Mutation of Asparagine 229 to Aspartate in Thymidylate Synthase Converts the Enzyme to a Deoxycytidylate Methylase†

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ABSTRACT: The conserved Asn 229 of thymidylate synthase (TS) forms a cyclic hydrogen bond network with the 3-NH and 4-O of the nucleotide substrate dUMP. The Asn 229 to Asp mutant of *Lactobacillus casei* thymidylate synthase (TS N229D) has been prepared, purified, and investigated. Steady-state kinetic parameters of TS N229D show 3.5- and 10-fold increases in the K_m values of CH_2H_4folate and dUMP, respectively, and a 1000-fold decrease in k_{cat}. Most important, the Asp 229 mutation changes the substrate specificity of TS to an enzyme which recognizes and methylates dCMP in preference to dUMP. With TS N229D the K_m for dCMP is bout 3-fold higher than for dUMP, and the K_m for CH_2H_4folate is increased about 5-fold; however, the k_{cat} for dCMP methylation is 120-fold higher than that for dUMP methylation. Specificity for dCMP versus dUMP, as measured by k_{cat}/K_m, changes from negligible with wild-type TS to about a 40-fold increase with TS N229D. TS N229D reacts with CH_2H_4folate and FdUMP or FdCMP to form ternary complexes which are analogous to the TS–FdUMP–CH_2H_4folate complex. From what is known of the mechanism and structure of TS, the dramatic change in substrate specificity of TS N229D is proposed to involve a hydrogen bond network between Asp 229 and the 3-N and 4-NH_2 of the cytosine heterocycle, causing protonation of the 3-N and stabilization of a reactive imino tautomer. A similar mechanism is proposed for related enzymes which catalyze one-carbon transfers to cytosine heterocycles.

Thymidylate synthase (TS,[1] EC 2.1.1.45) catalyzes the reductive methylation of dUMP by CH_2H_4folate to give dTMP and H_2folate. Much is known about the structure and function of TS. Over 20 sequences of TS have revealed that TS is the most conserved of known enzymes (Perry et al., 1990; Perryman et al., 1986), and three-dimensional structures of free and bound enzyme forms have been determined (Hardy et al., 1987; Matthews et al., 1990; Montfort et al., 1990; Perry et al., 1990). Basic features of the catalytic mechanism of TS have also been established (Scheme I) (Santi & Danenberg, 1984). After formation of reversible complexes, the thiol of Cys 198[2] in *Lactobacillus casei* TS adds to the 6-carbon of dUMP to activate the 5-carbon toward reactions with electrophiles. The resultant enolate or equivalent enol, intermediate **2**, reacts with the one-carbon unit of CH_2H_4folate to give the covalent intermediate **3**, which upon β-elimination of H_4folate, hydride transfer, and β-elimination gives the products dTMP and H_2folate.

Several enzymes catalyze related one-carbon-transfer reactions to the cytosine heterocycle. These include the DNA m^5C methyltransferases, which catalyze methylation of cytosine residues of DNA by AdoMet, and dCMP hydroxymethylase (dCMP Hmase) of T-even bacteriophage that catalyzes hydroxymethylation of dCMP by CH_2H_4folate. It is believed that these enzymes follow a similar catalytic path to TS since they also utilize a cysteine thiol as a nucleophilic catalyst and have analogous covalent intermediates (Santi & Danenberg, 1984; Wu & Santi, 1987; Basak et al., 1988; Chen, et al., 1991; Ivanetich & Santi, 1992). Further, the primary sequence of dCMP Hmase shows considerable homology with TS (Lamm et al., 1988).

With TS and related enzymes, it is likely that functional groups other than Cys are involved in facilitating recognition and chemistry at the pyrimidine ring. The crystal structure of the TS–dUMP complex shows that the completely conserved N229 and a highly ordered water molecule are in direct contact with the pyrimidine ring of dUMP. As depicted in Scheme I, N229 forms a hydrogen bond network bridging 3-NH and 4-O of the substrate dUMP; an ordered water molecular also forms a hydrogen bond with 4-O and with the highly conserved His 199. It is reasonable to suggest that Asn 229 may be involved in substrate recognition, stabilization of the enolate or equivalent enol, intermediate **2**, and facilitation of subsequent proton transfers on the pyrimidine ring. The same hydrogen bond network could not form with cytosine, so N229 also offers a rationalization for the specificity of TS for dUMP versus dCMP.

As an approach to understanding the structure and function of TS, the construction and analysis of multiple or "replacement sets" at highly conserved, putatively important residues have been undertaken (Climie et al., 1990; Michaels et al., 1990). Surprisingly, most residues studied tolerated one or more mutations without elimination of activity; indeed, several mutants of Asn 229 which cannot form the Asn 229–dUMP hydrogen bond network catalyzed dTMP formation.

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[1] Abbreviations: TS, thymidylate synthase; dCMP Hmase, 2′-deoxycytidine-5′-monophosphate hydroxymethylase; dUMP, 2′-deoxyuridine 5′-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dTMP, thymidine 5′-monophosphate; dCMP, 2′-deoxycytidine 5′-monophosphate; m^5dCMP, 5-methyl-2′-deoxycytidine 5′-monophosphate; H_2folate, 7,8-dihydrofolate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FdCMP, 5-fluoro-2′-deoxycytidine 5′-monophosphate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; $I/S_{50\%}$, ratio of inhibitor to substrate which gives 50% inhibition.

[2] The convention used follows the numbering system of *Lactobacillus casei* TS.

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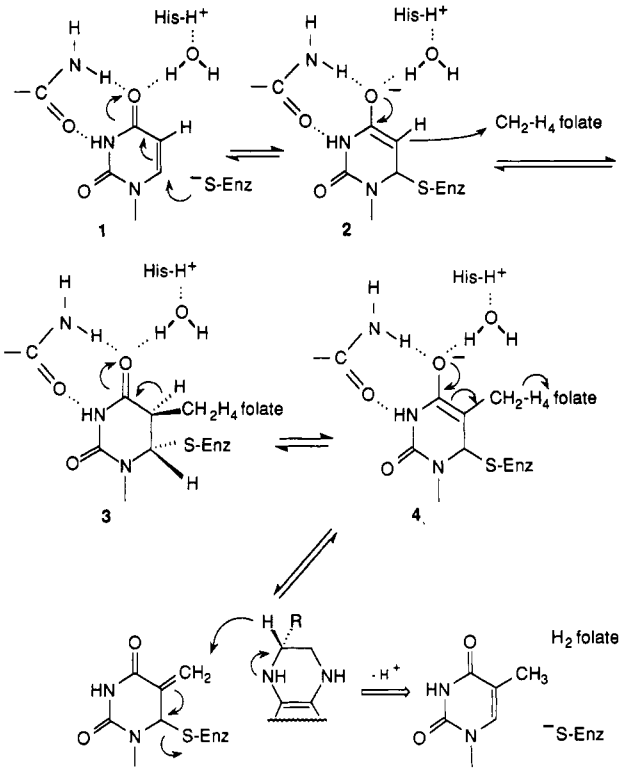
Scheme I



These findings have led us to the view that may residues of TS, including Asn 229, may assist in catalysis but are not essential.

In the present work, we describe the properties of *L. casei* TS N229D. Although the mutant is a poor catalyst for dTMP formation, it is an excellent catalyst for the methylation of dCMP. We propose a mechanism which accounts for the change in substrate specificity and a catalytic role for Asp 229 in the methylation of dCMP.

Materials and Methods

Materials. Escherichia coli strain χ2913 recA (Δ*thy*A572, *rec*A56) (Climie et al., 1992), the plasmid pSCTS9 (Climie & Santi, 1990), and (6*R*)-[6-^{3}H]CH_2H_4folate (26.6 mCi/mmol) (Bruice & Santi, 1982) have been reported. Oligonucleotide synthesis was performed at the UCSF Biomolecular Resource Center. FdCMP was obtained from Sierra Bioresearch and contained less than 1% FdUMP by HPLC analysis. Other materials have been described (Bruice & Santi, 1982) or were obtained from commercial sources.

Mutagenesis. General procedures for DNA manipulations (Ausubel et al., 1989; Sambrook et al., 1989) and the method of constructing TS mutants by cassette mutagenesis (Climie & Santi, 1990) have been described. Monomer forms of plasmids encoding mutants N229V, L, I, C, M, and A (Climie, et al., 1990) were purified by agarose gel electrophoresis, retransformed to χ2913 recA, and resequenced. The *Bgl*II/*Nhe*I fragment of pSCTS9 was replaced by a "stuffer" oligonucleotide 5′-GATCTGCGGCCGCATGCG-3′ to give pSCTS9(*Bgl*II–*Nhe*I); the stuffer does not code for TS and has a unique *Not*I site for restriction purification (Wells et al., 1986). Mutants N229D,G of *L. casei* TS were obtained by ligation of the *Bgl*II/*Nsi*I fragment of pSCTS9(*Bgl*II–*Nhe*I) with a DNA cassette which carried the coding sequence of pSCTS9 except for a mixture of four codons at position 229 [G(GA)(TA)] encoding Gly, Asp, and Glu. All other procedures were as described (Climie et al., 1990) except that the Thy$^-$ *E. coli* strain χ2913 recA was used.

Protein Purification. Enzymes were purified by sequential chromatography on phosphocellulose and hydroxyapatite as described (Kealey & Santi, 1992). The purification was monitored by SDS–PAGE using 12% gels (Laemmli, 1970) and, when possible, by specific activity determinations. Enzyme preparations were concentrated using Amicon Centriprep-30 concentrators and stored at −80 °C.

Enzyme Assays. The standard assay buffer contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 75 mM β-mercaptoethanol. Unless otherwise specified, the standard assay (1 mL) contained the above buffer plus 150 μM CH_2H_4folate and 150 μM dUMP for TS or 340 μM CH_2H_4folate and 400 μM dCMP for dCMP methylase. TS and dCMP methylase activities were monitored at 20 °C by the increase in absorbance at 340 nm which accompanies H_2folate production. One unit is the amount of enzyme necessary to produce 1 μmol of product in 1 min. Steady-state kinetic parameters were obtained by nonlinear least squares fit of the data to the Michaelis–Menten equation. $I/S_{50\%}$ values for FdCMP and FdUMP on dCMP methylase activity were obtained by initial velocity measurements with varying inhibitor concentrations after initiation of the reaction with enzyme. Assay solutions contained 200 μM dCMP and 345 μM CH_2H_4folate in the standard assay buffer.

HPLC Analysis of Nucleotide Products. HPLC was performed using a Hewlett-Packard 1090 HPLC equipped with a diode array detector and an Altex Ultrasphere IP column (4.6 mm × 25 cm); absorbance spectra between 220 and 400 nm were collected for all peaks. The eluant was 5 mM KH_2PO_4 and 5 mM tetra-*n*-butylammonium sulfate buffer, pH 7.0, with specified acetonitrile concentration. The flow rate was 1.0 mL/min. With a 0–5% acetonitrile gradient over 30 min, dCMP and m^{5}dCMP had retention times of 15 and 17.7 min, respectively; with a 0–30% acetonitrile gradient over 30 min, FdUMP and FdCMP had retention times of 9.6 and 13.6 min; with isocratic elution at 5% acetonitrile, retention times for dUMP and dTMP were 11.6 and 14.6 min, respectively. For analysis of ^{3}H-labeled reactants and products, fractions (1 mL) were collected and counted in 5 mL of Aquasol II.

SDS–PAGE of Ternary Complexes. Covalent TS–[6-^{3}H]FdUMP–CH_2H_4folate complexes were formed and analyzed by SDS–12% PAGE as described (Sirawaraporn et al., 1990). The TS N229D–FdCMP–(6*R*)–[6-^{3}H]CH_2H_4folate complex was formed with 5.0 μM TS N229D, 38 μM FdCMP, and 2.4 mM (6*R*)-[6-^{3}H]CH_2H_4folate in the standard TES assay buffer at room temperature for 4 h; the control reaction lacked FdCMP. Coomassie-stained gels were analyzed for radioactivity as described (Chamberlain, 1979).

Ultraviolet Difference Spectroscopy of the TS N229D–FdCMP (or FdUMP)–CH_2H_4folate Complexes. UV spectra were obtained using a Hewlett-Packard 8452A diode array spectrophotometer. The Δε at 330 nm for the complex was determined by titration of 5.6 μM FdCMP and 13 μM CH_2H_4folate with TS N229D. Corrections were made for dilution and light scattering, and difference spectra were obtained by subtracting the spectrum of enzyme plus CH_2H_4-folate from the spectrum of the complete mixture. Binding stoichiometries were determined by titration of 13 μM CH_2H_4folate and 4.2 μM TS N229D (dimer) with FdCMP or 3.8 μM TS N229D (dimer) with FdUMP.

Results

TS N229 mutants were prepared by cassette mutagenesis of pSCTS9(*Bgl*II–*Nhe*I), a derivative of the synthetic TS gene

Table I: Steady-State Kinetic Parameters of TS and N229D and N229A Mutants[a]

	k_{cat}[b] (s^{-1})	K_m (μM)		k_{cat}/K_m (s^{-1} μM^{-1})	
		dUMP or dCMP	CH_2H_4folate	dUMP or dCMP	CH_2H_4folate
TS activity					
wild type	3.6	5.0	10.0	0.71	0.36
N229D	0.0037	50.4	35.4	0.73×10^{-4}	0.10×10^{-4}
N229A	nd[c]				
dCMP methylase activity					
wild type	nd				
N229D	0.45	150	170	0.30×10^{-2}	0.26×10^{-2}
N229A	nd				

[a] Assays are as described under Materials and Methods. At least $10K_m$ dUMP and CH_2H_4folate were used for determining the kinetic constants for the TS reaction, while 340 μM ($2K_m$) CH_2H_4folate and 594 μM ($6K_m$) dCMP were used for dCMP methylation. [b] Calculation of k_{cat} was based on the MW of the dimer. [c] Not detectable by spectrophotometric assay at 0.5 mM dCMP or dUMP.

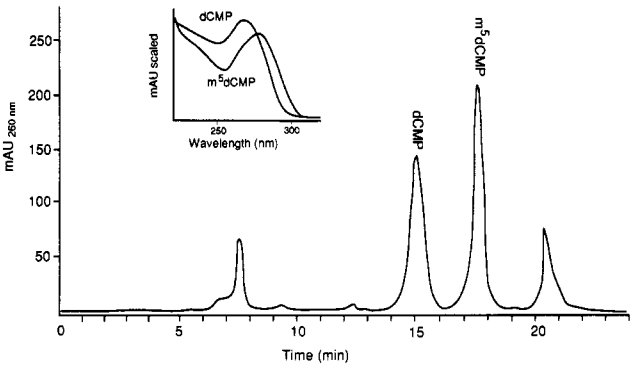


FIGURE 1: Partial HPLC profile of a reaction mixture of dCMP methylated by the TS N229D mutant. The insert shows the UV spectra of the HPLC peak indicated.

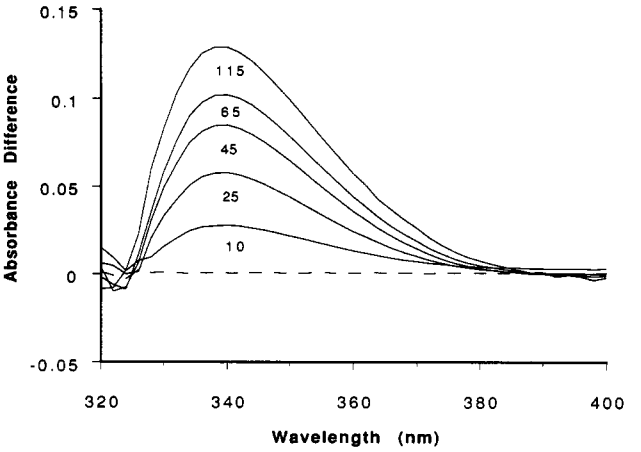


FIGURE 2: Difference spectra obtained during the TS N229D-catalyzed dCMP methylation reaction by scanning at times (minutes) indicated in the figure. The reaction solution contained 20 μM dCMP, 100 μM CH_2H_4folate, and 0.55 μM TS N229D mutant.

pSCTS9 (Climie & Santi, 1990). Plasmids were transformed into *E. coli* χ2913 recA, sequenced, retransformed and sequenced again through the cassette and flanking regions.

Initial assessment of TS activity was by growth of Thy$^-$ *E. coli* χ2913 recA harboring mutant plasmids on minimal media not containing thymine. TS N229A and N229D did not complement χ2913 recA, which indicates less than 0.002 unit of TS (mg of protein)$^{-1}$ in crude extracts (Climie et al., 1990). Several other N229 mutants (N229C, I, V, M, and L) have been shown to complement χ2913 and were presumed to have TS activity (Climie et al., 1990). These results have been verified with purified preparations of the mutant proteins by spectrophotometric assay (unpublished results).

TS N229A and N229D were expressed as about 10% and 20%, respectively, of the total soluble extract in *E. coli* and were purified to apparent homogeneity.

Purified N229A showed no TS activity under standard assay conditions, whereas TS N229D catalyzed a slow conversion of dUMP and CH_2H_4folate to dTMP and H_2folate (Table I). The production of dTMP was verified by HPLC, UV spectra analysis of the product peak, and transfer of the tritium from [6-^{3}H]CH_2H_4folate to the HPLC peak eluting as dTMP (data not shown). With TS N229D the K_m for dUMP was 10-fold higher than for wild-type TS, the K_m for CH_2H_4folate was 3.5-fold higher, k_{cat} was about 10^3-fold lower, and k_{cat}/K_m values were approximately 10^4-fold lower. When measured as an inhibitor of dCMP methylation (see below), dUMP showed a K_i of 10 μM, some 5-fold lower than its K_m.

When dCMP was used as substrate rather than dUMP, TS N229D catalyzed formation of m^{5}dCMP, as identified by HPLC and UV spectral analysis (Figure 1). Further, when [6-^{3}H]CH_2H_4folate was used in the reaction, the tritium was transferred to m^{5}dCMP. The latter experiment is analogous to that which established direct hydride transfer from CH_2H_4folate to dTMP in the TS reaction (Blakley; 1963; Pastore & Friedkin, 1962). Figure 2 shows the UV–vis absorbance difference spectral changes which occur upon conversion of dCMP to m^{5}dCMP. Using limiting dCMP, there was an increase in absorbance at 340 nm which upon completion of reaction corresponds to a $\Delta\epsilon_{max}(340)$ of 6300 M^{-1} cm^{-1}. This spectral change is qualitatively and quantitatively characteristic of the conversion of CH_2H_4folate to H_2folate [$\Delta\epsilon_{max}(340)$ of 6400 M^{-1} cm^{-1}] (Wahba, 1961). Together, these data provide convincing evidence that TS N229D catalyzes the reductive methylation of dCMP by CH_2H_4folate to give m^{5}dCMP and H_2folate.

Steady-state kinetic parameters for dCMP methylation and dTMP formation by TS N229D were determined (Table I). Compared to constants for dUMP as substrate, K_m for dCMP is 3-fold higher than that of dUMP, K_m for CH_2H_4folate is 5-fold higher, and k_{cat} is some 120-fold higher.

As reported for several other TSs (Rode et al., 1990), we found that dCMP is a poor inhibitor of *L. casei* TS (K_i = 3 mM). With a large amount of wild-type TS (100 μg) and a high concentration of dCMP (10 mM, $3K_i$), there was no detectable formation of m^{5}dCMP for up to 1 h; assuming we could detect 10^{-4} unit/mL, dCMP methylase activity is estimated to be under 10^{-3} unit/mg of TS, which is equivalent to a k_{cat} <0.001 s^{-1}. Several purified N229 mutant proteins (A, C, G, I, L, and V) also did not catalyze methylation of dCMP (unpublished results), and thus far the ability to catalyze the conversion of dCMP to m^{5}dCMP is limited to TS N229D.

To measure apparent inhibition constants of TS N229D-catalyzed dCMP methylation by FdUMP and FdCMP, we first made initial velocity determinations at varying inhibitor concentrations after initiation of the reaction with enzyme. We obtained $I/S_{50\%}$ values for FdCMP and FdUMP of 0.05

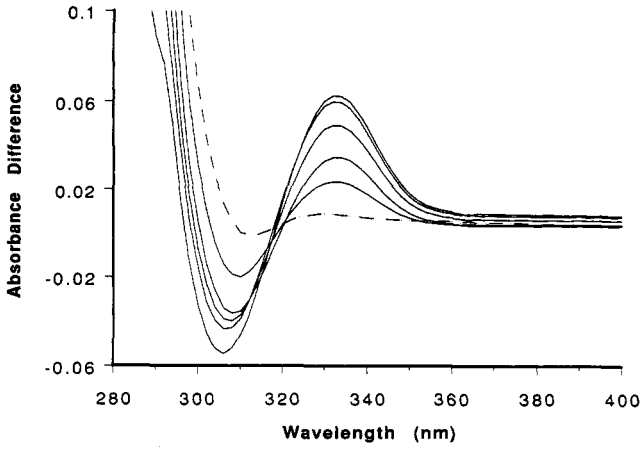


FIGURE 3: Difference spectra showing the loss of FdCMP and CH_2H_4folate absorbance and appearance of a new peak at 330 nm. For experimental conditions, refer to Materials and Methods. The dashed curve is for the mixture of TS N229D plus CH_2H_4folate. All the other curves show the spectral changes after additions of increasing amounts of FdCMP.

and 0.35, respectively, but because of the potential complexity of the interactions (see below), interpretation of these values is guarded. When TS N229D was treated with [6-^{3}H]-CH_2H_4folate and FdCMP or CH_2H_4folate and [6-^{3}H]-FdUMP, the radioactivity migrated with the 35-kDa protein subunit on SDS–PAGE. These results indicate that, as with wild-type TS, TS N229D forms covalent ternary complexes with either of these nucleotides which have structures analogous to intermediate **3** (Scheme I). Ultraviolet difference spectral analysis of TS N229D plus CH_2H_4folate plus FdCMP or FdUMP versus TS N229D plus CH_2H_4folate (Figure 3) shows a peak at 330 nm characteristic of the well-studied TS–FdUMP–CH_2H_4folate complex which has a $\Delta\epsilon_{330}$ of 17 700 M^{-1} cm^{-1} (Santi et al., 1976). Spectrophotometric titrating of limiting FdCMP and excess CH_2H_4folate with TS N229D gave $\Delta\epsilon_{330}$ = 16 130 M^{-1} cm^{-1}; titration of TS N229D and excess CH_2H_4folate with FdCMP or FdUMP showed that, at saturation of the enzyme, 1.0 mol of FdCMP or 0.85 mol of FdUMP is bound per mole of TS N229D dimer. These results indicate that only one of the two sites of dimeric TS N229D is fully occupied by the ligands. Reported half-site interactions of wild-type TS with various ligands add credence to this finding (Danenberg & Danenberg, 1979; Galivan et al., 1976; Pogolotti et al., 1986).

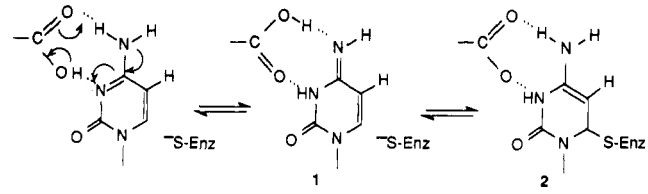
Discussion

A single Asn to Asp mutation at 229 of TS changes the substrate specificity of the enzyme from one which recognizes and methylates dUMP to one which preferentially recognizes and methylates dCMP.

TS N229D shows a greatly decreased ability to catalyze the normal TS reaction. dUMP and CH_2H_4folate show a 10- and 3.5-fold increase, respectively, in K_m values, and there is a 10^3-fold decrease in k_{cat}. Several other mutations of Asn 229 also show TS activity with lower k_{cat} values than that of wild-type TS (unpublished results), so we conclude that Asn 229 assists but is not essential for TS activity.

The most interesting property of TS N229D is its ability to catalyze redutive methylation of dCMP. In this reaction, the 6-hydrogen of the cofactor is transferred to the incipient methyl group of m^{5}dCMP and stoichiometric formation of H_2folate. Further, in the presence of CH_2H_4folate, both FdUMP and FdCMP form covalent ternary complexes with TS N229D which appear analogous to the well-studied TS–FdUMP–CH_2H_4folate complex. As with the latter, these

Scheme II



covalent complexes are stable toward SDS–PAGE and show characteristic UV–vis difference spectra with $\Delta\lambda_{max}$ at 330 nm. Interestingly, spectral studies of the interaction of FdCMP and FdUMP with TS N229D suggest that only one of the two sites of the dimer is fully occupied by ligands. From available data, the TS N229D-catalyzed methylation of dCMP appears directly analogous to the TS-catalyzed methylation of dUMP.

The kinetic properties of TS N229D reveal that the K_m values for the substrates dCMP and CH_2H_4folate are some 3–5-fold higher than the K_m values for dUMP and CH_2H_4-folate. The k_{cat} for TS N229D is some 120-fold higher using dCMP rather than dUMP and only 10-fold lower than dUMP methylation by wild-type TS. The k_{cat}/K_m values indicate that TS N229D has a 40-fold higher specificity for dCMP than for dUMP. In contrast, with wild-type TS, dCMP is not a substrate and binds very poorly.

We have not observed dCMP methylase activity with any of seven other mutants of Asn 229 studied, so the Asp residue is presumed to be necessary for this reaction. Curiously, Asn 229 of TS is not essential for methylation of dUMP, but Asp 229 of TS N229D appears essential for methylation of dCMP.

The ability of TS N229D to catalyze methylation of dCMP may be rationalized using the TS mechanism and structure as a framework. In TS, the amide of Asn 229 hydrogen bonds across the 3-NH and 4-O of the uracil moiety of dUMP (Scheme I) and may assist in substrate binding, orientation and stabilization of the initial covalent adduct, and subsequent proton transfers. dCMP cannot form the same hydrogen bond network with Asn 229 and is neither a substrate nor a significant inhibitor of the enzyme. With TS N229D, the Asp 229 residue could hydrogen bond to the 3-N and the 4-NH_2 of dCMP (Scheme II). Proton donation to the 3-N and acceptance from the 4-NH_2 of dCMP by Asp 229 would assist to stabilize the cytosine in its normally highly unfavorable, but presumably reactive imino tautomeric form. The latter is analogous to the stable, reactive keto tautomer of dUMP (**1**, Scheme I) and is proposed to be a reactive intermediate in the TS N229D-catalyzed methylation of dCMP. When the tautomeric equilibrium of dCMP is shifted toward the putative reactive imino species, the reactivity of dCMP in the active site could in theory be increased by the magnitude of the tautomeric equilibrium constant, or as much as 10^5-fold (Kenner et al., 1955). Subsequently, the Asp–dCMP hydrogen bond network could stabilize the covalent dihydropyrimidine adduct and facilitate proton transfers of the heterocycle in a manner similar to that of TS. The caveat in this proposal is that since the pK_a values of Asp and the 3-N of cytosine are both about 5, one of the two residues would require protonation to form the proposed reactive imino species. It is possible that the pK_a of Asp 229 is perturbed or that the small fraction of appropriately charged species of enzyme or substrate present at neutral pH is a highly reactive species. Future work will address this issue.

dCMP Hmase from T-even bacteriophage catalyzes a reaction similar to that of TS in that the one-carbon unit of CH_2H_4folate is transferred to the cytosine of dCMP; however, unlike TS, a subsequent redox reaction does not occur, and

	223								229							
L. casei TS	F	L	G	-	-	V	P	F	**N**	I	**A**	-	-	S	Y	A	L
T4 Hmase 1	V	F	G	F	R	N	**D**	Y	**A**	W	Q	-	-	K	Y	V	L
T4 Hmase 2	V	F	G	-	-	F	R	N	**D**	Y	**A**	W	Q	K	Y	V	L

FIGURE 4: Alignment of the amino acid sequence of T4 dCMP Hmase with *L. casei* TS. Alignments 1 and 2 of dCMP Hmase with *L. casei* TS differ in whether the two amino acid insert of dCMP Hmase is placed after residue 225 or after residue 231 of TS.

the products are Hm^5dCMP and H_4folate. Perhaps coincidentally, the K_m values for dCMP and CH_2H_4folate for TS N229D are similar to those of 150 and 100 μM, respectively, reported for T4 dCMP Hmase (Pizer, 1962). The primary sequence of dCMP Hmase has considerable homology with TS, particularly in residues involved in substrate binding (Lamm et al., 1988). However, the proposed alignment (1, Figure 4) places a two amino acid insert of dCMP Hmase between TS residues 225 and 226 and aligns Ala 181 of dCMP Hmase with Asn 229 of TS. We have shown that TS N229A has neither TS nor dCMP methylase activity. An alternate alignment which eliminates the 225–226 insert places Asp 179 of dCMP Hmase in alignment with Asn 229 of TS and gives an additional identity with the highly conserved Ala 231 of TS (2, Figure 4). The results described here support the alignment of Asn 179 of dCMP Hmase with Asn 229 of TS.[3]

With the exception of Asp 229, TS contains all the features necessary to catalyze one-carbon transfer at the 5-position of the cytosine heterocycle. We speculate that the structures of enzymes which catalyze similar reactions on the cytosine heterocycle will reveal a similar hydrogen bond network to assist catalysis. From the present work, we would suggest the involvement of Asp or Glu in many cases of enzymatic methylation of cytosine residues. It also occurs to us that the hydrogen bonds of G-C base pairs in DNA could play a similar "catalytic" role in the stabilization of intermediates and proton transfers necessary for methylation of cytosine residues.

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REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1989) *Current protocols in molecular biology*, John Wiley & Sons, New York.

Basak, A. K., Gautam-Basak, M., & Sander, E. G. (1988) *J. Cell Biol. 107*, 401a.

Blakley, R. L., Ramasastri, B. V., & McDougall, B. M. (1963) *J. Biol. Chem. 238*, 3075–3079.

Bruice, T. W., & Santi, D. V. (1982) *Biochemistry 21*, 6703–6709.

Chamberlain, J. P. (1979) *Anal. Biochem. 98*, 132–135.

Chen, L., MacMillan, A. M., Chang, W., Ezaz-Nikpay, K., Lane, W. S., & Verdine, G. L. (1991) *Biochemistry 30*, 11018–11025.

[3] It has been communicated to us that dCMP Hmase D179N catalyzes the hydroxymethylation of dUMP and that *E. coli* TS N177D catalyzes dCMP methylation (L. Hardy, unpublished results).

Climie, S., & Santi, D. V. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 633–637.

Climie, S., Ruiz, P. L., Gonzalez, P. D., Prapunwattana, P., Cho, S. W., Stroud, R., & Santi, D. V. (1990) *J. Biol. Chem. 265*, 18776–18779.

Climie, S. C., Carreras, C. W., & Santi, D. V. (1992) *Biochemistry* (in press).

Danenberg, K. D., & Danenberg, P. V. (1979) *J. Biol. Chem. 254*, 4345–4348.

Galivan, J. H., Maley, G. F., & Maley, F. (1976) *Biochemistry 15*, 356–362.

Hardy, L. W., Finer, M. J. S., Montfort, W. R., Jones, M. O., Santi, D. V., & Stroud, R. M. (1987) *Science 235*, 448–455.

Ivanetich, K. M., & Santi, D. V. (1992) *Prog. Nucleic Acid Res. Mol. Biol. 42*, 127–156.

Kealey, J., & Santi, D. V. (1992) *Protein Expression and Purification* (in press).

Kenner, G. W., Reese, C. B., & Todd, A. R. (1955) *J. Chem. Soc.*, 855–859.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lamm, N., Wang, Y., Mathews, C. K. & Ruger, W. (1988) *Eur. J. Biochem. 172*, 553–563.

Matthews, D. A., Appelt, K., Oatley, S. J., & Xuong, N. H. (1990) *J. Mol. Biol. 214*, 923–936.

Michaels, M. L., Kim, C. W., Matthews, D. A., & Miller, J. H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3957–3961.

Montfort, W. R., Perry, K. M., Fauman, E. B., Finer, M. J. S., Maley, G. F., Hardy, L., Maley, F., & Stroud, R. M. (1990) *Biochemistry 29*, 6964–6977.

Pastore, E. J., & Friedkin, M. (1962) *J. Biol. Chem. 237*, 3802–3810.

Perry, K. M., Fauman, E. B., Finer, M. J. S., Montfort, W. R., Maley, G. F., Maley, F., & Stroud, R. M. (1990) *Proteins 8*, 315–333.

Perryman, S. M., Rossana, C., Deng, T. L., Vanin, E. F., & Johnson, L. F. (1986) *Mol. Biol. Evol. 3*, 313–321.

Pizer, L. I., & Cohen, S. S. (1962) *J. Biol. Chem. 237*, 1251–1259.

Pogolotti, A. L. J., Danenberg, P. V., & Santi, D. V. (1986) *J. Med. Chem. 29*, 478–482.

Rode, W., Zielinski, Z., Dzik, J. M., Kulikowski, T., Bretner, M., Kierdaszuk, B., Ciesla, J., & Shugar, D. (1990) *Biochemistry 29*, 10835–10842.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, New York.

Santi, D. V., & Danenberg, P. V. (1984) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) pp 345–398, John Wiley & Sons, New York.

Santi, D. V., Pena, V. A., & Lam, S. S. (1976) *Biochim. Biophys. Acta 438*, 324–331.

Sirawaraporn, W., Sirawaraporn, R., Cowman, A. F., Yuthavong, Y., & Santi, D. V. (1990) *Biochemistry 29*, 10780–10785.

Wahba, A. J., & Friedkin, M. (1961) *J. Biol. Chem. 236*, pc11–pc12.

Wells, J., Cunningham, B., Graycar, T., & Estell, D. (1986) *Philos. Trans. R. Soc. London, A 317*, 415–423.

Wu, J. C., & Santi, D. V. (1987) *J. Biol. Chem. 262*, 4778–4786.