Active Site General Catalysts Are Not Necessary for Some Proton Transfer Reactions of Thymidylate Synthase†

Weidong Huang‡ and Daniel V. Santi*

Departments of Pharmaceutical Chemistry and of Biochemistry and Biophysics, University of California, San Francisco, California 94143-0448

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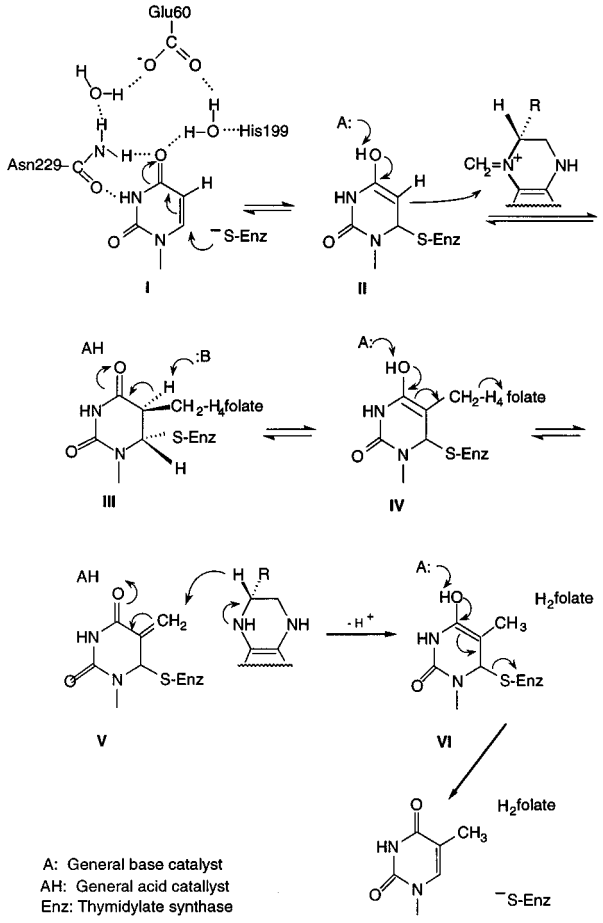
ABSTRACT: Several steps of the reaction catalyzed by thymidylate synthase (TS) require proton transfers to and from O-4 and C-5 of the pyrimidine moiety of substrate dUMP. It has been proposed that one or more of three active site residues—Glu60, His199, and Asn229—together with ordered water molecules serve as general catalysts in facilitating such proton transfers. These three residues, individually and together, were mutated to residues incapable of proton transfer, and the mutant enzymes were purified and tested for activity in the formation of dTMP and the dehalogenation of 5-bromo- and 5-iodo-dUMP. The dehalogenation reaction pathway shares at least two direct chemical counterparts with the TS reaction pathway which are believed to involve general acid/base catalysis—namely, the addition and elimination of the catalytic Cys of TS at C-6 of the pyrimidine substrate. Generally, the mutations had detrimental effects on dTMP synthesis with the triple mutant being completely inactive. In contrast, single mutants TS E60L and H199A and, interestingly, the triple mutant stripped of all three active site catalysts catalyzed the dehalogenation reaction as well as or better than the wild-type enzyme. It was concluded that addition and elimination reactions involving the 5,6-bond of pyrimidine substrates do not require general acid/base catalysis or, alternatively, the water molecules in the TS active site serve this role. The function(s) of the triad of general catalysts resides elsewhere in the reaction pathway leading to dTMP synthesis.

Thymidylate synthase (TS,[1] EC 2.1.1.45) catalyzes the reductive methylation of dUMP by 5,10-CH_2H_4folate to give dTMP and H_2folate. The structure, catalytic mechanism, and mutagenesis of TS have been of continuing interest to this laboratory [for a recent review, see Carreras and Santi (1995)].

An initiating event in the catalytic mechanism of TS involves nucleophilic attack of a conserved Cys residue of the enzyme at C-6 of dUMP to give a 5,6-dihydropyrimidine intermediate (**II** in Scheme 1) (Santi & Danenberg, 1984; Carreras & Santi, 1995). All subsequent covalent bond modifications involved in the reductive methylation at C-5 of dUMP occur on the covalently bound intermediate, and upon completion there is a β-elimination of the catalytic Cys from C-6 of the pyrimidine to give the product dTMP and regenerated enzyme.

The chemistry of the TS reaction requires several proton transfers to and from O-4 and C-5 of the pyrimidine of dUMP (Scheme 1). It has been proposed that three active site residues—Glu60,[2] His199, and Asn229—in a hydrogen bond network together with ordered water molecules serve as general catalysts in facilitating these proton transfers (Liu

Scheme 1



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* Correspondence should be addressed to this author.

‡ Current address: Department of Pathology, University of California, Irvine Medical Center, 101 The City Drive, Orange, CA 92668.

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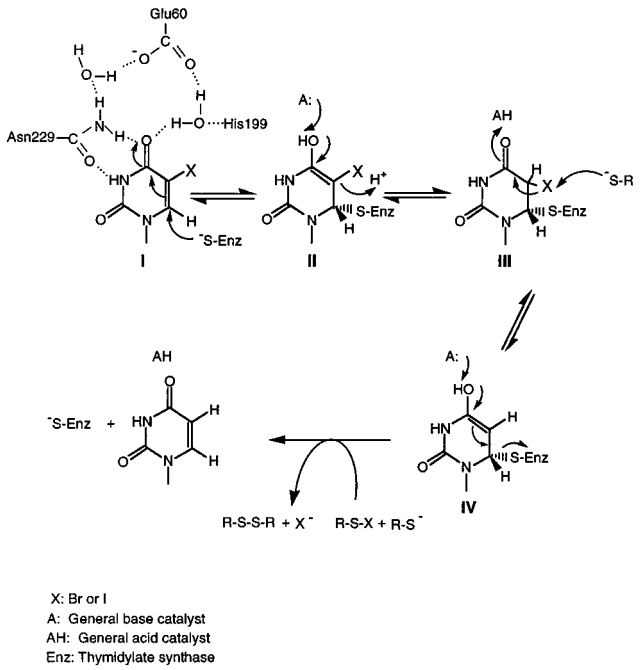
[1] Abbreviations: TS, thymidylate synthase; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; Br-dUMP, 5-bromo-2′-deoxyuridine 5′-monophosphate; IdUMP, 5-iodo-2′-deoxyuridine 5′-monophosphate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography.

[2] The numbering system of *L. casei* TS is used.

Scheme 2



& Santi, 1993; Huang & Santi, 1994; Carreras & Santi, 1995). Because of the multiple steps in the TS pathway, it has been difficult to identify the contributions of given residues to a particular step in the pathway. For this reason, "partial reactions", which undergo one or a few of the steps in the normal catalytic pathway, have been developed and used to study isolated steps in the TS pathway (Garrett *et al.*, 1979; Carreras & Santi, 1995). One such reaction is the CH_2H_4folate-independent dehalogenation of 5-Br- and 5-IdUMP (Scheme 2) (Garrett *et al.*, 1979), which shares at least two direct chemical counterparts with the TS pathway: (i) nucleophilic addition of the catalytic Cys to C-6 of the pyrimidine to form a 5-halo-5,6-dihydropyrimidine intermediate (**III** in Scheme 2), and (ii) elimination of the Cys from C-6 in the final step of the dehalogenation reaction.

The objective of the present work was to assess and compare the importance of residues of the hydrogen bond network in dTMP synthesis and in dehalogenation of 5-Br-(I)dUMP. We first mutated Glu60, His199, and Asn229 individually to residues incapable of proton transfer reactions; then we mutated all the residues together to produce an enzyme stripped of active site general catalysts. Our results indicate that the hydrogen bond network is essential for dTMP formation but not for the catalytic events that occur in the dehalogenation reaction.

MATERIALS AND METHODS

Materials. Escherichia coli χ2913recA (Δ*thy*A572, *rec*A56) (Climie *et al.*, 1992) and plasmid pSCTS13 and pSCTS9 have been described (Climie & Santi, 1990). Oligonucleotide synthesis and DNA sequencing were performed at the UCSF Biomolecular Resource Center. (6*R*)-CH_2H_4folate was a generous gift from SAPEC S.A. (Lugano, Switzerland) and EPROVA AG (Schaffhausen, Switzerland).

Mutagenesis and Protein Purification. The E60L, H199A, and N229V single mutants were constructed by cassette mutagenesis of the *Lactobacillus casei* TS synthetic gene in plasmid pSCTS13 or pSCTS9 as described (Huang & Santi, 1994; Climie *et al.*, 1990; Liu & Santi, 1993). The E60L/H199A/N229V triple mutant was constructed from the E60L single mutant and the H199A/N229V double mutant as follows. First, the H199A/N229V double mutant was constructed as follows: plasmid DNAs of TS H199A and N229V were each digested with *Eco*RI and *Bgl*II; the small DNA fragment containing the H199A mutation and the large DNA fragment containing the N229V mutation were purified by 1% agarose gel electrophoresis and ligated to give the H199A/N229 double mutant. The E60L/H199A/N229V triple mutant was then constructed as follows: plasmid DNAs of TS E60L and TS H199A/N229V were each digested with *Nco*I and *Eco*RI; the small DNA fragment containing the E60L mutation and the large DNA fragment containing the H199A/N229V mutations were purified and ligated to give the E60L/H199A/N229V triple mutant. The plasmid DNAs of the single and triple mutants were individually isolated and sequenced. Mutant enzymes were purified by chromatography on phosphocellulose and hydroxyapatite as described (Kealey & Santi, 1992). Enzyme preparations were concentrated using Centriprep-30 concentrators (Amicon) and stored at −80 °C in 10 mM KH_2PO_4, pH 7.0, 1 mM EDTA until use. The concentrations of the purified enzymes were determined spectrophotometrically using $\epsilon_{278} = 125\,600$ M^{-1} cm^{-1} (Carreras *et al.*, 1994), with the assumption that the mutations did not affect the extinction coefficient.

Enzyme Assays. dTMP formation was monitored spectrophotometrically at 340 nm as described (Pogolotti *et al.*, 1986). The standard TES buffer contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 75 mM β-mercaptoethanol. For the E60L mutant and the E60L/H199A/N229V triple mutant, dTMP formation was monitored by HPLC analysis (Huang & Santi, 1994). The steady-state kinetics for TS E60L were monitored by the TS-catalyzed release of tritium from [2-^{14}C, 5-^{3}H]dUMP as described (Huang & Santi, 1994).

TS-catalyzed dehalogenation of BrdUMP or IdUMP was monitored by the decrease in absorbance at 285 nm ($\Delta\epsilon_{285}$ = 5320 M^{-1} cm^{-1}) or 290 nm ($\Delta\epsilon_{290}$ = 6520 M^{-1} cm^{-1}) that accompanies dehalogenation of BrdUMP or IdUMP, respectively (Garrett *et al.*, 1979). Reaction mixtures contained TES/DTT assay buffer (50 mM TES, pH 7.4, 6.5 mM formaldehyde, 25 mM $MgCl_2$, 1 mM EDTA, and 10 mM DTT), 3−200 μM BrdUMP or IdUMP, and 0.12−3 μM of enzyme. Upon completion of the reaction, 0.5 μM wild-type TS and 200 μM CH_2H_4folate were added to the reaction mixture. The product of the dehalogenation reaction, dUMP, was then confirmed by conversion of dUMP to dTMP which was measured spectrophotometrically at 340 nm (Garrett *et al.*, 1979). For TS N229V, enzyme concentrations of up to 10 μM and incubations of up to 4 h were used in attempts to detect dehalogenation. The limit of detection for k_{cat} was 0.006 min^{-1} in this assay.

Steady-state kinetic parameters were obtained by a nonlinear least squares fit of the data to the Michaelis−Menten equation using the program Kaleidagraph (Abelbeck Software, Reading, PA, 1989). For kinetic measurements in which the enzyme concentration exceeded 1 μM, data were fit to an equation that corrects for ligand depletion by protein (Segel, 1975).

Table 1: dTMP Formation by Wild-type and Mutant TSs[a]

TS	k_{cat}, min^{-1}	Km, μM		k_{cat}/K_m, min^{-1} μM^{-1}	
		dUMP	CH_2H_4-folate	dUMP	CH_2H_4-folate
wild-type	540	6.8	19	79	28
E60L	2×10^{-2}	220	21	9×10^{-5}	10×10^{-3}
H199A	41	5.2	19	7.9	2.2
N229V	57	128	40	0.4	1.4
E60L/H199A/N229V	$<1 \times 10^{-4}$	ND[b]	ND		

[a] dTMP formation by wild-type TS, TS H199A, and TS N229V was monitored spectrophotometrically at 340 nm. k_{cat} values for TS E60L and TS E60L/H199A/N229V were obtained from HPLC analysis. K_m values for dUMP and CH2H4folate for TS E60L were obtained by the TS-catalyzed tritium release from [5-^{3}H]dUMP as previously described (Huang & Santi, 1994). The standard errors from nonlinear least squares fit of the experimental data are less than 20% for all values. [b] ND: not determined.

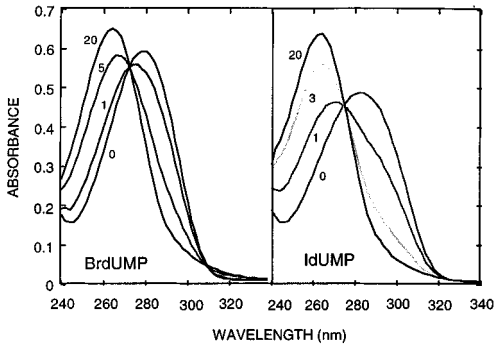


FIGURE 1: Ultraviolet spectral changes during TS E60L-catalyzed dehalogenation of BrdUMP and IdUMP. Reaction mixtures contained standard TES buffer, 0.18 μM TS E60L, 10 mM DTT, and 50 μM BrdUMP or IdUMP. The numbers shown indicate the reaction time in minutes.

RESULTS

TS mutants were constructed by cassette mutagenesis of the *L. casei* TS synthetic gene, and the mutations were confirmed by DNA sequencing. The TS mutants were expressed at levels of 10−30% of the total cellular protein in soluble crude extracts, as determined by 12% SDS−PAGE and comparison of the specific activity of the purified proteins to that of the crude extract. The mutant enzymes were purified by phosphocellulose and hydroxyapatite chromatography (Kealey & Santi, 1992) to apparent homogeneity as assessed by SDS−PAGE.

dTMP formation catalyzed by wild-type and mutant TSs was determined (Table 1). Decreases in rates of dTMP formation were observed for all mutant enzymes. There was an approximately 10-fold decrease in k_{cat} of dTMP formation for TS H199A and TS N229V. Similar data for TS N229V has been reported (Liu & Santi, 1993). There was a pronounced ($\sim 3 \times 10^4$-fold) decrease in k_{cat} value for TS E60L and no detectable dTMP formation (k_{cat} value $< 1 \times 10^{-4}$ min^{-1}) for the E60L/H199A/N229V triple mutant. For the TS mutants studied, the K_m for dUMP was either unchanged or moderately increased (<32-fold), and the K_m for CH_2H_4folate increased less than 3-fold, compared to wild-type TS (Table 1).

As described for wild-type TS (Garrett *et al.*, 1979), TS E60L, TS H199A, and TS E60L/H199A/N229V catalyzed the thiol dependent CH_2H_4folate-independent dehalogenation of BrdUMP and IdUMP to produce dUMP. When BrdUMP or IdUMP was reacted with TS E60L in the presence of DTT, the spectra were converted to those of dUMP (Figure 1). After completion of the reaction, wild-type TS and CH_2H_4-folate were added, and the product was verified to be >90% of dUMP by its conversion to dTMP. Thus, the major product of dehalogenation of either BrdUMP or IdUMP was dUMP. The conversion of BrdUMP to dUMP by TS E60L was also confirmed by HPLC analysis (data not shown). The rate of debromination of BrdUMP by TS E60L increased with increasing amount of DTT and reached half-maximum rate when the concentration of DTT was 0.7 mM. The concentration of DTT used in the standard reaction mixture was 10 mM, which is more than sufficient to saturate the enzyme for the dehalogenation reaction.

Table 2 shows the steady-state kinetic parameters for dehalogenation of BrdUMP and IdUMP by the wild-type and mutant enzymes. The dehalogenation reaction was monitored spectrophotometrically at 285 nm for BrdUMP and at 295 nm for IdUMP. For TS E60L, there were 70-fold and 22-fold increases in k_{cat} values for dehalogenation of BrdUMP and IdUMP, respectively, compared with those of wild-type TS. For TS H199A, k_{cat} values for dehalogenation of BrdUMP and IdUMP were similar to and 6-fold higher than those for wild-type TS, respectively. TS N229V was inactive in dehalogenation of BrdUMP or IdUMP (k_{cat} < 0.006 min^{-1}). The k_{cat} values for dehalogenation of BrdUMP and IdUMP for the E60L/H199A/N229V triple mutant were similar to those for wild-type TS. K_m values for BrdUMP and IdUMP for all mutant enzymes were either unchanged or moderately increased (<8-fold) compared to those for wild-type TS.

DISCUSSION

Several steps of the TS reaction require proton transfer to and from O-4 and C-5 of the pyrimidine of substrate dUMP (Scheme 1). These include (1) formation of the 5,6-dihydropyrimidine covalent intermediate **II** by nucleophilic attack of the catalytic Cys198 at C-6 of dUMP, (2) condensation of C-5 of the 5,6-dihydropyrimidine intermediate with cofactor CH_2H_4folate, (3) β-elimination of H_4folate from the intermediate **IV** to give a reactive exocyclic methylene intermediate **V**, (4) reduction of that intermediate, and finally (5) elimination of the enzyme from C-6 of intermediate **VI** to give dTMP and free enzyme. Chemical considerations of proton transfer reactions at such poorly basic or acidic sites[3] led to the proposal that general acid/base catalysts of the enzyme might assist in these reactions (Santi & Danenberg, 1984; Carreras & Santi, 1995). Structural evidence suggests that the side chains of three conserved residues—Glu60, His199, and Asn229—of the protein are contenders for the putative general catalysts. These three residues, together with at least two conserved water molecules, form a hydrogen bond network which positions labile protons within hydrogen-bonding distance of O-4 of dUMP and which is in close proximity to C-5 of dUMP (Finer-Moore *et al.*, 1990, 1993; Matthews *et al.*, 1990). As such, these residues, together with active site water molecules, are

[3] The pK_a of a protonated O-4 of a uracil is estimated to be *ca.* −4 (Sobell & Tomita, 1964; Shapiro & Danzig, 1972). The pK_a of the 5-H of the 5,6-dihydropyrimidine is estimated to be at least 20, and the pK_a of its corresponding enol is estimated to be at least 12 (Liu & Santi, 1993).

Table 2: Dehalogenation of BrdUMP and IdUMP by Wild-type and Mutant TSs[a]

	BrdUMP			IdUMP		
TS	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m, $min^{-1}\ \mu M^{-1}$	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m, $min^{-1}\ \mu M^{-1}$
wild-type	1.2	5.4	0.2	4.1	9	0.5
E60L	84	22	3.8	90	6.9	13
H199A	0.9	8.5	0.1	24	19	1.3
N229V	<0.006	ND[b]		<0.006	ND	
E60L/H199A/N229V	0.8	39	0.02	3.4	40	0.08

[a] Dehalogenation of BrdUMP and IdUMP was monitored spectrophotometrically at 285 and 290 nm, respectively. For TS N229V, the dehalgenation reaction was also monitored by HPLC. The standard errors from nonlinear least squares fit of the experimental data are less than 15% for all values. [b] ND: not determined.

the only contenders for the general catalyst(s) in the TS reaction.

In multistep pathways as complex as the TS reaction, it is difficult to assign the contributions of specific amino acids to individual steps. For this reason, several partial reactions of TS, which undergo one or a few steps in the normal catalytic pathway, have been developed (Garrett *et al.*, 1979). Studies of such reactions greatly simplify the chemistry and permit analysis of the effects of mutations on isolated steps of the TS pathway. One such reaction is the CH_2H_4folate-independent dehalogenation of Br(I)dUMP which shares two direct chemical counterparts with the TS pathway: (i) nucleophilic addition of the catalytic Cys to C-6 of the pyrimidine substrate to form a 5-halo-5,6-dihydropyrimidine intermediate and (ii) elimination of the enzyme from C-6 of the dihydropyrimidine intermediate in the final step of the reaction.

We have mutated each of the possible three general catalysts of TS to a residue incapable of proton transfer and then mutated all three residues together to strip the active site of all possible general catalysts. The mutants were examined for their effects on dTMP synthesis and the reactions involved in dehalogenation of Br- and IdUMP. The uncoupling of effects of the mutations on dTMP synthesis and the dehalogenation reactions allowed us to speculate on the contributions of these residues in the TS pathway.

TS E60L, H199A, and N229V were chosen for study because individually they provided catalytically active enzymes and side chains incapable of proton transfer. TS H199A showed the smallest alteration in the kinetics of dTMP formation compared to that of wild-type TS, with similar substrate K_m values, but about a 10-fold decrease in k_{cat}. TS N229V showed a similar K_m for the cofactor, a 20-fold increase in K_m of dUMP, and a 10-fold decrease in k_{cat} of dTMP formation compared to that of wild-type TS. The most affected single mutant was TS E60L which showed a large increase (30-fold) in the K_m for dUMP and a very large decrease (3×10^4-fold) in k_{cat}. The k_{cat} value for dTMP formation with the E60L/H199A/N229V triple mutant was decreased by at least 5×10^6-fold; the mutant was essentially inactive in catalyzing dTMP formation. It is not surprising that the triple mutant was inactive, since the active site has been stripped of all residues containing groups which could serve as general catalysts.

The detrimental effects of these mutations on dTMP formation contrasted with their effects on the partial reactions involving the dehalogenation of BrdUMP and IdUMP (Tables 1 and 2). Whereas TS E60L was an extremely poor catalyst for dTMP formation, it catalyzed the dehalogenation reaction *more* effectively than wild-type TS. Mutation of His199 to Ala resulted in a 10-fold decrease in k_{cat} for dTMP formation but no significant effects on k_{cat} for the dehalogenation reactions. Inexplicably, TS N229V, which showed only a 10-fold decrease in k_{cat} for dTMP formation, was inactive in the dehalogenation reactions (Liu & Santi, 1993). Possibly, this could be due to an altered orientation of the substrate in this mutant with consequent diminished formation of the thiol Michael adduct. Most importantly, the E60L/H199A/N229V triple mutant, which was inactive in dTMP formation, had k_{cat} values for dehalogenation of BrdUMP and IdUMP that were about the same as those for wild-type TS. Thus, completely stripping the active site of the putative general catalysts had little effect on the dehalogenation reaction.

Our results clearly show that the triad of active site residues—Glu60, His199, and Asn229—are required for dTMP formation but are not essential for the dehalogenation of BrdUMP or IdUMP. Since the dehalogenation reaction mimics certain catalytic events in dTMP synthesis, it is reasonable to conclude that these three residues of the active site may not be essential for the counterpart events in the normal TS pathway. Thus, nucleophilic attack of Cys198 at C-6 of the pyrimidine of the substrate and formation of a 5,6-dihydropyrimidine may only require juxtapositioning of the reactive moieties within the protected confines of the active site of TS and may not require other catalysts. The caveat to this proposal is that the 5-halopyrimidines may be sufficiently reactive toward nucleophiles that they might not require general acid assistance, whereas dUMP does. Alternatively, the structural water molecules of the active site may provide assistance with the 5-halopyrimidines but may be insufficient in doing so in dTMP synthesis. The other reaction common to pathways of both dTMP formation and Br(I)dUMP dehalogenation is the elimination of the enzyme from the 5,6-dihydropyrimidine adduct. Here, the elimination occurs from covalent intermediates which differ only by the 5-methyl group for dTMP formation, versus a 5-H for the dehalogenation reaction. Since β-elimination of the enzyme occurs in the absence of any active site general catalysts, we conclude that the elimination occurs independently of such catalysts but possibly with the assistance of water molecules in the active site.

In summary, we have shown that the TS-catalyzed dehalogenation of Br(I)dUMP, which has two direct counterparts to catalytic steps in dTMP synthesis, does not require the assistance of putative active site general catalysts. We propose that either these steps do not require general catalysis or, more likely, that water molecules of the active site of the enzyme serve such roles. Thus, at least some steps of the TS reaction—*i.e.*, nucleophilic attack of Cys and β-e-

limination of Cys to give products—may occur simply by the appropriate positioning of the substrate in an active site possessing strategically positioned water molecules and favorable thermodynamic properties of the reactions catalyzed. It will be of interest to determine whether the active site water molecules of native TS believed to directly donate to and accept protons from O-4 of dUMP (Finer-Moore *et al.*, 1990) are also present in the triple mutant which is completely stripped of putative active site general catalysts.

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