Byrd et al., 1978). The enzyme therefore stabilizes the higher energy trans-diaxial conformation. The relative three-dimensional orientation of the pterin ring of the cofactor and the pyrimidine of the substrate was predicted from the observed stereospecificity of the thymidylate synthase reaction and from the chemical steps postulated to comprise the enzyme mechanism (Sleicher & Benkovic, 1984) (Figure 2).

Perturbation of aromatic groups on the protein and of the folate chromophore is detected by Raman (Sharma et al., 1975; Fitzhugh et al., 1986), CD (Galivan et al., 1975; Donato et al., 1976), UV (Sharma & Kisliuk, 1973; Santi et al., 1974; Danenberg et al., 1974; Donato et al., 1976), and fluorescent spectroscopic analysis (Sharma & Kisliuk, 1975; Donato et al., 1976) of TS-FdUMP- $\text{CH}_2\text{-H}_4\text{folate}$ . Coupled with a



**FIGURE 2:** Proposed structure for the TS-dUMP- $\text{CH}_2\text{-H}_4\text{folate}$  complex (II in Figure 1). The trans-diaxial orientation of the catalytic sulphydryl and the methylene bridge is consistent with FNMR results for TS-FdUMP- $\text{CH}_2\text{-H}_4\text{folate}$  (Byrd et al., 1978). The pterin ring of the folate and the pyrimidine ring of dUMP are oriented such that antiperiplanar elimination could be followed by reduction via the C-6 hydrogen of the folate with the experimentally observed stereospecificity (Sleicher & Benkovic, 1984). This structure is not consistent with the crystal structure of dUMP and CB3717 in that the plane of the dUMP ring is rotated  $\sim 180^\circ$  about the glycosidic bond.

difference in Stokes radius (Lockshin & Danenberg, 1980), these observations show that there is a major conformational change in the protein on complex formation. Such conformational changes are consistent with kinetic analysis, which implies an ordered, synergistic binding of ligands.

We analyze the conformational changes upon ternary complex formation and identify residues involved in binding and catalysis from the crystal structure. As one step in this investigation, we determined the structure of the ternary complex of TS with dUMP and a potent anti-folate inhibitor, 10-propargyl-5,8-dideazafolate (CB3717) (Montfort et al., 1990). Designed by Jones et al. (1981), CB3717 has less internal constraint than  $\text{CH}_2\text{-H}_4\text{folate}$  since the imidazolidine ring is opened, and it has a propargyl substituent at N-10, which is effective because it slows the rates of association by 38-fold and dissociation by 70-fold relative to 5,8-dideazafolate that lacks this substituent (Pogolotti et al., 1986). CB3717 seems to induce a perturbation in enzyme structure during ternary complex formation which is similar in character and magnitude to the perturbations induced on formation of the TS-FdUMP- $\text{CH}_2\text{-H}_4\text{folate}$  complex (Pogolotti et al., 1986). It also induces covalent bond formation between enzyme and dUMP (Pogolotti et al., 1986); however, CB3717 cannot form a covalent bond to dUMP analogous to the covalent bond between cofactor and dUMP in intermediate II. Formation and dissociation of TS-dUMP-CB3717 are extremely slow compared to formation and dissociation of TS-FdUMP- $\text{CH}_2\text{-H}_4\text{folate}$  (Pogolotti et al., 1986). In examining the crystal structure of TS-dUMP-CB3717, we relate to the published structural data on the methyl transfer reaction and on the TS-FdUMP- $\text{CH}_2\text{-H}_4\text{folate}$  complex and correlate the crystal

structure with ligand binding, site-directed mutagenesis, and enzyme mechanism.

## EXPERIMENTAL PROCEDURES

**Structures for Reaction Intermediates.** Analysis of the structural basis for catalysis by TS is based on six highly refined structures: the structures of four species of unliganded<sup>2</sup> TS, *L. casei* TS (Hardy et al., 1987), *E. coli* TS (Perry et al., 1990), T4-TS (Finer-Moore et al., unpublished results), and human TS (Schiffer et al., unpublished results), and two structures for *E. coli* TS bound to substrate dUMP and CB3717 (Montfort et al., 1990). The four principal structures, those for native *L. casei* and *E. coli* and for the two ternary complexes, have been determined to 2.3-, 2.1-, 1.97-, and 1.97-Å resolution, respectively, and refined independently to *R* factors of 0.21, 0.25 (0.21), 0.22 (0.19), and 0.18 (0.16) for all nonzero (and for  $>3\sigma$ ) data. The structures were overlapped for comparison by minimizing the overall rms differences between their common cores, defined as the largest group of neighboring atoms whose distances from each other were the same  $\pm 0.5$  Å in the compared structures on the basis of difference distance matrices (Perry et al., 1990).

Models for steady-state intermediate II in Figure 1 were constructed on the basis of our crystal structure of an inhibited ternary complex formed between enzyme, substrate dUMP, and a folate analogue, 10-propargyl-5,8-dideazafolate (TS-dUMP-CB3717). In modeling of intermediate II, the substrate and cofactor were overlapped with the ligands in the crystal structure of the fully reduced ternary complex as closely as possible subject to the following constraints:

(1) The catalytic sulphydryl and methylene bridge are covalently bound to C-6 and C-5 of dUMP, respectively, in a trans-diaxial manner, as demonstrated by NMR (James et al., 1976; Byrd et al. 1978).

(2) There are no major conformational changes in the protein between TS-CB3717-dUMP and intermediate II.

(3) The PABA ring of CH<sub>2</sub>-H<sub>4</sub>folate and the phosphate moiety are close to the positions of the same groups in the crystal structure of the CB3717 ternary complex.

The phosphate and PABA ring binding sites are well-defined and composed of residues that are highly conserved in the TS sequences. The change in PABA ring environment upon binding to TS is most probably responsible for the intense absorption band around 325 nm (Sharma & Kisliuk, 1973; Santi et al., 1974), common to the UV spectra of many TS-nucleotide-folate complexes (Lockshin et al., 1984), as suggested from Raman spectroscopy by Fitzhugh et al. (1986). We expect that this PABA ring environment will be conserved as in the CB3717 complex. This orientation of the PABA ring places the glutamate close to the lysine/arginine-rich folate binding region 50(48)-58(56) (Maley et al., 1982). Therefore, one expects that the phosphate and PABA ring binding sites are the same in the covalent ternary complex and the TS-dUMP-CB3717 complex.

The molecular mechanics program AMBER (Singh et al., 1986) was used to optimize the conformation of the modeled ligands and eliminate steric clashes between modeled ligands and protein, but not as a means of evaluating the models. The covalent ternary complex was modeled into only one active site and refined along with 74 surrounding residues. Ordered water was not included. A distance-dependent dielectric value and a united-atom force field model (Weiner et al., 1984) were used for all residues in the protein except the active site

Table I: Summary of the Most Direct Ligand Interactions in TS<sup>a</sup>

role	residue	functional interaction <sup>b</sup>
nucleophile	Cys-198	covalent bond to C-5 of dUMP
phosphate binding	Arg-218	double donor to PO <sub>4</sub> , close to Cys-198
	Arg-178'	Tyr-261, donor to PO <sub>4</sub>
	Arg-179'	donor to PO <sub>4</sub>
	Arg-23	double donor to PO <sub>4</sub> and to C-terminus
ribose binding	Ser-219	donor to PO <sub>4</sub>
	Tyr-261	O-3' and Arg-178'
	His-259	close to O-3'
	Ser-219	close to O-3'
uridine ring	Asn-229	$\delta$ O---NH-3, $\delta$ NH <sub>2</sub> ---O-4, and H <sub>2</sub> O 5
	H <sub>2</sub> O 1	His-199 and O-4 through H <sub>2</sub> O 1 to O-4, 4-5 Å from C-5
	His-199	4-5 Å from C-5
	Tyr-146	N-H of Asp-221
	C=O of Ala-315	donor to O-2 acceptor from NH-2 and H <sub>2</sub> O 2
pterin ring	Asp-221	acceptor from NH-3 and H <sub>2</sub> O 3
	Trp-82	bonds to Glu-60, ring packing
	Trp-85	ring packing, C-terminus
PABA ring	Ile-81, Leu-224, Phe-228, Val-314	ring packing
alternate quinazoline site (Montfort et al., 1990)	Glu-60	N-1
	Ser-232	NH <sub>2</sub> 2
	Asn-229	NH-3 donor to $\delta$ O
	H <sub>2</sub> O 4	O-4

<sup>a</sup>Sequence numbers are for the *L. casei* sequence, as used in the alignments of TS sequences by Perry et al. (1990). <sup>b</sup>Interactions are hydrogen bonds unless stated otherwise. Hydrogen-bond donors or acceptors are defined where known. Packing refers to van der Waals contact. Residues described as close to a site are not hydrogen bonded but are close enough that they could play a role in catalysis.

cysteine. An all-atom force field model (Weiner et al., 1986) was used for the Cys-dUMP-CH<sub>2</sub>-H<sub>4</sub>folate moiety. The minimizations were done both without constraints and with the PABA-Glu moiety constrained with a 600-kcal harmonic force constant. In each case, 100 cycles of steepest descent minimization were followed by cycles of conjugate-gradient minimization, which were terminated when the difference in the energy gradients between successive steps was less than or equal to 0.10 kcal mol<sup>-1</sup> Å<sup>-1</sup>.

## RESULTS

(A) *CB3717 Complex as a Mimic of a Key Reaction Intermediate.* The crystal structure of the ligand-bound ternary complex of *E. coli* TS with substrate and the cofactor analogue CB3717 confirms that the cavity identified in the structure of P<sub>i</sub>-bound TS is indeed the ligand binding site (Hardy et al., 1987). The side chains of 25 residues, 15 of which are invariant in the 17 known TS sequences, line the cavity. Eleven of the 15 invariant residues originally assigned to the lining of the cavity are directly involved in ligand binding (see Figure 3, Table I). As we predicted from the structure of the free enzyme (Hardy et al., 1987), dUMP is bound with O-2 anti to the ribose ring ( $\chi \sim -170^\circ$ ) with the sugar 2'-endo, as suggested by Haertle et al. (1979), and with its phosphate group in the same position as P<sub>i</sub> in the *L. casei* structure. The catalytic sulphydryl is covalently bound to C-6 of dUMP (Montfort et al., 1990). The quinazoline ring lies over dUMP with its plane approximately parallel to the plane of dUMP,

<sup>2</sup> Unliganded structures for *E. coli*, *L. casei*, and T4 TS have inorganic phosphate bound at the site for phosphate in dUMP.

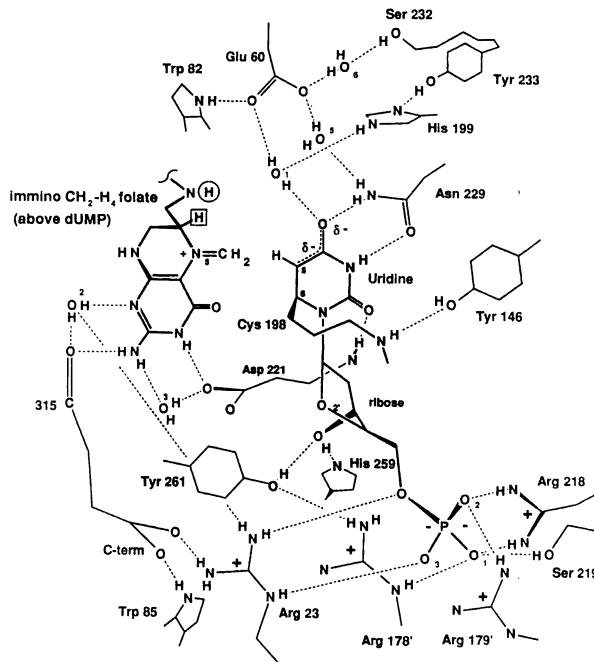


FIGURE 3: Schematic diagram of the side chains that surround the active center, indicating hydrogen bonds present in the model of the ternary complex, several of the conserved residues, and ordered water molecules. The numbering scheme is that of *L. casei* TS.

in a manner that could mimic intermediate II of Figure 1.

The crystal structure of TS-dUMP-CB3717 contains a dimer in the asymmetric unit; thus, monomers I and II, which are identical in all four unliganded structures (*L. casei*, *E. coli*, human, and T4), were built and refined independently and show some minor differences in their active sites, and the temperature factors in one monomer are systematically lower than in the other (Montfort et al., 1990). The largest deviations between the protein backbones in the active sites are in the regions with the higher temperature factors, namely, the loop residues 21–26(19–24)<sup>3</sup> and C-terminal residue Ile-316(264). The backbone atoms for the remainder of the active site residues are the same in the two monomers, and the orientations of only a few residues are different.

(B) *Functional Correlates of the Models.* The model which most closely matches the crystal structure subject to the constraints is shown in Figures 4, 5, and 8. In this model the pterin ring of the cofactor and the pyrimidine ring of dUMP are nearly superimposed on the positions of the quinazoline ring of CB3717 and the pyrimidine ring of dUMP in the crystal structure. The methyl group covalently bound to C-5 of dUMP is attached to N-5 of the cofactor, in agreement with chemical studies (Pogolotti et al., 1976; Pellino & Danenberg, 1985). N-10 in CB3717 is 4.75 Å from C-5 of dUMP and a methylene bridge could not joint these two atoms without large shifts in the ligand positions.

As predicted by Slieker and Benkovic (1984), the pterin ring of CH<sub>2</sub>-H<sub>4</sub>folate and the pyrimidine ring of dUMP overlay one another, with the planes of the two ring systems nearly parallel. However, the pyrimidine ring is rotated approximately 180° from their suggested orientation, Figure 2, and our absolute configuration at C-6 of dUMP is opposite to theirs

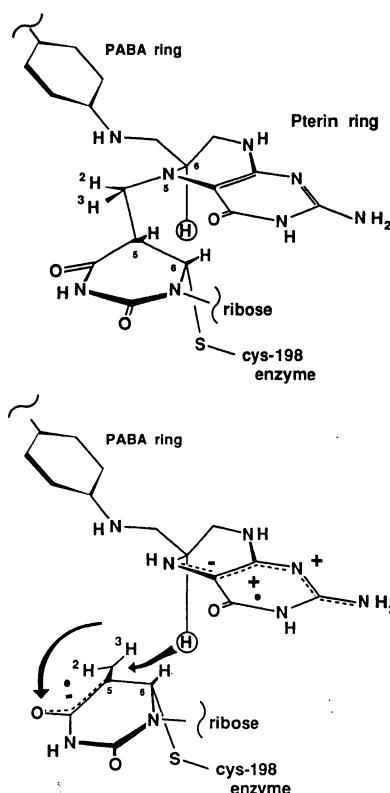


FIGURE 4: Proposed structure for the TS-dUMP-CH<sub>2</sub>-H<sub>4</sub>folate complex (II in Figure 1) which is consistent with the dUMP conformation seen in the crystal structure of TS-dUMP-CB3717. The catalytic sulphydryl and methylene bridge are trans-diaxial substituents of C-6 and C-5, respectively, of dUMP. The sulphydryl is bound to the opposite side of the pyrimidine ring as in Figure 2, but reduction by the C-6 hydrogen must occur on the same side of the exocyclic methylene as in Figure 2 in order to give the correct stereochemistry for the labeled methyl in the experiments discussed by Slieker and Benkovic (1984).

(S instead of R). As a result, the pterin ring of the cofactor does not overlap so completely with the pyrimidine ring of dUMP as in Figure 2.

Chemical model studies have shown that the ternary complex, intermediate II, most likely collapses to a reactive exocyclic methylene derivative of dUMP and tetrahydrofolate (Pogolotti & Santi, 1977) (Figure 1). An antiperiplanar arrangement of the two leaving groups, H<sub>5</sub> of dUMP and tetrahydrofolate, around the C-5 (dUMP)-C-11 (CH<sub>2</sub>-H<sub>4</sub>folate) bond is the most favored configuration for both a concerted E2 elimination and an E1CB mechanism (Albright et al., 1985). In our model the leaving groups are not so aligned. They are in a gauche orientation with the dihedral angle H-5-C-5-C-11-N-5 = 80° (see Figure 6). However, in contrast to concerted eliminations, the E1CB mechanism, where a proton is first abstracted from C-5 of dUMP to give a carbanion at C-5 before the H<sub>4</sub>folate leaves, does not require alignment of leaving groups in either a synperiplanar or antiperiplanar manner. Therefore, our model is most consistent with an E1CB mechanism for collapse to the exocyclic methylene species, as had been proposed (Pogolotti & Santi, 1977).

Because the binding cavity is spacious and the protein is known to undergo conformational changes during the reaction, we cannot discount the possibility that the tetrahydrofolate and the C-5 hydrogen of dUMP become aligned sometime prior to elimination and then could utilize an E2 mechanism. Besides our favored model which is closely based on the crystal structure of TS-dUMP-CB3717, we were able to construct such a model of the complex which is aligned for an E2 mechanism,

<sup>3</sup> The numbering scheme used here is that of the *L. casei* sequence; *E. coli* numbering is given in parentheses following the *L. casei* residue number. After alignment of the *E. coli* and *L. casei* structures, the relation between the numbering of their sequences is as follows:  $N_{L.c.} = N_{E.c.} + 2$  for residues 1–89 in the *L. casei* sequence;  $N_{L.c.} = N_{E.c.} + 52$  for residues after 89.

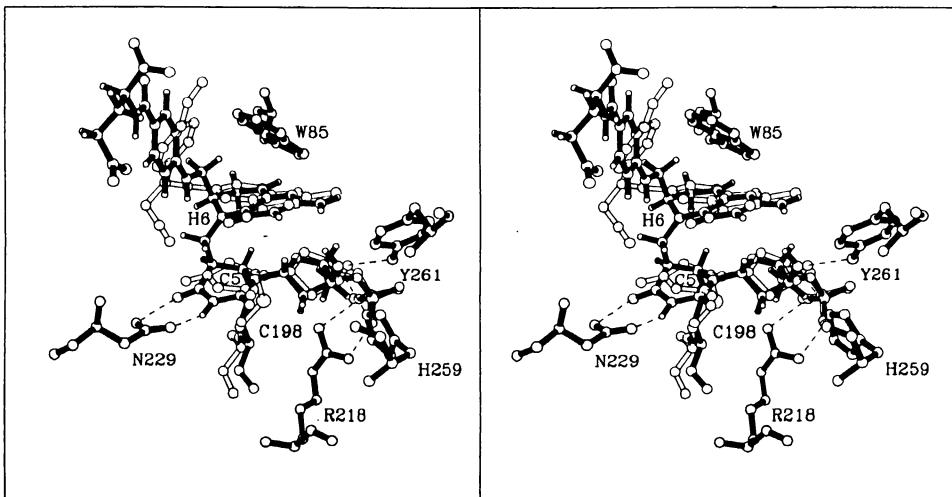


FIGURE 5: "Crossed-eyes" stereo plot of the model for ternary complex II overlaid on dUMP and CB3717 from the crystal structure of TS-dUMP-CB3717 (open bonds). The Glu moiety from the crystal structure has been omitted for clarity. dUMP,  $\text{CH}_2\text{-H}_4\text{folate}$ , and a few ligand binding residues from the model are plotted with filled-in bonds. Hydrogen bonds are denoted by dashed lines. Residue labels use the *L. casei* numbering.

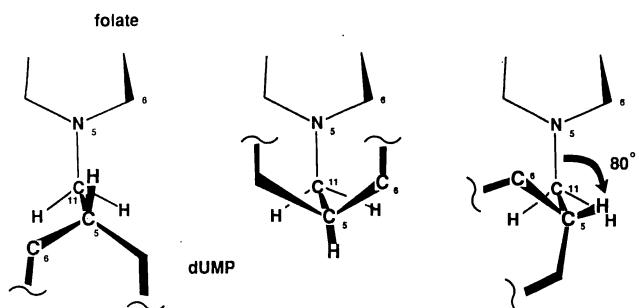


FIGURE 6: The conformation that favors a concerted E2 elimination requires that the C-5-H-5 bond of dUMP is antiparallel (antiperiplanar) or parallel (synperiplanar) to the C-11-N-5 bond of  $\text{CH}_2\text{-H}_4\text{folate}$ , when viewed down the C-5 (dUMP)-C-11 (folate) bond. In the modeled complex based on the crystal structure of TS-dUMP-CB3717, the angle is gauche,  $80^\circ$ .

one in which the tetrahydrofolate and the C-5 hydrogen of dUMP were synperiplanar and dUMP C-6 had the same absolute configuration seen in the crystal structure.

We could also build a model of the most favored antiperiplanar arrangement. This alignment requires opposite absolute configuration at C-6 from that seen in the TS-dUMP-CB3717 crystal structure. Since we are basing the model of the reaction intermediate on an inhibitor, we cannot yet be certain that dUMP does not bind with the opposite configuration in the enzymatic reaction. This possibility deserves consideration because it accounts for the stereospecificity of the subsequent hydride transfer step in which the C-6 hydrogen of tetrahydrofolate is transferred to the dUMP exocyclic methylene group (Pastore & Friedkin, 1962; Blakely et al., 1963; Lorenson et al., 1967; Santi et al., 1976). As illustrated in Figure 2, experiments in which the one-carbon unit transferred to dUMP was labeled with different isotopes of hydrogen have shown that (*6R,11S*)-5,10-methylene[11- $^2\text{H}$ , $^3\text{H}$ ]tetrahydrofolate is converted to (*S*)-methyl[ $^1\text{H}$ , $^2\text{H}$ , $^3\text{H}$ ]TMP. The C-6 hydrogen of the cofactor in the model for intermediate II in Figure 2 would be oriented over the *re* face of the exocyclic methylene, as shown in Figure 2; thus, hydride transfer would give the observed absolute configuration at the labeled methyl of dTMP with only small changes in the relative orientations of the dUMP pyrimidine ring and the pterin ring of  $\text{CH}_2\text{-H}_4\text{folate}$ .

In our favored model, hydride transfer must take place to the face of the dUMP pyrimidine ring opposite that facing the

pterin ring in order to give the correct stereochemistry at the labeled methyl transferred to dUMP. Thus, reorientation of the dUMP pyrimidine ring by, for example, rotation about the bond between N-1 and the ribose ring, as shown in Figure 5, would be required before the hydride-transfer step.

In all models, the phosphate binding site and PABA-Glu binding sites seen in the crystal structure were preserved. AMBER refinement served to eliminate bad protein-ligand interactions with very few changes to the protein structure. The results of refinement were essentially the same with and without constraints on the PABA-Glu position. The energy of the refined model based on the crystal structure was, as expected, somewhat lower than the energies of the other two, ca. -2080 vs -2060 kcal, but no model had obviously bad geometry. The invariant Asn-229(177), which provides the primary interaction between dUMP and TS in the crystal structure of TS-dUMP-CB3717, hydrogen bonds to dUMP only in our favored model.

The cofactor is normally linked to a linear sequence of as many as seven glutamate residues that are  $\gamma$ -linked or  $\alpha$ -linked to the PABA ring and to each other (Ferone et al., 1986). A cleft on the surface of the enzyme close to the active center contains several of the residues known to be involved in binding of glutamates. The crystal structure is of a monoglutamylated cofactor analogue; however, a tentative model of seven glutamates, the first two with  $\gamma$ -linkages and the next five with  $\alpha$ -linkages, was built to fit the structure as shown in Figure 7.

**(C) Conservation and Hydrophobicity in the Ligand Environment.** In the ternary complex model, the pterin ring lies over the dUMP and close contacts between the two ligands are themselves important binding determinants. Contacts between the ligands and the protein are nearly identical with those of the ligand-protein interaction seen in the crystal structure (Montfort et al., 1990) and are shown in Figure 8. Four highly conserved arginines, two from each monomer, provide a favorable, positively charged binding surface for the phosphate anion. These four arginines, Arg-23(21), Arg-218(166), Arg-178'(126'), and Arg-179'(127'), as well as Ser-219(167) are involved in an extensive hydrogen-bonding network around the phosphate. Each arginine forms at least one hydrogen bond to the phosphate oxygens. Arg-23(21) is also hydrogen bonded to the C-terminus of the protein. Arg-218(166) is hydrogen bonded to the carbonyls of residues

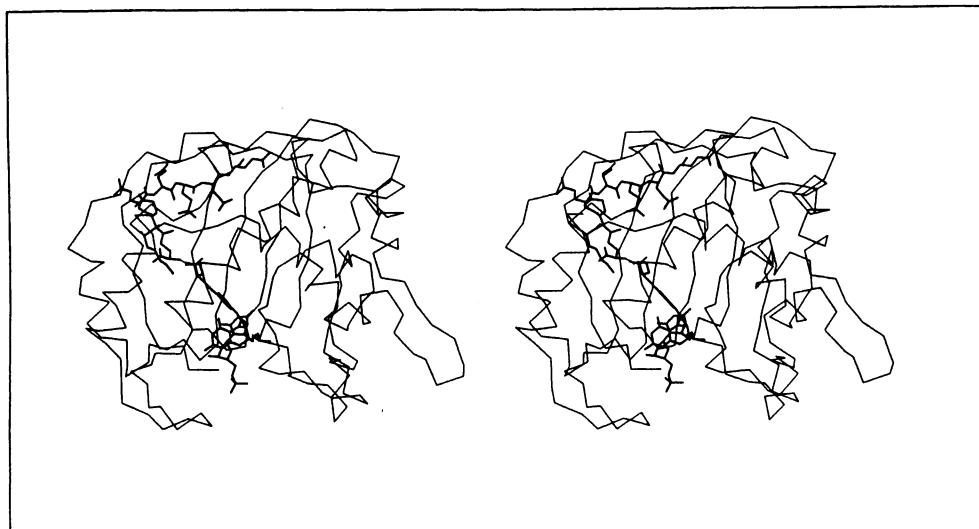


FIGURE 7: Crossed eyes stereo plot of a covalent ternary complex model between TS, dUMP, and a polyglutamylated  $\text{CH}_2\text{-H}_4\text{folate}$ . Seven glutamates have been attached, the first two through  $\gamma$ -peptide linkages and the next five through normal  $\alpha$ -peptide linkages as is known to occur in *E. coli* (Ferone et al., 1986). In addition to the *E. coli* TS  $\alpha$ -carbon backbone, lysine-50(48) is shown.

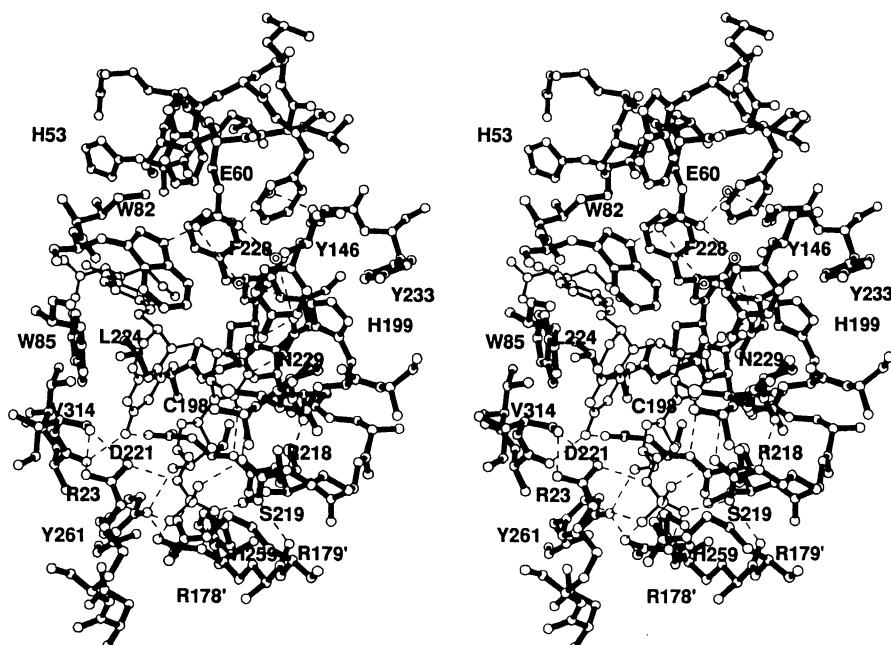


FIGURE 8: Crossed eyes stereo plot of a model for II of Figure 1. The model was derived from the TS-dUMP-CB3717 crystal structure by modeling the ligands and refining the structure of the ligands and surrounding residues with energy minimization (Singh et al., 1986). Ligand binding residues are labeled, and hydrogen bonds are shown with dashed lines. Concentric circles represent ordered waters from the crystal structure. The residue numbers correspond to the *L. casei* sequence. The hydrogen bonds to the pterin ring of  $\text{CH}_2\text{-H}_4\text{folate}$  are slightly different from those to the quinazoline ring of CB3717, probably due to omission of ordered water from AMBER refinement. In the crystal structure Asp-221(177) is a hydrogen-bond acceptor from N-3 and from ordered water 3 which is also hydrogen bonded to N-2. The NH<sub>2</sub> group of Arg-23(21), which is hydrogen bonded to the dUMP phosphate, is hydrogen bonded to ordered water 2 rather than to the C-terminus (Figure 3) (Montfort et al., 1990).

Pro-196(144), Pro-197(145), and Arg-178'(126'). Arg-178'(126') is hydrogen bonded to the hydroxyl of Tyr-261(209).

Invariant Tyr-261(209) and His-259(207) are the primary contacts to the ribose ring of dUMP: they are hydrogen bonded to O-3' on the ribose ring. Hydrogen bonds between the pyrimidine of dUMP and invariant Asn-229(177) and the NH of Asp-221(169) are the only other hydrogen-bonded contacts between dUMP and the protein. Asn-229(177) is hydrogen bonded to both O-4 and N-3 of dUMP.

The only protein side chain to hydrogen bond to the cofactor is that of invariant Asp-221(169). Other hydrogen bonds involving the cofactor are made with either water or backbone atoms. In particular, the exocyclic amino group on the pterin

ring is hydrogen bonded to the terminal carbonyl [residue 315(263)] in the protein.

The PABA ring lies in a hydrophobic pocket surrounded by the side chains of the highly conserved residues Ile-81(79), Leu-224(172), Phe-228(176), and Val-314(262). The pocket is formed in part by movement of the C-terminus on ligand binding. Phe-228(176) and Leu-224(172) are within van der Waals contact of the PABA ring. Trp-85(83) and Trp-82(80) are just below the PABA ring, stacked against the pterin ring.

## DISCUSSION

(A) *Modeling the Reaction Intermediate.* In modeling II we chose to be as conservative with respect to the crystal structure of the TS-dUMP-CB3717 complex as possible. It

is, for example, possible to model intermediate II as in Figure 2 while preserving both PABA and phosphate binding sites as well as protein structure. However, in our favored model dUMP is bound in the anticonformation with *S* absolute configuration at C-6, as seen in the crystal structure, and the pyrimidine ring of dUMP and pterin ring of cofactor are rotated only slightly from the pyrimidine and quinazoline ring binding sites in the crystal structure (Figure 5). This intermediate would probably collapse to tetrahydrofolate and the exocyclic methylene derivative of dUMP by an E1CB mechanism. A conformational change is required to bring the exocyclic methylene into the plane of the pyrimidine ring. This change could involve a change in conformation of the C-5 hydrogen of dUMP from equatorial to axial prior to its abstraction (James et al., 1976). This change could twist the pyrimidine ring of dUMP about the bond between N-1 and the ribose ring and leave the C-6 hydrogen of cofactor oriented for hydride transfer to the *re* face of the methylene (the face the C-5 hydrogen is abstracted from) as required by the stereochemical results of Slicker and Benkovic (1984). That we can model the ligands in several different binding modes with little difference in energy suggests that the reactants can undergo the conformation changes necessary during the reaction relatively easily.

(B) *Roles of Catalytic and Ligand Binding Residues: Species Variation and Site-Directed Mutagenesis.* Precise alignment of substrate and cofactor in the cavernous active site of TS is an important part of catalysis. TS has 46 residues (18% of all residues in *E. coli* TS) which are invariant in the 17 published sequences [see Perry et al. (1990) for alignment], and 11 of these are directly involved in ligand binding. The orientation of substrate and cofactor is a complex and dynamic process involving many factors, including protein conformation change. Key ligand binding residues, the effects of chemical modification, and site-directed mutagenesis of these residues are discussed in relation to the structure.

(1) *The Reactive Nucleophile, Cys-198.* Invariant Cys-198, the nucleophile that attacks C-6 of dUMP, activates C-5 of dUMP for condensation with the cofactor. Mutation of Cys-198 to Gly, Ala, or Thr inactivates *E. coli* TS, while mutation to Ser reduces  $k_{cat}$  by a factor of 5000 (Dev et al., 1988). The Cys-198 side chain accounts for some of the binding specificity since it is believed to make van der Waals contacts with dUMP in the TS-dUMP binary complex (Dev et al., 1988). In the steady-state intermediate analogue TS-FdUMP-CH<sub>2</sub>-H<sub>4</sub>folate, Cys-198 is covalently bound to C-6 of dUMP (Bellisario et al., 1979).

(2) *Need for Catalytic Acid near N-10 of the Cofactor and a Base to Abstract the Proton at C-5 of dUMP.* In addition to Cys-198, a general acid and a general base have been postulated to help catalyze the TS reaction. The general acid has been postulated to protonate N-10 of CH<sub>2</sub>-H<sub>4</sub>folate, facilitating opening of the imidazolidine ring (Benkovic, 1980; Fife & Pellino, 1981). The general base has been postulated to abstract a proton from dUMP C-5 in the covalent ternary complex prior to elimination of oxidized cofactor. It is not clear from our structure of liganded *E. coli* TS which residues, if any, fulfill either of these roles. Histidines are good candidates for either function, but the only histidine in the vicinity of dUMP C-5 or folate N-10 is His-199(147), which while conserved in 16/17 sequences is not totally invariant, since it is valine in the gene from subtilisin phage  $\phi$ 3T. It is not likely, therefore, to have an essential role in catalysis.

In a detailed analysis, Dev et al. (1989) changed the histidine residue in *E. coli* TS for several other residues and

analyzed effects on the kinetics of the reaction versus pH. Substitution by glycine or neutral or small nonpolar amino acids reduced  $k_{cat}$  10-fold, suggesting that while His-199(147) is dispensable it has some role if only a conformational one in retaining a competent active site. Replacement by negatively charged amino acids, Asp or Glu, reduced  $k_{cat}$  by a factor of 10<sup>3</sup>, and replacement by the positive amino acids lysine or arginine reduced  $k_{cat}$  by 10<sup>4</sup>. Further, the effect was localized to the initial rate of association of the substrate and cofactor, and His-199(147) is not required for proton abstraction. More informative, when these authors measured activity versus pH in the wild type and mutants, the pH profile for wild type shows activity rising with a controlling pK<sub>a</sub> of 5.60 ± 0.7, with no fall in activity up to at least pH 9.5. Yet in mutants where His-199(147) is changed for Gly or for neutral or hydrophobic amino acids, loss of activity coincided with the deprotonation of a residue with an apparent pK<sub>a</sub> of 9.10–9.50 (Dev et al., 1989). The enthalpy of ionization was determined from the temperature dependence of pK<sub>a</sub> for dissociation, giving a value of 12.1 ± 0.9 kcal for the His-199(147)Gly-catalyzed reaction. Comparison with the values for  $\Delta H_{ionization}$  for Asn or Tyr (6–7 kcal/mol) suggested that these nearby residues were not likely to be responsible but that Lys or Arg residues are more likely implicated. Since the effect is on the association rate of complex formation,  $k_{on}$ , Dev et al. (1989) conclude that His-199(147) may control the pK<sub>a</sub> of Lys or Arg, which in turn controls association with the cofactor CH<sub>2</sub>-H<sub>4</sub>folate during the formation of a transition-state complex that may be stabilized by His-199(147). His-199(147) is not close to a Lys or Arg in the structure; however, it is hydrogen bonded to invariant Tyr-233(181). Tyrosine has a normal pK<sub>a</sub> of ~10, and hydrogen bonding to His might stabilize the protonated form more than in the mutants.

One explanation of the effects of the His-199(147) mutations on  $k_{cat}$  might be that the conformation of the enzyme, affecting the  $k_{on}$  rate, depends on His-199(147) within the active center. If so, these effects should be apparent in the crystal structures of these mutants. His-199(147) bonds to a very well ordered water molecule that in turn bonds to O-4 on dUMP and Glu-60(58). Glu-60(58) is also bonded to Trp-82(80), which is closely packed against the pterin ring of the cofactor. Thus His-199(147) could be very important in maintaining enzyme conformation, one of the ideas suggested by Dev et al. (1989).

Other functional groups in the vicinity of folate N-10 or dUMP C-5 include invariant residues Trp-82(80), Glu-60(58), and Tyr-146(94). These residues do not have appropriate pK<sub>a</sub>'s for the role of general acid or base. Like His-199(146), they are too far from the ligands to have a direct role in catalysis, although they could act either alone or together by ordering water. Glu-60(58) and Trp-82(80) are 5 Å from N-10 of both CB3717 in the crystal structure and CH<sub>2</sub>-H<sub>4</sub>folate in our models for the ternary complex and are involved in an extensive hydrogen-bonding network involving Asn-229(177), His-199(147), and at least three water molecules. Tyr-146(94) and His-199(147) are 4–5 Å from C-5 of dUMP in models of the ternary complex, and the hydroxyl of Tyr-146(94) is hydrogen bonded to the amide of active site Cys-198(146). Site-directed mutagenesis experiments may help to elucidate roles of these conserved residues.

(3) *Activation of the Active Site Sulfhydryl.* Arg-218 may activate Cys-198(146) through charge interactions or hydrogen bonding with the catalytic sulfhydryl (Hardy et al., 1987). In P<sub>i</sub>-bound *E. coli* TS, the guanidinium of Arg-218(166) is 3.5 Å from Cys-198, too far to form a good hydrogen bond to the

SH group but near enough that the charge on Arg-218 could stabilize the more nucleophilic thiolate anion S<sup>-</sup> form of Cys-198 and lower the pK<sub>a</sub> of Cys-198. However, the pK<sub>a</sub> of Cys-198(146), determined from the pH dependence of inactivation of *L. casei* TS by dithionitrobenzoic acid (DTNB), appears to be fairly normal for cysteine at ~8.0 (Munroe et al., 1978). A higher than expected inactivation rate at low pH, where the cysteine is presumably not ionized, was attributed to activation by a general base on the protein (Munroe et al., 1978), which may be Arg-218(166).

Dev et al. (1989) recorded the pH/activity profile, which shows a rate-controlling pK<sub>a</sub> of 5.60 ± 0.7 assigned to deprotonation of N-1 of the pteridine ring. There was no fall-off at high pH up to ~9.1, and no group around pK<sub>a</sub> = 8 was seen in the profile, suggesting that nucleophilic attack is not in the rate-limiting step or that the pK<sub>a</sub> of Cys-198(146) is at or below 5.6. Dev et al. point out that more than one group contributes to the low limb of the pH, and it is possible that the second group could be Cys-198(146) with a pK<sub>a</sub> lowered by interaction with Arg-218.

Arg-218(166) is probably the reactive active site arginine identified by chemical modification (Cipollo & Dunlap, 1979; Belfort et al., 1980). This essential arginine was shown to be close to Cys-198, and its modification was blocked by dUMP binding. Modification of the arginine was pH sensitive, with the inactivation constant of the protein 100-fold faster at pH 8.4 than at pH 7.4 (Belfort et al., 1980).

(4) *The Phosphate Binding Tetrahedron.* The phosphate anion is surrounded by four positively charged arginine side chains, each of which contributes electrostatic and hydrogen-bonding stabilization. TS is relatively insensitive to substitution by mutation of invariant residue Arg-179'(127') to several other residues in *L. casei*, including glutamic acid of opposite charge (Santi et al., 1990). Thus, Arg-179'(127') plays a role in dUMP binding but is not an essential binding determinant.

Arg-178'(126') and Arg-23(21) are hydrogen bonded to the phosphate in the liganded structure of *E. coli* TS. In addition, Arg-178'(126') is hydrogen bonded to the invariant Tyr-261(209) and Arg-23(21) is hydrogen bonded to the carboxy terminus of the protein. These arginines may have multiple roles in stabilizing the ligand binding sites in the protein.

Enzyme activity is more sensitive to mutations at Arg-218(166), which so far has not been substituted for any other amino acid without a loss of at least 10<sup>-4</sup> in activity (Climie et al., unpublished results). Arg-218 may function not only to bind phosphate but also to help orient the catalytic Cys-198(146) by hydrogen bonding to the backbone carbonyls of residue 196(144) and Pro-197(145).

(5) *Is Orientation of Ribose Important to Catalysis?* Besides the phosphate-binding arginines, the primary dUMP-binding residues are the totally conserved Tyr-261(209), His-259(207), and Asn-229(177). Tyr-261(209) is probably the tyrosine whose chemical modification inactivates the enzyme (Rosson et al., 1980). The obvious role of Tyr-261(209) is binding O-3' of dUMP. However, conserved residue His-259(207) may also hydrogen bond to O-3', and Ser-219(167) in the vicinity of Tyr-261(209) could hydrogen bond to O-3' if the conformation changed slightly. Tyr-261(209) may be essential for precisely orienting dUMP in the ternary complex, both by hydrogen bonding and by shaping the binding cavity.

(6) *Hydrophobic Orientation at the PABA-Glu Binding Site.* There are no hydrogen bonds to the PABA-Glu moiety in the crystal structure of TS-dUMP-CB3717, but there is a well-defined hydrophobic binding site for the PABA ring which

is composed of highly conserved residues including residues Ile-81(79), Leu-224(172), and Phe-228(176). When Cys-52(50), which is about 5.5 Å from the glutamate moiety of CB3717 in the crystal structure, is mutated to a tyrosine in *E. coli* TS, binding of ligands is significantly impaired (Maley & Maley, 1988). The mutant forms a ternary complex with FdUMP and CH<sub>2</sub>H<sub>4</sub>folate but to a much lesser extent than the wild-type enzyme. Formation of a complex between mutant protein, dUMP, and CB3717 was 15-fold slower than in wild-type enzyme, and binding of CB3717 or dUMP by themselves was diminished. When a tyrosine side chain for residue 52(50) is modeled into the structure of TS-dUMP-CB3717, it not only overlaps the binding site for the glutamate moiety, but it is also in close contact with the side chain of Phe-228(176). The phenyl group of Phe-228(176) is in van der Waals contact with the PABA ring, and its reorientation to alleviate steric clashes with the substituted tyrosine might disrupt the PABA ring binding site, preventing folate from binding or causing it to bind incorrectly.

(7) *The C-Terminal Carboxyl Group Is Dynamically Involved in Ligand Binding.* During formation of II the C-terminal four residues move toward the active site cavity and become more ordered (Montfort et al., 1990). The C-terminal residues help form the PABA ring binding site and hydrogen bond to the quinazoline ring of CB3717, as well as the pterin ring of CH<sub>2</sub>H<sub>4</sub>folate in our model of the covalent intermediate.

Trp-85(83) hydrogen bonds to the terminal carboxyl group of the liganded protein. Although not conserved in the TS sequences, residue 85(83) is always a hydrogen-bonding residue and in 16 out of 17 sequences is either an Asn or a Trp, which have hydrogen-donating nitrogens in equivalent positions. A hydrogen bond between the carboxy terminus and Arg-23(21) may also help stabilize the conformation of the liganded protein.

Removal of the terminal *L. casei* TS residue, Val-316, with carboxypeptidase completely inactivates the enzyme (Aull et al., 1974), although this residue is not conserved in the TS sequences. This result is consistent with a mechanism requiring the C-terminal carboxyl group, but not the side chain, for ligand binding or catalysis. Inactivation by carboxypeptidase does not affect dUMP binding (Galivan et al., 1977). Our models of the covalent ternary complex, where the pterin ring of CH<sub>2</sub>H<sub>4</sub>folate is hydrogen bonded to the C-terminus, may explain this observation.

Folates in vivo are modified to contain polyglutamate tails, where the glutamate moiety of the cofactor is extended with four to seven additional glutamates through peptide bonds to either the α- or γ-carboxyl groups (Ferone et al., 1986). Formation of ternary complexes with dUMP and pteroylpolyglutamates protects the enzyme from carboxypeptidase inactivation (Galivan et al., 1977). The C-terminus lies over residue 50(48), near the lysine- and arginine-rich stretch 50–58(48–56) known to interact with polyglutamylated folates (Maley et al., 1982). Shown in Figure 7 is a covalent ternary complex model in which a polyglutamylated folate has been modeled to fit a cavity in TS between residues 50–58(48–56) and 299–311(247–259). The polyglutamate tail lies over the C-terminal peptide and interacts with a number of lysines and arginines. Binding in this manner might block access of carboxypeptidase to the C-terminus.

(C) *Segmental Accommodation: Role of Conformational Change in Enzyme Mechanism.* The dramatic changes in the spectra and hydrodynamic behavior of *L. casei* TS on formation of ternary complexes have suggested large conformational differences between unliganded TS and TS in ternary

complexes with substrate and cofactor analogues. In fact, the differences between P<sub>i</sub>-bound *E. coli* TS and TS-dUMP-CB3717, although quite extensive, are not particularly large (Montfort et al., 1990). The rms deviations between  $\alpha$ -carbon positions is 0.83 Å, with the larger shifts occurring predominantly on the left side of the molecule as displayed in Figure 7. The shifts result in segments of helices,  $\beta$ -sheet, and loops moving toward the active site to accommodate ligand binding. The greatest shift is in residues 312–316(260–264) at the C-terminus. These residues become more ordered in the ternary complex and move over 4.0 Å into the active site, forming an important binding determinant for intermediate II.

The binding sites for dUMP and CB3717 in the crystal structure are also present in the P<sub>i</sub>-bound structure. Most of the residues at these sites are well-ordered and correctly oriented for ligand binding once the active site shifts to accommodate the ligands. Two exceptions are Phe-228(176) at the PABA ring binding site whose phenyl ring is oriented approximately 50° from its position in P<sub>i</sub>-bound *E. coli* TS and Arg-23(21) whose guanidinium group moves into the active site only after ligand binding. Ligand synergism must then be attributed to contacts between substrate and cofactor as well as to increased protein contacts for both ligands, which result from the segmental shifts that accompany ternary complex formation. An example of such cooperative binding is the movement of the C-terminal four residues to make contacts with the PABA and pterin rings of folate and also to hydrogen bond to Arg-23(21), which becomes part of the phosphate binding site of dUMP.

dUMP binding to the FdUMP-enzyme complex indicates a 10-fold difference in the affinities of the second site for dUMP (Danenberg & Danenberg, 1978). The native enzyme is symmetric (Hardy et al., 1987; Perry et al., 1990). Therefore, asymmetry would necessarily follow ligation to give the putative nonequivalent active sites. The asymmetry seen in the TS-dUMP-CB3717 crystal structure suggests, but does not demonstrate, that an allosteric effect is relayed from one active site to the other. The very small differences in the structures of the two active sites offer no explanation for the putative differences in their reactivity.

(D) *Ordered bi-bi Sequence in Binding of Substrates and Cofactor.* TS can be modified to prevent binding of either dUMP or polyglutamylated CH<sub>2</sub>-H<sub>4</sub>folate analogues without interfering with binding of the other ligand, demonstrating that the binding sites for substrate and cofactor are independent (Galivan et al., 1976a). This result is consistent with the crystal structures of TS, in which the binding sites for CB3717 and dUMP as well as the deduced binding site for CH<sub>2</sub>-H<sub>4</sub>folate in the ternary complex are preformed in the P<sub>i</sub>-bound enzyme. Binding of one ligand is not required to open a binding site for the second. A random component to the kinetics of ligand binding, as observed by Lu et al. (1984) with polyglutamylated cofactor, cannot be excluded on the basis of crystallographic results.

When monoglutamated cofactor is used, there is evidence that the ligands bind in an ordered fashion in which dUMP binds first followed by cofactor and that cofactor leaves before dTMP in a bi-bi reaction sequence (Lorenson et al., 1967; Danenberg & Danenberg, 1978; Santi & Danenberg, 1984). These results may be explained in part by the fact that the productive binding site for CH<sub>2</sub>-H<sub>4</sub>folate is formed in large part by the nucleotide, and the dUMP binding site is relatively inaccessible once cofactor is bound. Galivan et al. (1976b) show that folates which exhibit no detectable binding without

nucleotide bind tightly to the enzyme-nucleotide complex. The K<sub>d</sub>'s are at least several orders of magnitude lower in the presence of nucleotide. This is consistent with the nucleotide forming a large part of the productive binding site for folates.

When polyglutamylated folates are used, there is evidence for a change in the order of binding (Lu et al., 1984). Dissociation constants improve at least 30-fold over monoglutamylated folate, with a maximal binding for the tetraglutamyl form which is over 150-fold lower. Concomitant with the increased affinity there appears to be a change in order of binding in which dUMP follows binding of folate and dTMP leaves before H<sub>2</sub>folate. The model for septaglutamated TS (Figure 7) may help explain the slower off-rates.

## CONCLUSIONS

Methyl transfer by TS involves many steps. The catalytic site contains at least 25 side chains of which 15 are totally conserved, and many of the remainder are homologous but potentially useful in species-specific drug design. Conformational dynamics of protein and ligands involved in binding the substrate and cofactor are revealed in comparing the unliganded with liganded TS (Montfort et al., 1990). The difference in structures is not an interdomain shift; rather, it involves segmental accommodation of many conserved residues and functional loops.

While there is no recognized cooperativity between the two active sites of the obligate dimer, there is asymmetry in reactivity of the two distant active sites (Danenberg & Danenberg, 1979). A conformational change following ligation may be responsible for the asymmetric behavior, as suggested by an asymmetry we see in the TS-dUMP-CB3717 structure. Reasonably similar segmental accommodation is probably key to the intersubunit feedback. It is notable that the overall temperature factor of one of the monomers is higher than for the other.

The ordered binding of substrate and then cofactor can be understood as dUMP forming a large part of the binding site for the only functional conformer accessible to CH<sub>2</sub>-H<sub>4</sub>folate. Synergistic binding of ligands can be attributed both to the fact that the ligands provide binding sites for one another and to the fact that segmental accommodation, which is presumably most pronounced on ternary complex formation, increases protein contacts to both ligands.

Thus, a new enzymatic function emerges. The active site is a cavernous cleft that closes down on the reactive components yet retains space enough for many partially ordered water molecules. No group on the protein seems close enough to C-5H to act directly as an acid/base catalyst for facilitated proton transfer. The orientation of substrate and cofactor presumably provides for some of the necessary stabilization of the transition state. Highly ordered water molecules within the active site, especially H<sub>2</sub>O 1 between O-4 of dUMP, Glu-60(58), and His-199(147) presumably assist in mediating both proton transfer and transition-state stabilization.

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