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Structural aspects of the inhibition and catalytic mechanism of thymidylate synthase*

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Thymidylate synthase (TS) is a target for anticancer drugs, due to its unique role in the biosynthesis of an essential DNA precursor. The X-ray structures available for several bacterial enzymes have been used to design novel inhibitors of TS, to structurally analyze the binding mode of existing inhibitors, and to propose catalytic roles for amino-acid residues on the protein. The first part of this paper describes some aspects of structure-based drug design, including a recent result from the groups of Montfort and Maley emphasizing the importance of conformational changes in inhibitor binding. The second part of the paper describes the work of the author on the TS mechanism, especially the catalytic roles of active site amino acids Asn177 and Glu58 in TS from *Escherichia coli*. An important function for Glu58 is proposed to be preventing the excessive stabilization of a covalent intermediate. The use of isotope effects to probe the mechanistic basis for stimulation of *E. coli* TS by magnesium ions, and to identify differences between the *E. coli* and human enzymes, is described. The hypothesis that N5 of tetrahydrofolate provides the basicity for deprotonation of the nucleotide is also discussed.

In all known organisms, thymidylate synthase (TS; EC 2.1.1.45) is responsible for supplying an essential precursor for the DNA synthesis which accompanies cell proliferation. Inhibition of TS leads to the death of rapidly growing cells, such as tumor cells, probably due to accumulation of dU residues in chromosomal DNA. Currently, fluorouracil is the only anticancer agent in routine clinical use which directly targets TS. *In vivo*, fluorouracil is converted to 5-fluoro-2′-deoxyuridylate (FdUMP). FdUMP, an analogue of the TS substrate dUMP, inhibits TS by formation of a covalent complex which also contains the other substrate, *N*5,*N*10-methylenetetrahydrofolate (CH_2-THF). This inhibition arises due to the replace-

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Abbreviations: CH_2THF, *N*5,*N*10-methylene-5,6,7,8-tetrahydrofolate; dCMP, 2′-deoxycytidylate; DHF, dihydrofolate; dGMP, 2′-deoxyguanylate; dTMP, thymidylate; dUMP, 2′-deoxyuridylate; FdUMP, 5-fluoro-2′-deoxyuridylate; GMP, guanylate; ^{D}V, deuterium kinetic isotope effect upon k_{cat}; PDDF, 10-(3-propynyl)-5,8-dideazafolate; THF, tetrahydrofolate; TS, thymidylate synthase. Variants of *Escherichia coli* TS created by site-directed mutagenesis are indicated by the amino-acid residue number, preceded by the single letter code for the wild-type residue, and followed by the single letter code for the new residue.

ment of the nucleotide's C5 hydrogen by fluorine. The C5 hydrogen which is normally present is lost as a proton during C5 methylation. (During the steady state turnover of the enzyme, the rate at which this proton is transferred to water is nearly identical to the rate at which thymidylate is formed.) Because a fluorine cation is too unstable to be formed, the inhibited complex which accumulates with FdUMP mimics the intermediate in the TS catalytic cycle which occurs just prior to C5 deprotonation. The FdUMP complex, and similar ones with analogues of CH_2THF, have been useful in both solution and crystallographic studies of TS.

TS is a homodimer with two active sites. X-ray crystallographic studies revealed symetrical active sites, each composed mainly of residues from one subunit and a few residues from the other [1–3]. Catalytic roles of various active site residues have been proposed on the basis of the X-ray structures [4, 5]. We and others have been testing these proposals by characterizing variants of bacterial TSs in which one or more of these active site residues has been replaced by another amino acid *via* site-directed mutagenesis.

Because of the limited or lack of response of some neoplasms to fluorouracil, the toxicity of the drug, and the potential for resistance, TS has been the target of efforts to design anticancer agents as alternatives to fluorouracil. Although my research group has not been involved in these efforts, I will begin by reviewing some of the issues involved, and describe an interesting recent finding by Montfort, Maley, and coworkers [6] which has important implications for structure based inhibitor design. I will then focus upon our own efforts to understand the stereoelectronic details of how TS catalyzes its complex, multi-step reaction. Certain aspects of the recent biochemical and structural studies of TS have been reviewed this year in an article by Carreras and Santi [7].

STRUCTURE BASED INHIBITOR DESIGN

Utility of a homologous structure

Many current mechanistic investigations and structure-based drug design efforts have benefitted from X-ray structural studies of TS. Structures have been solved for the apoenzyme, as well as binary and ternary complexes, for the enzymes from several prokaryotic sources. Those coordinate sets which are available from the Brookhaven Protein Data Bank are listed in Table 1. The target for an anticancer agent would be human TS. Although the human apoenzyme was crystallized several years ago by two different groups [14, 15], difficulties have been encountered in the determination of an interpretable, relevant structure (Dr. J. Finer-Moore, personal communication). Neverthe-

Table 1

Coordinates for thymidylate synthase structures deposited at Brookhaven Protein Data Bank

PDB code	Species (mutations)	Ligands	Reference
3TMS	*E. coli*	phosphate	[8]
2TSC	*E. coli*	dUMP + 5,8-dideaza-10-propynyl-folate (CB3717)	[2]
2BBQ	*E. coli*	dUMP + 5,8-dideaza-10-propynyl-pteroyl-Glu_4	[9]
1TLC	*E. coli*	dGMP + BW 1843U89	[6]
1TYS	*E. coli* (Cys146Ser)	dTMP + DHF	[10]
4TMS	*L. casei*	phosphate	[1, 11]
1TDM	*L. casei*	dUMP	[11]
1TDA	*L. casei* (Val316Am)	phosphate	[12]
1TDB	*L. casei* (Val316Am)	FdUMP	[12]
1TDC	*L. casei* (Val316Am)	dUMP	[12]
2TDD	*L. casei* (Val316Am)	FdUMP + 5-hydroxymethyl-THF	[12]
1TIS	bacteriophage T4	phosphate	[13]

less, the structure of the *E. coli* enzyme has been utilized successfully as a model for the human enzyme [16]. This was made possible by the extreme conservation of the protein, especially in the active site. The amino-acid sequences of *Escherichia coli* and human TS are 46% identical. TS is one of the most highly conserved of any protein for which data is available [8]. (The mechanisms of human and bacterial enzymes are likely very similar, although some differences in kinetic details are described below.) In an efficient approach pioneered by workers at Agouron Pharmaceuticals [16], iterative cycles of structural analysis, inhibitor design and synthesis, evaluation of target enzyme inhibition and enzyme-inhibitor structure, and re-design of the inhibitor structure yielded several inhibitors of human TS with dissociation constants of about 10 nM. These compounds have no signi-

ficant structural resemblance to either substrate for TS, nor was such resemblance intended. The success of this approach provided a model for rational, structure-based design of inhibitors of therapeutically important enzymes.

Flexibility of the target enzyme as a consideration

When an enzyme which is the target for structure-based inhibitor design undergoes conformational changes during ligand binding or catalytic turnover, the designer must choose which of these conformations to target. The apoenzyme form of TS undergoes extensive conformational changes (see Fig. 1) upon formation of ternary complexes with dUMP (or dUMP analogues) and various folate derivatives [2, 3]. Much of this conformational change can be induced by the binding of a folate deri-

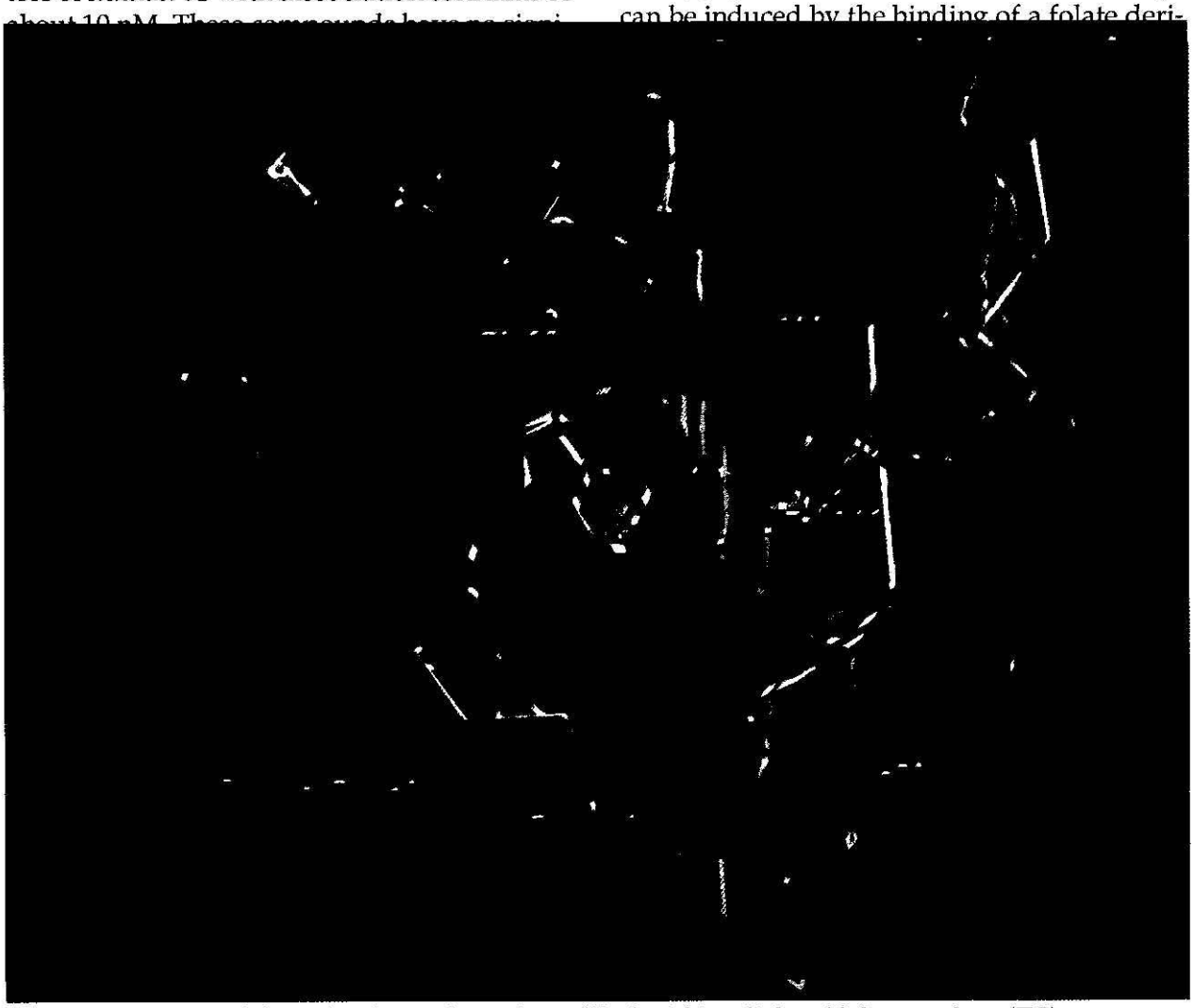

Fig. 1. Comparison of the active site conformations of Escherichia coli thymidylate synthase (TS) apoenzyme (Brookhaven Protein Data Bank coordinate file code 3TMS) and ternary complex (PDB code 2TSC).
Side chains are shown explicitly for a few active site amino acids (Arg21, Glu58, Cys146 and Asn177); α-carbon tracings depict the other portions of the protein shown. In the ternary complex structure, CB3717 is shown in red and dUMP, in yellow.

vative alone to the enzyme [9]. However, the avidity of folate derivatives for binary TS-nucleotide complexes is much greater than for the naked enzyme. Much of the interaction surface for the pteridine is provided by the enzyme-bound nucleotide. Moreover, folate derivatives have several binding modes available to them [2, 12]. Which binding site is most occupied probably depends on the relative stabilities of the conformations accessible to the ternary complex. The choice of which conformation to target is a complex but important consideration in such a system.

In addition to the structure-based approach described above, other more traditional programs of drug development have yielded potent inhibitors of TS which are analogues of the folate substrate (e.g. Burroughs Wellcome compound 1843U89 [17] and the Zeneca compound ICI D1694 [18], shown in Fig. 2). ICI D1694, which has advanced into phase II clinical trials, was designed by structural modification of the quinazoline folate analogue, CB3717. The latter compound, 5,8-dideaza-*N*10-(3-propynyl)-folate, is a potent TS inhibitor, but was found to be nephrotoxic due to its precipitation in the kidney. Several crystal structures have been solved of complexes between TS and CB3717, providing much of the information regarding the conformational changes described above. CB3717 has also occasionally been referred to as PDDF (propynyl-dideaza-folate).

The X-ray structural studies of ternary complexes between *E. coli* TS, nucleotides, and 1843U89 revealed an unexpected structural response of the target enzyme to the agent (Dr. W. Montfort, personal communication). 1843U89 is a benzoquinazoline type folate analogue, in which the *p*-aminobenzoyl moiety is converted to an isoindolinone by the presence of an additional methylene (see Fig. 2). This drug inhibits human TS at subnanomolar concentrations [17]. The binding mode of 1843U89 involves the insertion of the isoindolinone ring between several amino-acids residues which form a hydrophobic cluster in the TS apoenzyme, the dUMP binary complex, and the complex between TS, dUMP, and CB3717. As a result of 1843U89 binding, many of the van der Waals contacts between Ser54, Glu58, Val77, Ile79, and Phe176 are disrupted. To accomodate the creation of this novel binding site, unique translational and rotational movements of the protein backbone also occur (Dr. W. Montfort, personal communication). The structure of the complex containing 1843U89 is different enough from that which occurs with other folate analogues that the compound will enhance the binding of purine nucleotides, dGMP and GMP [6], as well as dUMP. This was the first report of binding of purine nucleotides to TS. Before the structures of these complexes were determined experimentally, the novel binding mode of 1843U89 might only have been anticipated by the addition of robust molecular dynamics to the theoretical structural analysis. This indicates the likely profitability of sampling an en-

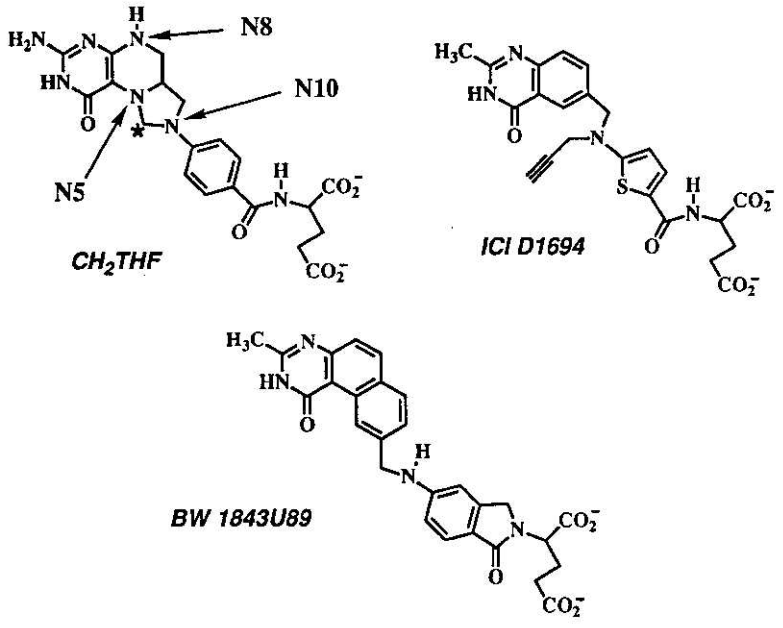

Fig. 2. Structures of N5,N10-methylenetetrahydrofolate (CH_2-THF), the one carbon donor and reductant in the TS-catalyzed reaction and two analogues, ICI D1694 and BW1843U89, which are prospective anticancer agents due to their potent inhibition of TS.

The asterisked methylene is donated to C5 of dUMP during the TS-catalyzed reaction and is replaced by protons on N5 and N10 in tetrahydrofolate. The positions of N5, N8 and N10 are indicated.

semble of inhibitor structures, once a pharmacophore has been established.

CATALYTIC ROLES OF ACTIVE SITE GROUPS

Asparagine 177 determines the pyrimidine specificity of TS

Our attention will now turn to questions involving the structural basis for catalysis by TS. The first question about the function of TS which my research group addressed by site-directed mutagenesis was the structural origin of the pyrimidine substrate specificity [19]. For the *E. coli* enzyme assayed at 30°C, the ratio of the values of k_{cat}/K_m for dUMP and dCMP exceeds 10^{14}. This corresponds to about 21 kcal mol^{-1} lowering of the transition state energy for the TS-catalyzed methylation of dUMP, compared to that for dCMP. The main structural differences in the two nucleotides are boxed in Fig. 3. (There are probably additional slight differences in electron distribution in the other portions of the heterocycles.) The difference in the abilities of dUMP and dCMP to serve as TS substrates arises from the way in which the anionic intermediates occuring during catalytic turnover of dUMP are stabilized. The initial chemical step in the catalytic mechanism (Fig. 4) is attack of an enzymic nucleophile (Cys146 in *E. coli* TS) upon C6 of the pyrimidine of dUMP, to form the first of these anionic intermediates (I). This creates a carbanion at C5, which can be delocalized into O4. The C5 anion is alkylated by the methylene of CH_2THF, forming intermediate II (mimicked by the FdUMP complex). Deprotonation of intermediate II at C5 of the pyrimidine results again in the formation of an oxyanion at O4 (intermediate III). These intermediates are stabilized by a hydrogen bonding network involving several tightly bound water molecules and the side chain of Asn 177. Asn177 is an invariant amino acid in all TS sequences reported to date. The amide side chain of Asn177 forms hydrogen bonds to both N3 and O4 of dUMP (Fig. 5). However, such hydrogen bonds are apparently insufficient to stabilize the N3 amine anion which would be present in the corresponding intermediates formed from dCMP, such as

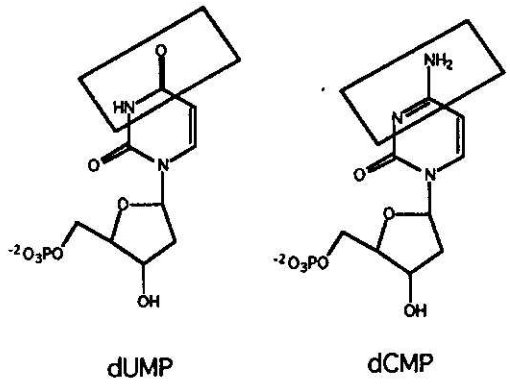

Fig. 3. Structural differences between dUMP and dCMP.

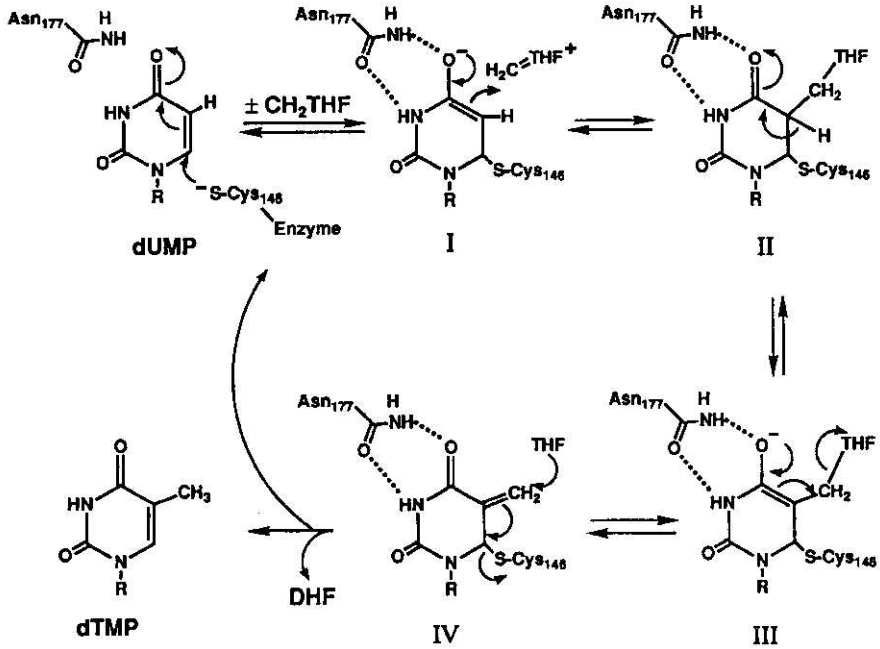

Fig. 4. Proposed catalytic mechanism for thymidylate synthase [7].
The residue numbering is for the *E. coli* enzyme. R represents 1'-(5'-phospho-2'-deoxyribofuranosyl). Although the recycling of the enzyme is shown, the initial binding of dUMP is not shown explicitly. The use of double arrows at all steps until the final (hydride transfer) step is meant only to imply reversability; the sizes of the arrows do not imply anything about the relative rates of the steps indicated.

shown in Fig. 6. Instead, protonation of N3 in such intermediates would be necessary to circumvent the formation of an amine anion. Indeed, Nature has already used this ploy in an

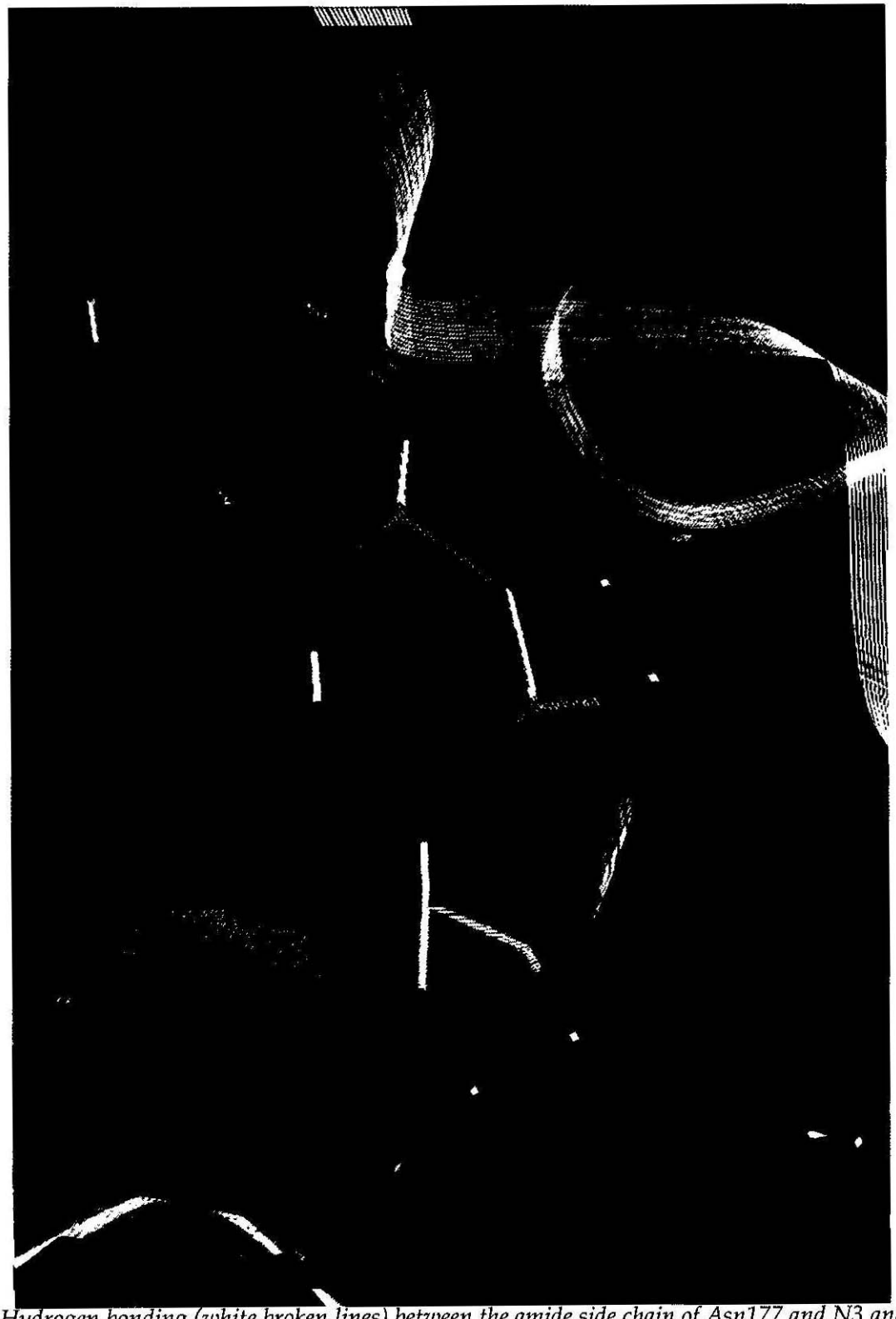

Fig. 5. Hydrogen bonding (white broken lines) between the amide side chain of Asn177 and N3 and O4 of dUMP, in the TS-dUMP-CB3717 X-ray structure.

The lengths in ångstroms of the hydrogen bonds are indicated. The nucleotide is covalently bound to TS *via* a bond between the side chain sulfur of Cys146 and C6 of the pyrimidine. The white ribbon represents a tracing of the backbone of the protein.

enzyme which is homologous to TS, but which utilizes dCMP as its preferred substrate. This is the T even bacteriophage enzyme, dCMP hydroxymethylase (EC 2.1.2.8). In this hydroxymethylase, the residue corresponding to Asn177 is an aspartic acid, which suggested that conversion of this Asn to Asp in TS would alter the enzyme's pyrimidine specificity.

These ideas were confirmed using site-directed mutagenesis [19, 20] to alter the recombinant products of plasmid-born genes for TS and the hydroxymethylase. Replacement of Asn177 by Asp in *E. coli* TS yielded an enzyme variant with 430-fold lower k_{cat} on dUMP and at least 33000 higher k_{cat} on dCMP, compared to the wild type enzyme [19]. Moreover, the ratio of the values of k_{cat}/K_m for dUMP and dCMP was reduced from 10^{14} to 0.04. Formation of 5-methyl-dCMP by the Asn to Asp variant of TS (TS(N177D)) was confirmed by NMR analysis of a purified sample of the product nucleotide. The pyrimidine specificity of the enzyme was therefore reversed by the Asn to Asp substitution. The reciprocal (Asp to Asn) mutation in phage T4 dCMP hydroxymethylase altered the pyrimidine specificity of that enzyme in the expected (opposite) direction, as well as increasing the rate constant for FdUMP binding [20]. These confirmations of our ideas regarding the structural and mechanistic bases for pyrimidine specificity led us to suggest [20] that the carboxylic acid of an Asp or Glu residue might be responsible for protonating N3 of cytosine moieties during the methylation reactions catalyzed by DNA (5-Ctd) methyltransferases. Support for this notion has been recently provided by X-ray structures of *Hha*I and *Hae*III DNA methylases covalently bound to synthetic DNA containing 5-fluorocytosine [21, 22]. Both methylase structures show Glu residues appropriately positioned to protonate N3 and form a hydrogen bond to the N4 amino group of the methylated 5-fluorocytosine.

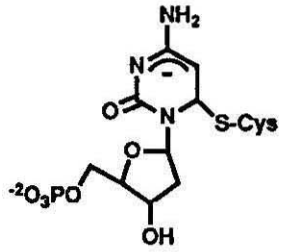

Fig. 6. Hypothetical Michael adduct between dCMP and an active site Cys nucleophile.

An anomaly in the behavior of TS(N177D) was suggested by the biphasic kinetics of the absorbance changes observed at 340 nm (Fig. 7) when dUMP was used as a substrate at a satu-

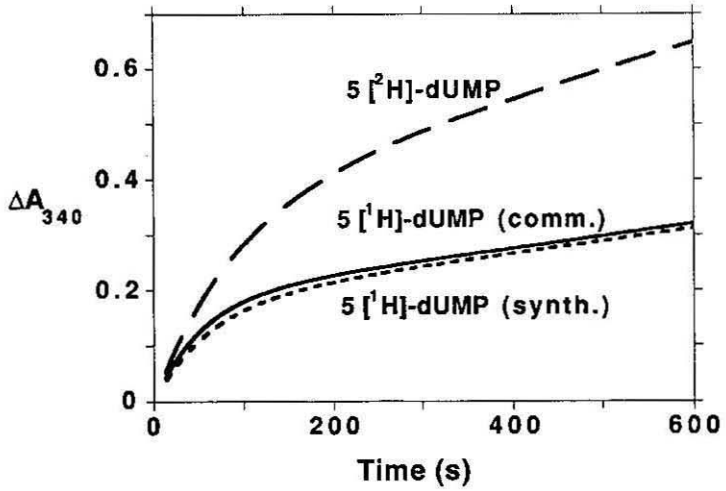

Fig. 7. Biphasic kinetics of absorbance changes during the interaction of TS(N177D) with dUMP and CH_2THF.

Assays were performed at 30°C and contained 0.25 mM dUMP, 1mM CH_2THF and 2.7 μM TS(N177D) dimer, in aqueous solution containing 0.1 M Tris hydrochloride, pH 7.0, 30 mM magnesium chloride, 20mM β-mercaptoethanol. These assays were done with synthetic 5[^{1}H]dUMP (dashed line) and 5[^{2}H]dUMP (broken line) and a commercial sample of 5[^{1}H]dUMP, purchased from Sigma (solid line). Samples of 5[^{1}H]deoxyuridine and 5[^{2}H]deoxyuridine were prepared by reduction of 5-iodo-2'-deoxyuridine (with Zn dust in H_2O/acetic acid and D_2O/perdeuteroacetic acid, respectively) and purified by flash chromatography on silica gel. These 2'-deoxyuridines were then phosphorylated (employing phosphorus oxychloride in trimethylphosphate at −10°C) to provide the synthetic samples of 5[^{1}H]dUMP and 5[^{2}H]-dUMP, which were purified by anion exchange HPLC before use. NMR analysis of the synthetic samples verified their structures and confirmed > 99% substitution of C5 by deuterium in the 5[^{2}H]dUMP.

rating concentration. (Absorbance changes at 340 nm monitor the generation of dihydrofolate (DHF) concomitant with production of dTMP, but can also occur upon the stoichiometric binding of CH_2THF or analogues to TS. Detection in the latter case requires substrate level enzyme concentrations.) TS(N177D) exhibited linear, not biphasic, kinetic behavior with saturating dCMP as substrate. However, the ability of TS(N177D) to utilize dCMP as a substrate was transiently inactivated by prior treatment with dUMP. The transient inactivation of TS(N177D) by dUMP was time-dependent, required the presence of CH_2THF, and was completely reversible following total conversion of the dUMP to dTMP. These features suggested that a catalytic intermediate was accumulating during the turnover of dUMP by the mutant enzyme. The initial "burst" of absorbance change at 340 nm would correspond to the formation of this intermediate, consisting of both the nucleotide and folate substrates bound to the enzyme. Evidence in support of this idea was obtained by measuring the binding of radioactively labelled nucleotides to TS using a nitrocellulose filter binding assay. 6[^{3}H]dUMP, like 6[^{3}H]-5-fluoro-dUMP, was tightly bound to TS(N177D) in a CH_2THF-dependent manner. However, binding assays with dUMP labelled by tritium at C5 did not yield CH_2THF-dependent tritium-labelling of TS(N177D). The latter result indicated that the catalytic intermediate which accumulates has already lost the C5 proton, perhaps resembling intermediate III in Fig. 4. This was further tested by measuring isotope effects on both the inactivation rate and steady state turnover rate of TS(N177D), arising from deuterium substitution of dUMP at C5. If the enzyme form which is primarily responsible for the inactivation requires C5 deprotonation, deuterium substitution might slow the inactivation and allow less of this form to accrue in the steady state. The observed normal deuterium isotope effect of 1.6 upon the first order inactivation rate and the inverse deuterium isotope effect of 0.6 upon the steady state turnover rate (Fig. 7 and unpublished data of Mr. C. Thompson and the author) are therefore consistent with accumulation of intermediate III during TS(N177D)-catalyzed turnover of dUMP.

In the original 1992 report [19] describing the altered pyrimidine specificity of TS(N177D),

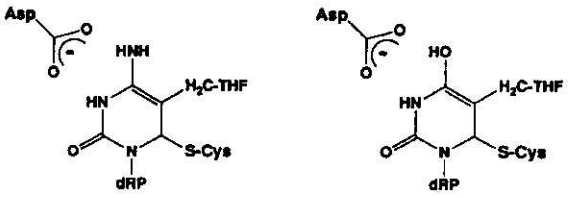

Fig. 8. Protonation of dCMP adduct and dUMP adduct by the carboxylic acid of an active site Asp residue.
dRP = 1′-(2′-deoxy-5′-phosphoribofuranosyl). From ref. [19].

we proposed an explanation of the accumulation of intermediate III with TS(N177D). The hypothesis was that this intermediate becomes adventitiously protonated by the carboxylic acid of Asp177. Thus the carboxylic acid is necessary to stabilize intermediates involving dCMP (see Fig. 8) by acting as a charge sink, but protonation of O4 in an intermediate derived from dUMP was suggested to stabilize the intermediate excessively. However, we have since discovered that the inactivation of TS(N177D) by dUMP requires the presence of Mg^{2+}. The original experiments with TS(N177D) were performed at pH 7 in the presence of 30 mM magnesium chloride. Omission of Mg^{2+} from the assay solutions prevents the inactivation of TS(N177D) by dUMP. The Mg^{2+} requirement for the inactivation of TS(N177D) by dUMP suggests an alternative explanation for the stabilization of intermediate III (Fig. 9). The side chain of Asn177 and O4 of enzyme-bound dUMP both face a cavity within the active site of TS which also contains

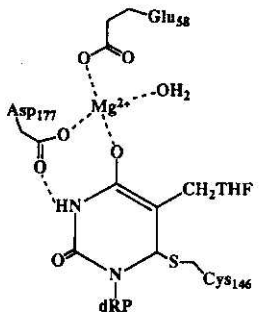

Fig. 9. Mg^{2+} complex of catalytic intermediate III, proposed to explain the transient inactivation of TS(N177D) by dUMP.
Note that the position of Mg^{2+} shown is hypothetical, since no X-ray structures of TS with magnesium ion present have been solved to date. dRP = 1′-(2′-deoxy-5′-phosphoribofuranosyl).

several water molecules. This cavity in the ternary complex is isolated from the bulk solvent by the bound substrates and protein moieties. The side chains of several other residues, including Glu58 and His147, protrude into this cavity. The relative orientations and interatomic distances of the O4 oxyanion and carboxylates from Glu58 and Asp177 would allow these groups to provide ligands for a magnesium ion, with additional ligands possibly provided by internal water molecules. This could provide the charge neutralization needed to stabilize intermediate III.

Magnesium ions are routinely included in assays of TS because of the stimulatory effect on the wild type enzyme's activity [23]. With wild type TS from *E. coli*, this is not merely an effect upon substrate binding affinities, because the values of K_m for either dUMP or CH_2THF are altered no more than two-fold, whereas k_{cat} is elevated 4.3-fold by a saturating concentration of Mg^{2+} (Table 2). The structural origin of the stimulatory effect of Mg^{2+} is unknown, since this ion is incompatible with any of the crystallization solvents used in the previous X-ray structural studies of TS. Some clues about which chemical step in the TS reaction pathway must be affected by Mg^{2+} are provided by kinetic isotope effects upon the deprotonation and hydride transfer steps. In the presence of magnesium ions, wild type TS shows a deuterium isotope effect upon k_{cat} (^{D}V) arising from ^{2}H substitution at C5 of dUMP only slightly greater than unity (Table 3), whereas in the absence of magnesium ions, this isotope effect is 2.3. In contrast, the deuterium kinetic isotope effect upon k_{cat} arising from ^{2}H substitution at C6 of CH_2THF is about 4.5 in the presence of magnesium ions (Table 3; [24]). The value of ^{D}V arising from the deuterium isotope effect on hydride transfer decreases slightly in the absence of magnesium ions (Table 3). Together, these results suggest strongly that the elevation of k_{cat} by Mg^{2+} is due to an enhancement in the rate of C5 deprotonation. If the deprotonation of dUMP becomes less rate-limiting in the presence of Mg^{2+}, the overall turnover rate should be less sensitive to C5 deuteration, as

Table 2
Effect of Mg^{2+} on the kinetic parameters for E. coli thymidylate synthase[a]

	K_m, dUMP (μM)	K_m, 6R-CH_2THF (μM)	k_{cat} (s^{-1})
150 mM Mg^{2+}	15	10	11.7 (±0.6)
$-Mg^{2+}$	27	5	2.7 (±0.07)

[a]All values were determined from spectrophotometric assays done at pH 7.0, 30°C, 0.1 M Tris hydrochloride. The apparent K_s for Mg^{2+}, i.e., that concentration which produces half-maximal stimulation of k_{cat}, is 7 mM. The indicated uncertainties in the k_{cat} values are standard errors.

Table 3
Deuterium kinetic isotope effects on deprotonation and hydride transfer catalyzed by E. coli thymidylate synthase[a]

Enzyme	5-[^{2}H]dUMP		6-[^{2}H]CH_2THF
	^{D}V[b]	$^{D}(V/K)$[c]	^{D}V
Wild type + Mg^{2+}	1.32 (±0.02)	1.2 (± 0.3)	4.5 (± 0.1) 4.4 (± 0.2)[d]
$-Mg^{2+}$	2.3 (±0.1)	not done	3.7 (± 0.1)
N177D – Mg^{2+}	3.8 (± 0.5) 4.1 (± 0.4)[d]	7.1 (±2.5)	3.4 (± 0.3) 4.1 (± 0.2)[d]
E58Q + Mg^{2+}	–	–	1.5 (± 0.1)[d, e]

[a]All values were determined using the spectrophotometric assay unless otherwise indicated. Conditions were as listed in Table 2. The indicated uncertainties are standard errors.

[b]The term ^{D}V is the ratio of k_{cat} measured with the ^{1}H-containing substrate to k_{cat} with the ^{2}H-containing substrate.

[c]The term $^{D}(V/K)$ is the ratio of k_{cat}/K_m measured with the ^{1}H-containing substrate to k_{cat}/K_m with the ^{2}H-containing substrate.

[d]Determined by HPLC assays of dTMP formation rates.

[e]From ref. [25].

observed. The slight decrease in ^{D}V arising from the deuterium isotope effect on hydride transfer probably stems from larger values of committment factors, due to the increased contribution of C5 deprotonation to the limitation of k_{cat}. The dramatic increase in ^{D}V arising from ^{2}H substitution at C5 of dUMP with TS(N177D) in the absence of magnesium ions suggests that, with this variant of TS, C5 deprotonation limits the overall rate at least as much as hydride transfer does. (These conclusions assume that neither Mg^{2+} nor the Asn177Asp mutation cause significant changes in intrinsic isotope effects or the transition state structures for the deprotonation or hydride transfer steps.)

As mentioned above, TSs from humans and *E. coli* are similar enough to allow the X-ray structure of the bacterial enzyme to be used successfully to design potent inhibitors of the human enzyme. Although there have been no reports of any major differences in the mechanisms of the two enzymes, minor differences do exist. In contrast to *E. coli* TS, the recombinant human enzyme is only slightly stimulated by Mg^{2+} (35% increase in k_{cat}). The turnover number for human TS is at least five lower than that observed for *E. coli* TS in the presence of Mg^{2+}. The values of ^{D}V arising from deuterium isotope effects on deprotonation and hydride transfer measured at pH 7 using human TS are 1.0 and 2.1, respectively, and are insensitive to magnesium ion concentrations. These differences indicate that the catalytic rate of human TS may be less limited by intrinsic chemistry than the catalytic rate of *E. coli* TS is.

Stability of a catalytic intermediate is moderated by glutamate 58

Glutamic acid 58 is another active site amino acid which plays an important role in catalysis. Like Asn177, Glu58 is an invariant residue in all TS sequences determined to date. Two roles were proposed for Glu58 on the basis of the X-ray structure of the covalent complex between *E. coli* TS, FdUMP, and CH_2THF [5]. These were: (1) activation of N5,N10-CH_2THF by protonation of N10, leading to the formation of an N5 methylene iminium ion (Fig. 10), and (2) deprotonation of C5 of dUMP. To test these roles, variants of *E. coli* TS were generated by site-directed mutagenesis in which Glu58 was replaced by Gln and Asp [25]. The rate of dTMP formation was decreased 50- and 2000-fold, respectively, by the Asp and Gln substitutions of Glu58 (Table 4). However, the rate of tritium release into solvent water from 5[^{3}H]dUMP was not as severely affected by the Glu58 substitutions. As a result, the Glu58 variants of TS catalyze C5 deprotonation at a substantially

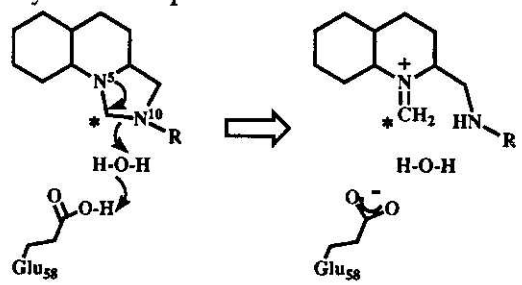

Fig. 10. Proposed role of Glu58 in the conversion of the N5,N10-bridged form of CH_2THF to the 5-iminium ion [5].
The structural details of the pteridine are simplified for clarity. R = 4-(*N*-glutamyl)benzoyl. From ref. [25].

Table 4
Effect of Glu58 substitutions in E. coli TS upon rate constants for tritium exchange and alkylation reactions[a]

Enzyme	k_{lim} (s^{-1}) for ^{3}H-exchange with:				k_{cat} (s^{-1}) for thymidylate	
	THF		CH_2THF		Formation	
Wild type	0.8	(1.0)[b]	3.6	(1.0)	3.6	(1.0)
Glu58Asp	0.08	(0.1)	0.43	(0.12)	0.07	(0.02)
Glu58Gln	0.04	(0.05)	0.010	(0.003)	0.0018	(0.0005)

[a]From ref. [25].

[b]The numbers in parenthesis are the ratio of the indicated rate constant to the value observed with the wild type enzyme.

higher rate than alkylation. Hence the tritium release from 5[^{3}H]dUMP catalyzed by TS(E-58D) and TS(E58Q) in the presence of CH_2THF is a sum of turnover and solvent exchange reactions. (Similar behavior has been observed with mutants of recombinant *Lactobacillus casei* TS [26].) This contrasts with the wild type enzyme, for which intermediate III has a high forward catalytic commitment, leading to nearly equal rates of C5 deprotonation and dTMP formation. These results eliminate role (2) for Glu58. Like many other candidate amino acids in the active site of TS which have been examined using site-directed mutagenesis, Glu58 cannot be the base which deprotonates C5 of dUMP (see below).

The N5 methylene iminium ion has been suggested to be the enzyme-bound electrophile which adds to C5 of dUMP to form intermediate II during catalysis by TS [27]. This proposal was based upon studies of the reversible, non-enzymatic condensation of formaldehyde with THF and analogues [27, 28]. A recent study of model reactions suggests that nucleophilic addition to the methylene may be concerted with cleavage of the methylene-N10 bond [29]. The suggestion that Glu58 may protonate N10 to assist the cleavage of the methylene-N10 bond was based upon the proximity of these groups in the X-ray structures of TS ternary complexes, and upon the fact that Glu58 resides in a hydrophobic pocket in the TS active site. The conserved hydrophobic nature of the Glu58 environment are consistent with a substantially elevated pK_a for the side chain carboxylic acid of this residue. This would mean that Glu58 would be in the appropriate state to protonate N10 upon the binding of CH_2THF to TS at pH 7. To test whether Glu58 has a role in the activation of N5,N10-CH_2THF by protonation of N10, the effects of the Glu58 mutations on rates of CH_2THF-dependent tritium release were compared to their effects on rates of THF-dependent tritium exchange. Since THF already has N10 protonated, the latter rates should be less affected by the Glu58 substitutions than the former. This was true for the Glu58Gln variant of TS (Table 4), and also for similar mutants of dCMP hydroxymethylase [25]. These results are consistent with an auxiliary function for Glu58 in the activation of CH_2THF (role (1) listed above). This cannot, however, explain why substitutions of Glu58 in TS (and of the corresponding glutamic acid in dCMP hydroxymethylase) yields enzyme variants in which C5 deprotonation and alkylation are "uncoupled." The uncoupling requires a significant role for Glu58 either in the partitioning of catalytic intermediate III or in the final catalytic step, hydride transfer to intermediate IV.

Several lines of evidence suggest that Glu58 plays a major role in the partitioning of intermediate III. If the final step in TS catalysis, hydride transfer, were decreased severely by the Glu58 mutations, then the value of ^DV arising from the deuterium isotope effect on this step should either increase or remain constant. The observed three-fold *decrease* in the observed isotope effect on hydride transfer with TS(E58Q) (Table 3) indicates instead that an earlier step has become more rate-limiting. This earlier step is probably conversion of intermediate III to IV.

Evidence that eliminates any important role for the corresponding Glu in the final catalytic step for dCMP hydroxymethylase was obtained by measuring the rate of solvent ^{18}O exchange into the hydroxymethyl group of [^{16}O]-5-hydroxymethyl-dCMP. For the hydroxymethylase, the final step is a reversible hydration of intermediate IV, rather than hydride transfer. The mutations of the hydroxymethylase decreased the rate of the $^{18}O/^{16}O$-exchange reaction by less than six-fold [25]. Again, this indicates that the dramatic effects on rates of alkylated product formation are due to an earlier step, consistent with slower conversion of intermediate III to IV. Variants of both TS and dCMP hydroxymethylase give rise to UV chromophores in the presence of CH_2THF and nucleotides which are likely due to the accumulation of intermediate III during turnover [25]. It is noteworthy that this is the same catalytic intermediate which was proposed to accumulate during turnover of dUMP by TS(N177D) in the presence of Mg^{2+}.

The behavior of the Glu58 variants of TS can be explained on the basis of the enzyme's need to maintain a balance between stability and reactivity of catalytic intermediates. Intermediate III is evidently still formed by the Glu58 variants of TS, since the TS-catalyzed tritium exchange reactions still occur at substantial rates. This intermediate must have a high forward catalytic commitment when formed by wild type TS, since thymidylate formation and

C5 proton removal occur at nearly identical rates. However, with the Glu58 variants, intermediate III no longer has a high forward commitment. At the point in the TS catalytic cycle where intermediate III occurs, the side chain of Glu58 should exist as the carboxylate anion. (Recall that Glu58 earlier protonated N10 of CH_2THF). This anion must be within about 5 Å of the dUMP-derived O4 oxyanion of intermediate III, within a cavity sequestered from bulk solvent (Fig. 11). With wild type TS, the unfavorable electrostatic interaction between these two anions is proposed to moderate the stability of intermediate III, increasing its forward catalytic commitment. This interaction would be decreased or removed by the substitutions in TS(E58D) and TS(E58Q), explaining why intermediate III can form but is sluggish in turning over to yield alkylated products.

Our results with TS variants suggest that catalytic intermediate III is particularly vulnerable to excessive stabilization by the enzyme. This observation has potential relevance to the design of TS inhibitors. It might be possible to design a compound which would exploit the vulnerability of intermediate III, and inhibit TS by stabilizing this intermediate.

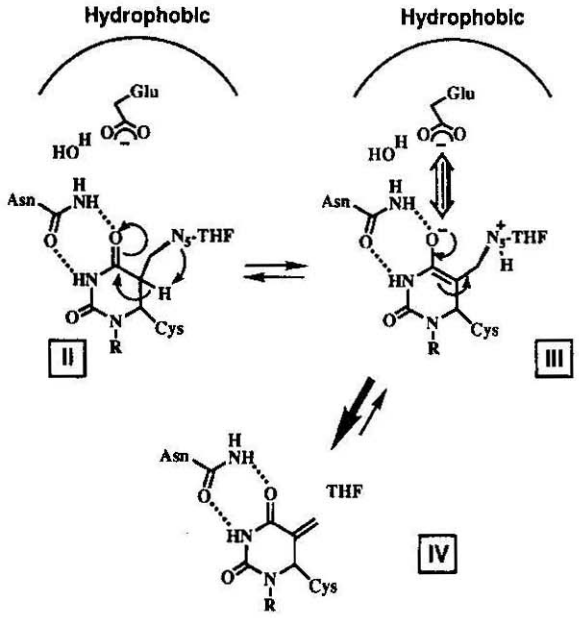

Fig. 11. Concerted removal of the C5 proton from the dUMP heterocycle and protonation of N5 of THF, followed by β-elimination of the THF cation. The proton transfer must occur *via* at least one intermediary water molecule (not shown explicitly), since N5 of THF is on the opposite side of the pyrimidine from the proton being removed. An electrostatic repulsion (double arrow) between the O4 oxyanion and the Glu58 carboxylate is proposed to moderate the stability of intermediate III, increasing its forward catalytic commitment.

Substrate participation in catalysis: N5 of tetrahydrofolate as a base

The identity of the base responsible for deprotonating C5 of dUMP during catalysis by TS has been elusive. Site-directed mutagenesis has been used to eliminate the candidacy of all the amino acids within proximity of C5 of dUMP (or FdUMP) in the crystal structures of various ternary complexes, including Glu58 [25, 26], His147 [30], and Tyr94 (Dr. Frank Maley, personal communication). Prior to the deprotonation of C5, the pyrimidine heterocycle must undergo a conformational isomerization [5], involving axial-equitorial interconversion of the C5 and C6 substituents, in order to yield the observed stereochemistry of hydride transfer to the incipient methyl group [31]. This conformation for intermediate II, with an axial C5 hydrogen, increases the acidity of the C5 hydrogen. The coplanarity of C5, C4, and O4 of the pyrimidine in this conformation allows the delocalization of the charge into O4 after proton loss. The next step, β-elimination of N5 of THF, would be assisted by prior protonation of N5, since the cation would be a better leaving group.

The considerations above led to the hypothesis that the ultimate basicity for deprotonation of C5 of dUMP is provided by N5 of THF. This proton transfer would require one or two bridging water molecules, since the acceptor (N5 of THF) is on the opposite side of the pyrimidine from the axial proton being donated. Several water molecules sequestered within the active site cavity provide candidates for this bridge. To test the hypothesis that N5 is the base responsible for labilizing the C5 proton, the exchange reaction between 5[^{3}H]-dUMP and solvent water was employed. The ability of TS to catalyze this exchange reaction is dependent on the presence of THF. Whereas 10-deaza-THF was nearly as effective as THF in enhancing rate of the TS-catalyzed tritium exchange reaction, 5-deaza-THF was completely incapable of accelerating the exchange reaction [25]. Similar observations were made with dCMP hydroxymethylase, using 5[^{3}H]dCMP

[25]. (8-Deaza-*N*5,*N*10-methylene-5,6,7,8-tetrahydrohomofolate has been previously shown to be a substrate for *L. casei* TS [32], ruling out any essential role for N8.) The inability of 5-deaza-THF to enhance the tritium exchange reaction catalyzed by TS, or dCMP hydroxymethylase, is not due to a lack of enzyme binding, since 5-deaza-THF inhibits both TS and the hydroxymethylase. Futhermore, 5-deaza-THF effectively enhances the rate of the hydroxymethylase-catalyzed $^{18}O/^{16}O$ exchange reaction between solvent and the hydroxymethyl group of hydroxymethyl-dCMP described above [33], indicating that it is a good structural analogue of THF. These results support the hypothesis that N5 of THF is the elusive base which removes the C5 proton of dUMP during TS catalysis.

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