Electrostatic Guidance of Catalysis by a Conserved Glutamic Acid in *Escherichia coli* dTMP Synthase and Bacteriophage T4 dCMP Hydroxymethylase†

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ABSTRACT: Thymidylate synthase (TS) and dCMP hydroxymethylase (CH) are homologous enzymes which catalyze the alkylation of C5 of pyrimidine nucleotides. One of the first catalytic steps is isomerization of the alkyl donor, methylenetetrahydrofolate, from its N5,N10 bridged form to the N5 iminium ion upon enzyme binding. Glu58 in TS has been postulated [Matthews et al. (1990) *J. Mol. Biol. 214,* 937−948] to be involved in this isomerization and the deprotonation of C5 of the nucleotide. Substitution by Asp or Gln of Glu58 in *Escherichia coli* TS, or of the corresponding Glu60 in CH from phage T4, decreases the activity of either enzyme. Alkylation is slowed much more than deprotonation, indicating uncoupling of steps which are tightly coupled for the wild-type enzymes. The data support minor roles for Glu58/60 in nucleotide binding and in isomerization of methylenetetrahydrofolate, but no major roles in nucleotide deprotonation, product dissociation, or hydration catalyzed by CH. The primary role of Glu58/60 is to accelerate bond cleavage between N5 of tetrahydrofolate and the methylene being transferred. The influence of Glu58/60 on the rate of bond cleavage is proposed to arise from electrostatic destabilization, due to the proximity of the glutamyl carboxylate, of the anionic species formed when C5 of the nucleotide is deprotonated. The proposal explains the uncoupling of deprotonation and alkylation with the Glu58/60 variants and the reduced kinetic isotope effect on hydride transfer for TS(Glu58Gln). The inability of 5-deazatetrahydrofolate to stimulate enzyme-catalyzed tritium exchange from [5-^{3}H]nucleotides into solvent suggests that N5 of tetrahydrofolate is the base which deprotonates the nucleotide.

Elucidation of the catalytic role of a particular component of an enzyme requires combined information from a variety of sources. The three-dimensional model of the enzyme bound to substrates or inhibitors is usually an essential prerequisite to informative studies of active site structure. However, the static information embodied in such a structure cannot alone reveal the dynamic chemical processes which accompany catalysis. Detailed genetic and kinetic studies, based upon the mechanistic hypotheses suggested by the structure, are important complementary tools in the testing of proposed catalytic roles of active site amino acid residues or cofactors. We here describe parallel kinetic studies of site-directed mutant variants of two homologous enzymes, for one of which several X-ray structures are available.

Thymidylate (dTMP)[1] synthase (EC 2.1.1.45, TS) and deoxycytidylate (dCMP) hydroxymethylase (EC 2.1.2.8, CH) are homologues (Graves, 1994) which catalyze analogous alkylation reactions. Both are involved in the biosynthesis of DNA precursors. Both enzymes are homodimers. TS is biologically ubiquitous and catalyzes the conversion of deoxyuridylate (dUMP) and *N*5,*N*10-methylene-5,6,7,8-tetrahydrofolate (CH_2THF) to dTMP and dihydrofolate (DHF). This is essentially an irreversible reaction, due to the oxidation of the folate substrate. CH, encoded by the genome of T even bacteriophages, catalyzes the reversible conversion of dCMP and CH_2THF to 5-(hydroxymethyl)-dCMP (HMdCMP) and THF.

Both TS and CH are covalently inactivated in the presence of CH_2THF by the mechanism-based inhibitor 5-fluoro-dUMP (FdUMP), the metabolically activated form of the anticancer agent fluorouracil. The essential role of TS in DNA synthesis has made the enzyme an attractive target for the design of new therapeutic agents which are less toxic than fluorouracil, or which are specific for infectious microbes. As a result TS has been extensively studied structurally and kinetically, as well as by site-directed mutagenesis. We have proposed a catalytic mechanism for CH (Scheme 1) which is based upon a similar previous proposal for TS (Scheme 2). The essential similarity of the early steps in catalysis by the two enzymes has been established in our previous studies of TS from *Escherichia coli* and CH from bacteriophage T4 (Graves et al., 1992; Hardy & Nalivaika, 1992; Graves & Hardy, 1994). We recently provided evidence from $^{18}O/^{16}O$ exchange experiments (Butler et al., 1994) for a key late intermediate in catalysis by CH (IV in Scheme 1).

One of the earliest indications that catalysis by CH proceeds via a mechanism similar to that of TS was the facile tritium−protium exchange reaction which CH catalyzes

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[1] Abbreviations used are: CH, dCMP hydroxymethylase; CH_2THF, *N*5,*N*10-methylene-5,6,7,8-tetrahydrofolate; dCMP, 2′-deoxycytidylate; DHF, dihydrofolate; dTMP, thymidylate; dUMP, 2′-deoxyuridylate; FdUMP, 5-fluoro-2′-deoxyuridylate; HMdCMP, 5-(hydroxymethyl)-2′-deoxycytidylate; HPLC, high-pressure liquid chromatography; ^DV, deuterium kinetic isotope effect upon k_{cat}; PDDF, 10-(3-propynyl)-5,8-dideazafolate; RV, retention volume; THF, tetrahydrofolate; TS, thymidylate synthase.

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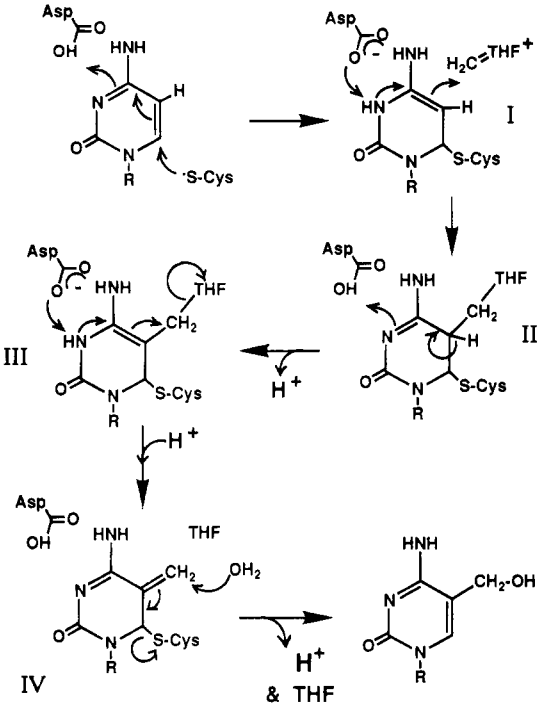
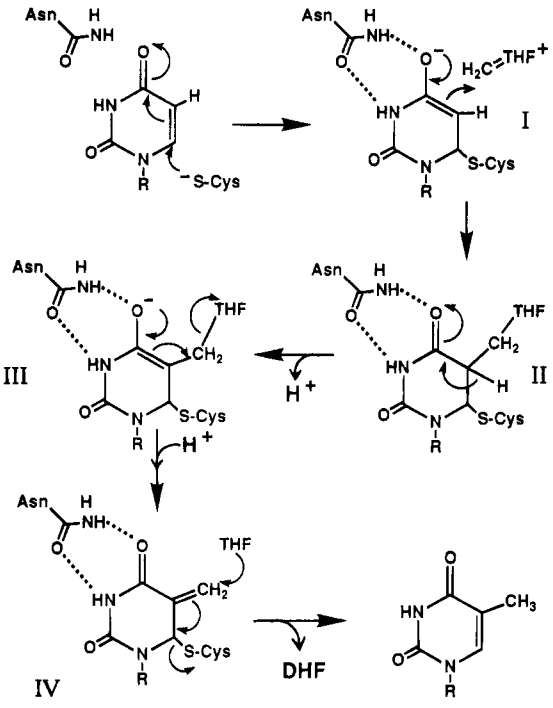
Scheme 1: Proposed Catalytic Mechanism for CH (Graves et al., 1992), Indicating Roles Suggested for Cys148 and Asp179 from Previous Studies

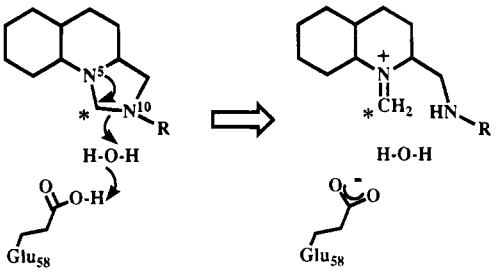


Scheme 2: Proposed Catalytic Mechanism for TS (Santi & Danenberg, 1984; Matthews et al., 1992), Indicating Roles Suggested for Cys146 and Asn177 from Previous Studies



between [5-^{3}H]dCMP and solvent water in the presence of THF (Yeh & Greenberg, 1967). With wild-type CH, this THF-dependent exchange reaction occurs faster than the CH_2-THF-dependent release of tritium from [5-^{3}H]dCMP accompanying alkylation. TS will also catalyze a similar exchange reaction between solvent water and [5-^{3}H]dUMP, which is accelerated by THF (Lomax & Greenberg, 1967) or folate (Pogolotti et al., 1979). The TS-catalyzed exchange reaction is much slower than alkylation. These exchange

Scheme 3: Protonation of N10 of CH_2THF by Glu58 [R = 4-(N-Glutamyl)benzoyl]



reactions provide strong evidence for the participation of 5,6-saturated pyrimidine intermediates in catalysis by both CH and TS (Ivanetich & Santi, 1992).

Proposals for the catalytic roles of invariant active site amino acid residues in TS and CH are based upon analysis of the several X-ray structures of crystalline TS. One such residue is Glu58 in *E. coli* TS, which corresponds to Glu60 in T4 phage CH. Two roles for this residue's side-chain carboxylic acid or carboxylate in TS catalysis were suggested by Matthews and colleagues, based upon their structure for the covalent complex between *E. coli* TS, FdUMP, and CH_2-THF (Matthews *et al.*, 1992a). These were (i) to protonate N10 of the N5,N10-bridged form of CH_2THF, to promote the generation of the N5-iminium ion (Scheme 3), and (ii) to assist the water-mediated abstraction of the proton from C5 of dUMP. The N5-iminium ion of CH_2THF is likely to be the enzyme-bound form of the folate substrate which undergoes attack by the C5 anion of dUMP, on the basis of chemical precedent (Kallen & Jencks, 1967b), inability of the structure of TS to accommodate the N5,N10-bridged form (Hardy et al., 1987; Montfort et al., 1992; Matthews et al., 1992), and the structure of a crippled variant of TS bound to FdUMP and 5-(hydroxymethyl)-THF (Perry et al., 1993). The first role suggested requires an elevated pK_a for the side group of Glu58. This is plausible, since Glu58 is buried in the fully liganded enzyme and resides within a cluster of residues whose hydrophobicity is highly conserved. An elevated pK_a for Glu58 would make the carboxylate a stronger base, which is also consistent with a role for this group in activating a water molecule to deprotonate C5 of dUMP.

The proximity of N10 (of folate derivatives bound to crystalline TS) to the carboxyl oxygens of Glu58 was a key factor in the suggestion that this residue might catalyze the formation of the 5-iminium ion. In two ternary complex structures (Matthews et al., 1992; Montfort et al., 1992) this distance is about 4 Å. However, N10 of the folate is more distant (>7Å) from the carboxylic acid side chain of Glu58 in the X-ray structure of the crystalline complex formed between FdUMP, CH_2THF, and a variant of *Lactobacillus casei* TS with a one-residue C-terminal truncation which prevents catalytic turnover (Perry et al., 1993). In this complex, the N5,N10-bridged form of the folate substrate has already rearranged to 5-hydroxymethyl-THF, in spite of the fact that the enzyme is prevented from alkylating dUMP by the C-terminal truncation. In this structure, which should be a good model of the initial complex formed upon precatalytic ligand binding, the conformations of both the nucleotide and the folate are rather different from those observed in earlier structures (see Figure 1). N10 is close in this structure to C5 of dUMP (3.1 Å), but the carboxylate of *L. casei* TS residue Glu60 (which corresponds to *E. coli*

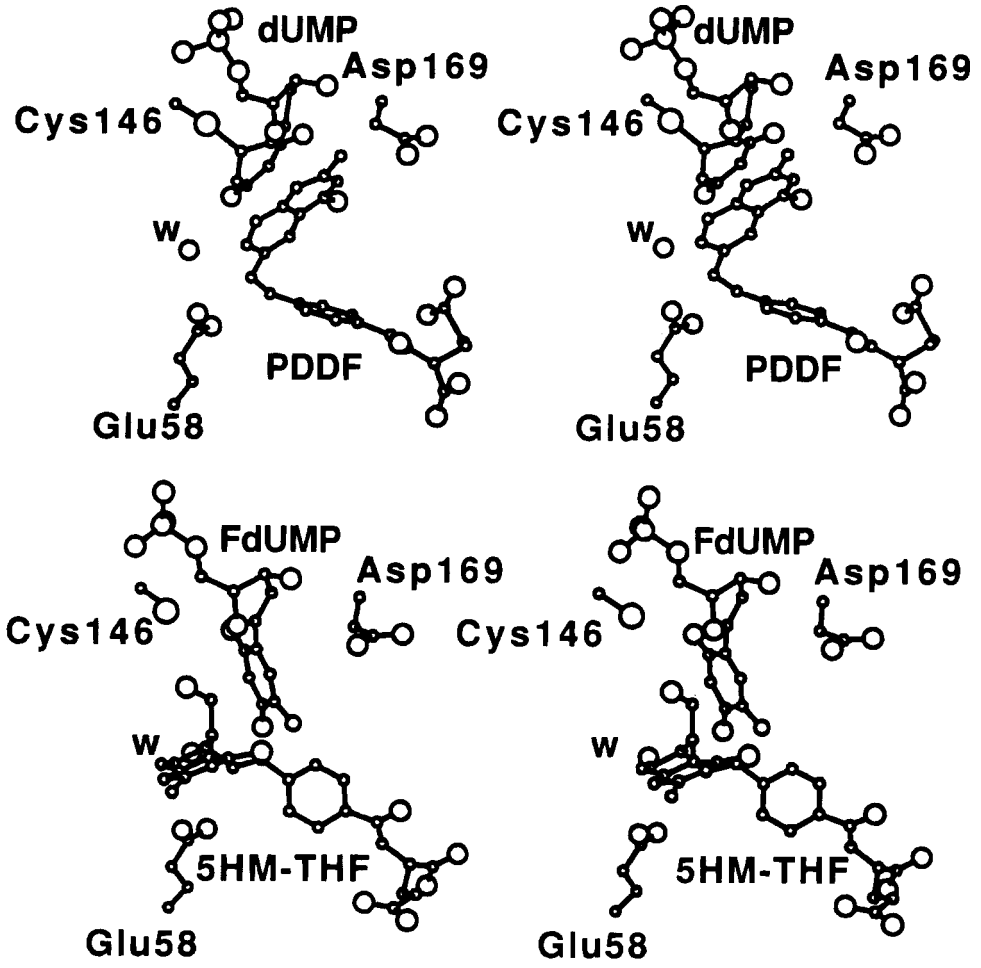


FIGURE 1: Convergent stereodiagrams of the conformations of bound folate derivatives in the active sites of (top) wild-type *E. coli* TS and (bottom) the one-residue-truncated version of *L. casei* TS, TS(V316Am). The top structure, from Brookhaven PDB coordinate set 2TSC, is the complex between *E. coli* TS(wt), dUMP, and 10-(3-propynyl)-5,8-dideazafolate (PDDF) (Montfort et al., 1992). The bottom structure, from Brookhaven PDB coordinate set 2TDD, is the complex between *L. casei* TS(V316Am), FdUMP, and CH_2THF (Perry et al., 1993). To simplify viewing, the 10-(3-propynyl) moiety of the PDDF in the former structure is not shown. In the latter structure, the folate derivative has rearranged to 5-(hydroxymethyl)-THF (5HM-THF). The structures were aligned by least squares superposition of the Cα positions of residues 2−82 and 136−192 of the *E. coli* enzyme with the corresponding residues of the *L. casei* enzyme, using the Quanta molecular modeling software package. In addition to the ligands, the Cα and side-chain atoms of residues Glu58, Cys146, and Asp169 of *E. coli* TS, a bound solvent molecule (w), and corresponding portions of the *L. casei* structure are shown.

TS residue Glu58) is not. These differences, which were thoroughly described by Perry and co-workers (Perry et al., 1993), are likely due to the fact that the truncated enzyme cannot undergo the conformational changes normally caused by ligand binding.

Because of the uncertainty raised by the differences in the X-ray structural information, and the need in any event to test the hypothetical roles of Glu58 in TS and Glu60 in CH, we have generated site-directed mutant variants of both enzymes and compared their kinetic behaviors with those of the wild-type enzymes in several assays. One of these proteins, TS(E58Q),[2] was examined previously by another group (Zapf et al., 1993), who concluded that their data supported a role for TS residue Glu58 in the formation of the 5-iminium ion form of CH_2THF. We compare our results with those previously reported and come to the rather different conclusion that replacement of Glu58 in TS or Glu60 in CH leads to severe uncoupling of the early and late steps of catalysis. This observation has also been made recently with similar mutants of *Lactobacillus casei* TS (Huang & Santi, 1994). The most significant catalytic role for this active site Glu in CH and TS probably involves cleavage of the bond which links N5 of THF to the C11 methylene being transferred to C5 of dCMP or dUMP.

MATERIALS AND METHODS

Materials. Tritium-labeled nucleotides were obtained from Moravek Biochemicals, Brea, CA. dCMP, dUMP, dTMP, and folic acid were obtained from Sigma Biochemicals. CH_2-THF, routinely prepared from commercial THF (Sigma or Fluka), was quantitated and checked for purity as described (Graves *et al.*, 1992). The concentrations of CH_2THF have been corrected for the purity of the L-(6*R*) isomer; concentrations of THF and THF analogues are uncorrected. A sample of the 5-deaza analogue of THF was provided by Dr. C. Shih of Eli Lilly Research Laboratories.

The 10-deaza analogue of THF was synthesized from 10-deazaaminopterin, a gift from Dr. J. R. Piper of the Southern Research Institute. A sample of 10-deazaaminopterin was refluxed for 4 h in 0.1 N aqueous sodium hydroxide under

[2] Variants of CH or TS created by 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.

nitrogen, and the resulting 10-deazafolic acid (>99% conversion by HPLC assay) was precipitated with hydrochloric acid. Reduction of the 10-deazafolic acid to the corresponding 5,6,7,8-tetrahydro compound was accomplished with sodium borohydride, as described for folic acid (Scrimgeour & Vitols, 1966). The 10-deaza-THF product was purified by chromatography on DEAE-cellulose and quantitated using an extinction coefficient at 256 nm of 31 300 M^{-1} at pH 12 (Struck et al., 1971). Reduction of the 10-deaza analogue was accompanied by loss of the absorbance at longer wavelength (λ_{max} 363 nm) characteristic of oxidized folates.

[6-^{1}H]CH_2THF and [6-^{2}H]CH_2THF for kinetic isotope effect measurements were synthesized by reduction of folic acid with, respectively, sodium borohydride and sodium borodeuteride (Aldrich), using a published method (Scrimgeour & Vitols, 1966). These samples of CH_2THF were purified by chromatography on DEAE-cellulose using a gradient of triethylammonium bicarbonate, as previously described (Butler & Hardy, 1994; Curthoys et al., 1972). This purification method partially resolved the 6*R* and 6*S* isomers of CH_2THF (Kaufman et al., 1963). Preparations of [6-^{1}H]- and [6-^{2}H]CH_2THF obtained by this procedure were 70–80% pure 6*R* isomer.

Bacteriology, Cloning, and Mutagenesis. Conventional recombinant methods were used for plasmid construction (Sambrook et al., 1989). *E. coli* XAC25 (Kim et al., 1993), which is deleted for the entire TS gene (*thyA*), was used for all recombinant methods with plasmids carrying mutant alleles of *thyA*. This strain was also used for complementation tests of mutant *thyA* alleles. The ability of TS(wt) or TS(E58D) to support the growth of *E. coli* XAC25 in the absence of thymidine at 37 °C was measured using the same Bluescript-derived plasmids that were used to overexpress these proteins (see below), but without IPTG induction. The minimal medium used for complementation tests was described previously (Hardy & Nalivaika, 1992).

Complementation tests of mutant alleles of the T4 gene for CH (gene *42*) utilized a T4 phage strain from which gene *42* is deleted in its entirety and a pBR322-derived plasmid, pKG27, which contains a copy of *42* without flanking T4 DNA sequences, under transcriptional control of P_{lac} (Graves, 1994). T4Δ42 and pKG27 allowed straightforward complementation tests of CH variants, without the complications which arose in earlier work (Graves et al., 1992) due to phage–plasmid recombinants.

The alleles encoding the CH(E60Q) and CH(E60D) enzyme variants were constructed by cassette mutagenesis using synthetic DNA duplexes. Each duplex contained the desired sequence changes and ended with *Xmn*I and *Hin*dIII endonuclease recognition sequences to allow reconstruction of gene *42* by ligation with the appropriate plasmid fragments (Graves, 1994). The alleles encoding the TS variants were generated with the Amersham Sculptor kit, by mismatched oligonucleotide primer-directed DNA synthesis, using as a template the single-stranded form of pLHTS1 (Hardy & Nalivaika, 1992). All mutated alleles of genes *42* and *thyA* were sequenced in their entirety to ensure that only the desired mutations were present.

Enzyme Preparation. CH(wt), CH(E60D), and CH(E60Q) were overproduced in *E. coli* strain BL21 from Bluescript plasmids (Stratagene) carrying the appropriate *42* alleles under P_{T7} transcriptional control. Overexpression of CH was induced by infection with λCE6 (Studier et al., 1990); wild type CH and variants of it were purified to homogeneity as previously described (Graves et al., 1992; Graves, 1994). TS was produced in *E. coli strain* XAC25, to prevent contamination from endogenous TS(wt), from Bluescript plasmids (Stratagene) carrying the appropriate *thyA* alleles under P_{lac} transcriptional control. Overexpression of TS was induced with IPTG. This approach gives better overproduction of TS than the T7 system does (E. Nalivaika, unpublished; F. Maley, personal communication). Protein concentrations were quantitated by Coomassie blue dye staining, standardized with bovine serum albumin. All enzyme concentrations are reported as the dimer, calculated from protein concentrations with monomer molecular weights of 30 441 for TS and 28 487 for CH, except as noted otherwise.

Isotope-Exchange Reactions. The details of the conditions and procedures used for these assays were described previously (Hardy & Nalivaika, 1992; Butler et al., 1994). All kinetic measurements were made in duplicate or triplicate at 30 °C. Control experiments indicated that none of the enzymes studied lost significant activity under the assay conditions and times. Initial velocities of the transfer of tritium from [5-^{3}H]nucleotides to solvent water were calculated using data measured at less than 20% transfer. The values of kinetic parameters were calculated from the variation of the initial velocities with substrate concentration at a fixed concentration (usually saturating) of the second substrate. In most cases, the data were fit directly to the Michaelis–Menten equation, using the program Kaleidagraph. The dependence upon dUMP concentration of initial velocities for the THF-dependent exchange were not simple hyperbolics, for TS(wt) or either of the Glu58 variants of TS. At higher dUMP concentrations, this substrate is inhibitory (Figure 2). These data were fit to eq 1:

$$v_i = \frac{E_T k_{lim}[\text{dUMP}]}{K_M + [\text{dUMP}] + ([\text{dUMP}]^2/K_I)} \quad (1)$$

where E_T is the total enzyme concentration, k_{lim} is the limiting rate constant, K_M is the Michaelis constant, and K_I is the substrate inhibition constant.

The data for the K_M of dCMP for CH_2THF-dependent tritium release catalyzed by CH were obtained using 1.8 mM CH_2THF and dCMP concentrations from 1 μM to 4 mM. The data for the K_M of CH_2THF for CH-catalyzed tritium release were obtained using 2 mM dCMP and CH_2THF concentrations from 3 to 300 μM. Enzyme concentrations were 0.23–0.9 μM for CH(E60D) and 0.32 μM for CH(E60Q). Values for CH(wt) were reported previously (Graves et al., 1992).

The values of K_M of dCMP for THF-dependent tritium release catalyzed by CH(wt) and variants were determined to be ≪1 mM, from the constancy of initial velocities at dCMP concentrations from 1 to 4 mM (with 2 mM THF). The values of K_M of THF for CH-catalyzed tritium release were measured at 2 mM dCMP, using data obtained from 1 μM to 6 mM THF. Enzyme concentrations were 0.01 μM for CH(wt), 0.84 μM for CH(E60D), and 0.21 μM for CH(E60Q).

The data for the K_M of dUMP for CH_2THF-dependent tritium release catalyzed by TS were obtained using dUMP concentrations from 2 μM to 1 mM and CH_2THF concentrations of 0.1 mM for TS(wt), 6.8 mM for TS(E58D), and 4.5 mM for TS(E58Q). The values of K_M of CH_2THF for TS-

catalyzed tritium release were measured at 1 mM dUMP, using CH_2THF concentrations from 0.023 to 9 mM. Enzyme concentrations were 0.13−3.2 μM for TS(E58D) and 0.33−11.5 μM for TS(E58Q).

The data for the K_M of dUMP for THF-dependent tritium release catalyzed by TS were measured at 6 mM THF, using dUMP concentrations from 2 μM to 1 mM. The values of K_M of THF were measured at 1 mM dUMP, using THF concentration ranges of 0.1 to 20 mM with TS(wt) and 0.05 to 15 mM with TS(E58D) and TS(E58Q). Enzyme concentrations were 6.6 μM for TS(wt), 3.3−13 μM for TS(E58D), and 5.3−6.6 μM for TS(E58Q).

The ability of 5-deaza-THF (1 mM) or 10-deaza-THF (1 mM) to promote the tritium-exchange reactions catalyzed by CH and TS was measured with 1 mM dCMP and 1 mM dUMP, respectively, using the same assay conditions and procedures as employed with THF and CH_2THF. Enzyme concentrations used with the deaza-THF analogues were 6.6 μM TS(wt) and TS(E58Q), 26 μM CH(wt) with 5-deaza-THF, 35 nM CH(wt) with 10-deaza-THF, 0.7 μM CH(E60D), and 0.2 μM CH(E60Q). (The high K_M values for reduced folates exhibited by TS(E58D) prevented meaningful measurements with that TS variant and the deaza THF analogues.) Control reaction mixtures contained either *bona fide* THF (7 mM in TS reactions, 2 mM in CH reactions) or CH_2THF [1 mM in TS(wt) reactions, 10 mM in TS(E58Q) reactions, and 1 mM in CH reactions]. Appropriate concentrations of enzymes were used in the control reactions, to allow measurement of initial velocities. The inhibition of the CH_2THF-dependent tritium release activity of CH(wt) and TS(wt) was measured using 1 mM [5-^{3}H]nucleotide, CH_2THF (0.09 mM for TS and 1 mM for CH), and 1 mM 5-deaza-THF.

The oxygen isotope exchange between solvent water and the hydroxymethyl group of HMdCMP was monitored using the ^{13}C NMR signal arising from isotopically enriched HMdCMP, using the reaction conditions and procedures previously described (Butler et al., 1994). Reactant concentrations are given in the caption of Figure 4.

Product Formation and Kinetic Isotope Effects. The rates of dTMP formation by TS and of HMdCMP formation by CH were determined by quantitating the nucleotides in timed samples of reaction mixtures using reversed-phase HPLC. The application of this method for assays of CH has been described in detail elsewhere (Graves & Hardy, 1994; Graves, 1994). Similar methods were used for TS, except that 10% methanol was present in the HPLC elution buffer to decrease the retention volumes of dUMP and dTMP. Quantitation of dTMP production by scintillation counting using [2-^{14}C]dUMP and by quantitative UV detection gave identical results. Spectrophotometric assays of DHF production (Hardy & Nalivaika, 1992) gave the same rates as HPLC assays of dTMP production for TS(wt) and TS(E58D).

Reaction mixtures used for the measurement of kinetic isotope effects contained 1 mM dUMP, 1 mM (6*R*)-[6-^{1}H]CH_2THF or (6*R*)-[6-^{2}H]CH_2THF, and 0.14−1.2 μM TS(wt), or 1 mM dUMP, 5 mM (6*R*)-[6-^{1}H]CH_2THF or (6*R*)-[6-^{2}H]CH_2THF, and 28−55 μM TS(E58Q). Quadruplicate reactions were allowed to proceed to between 10% and 20% conversion and were quenched and analyzed as described above.

Formation of Complexes with FdUMP, dUMP, and dCMP. Formation of complexes between TS or CH, [6-^{3}H]FdUMP, and CH_2THF and analysis by SDS−PAGE and autoradiography were done as previously described for CH (Graves & Hardy, 1994). The reactant concentrations and reaction times are described in the caption to Figure 3.

Table 1: Kinetic Parameters for CH_2THF-Dependent Alkylation (k_{cat}) and Tritium Release (k_{lim}) Catalyzed by TS and CH, with dUMP and dCMP, Respectively[a]

enzyme	k_{cat} (s^{-1})	k_{lim} (s^{-1})	k_{lim}/K_M for dUMP or dCMP (s^{-1} mM^{-1})	k_{lim}/K_M for CH_2THF (s^{-1} mM^{-1})
TS				
wt[b]	3.6	3.6	800	380
	[1][c]	[1]	[1]	[1]
E58D	0.07	0.43 ± 0.03	23 ± 5	0.31 ± 0.09
	[0.02]	[0.12]	[0.03]	[0.0008]
E58Q	0.0018	0.010 ± 0.002	0.26 ± 0.06	0.013 ± 0.005
	[0.0005]	[0.003]	[0.0003]	[0.00003]
CH				
wt[b]	12.7	15 ± 1.5	107 ± 52	1360 ± 270
	[1]	[1]	[1]	[1]
E60D	0.007	0.86 ± 0.3	1.4 ± 0.6	96 ± 57
	[0.0006]	[0.06]	[0.01]	[0.07]
E60Q	0.0008	0.38 ± 0.3	1.5 ± 0.4	25 ± 9
	[0.00006]	[0.03]	[0.01]	[0.02]

[a] Determined using the substrate and enzyme concentrations described in Materials and Methods. [b] The parameters for the wild-type enzymes are for (full) turnover reactions, not (partial) isotope-exchange reactions. See the text for details. [c] The numbers in brackets are the indicated parameters divided by the values for the wild-type enzymes.

To measure the absorbance changes which accompany the formation of ternary complexes between TS(E58Q) or CH(E60Q), nucleotides, and THF or CH_2THF, UV spectra were measured on a Hewlett-Packard 8254A spectrophotometer in 1-cm path length quartz cuvettes and processed using an online Hewlett-Packard computer. In a typical experiment, enzyme (final concentration, 0.1 - 0.2 mM monomer) was mixed with 0.3 mM CH_2THF in the standard assay buffer for that enzyme, in a final volume of 1.0 mL, and a reference spectrum was taken. Nucleotide was then added (2−5 μL of a concentrated stock solution) to give a final concentration of 0.3 mM, and the solution was mixed. A second spectrum was taken, from which the reference spectrum was subtracted. For titrations, increasing volumes of dCMP or dUMP (up to 2% of the total volume in the cuvette) were added, and absorbance measurements (versus an air blank) were taken of the reference solution and after each addition of nucleotide. The absorbance values were then subtracted manually, corrected for the slight dilutions caused by the volume changes, and plotted as a function of the nucleotide concentration or of the molar ratio between nucleotide and enzyme.

RESULTS

Replacement of Glu58 in TS and Glu60 in CH Decreases Function in Vivo and in Vitro. The replacement of Glu58 in TS and Glu60 in CH by either Asp or Gln impaired the function of these enzymes *in vitro* (Table 1) and *in vivo*. Of the CH and TS variants, only TS(E58D) was capable of producing enough 5-alkylated nucleotide (dTMP) *in vivo* to allow the growth of organisms deleted for the wild-type gene. *E. coli* ΔthyA containing the plasmid-borne gene for TS(E58D) had a doubling time (in the absence of added thymidine) which was three times longer than ΔthyA bacteria containing the same plasmid with the wild-type TS gene.

Table 2: Kinetic Parameters for THF-Dependent Tritium-Exchange Reactions Catalyzed by TS and CH with dUMP and dCMP, Respectively[a]

enzyme	k_{lim} (s^{-1})	k_{lim}/K_M for dUMP[b] (s^{-1} mM^{-1})	k_{lim}/K_M for THF (s^{-1} mM^{-1})
TS			
wt	0.80 ± 0.03	22 ± 10	1.1 ± 0.5
	[1][c]	[1]	[1]
E58D	0.078 ± 0.003	3.3 ± 0.8	0.016 ± 0.002
	[0.1]	[0.1]	[0.01]
E58Q	0.04 ± 0.01	3 ± 1	0.07 ± 0.03
	[0.05]	[0.1]	[0.06]
CH			
wt	36 ± 5	ND[d]	171 ± 52
	[1]		[1]
E60D	10.8 ± 0.7	ND	33 ± 6
	[0.3]		[0.2]
E60Q	3.6 ± 0.2	ND	21 ± 5
	[0.1]		[0.1]

[a] Determined using the substrate and enzyme concentrations described in Materials and Methods. [b] Calculated with k_{lim} and K_M values determined by regression of the data on eq 1. The data for TS(wt) and TS(E58D) are shown in Figure 2. [c] The numbers in brackets are the indicated parameters divided by the values for the wild-type enzymes. [d] Not determined.

The rate of dTMP formation catalyzed by purified TS(E58D) with saturating substrates was 50-fold lower than the rate of dTMP formation by TS(wt). The rates of nucleotide alkylation by the other three enzyme variants were decreased by 1000-fold or more.

Uncoupling of Proton Removal from Alkylation. The limiting rate constants for the transfer of tritium from [5-^{3}H]-nucleotides into solvent water were also lowered by the Glu58/60 mutations (Tables 1 and 2). The magnitude of these decreases were less than those observed for product formation. For the Glu58/60 variant enzymes, the CH_2THF-dependent formation of alkylated nucleotides is significantly slower than the CH_2THF-dependent transfer of tritium from [5-^{3}H]nucleotides into solvent water (Table 1). Hence the latter process, like the THF-dependent[3] transfer of tritium from [5-^{3}H]nucleotides into solvent (Table 2), is an isotope-exchange (partial) reaction. This contrasts with either wild-type enzyme, for which the CH_2THF-dependent formation of alkylated nucleotides occurred at the same rate as the CH_2-THF-dependent transfer of tritium from [5-^{3}H]nucleotides into solvent.

The values of k_{lim}, $k_{lim}/K_{M,folate}$, and $k_{lim}/K_{M,nucleotide}$ for the CH_2THF-dependent tritium-exchange reactions (Table 1) were more affected by the Glu58/60 mutations than were the values for THF-dependent tritium exchange (Table 2), in most cases. A notable exception was that identical k_{lim} values were observed for the two exchange reactions catalyzed by TS(E58D). For CH_2THF-dependent tritium exchange catalyzed by the TS variants, the values of $K_{M,folate}$ were increased 80−140-fold, a greater effect than the 5−10-fold increases seen in $K_{M,dUMP}$. In contrast, for CH_2THF-dependent tritium exchange catalyzed by the CH variants, the values of $K_{M,folate}$ were unaffected and the values of $K_{M,dCMP}$ increased 2−4 fold.

[3] HPLC assays of reaction mixtures were used to demonstrate that THF preparations were not contaminated with any CH_2THF. In the presence of THF, no alkylated nucleotides were detectable by HPLC, under conditions where >90% of the tritium from [5-^{3}H]nucleotide was transferred to solvent by the action of CH or TS.

Table 3: Effects of THF and Analogues on Rates of Exchange Reactions between Solvent and [5-^{3}H]Nucleotides Catalyzed by TS and CH[a]

	initial velocity (μmol min^{-1} mg^{-1}) with:				
enzyme	CH_2THF	THF	10-deaza-THF	5-deaza-THF	CH_2THF + 5-deaza-THF
TS(wt)	3.6	0.073	0.073	<0.001	0.5
TS(E58Q)	0.01	0.07	0.088	ND[b]	ND
CH(wt)	11	36	4.7	<0.001	5
CH(E60D)	0.64	3.4	0.68	ND	ND
CH(E60Q)	0.47	1.2	0.44	ND	ND

[a] Duplicate or triplicate reactions were performed and analyzed as described in Materials and Methods. [b] Not determined.

The ability of THF analogues which lack N5 and N10 to promote the tritium-exchange reactions catalyzed by TS and CH was measured (Table 3), in order to further explore the possible interactions of Glu58 in TS and Glu60 in CH with these nitrogens of THF or CH_2THF. At a concentration of 1 mM, 10-deazatetrahydrofolate was nearly as effective as similar or higher concentrations of THF in promoting the tritium-exchange reactions catalyzed by TS(wt) and TS-(E58Q). The 10-deaza analogue of THF also promoted the tritium-exchange reactions catalyzed by CH(wt), CH(E58D), and CH(E58Q), although at 1 mM the analogue was slightly less effective than THF. (HPLC assays revealed that 10-deaza-THF was not a viable substrate for dTMP formation from dUMP and formaldehyde.) The data in Table 3 eliminate the candidacy of N10 of THF for any significant role in proton removal. The Glu58/60 mutations had no significant effect on the ability of 10-deaza-THF to accelerate the tritium-exchange reactions, which indicates that neither Glu58/60 nor N10 of THF is essential for binding of THF to CH and TS.

The 5-deaza analogue of THF was totally incapable of accelerating the tritium-exchange reactions catalyzed by either TS(wt) or CH(wt). Binding of 5-deaza-THF to TS-(wt) and CH(wt) was demonstrated by the fact that 1 mM of the 5-deaza analogue inhibited these enzymes by 86% and 54%, respectively. The relevance of the results obtained with 5-deaza-THF is elaborated in the Discussion.

Substrate Inhibition of TS-Catalyzed Exchange Reaction. The initial velocities for the THF-dependent tritium-exchange reaction catalyzed by TS(wt) and both Glu58 variants were not simple hyperbolic functions of the dUMP concentration. The decreased initial velocities at higher dUMP concentrations (Figure 2) were not due to lower specific radioactivity of the nucleotide, nor was inhibition by high dUMP concentrations observed for the CH_2THF-dependent tritium-exchange reaction. Values of k_{lim} and $k_{lim}/K_{M,dUMP}$ for the THF-dependent tritium exchange catalyzed by TS (Table 2) were calculated using an equation which included a K_I term for substrate inhibition (eq 1). The values of K_I are 53 μM, 0.6 mM, and 3 mM for TS(wt), TS(E58D), and TS(E58Q), respectively. The values of K_I and K_M from the data obtained with TS(wt) are identical, within experimental error. For either TS(E58Q) or TS(E58D), K_I differs significantly from K_M.

Effect of Glu58/60 Mutations on Formation of Ternary Complexes. The replacement of the active site glutamic acid in either TS or CH reduced the ability of the enzymes to form covalent complexes with CH_2THF and [6-^{3}H]FdUMP. Such complexes form readily with either wild-type enzyme.

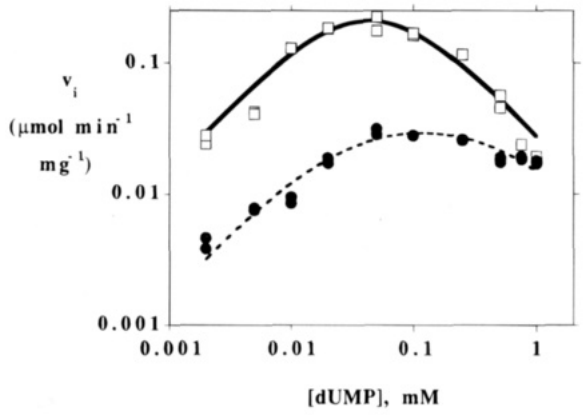


FIGURE 2: Dependence upon total dUMP concentration of the initial velocities of THF-dependent tritium exchange between [5-^{3}H]dUMP and solvent water, catalyzed by TS(wt) (□) and TS(E58D) (●). The lines are the theoretical fits of the data to eq 1.

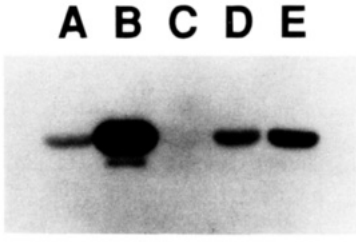


FIGURE 3: Autoradiograms of the complexes formed by TS(wt) and TS(E58Q) with [6-^{3}H]FdUMP after SDS−PAGE. (A) TS(wt) + FdUMP; (B) TS(wt) + FdUMP + CH_2THF; (C) TS(E58Q) + FdUMP; (D and E) TS(E58Q) + FdUMP + CH_2THF. Complexes were formed and analyzed as previously described (Hardy & Nalivaika, 1992; Graves & Hardy, 1994). The samples, containing 14.8 mM enzyme, 11 mM [6-^{3}H]FdUMP, and 0.5 mM CH_2THF, if present, were reacted at 30 °C for 10 min before SDS−PAGE, except the sample in lane E, which was reacted for 20 min. The major and minor bands in lane B migrate with apparent molecular masses of 35 and 30 kDa, respectively.

With TS(wt), a small amount of binary FdUMP−enzyme complex can be detected by SDS−PAGE (Figure 3, lane A). No interaction of FdUMP with CH(E60D) or with CH(E60Q), in the presence or absence of CH_2THF, could be detected by nitrocellulose filter binding or by SDS−PAGE/autoradiographic assay. The covalent binding of FdUMP to TS(E58Q) (Figure 3) or TS(E58D) could still be observed, but the binding occurred much more slowly than it did to TS(wt). The amount of FdUMP covalently bound to TS(E58Q) increased with time (compare lanes D and E, Figure 3). Although the amount of FdUMP bound to TS(E58Q) continued to increase for up to 2 h, this amount was still far less than that bound to TS(wt) after 10 min (at which time binding to TS(wt) was complete). At 11 μM FdUMP, the covalent inactivations of TS(E58Q) (Figure 3) and TS(E58D) (data not shown) were dependent upon the presence of CH_2THF.

Binding interactions between TS(E58Q), dUMP, and CH_2THF and between CH(E58Q), dCMP, and CH_2THF could be detected by UV difference spectroscopy (*e.g.*, Figure 4A). Titration of 0.1−0.2 mM solutions of either enzyme with its cognate nucleotide, in the presence of excess CH_2THF, gave an increasing difference signal which plateaued at a nucleotide concentration approximately equal to the concentration of enzyme monomer (*e.g.*, Figure 4B). The maximum difference absorbance was observed in both cases at wavelength 334−336 nm, with extinction coefficients of

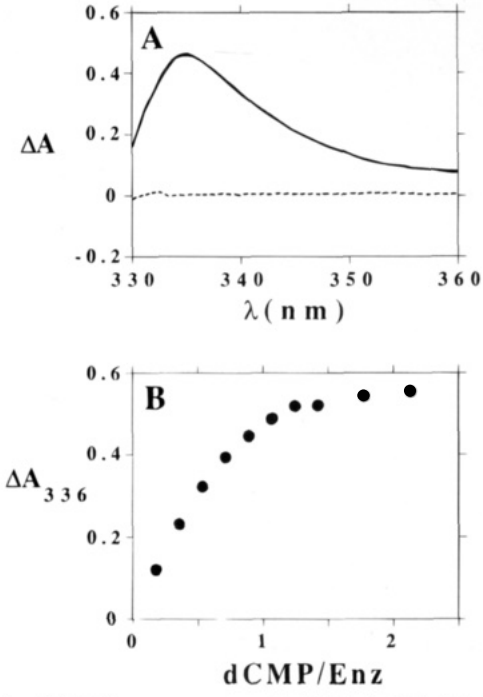


FIGURE 4: (A) Difference spectrum (solid line) elicited by interaction between CH(E60Q) (180 μM monomer), dCMP (0.2 mM), and CH_2THF (0.27 mM), determined as described in Materials and Methods. The dashed line is the "difference spectrum" obtained before addition of dCMP. (B) Spectrophotometric titration of CH(E60Q) (180 μM monomer) with dCMP in the presence of 0.3 mM CH_2THF. The abscissa indicates the concentration ratio of dCMP to CH dimer.

2400 and 3300 AU cm^{-1} M^{-1}, respectively, for the TS(E58Q) and CH(E60Q) monomers. The wavelengths of the difference absorbance maxima were too long to emanate from Trp or Tyr residues, and they probably arise from a change in the environment of the folate chromophore upon binding to the enzymes. The absorbance changes occurred very rapidly and were essentially complete within the manual mixing time (*ca.* 10 s). With TS(E58Q), significant absorbance changes were observed only with dUMP and CH_2THF. With CH(E60Q), similar difference spectra were obtained using dUMP instead of dCMP (with CH_2THF), or using THF instead of CH_2THF (with dUMP). No absorbance changes were observed with either TS(E58Q) or CH(E60Q) in response to addition of nucleotide alone or reduced folate alone, or with deoxycytidine or deoxyuridine instead of dCMP or dUMP.

Effect of Glu58/60 Mutations on Processing of 5-Methylene Nucleotide Intermediates. The final steps in catalysis by TS and CH are postulated to be reduction and hydration, respectively, of 5-methylene pyrimidine nucleotide intermediates covalently linked to the enzymes. The effect of altering Glu58 in TS on this final catalytic step was assessed by measuring the deuterium kinetic isotope effect (^DV) on hydride transfer, arising from the substitution by deuterium of the transferred C6 hydrogen of CH_2THF. The value of ^DV measured by an HPLC assay with TS(wt) was 4.4 (SE 0.2), which is identical to the value obtained using a spectrophotometric assay (Appleman et al., 1990; L. W. Hardy, unpublished). A significantly lower value, 1.47 (SE 0.14), was obtained for ^DV measured with TS(E58Q). The effect of altering Glu60 in CH upon the hydration step was assessed from changes in the rate of $^{16}O/^{18}O$ exchange between [^{18}O-hydroxymethyl]HMdCMP and solvent [^{16}O]-

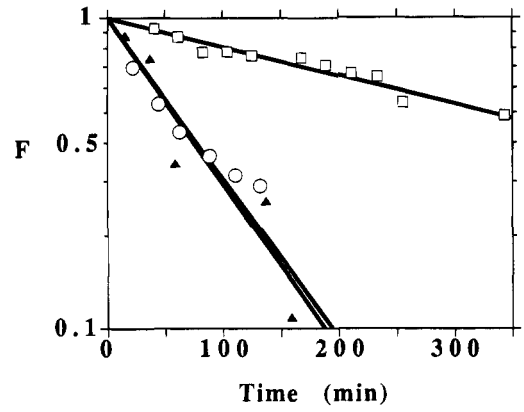


FIGURE 5: Effect of Glu60 mutations upon the rate of CH-catalyzed exchange of ^{18}O from solvent water into [hydroxymethyl-^{13}C,^{16}O]-hydroxymethyl-dCMP, analyzed by ^{13}C NMR as previously described (Butler & Hardy, 1994). F is the fraction of [^{13}C,^{16}O]-HmdCMP remaining (not exchanged). The reactions were performed and monitored using ^{13}C NMR. Reactions were performed at 0.5 mM THF, and reaction mixtures contained (□) 0.26 μM CH(E60Q) and 6.8 mM HMdCMP; (○) 1.76 μM CH(E60D) and 5.75 mM HMdCMP; and (▲) 0.26 μM CH(wt) and 3.8 mM HMdCMP. The lines describe the fits of the data to first-order equations, with the following pseudo-first-order rate constants: E60Q, 0.0021 min^{-1} (SE 0.001); E58D, 0.012 min^{-1} (SE 0.001); wild type, 0.012 min^{-1} (SE 0.002). Each value was multiplied by the HMdCMP concentration and divided by enzyme concentration to calculate the initial velocity of the exchange reaction.

H_2O. The $^{16}O/^{18}O$-exchange reactions catalyzed by CH(wt) and the Glu60 variants were monitored (Figure 5) using the ^{13}C NMR signal of [^{13}C-hydroxymethyl]HMdCMP (Butler et al., 1994). The rate of this exchange reaction was decreased less than 10-fold by the Glu60 mutations. The initial velocities of $^{16}O/^{18}O$ exchange for CH(wt), CH(E58D), and CH(E58Q), respectively, were 3.0, 0.7, and 0.5 μmol min^{-1} mg^{-1}.

DISCUSSION

Uncoupling of Nucleotide Deprotonation from Alkylated Product Formation. The replacement, by Gln or Asp, of Glu58 in TS and Glu60 in CH alters the kinetic characteristics of these enzymes. The severity of the effect varies, depending upon the assay. The most pronounced result of the Glu58/60 mutations is a severe uncoupling of the process of deprotonation of C5 of the nucleotide from the process of alkylated nucleotide formation. This uncoupling is more severe for the CH variants than it is for the TS variants (Table 1). For either TS(wt) or CH(wt), the rate of tritium release is very close to the rate of alkylation. The exchange of tritium from solvent [^3H]H_2O into substrate dUMP, catalyzed by *E. coli* TS(wt) under turnover conditions (*i.e.*, in the presence of CH_2THF), occurs at no more than 10% of the rate of dTMP formation (Lomax & Greenberg, 1967). For the Glu58/60 variant enzymes, the ratio of the CH_2THF-dependent tritium release rate to the alkylation rate (R) ranges from 5.6 to 475. An analysis based on Schemes 1 and 2 suggests that some step after intermediate III has become so slow that the reverse reaction effectively competes. The binding of substrates to TS and CH is ordered bi-bi (Scheme 4), with nucleotide substrate binding first and nucleotide product released last (Danenberg & Danenberg, 1978; Lee et al., 1988; Ghose et al., 1990). With saturating nucleotide, the expression for R (based on Scheme 4) is

$$R = \frac{k_{exchange} + k_{cat}}{k_{cat}} = 1 + \frac{1 + k_8/k_9'}{1 + k_9'/k_8'} \quad (2)$$

where k_8' and k_9' are the net rate constants for the back and forward reactions of intermediate III, derived by Cleland's method (Cleland, 1975):

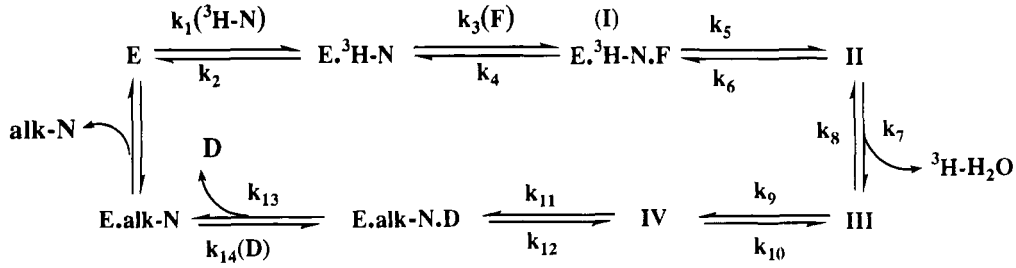
$$k_8' = \frac{k_2k_4k_6k_8}{k_2k_4(k_6 + k_7) + k_5k_7(k_2 + k_3F)} \quad (3)$$

$$k_9' = \frac{k_9k_{11}}{k_{10} + k_{11}} \quad (4)$$

Note that CH_2THF-dependent tritium release (k_{lim}) includes both exchange ($k_{exchange}$) and turnover (k_{cat}). The $k_3k_5k_7F$ term in the denominator of eq 3 must increase rather slowly; otherwise, CH_2THF would effectively suppress tritium exchange, which did not occur at the concentrations used in our studies. In catalysis by the wild-type enzymes, III must primarily partition forward, and k_9' must be much greater than k_8', since $R = 1$. In contrast, k_9' must be much less than k_8', in catalysis by the Glu58/60 variant enzymes. An alternate explanation is that, in the wild-type enzymes, C5 deprotonation largely occurs concurrently with the completion of the one-carbon transfer (*i.e.*, cleavage of the methylene-folyl N5 bond), and the Glu58/60 mutations allow deprotonation to occur without simultaneous cleavage of the methylene-N5 linkage. This alternative seems less likely, since the exchange of tritium from solvent into dUMP implies that at least a fraction of turnover occurs with C5 deprotonation preceding cleavage of the methylene-folyl N5 bond.

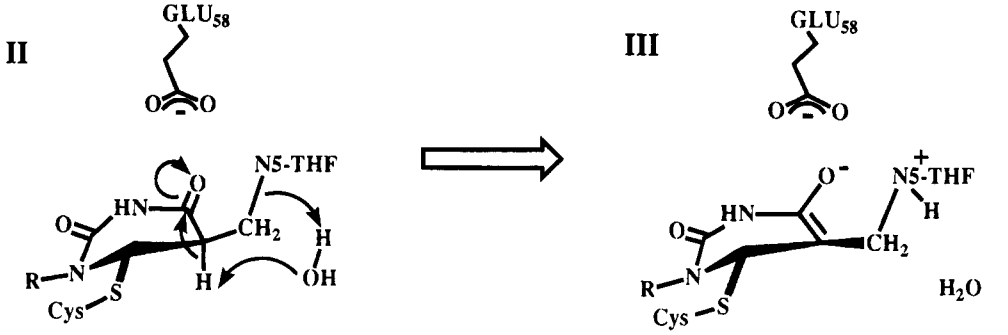
The expression for $k_{lim}/K_{M,nuc}$ for the tritium-exchange reactions, based upon Scheme 4, is the same as that for the

Scheme 4: Kinetic Mechanism for the Reaction Catalyzed by TS, Based upon the Catalytic Pathway Shown in Scheme 2[a]



[a] This mechanism also applies to CH (based upon Scheme 1), except that THF is produced instead of dihydrofolate (D). alk-N = HMdCMP or dTMP; E = enzyme (TS or CH); F = CH_2THF; and ^3H-N = [5-^3H]dCMP or [5-^3H]dUMP. The tritium-exchange reactions constitute the portion of the scheme up to the step governed by rate constant k_7. Species I, II, III, and IV are shown in Schemes 1 and 2; substrate binding and product dissociation steps are not indicated in Schemes 1 and 2. Forward and reverse rate constants are even and odd numbered, respectively.

Scheme 5: Concerted Abstraction of the C5 Proton of dUMP and Protonation of N5 of THF, Covalently Tethered to C5 via the Methylene Being Transferred[a]



[a] At least one intermediary water molecule must be involved in the proton transfer, since N5 must be on the opposite face of the pyrimidine from the abstracted proton. R = 1′-[2′-deoxy-5′-phosphoribofuranosyl].

entire reaction (Santi et al., 1990):

$$\frac{k_{lim}}{K_{M,nuc}} = k_1 \quad (5)$$

This expression differs from one proposed previously (Zapf et al., 1993) for TS(E58Q), which would predict that the rate of catalysis by TS(E58Q) is not saturable with CH_2-THF; our data show that this clearly is not the case.

Substrate Inhibition in THF-Dependent Partial Reaction. The inhibition by dUMP of the TS-catalyzed THF-dependent tritium-exchange reaction may indicate that only one active site of the TS dimer is active in the exchange reaction, and that binding of dUMP at the second active site actually inhibits the catalysis occurring at the first site. This is consistent with earlier reports of negative cooperativity in the interactions of the two TS active sites [reviewed in Hardy and Nalivaika (1994)]. The apparent binding constants for exchange and inhibition are identical with TS(wt), but the Glu58 mutations decrease the negative cooperativity of the enzyme by lowering the apparent affinity of the second site for dUMP.

Role of Glu58/60 in 5-Iminium Ion Formation. To test whether Glu58/60 promotes the generation of a 5-iminium ion form of CH_2THF by protonating N10 of the N5,N10-bridged form, we compared reactions using CH_2THF with ones using THF. THF has no N5,N10-bridge and therefore should not require protonation of N10. The kinetic parameters for CH_2THF-dependent tritium exchange (Table 1) were decreased more by the Glu58/60 mutations than were the parameters for THF-dependent tritium exchange (Table 2). An exception to this trend was seen for TS(E58D), perhaps due to the fact that TS(E58D) is quite active (2% of wild-type activity). The trend is consistent with some role for Glu58/60 in the activation of CH_2THF, although the magnitude of the effects on k_{lim} for the CH_2THF-dependent tritium-exchange reactions indicates an auxiliary rather than an essential role. The decreased rates of covalent inactivation of TS and CH by FdUMP and CH_2THF (Figure 3) also indicate a role for Glu58/60 in an early step of catalysis, since the FdUMP complex mimics intermediate II (Schemes 1 and 2). However, the 30−3000-fold lower rates of nucleotide binding (k_1; *cf.* eq 5) may be partially responsible for the loss of FdUMP sensitivity. The effect on k_1 indicates weakening or loss of the water-mediated hydrogen bond donated by the side-chain carboxylic acid of Glu58 to O4 of dUMP or FdUMP (Matthews et al., 1990; Montfort et al., 1990).

Deprotonation of C5 of Nucleotide: Identity of the Base. Like other residues in the TS active site which have been tested (Matthews et al., 1992; Stroud & Finer-Moore, 1993), Glu58 in TS (or Glu60 in CH) is not essential for the C5 deprotonation step. Interestingly, 5-deaza-THF fails to support tritium exchange by either TS(wt) or CH(wt), although this analogue clearly binds to both enzymes (Table 3). The K_I for 5-deaza-THF with CH is 0.11 mM (Butler et al., 1994), similar to the K_M for THF. The failure of 5-deaza-THF to accelerate tritium exchange contrasts sharply with this analogue's ability to strongly enhance the CH-catalyzed $^{18}O/^{16}O$ exchange between water and HMdCMP (Butler et al., 1994). This failure suggests that N5 of THF is the elusive base which deprotonates C5 of the nucleotide (Scheme 5). This idea is attractive for two reasons: (i) no amino acid candidate for this catalytic role has survived the scrutiny of site-directed mutagenesis, and (ii) it suggests an explanation for the tight coupling of proton abstraction and alkylation seen for the wild-type enzymes. Protonation of N5 concomitant with C5 deprotonation would increase the catalytic commitment to the next step, *i.e.*, cleavage of the N5−methylene bond. At least one water would be required to mediate the proton transfer during turnover with CH_2THF, since N5 would be on the opposite face of the pyrimidine from the abstracted proton. Several bound waters in the TS active site (Matthews et al., 1990; Montfort et al., 1990) are candidates for the proton shuttle. An apparent problem with the proposal is that the pK_a of 4.8 for N5 of THF (Kallen & Jencks, 1967a) is too low to abstract a proton from a carbon acid. However, pK_a values can be quite perturbed in the active site of an enzyme, as has been recently reported for N5 of DHF in the active site of dihydrofolate reductase (Chen et al., 1994). Although further studies are required to test this proposed role for N5 of THF, the notion that intermediate III is highly committed to forward catalysis in the reactions by CH(wt) and TS(wt) suggests a structural mechanism by which Glu58/60 could enforce the coupling of 5-proton removal and alkyl transfer (*vide infra*).

Glu58/60 Enforces Coupling via Electrostatic Destabilization. The expression for k_{cat}, based upon Scheme 4, is

$$k_{cat} = k_5k_7k_9k_{11}k_{13}/ [(k_6k_8 + k_5k_8 + k_5k_7)(k_{10}k_{12} + k_{10}k_{13} + k_{11}k_{13}) + (k_5 + k_6 + k_7)k_9k_{11}k_{13} + k_5k_7k_9(k_{11} + k_{12} + k_{13})] \quad (6)$$

The reduced value of k_{cat} for product formation could be due

to effects on k_5, k_7, k_9, k_{11}, or k_{13}. However, the effect on CH_2THF activation or on C5 deprotonation, or the product of both effects, are all insufficient to explain the large decrease in k_{cat}. Glu58/60 must play a more significant role in a catalytic step later than that governed by k_7, such as the cleavage of the bond linking N5 of THF to the methylene group being transferred (k_9). This would explain the decrease in k_9'.

Glu60 in CH probably has no significant role in the final (hydration) step of CH catalysis (k_{11}), or in product HMd-CMP or THF dissociation (k_{13}), since the rates of the $^{16}O/^{18}O$-exchange reactions catalyzed by CH, CH(E60D), and CH(E60Q) differ by a factor of 6 at most (Figure 5). Glu58 in TS also has no significant role in TS-catalyzed hydride transfer (k_{11}), since the value of ^{D}V would be expected to increase or remain constant if the hydride-transfer step were slowed by the E58Q mutation. However, the 3-fold *lower* value of ^{D}V for hydride transfer catalyzed by TS(E58Q) is consistent with a significant decrease in k_9. The expression for ^{D}V is

$$^{D}V = \frac{k_{cat,H}}{k_{cat,D}} = \frac{(k_{11H}/k_{11D}) + C_{Vf}}{1 + C_{Vf}} \quad (7)$$

The C_r terms ($= k_{12}/k_{13}$) normally present in ^{D}V (Cook & Cleland, 1981) are negligible since the hydride-transfer step is essentially irreversible. For TS(wt), the magnitude of ^{D}V, 4.4 (Appleman et al., 1990), has led to the supposition that k_{cat} for TS is equal to k_{11}, the rate constant for hydride transfer (Santi et al., 1990). This means that, for the wild-type enzyme, C_{Vf} must be much less than 1. The 3-fold lower value of ^{D}V for TS(E58Q) indicates that C_{Vf} must have increased to 6. Based on eq 6, the C_{Vf} term is

$$C_{Vf} = k_{11} \times \frac{(k_5k_7 + k_5k_8 + k_6k_8)k_{13} + \{(k_5 + k_6 + k_7)k_{13} + k_5k_7\}k_9}{(k_5k_7 + k_5k_8 + k_6k_8)k_{13}k_{10} + k_5k_7k_{13}k_9} \quad (8)$$

If the decrease in k_{cat} arising from the Glu58Gln mutation is mostly due to a much lowered value of k_9, then the expression for C_{Vf} simplifies to k_{11}/k_{10}. Thus, the hydride-transfer step (k_{11}) would have to be 6 times greater than reversal of the β-elimination step (k_{10}), which seems reasonable.

How might the replacement of Glu58/60 slow the step governed by k_9, cleavage of the folyl N5−methylene bond? Consider the two following possibilities. (i) Protonation of N5, which is essential to prevent the expulsion of a highly unfavorable amine anion, is one possible means by which Glu58/60 could accelerate the cleavage of this bond. However, this is improbable for several reasons. After the initial protonation of N10 by the side-chain carboxylic acid of Glu58/60 (*cf.* Scheme 3), this side chain would be in its anionic carboylate form. The return of this carboxylic acid to its acid form by proton transfer from the pyrimidine C5 has already been excluded (*vide supra*). Moreover, this possibility precludes the utilization of the basicity of the folyl N5 to deprotonate the pyrimidine C5. (ii) The second possibility is that the presence of the carboxylate anion of Glu58/60 shortens the lifetime of the anionic intermediate III, by electrostatic repulsion. The Glu58 carboxylate is 5.5 Å from O4 of bound dUMP (Matthews et al., 1990; Montfort et al., 1990). Although the cavity containing these groups also contains several waters, it is isolated from bulk solvent and surrounded by hydrophobic residues. This passive/aggressive role for the carboxylate anion of Glu58/60 is also applicable in the CH mechanism. In that case, the anion destabilized by the Glu60 carboxylate would arise from the side chain of Asp179 (Graves et al., 1992), which is likely to be in close proximity to N3 and the 4-amino group of dCMP. It cannot be excluded that, for CH(wt) and TS(wt), catalysis may proceed *via* concerted deprotonation of the pyrimidine C5 and cleavage of the folyl N5−methylene bond because of this electrostatic repulsion. If this is true, the Glu58/60 mutations must have altered the catalytic mechanism to allow these steps to take place sequentially. (The effective value of k_9 cannot have decreased due to siphoning of enzyme into a nonproductive form (*e.g.*, intermediate III), since this would lower all the steady state rates in parallel, without affecting ^{D}V.)

Comparison with Previous Work. Most current models for the catalytic mechanisms of TS (Stroud & Finer-Moore, 1993; Santi & Ivanetich, 1993) and CH (Graves et al., 1992) propose that the deprotonation of the nucleotide (dUMP or dCMP) precedes the cleavage of the folyl N5−methylene bond, with an intervening oxyanion-containing intermediate III. However, Matthews and colleagues (Matthews et al., 1990) proposed a mechanism which employed β-elimination of the C5 proton of dUMP from intermediate II to directly produce the 5-methylene-dUMP species covalently linked to the enzyme (IV). We suggest that protonation of N5 of THF, in order to promote subsequent cleavage of the bond to the methylene, is concerted with the abstraction of the C5 proton of the nucleotide.

A recent study with the Glu60Ala and Glu60Leu variants of *Lactobacillus casei* TS (Huang & Santi, 1994) led to the conclusion that the primary role of this active site residue is to influence the partitioning of intermediate III. These mutations in *L. casei* TS also led to uncoupling of dUMP deprotonation and dTMP formation (R = 20−40 for the variant enzymes). The transient formation of a complex between the mutated *L. casei* TS, dUMP, and CH_2THF which is stable to denaturation by sodium dodecyl sulfate is consistent with a severe impairment in the conversion of intermediate III to IV. Similar trapping experiments with Glu58 variants of *E. coli* TS have been unsuccessful (L. W. Hardy and E. Nalivaika, unpublished).

Some of the results reported here differ considerably from those reported earlier by another group (Zapf et al., 1993) in a study of TS(E58Q). In the earlier report, the limiting rate constant for CH_2THF-dependent tritium exchange catalyzed by TS(E58Q) was found to be 0.005 s^{-1}, within experimental error of the value found in the present study (Table 1). However, the value of k_{cat} for product formation, measured for TS(E58Q) using a spectrophotometric assay (Zapf et al., 1993), was reported to be *ca.* 2-fold *higher* than k_{lim} for CH_2THF-dependent tritium exchange. This discrepancy between the rate constants measured by the different assays was ascribed to a combination of a possible tritium kinetic isotope effect on C5 deprotonation, insensitivity of the spectrophotometric assay, and experimental error. Our direct measurements of dTMP formation using an HPLC assay indicate a value for k_{cat} for product formation by TS-(E58Q) which is much lower than the value earlier reported (Zapf et al., 1993) and which is 20-fold *lower* than k_{lim} for CH_2THF-dependent tritium exchange. It seems unlikely that

these large differences are due to different assay conditions, which were very similar in the previous and present studies. Our spectroscopic studies (*e.g.*, Figure 4) of the binding of nucleotides and CH_2THF to TS(E58Q) and to CH(E60Q) strongly suggest an alternative explanation. The absorbance changes observed by Villafranca, Dunlap, and colleagues (Zapf et al., 1993) were probably those which accompany formation of a ternary complex[4] between dUMP, CH_2THF, and TS(E58Q), rather than the production of DHF. This ternary complex is likely to be species III.

Summary of Conclusions. Substitution of Glu58 in TS and Glu60 in CH by site-directed mutagenesis uncouples early steps in catalysis by TS and CH from later steps. These residues probably play accessory roles in initial nucleotide binding, and in the activation of CH_2THF, but are likely not involved in the deprotonation of C5 which accompanies alkylation. The ultimate basicity for the deprotonation may be substrate-derived, *i.e.*, N5 of THF. It is also unlikely that Glu58/60 is involved in the final steps of catalysis by CH and TS, hydration and hydride transfer, respectively. The most important role for Glu58/60 is in the cleavage of the bond linking N5 of THF to the transferred methylene moiety. Although we have not been able to directly test whether this step has been affected by the Glu58/60 mutations, the observed effects upon none of the other microscopic steps can explain the pronounced decrease in the value of k_{cat} for alkylated product formation. Further, the lowered ^{D}V arising from a step subsequent to the cleavage of the folyl N5–methylene bond, hydride transfer catalyzed by TS, is consistent with a significant decrease in the rate of that bond cleavage. We propose that the decreased rate of this bond cleavage is due to the loss of electrostatic destabilization, by the side-chain carboxylate of Glu58/60, of the anionic intermediates resulting from deprotonation of the nucleotide. A lower rate of N5–methylene bond cleavage would explain the significant uncoupling of deprotonation and alkylation for all four enzyme variants examined.

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[4] Similar difference UV signals with maxima in the range 320–340 nm have been reported previously for stable ternary complexes between TS(wt), dUMP, or FdUMP and folate derivatives (Lockshin *et al.*, 1984).