A Novel dCMP Methylase by Engineering Thymidylate Synthase†

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ABSTRACT: X-ray crystal structures of binary complexes of dUMP or dCMP with the *Lactobacillus casei* TS mutant N229D, a dCMP methylase, revealed that there is a steric clash between the 4-NH_2 of dCMP and His 199, a residue which normally H-bonds to the 4-O of dUMP but is not essential for activity. As a result, the cytosine moiety of dCMP is displaced from the active site and the catalytic thiol is moved from the C6 of the substrate about 0.5 Å further than in the wild-type TS−dUMP complex. We reasoned that combining the N229D mutation with mutations at residue 199 which did not impinge on the 4-NH_2 of dCMP should correct the displacements and further favor methylation of dCMP. We therefore prepared several TS N229D mutants and characterized their steady state kinetic parameters. TS H199A/N229D showed a 10^{11} change in specificity for methylation of dCMP *versus* dUMP. The structures of TS H199A/N229D in complex with dCMP and dUMP confirmed that the position and orientation of bound dCMP closely approaches that of dUMP in wild-type TS, whereas dUMP was displaced from the optimal catalytic binding site.

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) by 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4-folate) to form deoxythymidine-5′-monophosphate (dTMP) and 7,8-dihydrofolate (H_2folate). Comparison of sequences of TS from ∼30 different organisms reveals that it is one of the most highly conserved enzymes (*1*, *2*). A large number of three-dimensional structures of free and ligand-bound TSs and TS mutants have been determined and provide a structural understanding of substrate recognition, stereochemical aspects of the reaction pathway (*3*), and roles of specific residues in the reaction chemistry (*4*).

Asn 229 is invariant in all TS's sequenced to date, and forms a cyclic hydrogen bond network with the 4-O and 3-NH of the substrate dUMP (Figure 2a) (*5*−*7*). Nevertheless, it can be replaced by numerous other residues, and the resultant mutants retain TS activity and also gain the capability to bind 2′-deoxycytidine 5′-monophosphate (dCMP) tightly (*8*, *9*). Moreover, alteration of Asn 229 to aspartate provides an enzyme which methylates dCMP more efficiently than dUMP, although the catalytic efficiency is still significantly lower than is the wild-type TS for its natural substrate dUMP (*10*, *11*).

The mechanism for the methylation of dCMP by TS N229D (Figure 1) is believed to involve protonation of 3-N of dCMP by the Asp 229 side chain and concomitant or subsequent proton acceptance from the exocyclic 4-NH_2 of dCMP to stabilize a reactive imino tautomer of the nucleotide (*10*). The imino form of dCMP could then undergo nucleophilic attack by Cys-198 and chemical conversions in a manner directly analogous to the normal TS-catalyzed methylation of dUMP.

The absence of dCMP methylation activity by wild-type Asn 229 TS and the remarkable change in specificity upon mutation of Asn 229 to Asp are explained by the structures of relevant binary complexes (Figure 2). In principle, the side chain of Asn 229 could provide the requisite acceptor and donor hydrogen bonds to dCMP by 180° rotation about the Cβ−Cγ bond. That is, Asn 229 Nδ2 could hydrogen bond to 3-N of dCMP and Asn-229 Oδ1 could hydrogen bond to 4-NH2 of dCMP. However, the crystal structure of the TS−dCMP complex shows that a hydrogen bond from Gln 217 Nϵ2 to the Asn 229 Oδ1 maintains Asn 229 in the same tautomeric form as in the TS−dUMP complex, and that dCMP is dislodged from the pyrimidine binding site (Figure 2d) (*12*). In contrast, the crystal structure of TS N229D shows that the 229 Asp forms the appropriate cyclic hydrogen bond network with the 4-NH_2 and 3-N of dCMP needed for conversion of the pyrimidine to its reactive imino form (Figure 2e).

In the *Lactobacillus casei* TS−dUMP complex the pyrimidine ring of dUMP is nestled against the His 199 imidazole. The close distance between His 199 Cϵ1 and 4-O of dUMP suggests these two atoms are hydrogen bonded (Figure 2a), although His 199 is not necessary for optimal activity (*13*, *14*). The 4-NH2 substituent on dCMP is not only bulkier than the dUMP carbonyl, but also would not function as a hydrogen bond acceptor from His 199 Cϵ1-H. Thus steric effects in the N229D single mutant force dCMP slightly away from the wall of the dUMP binding site (Figure 4, light grey) where it may not be optimally oriented for catalysis (*12*).

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[1] Abbreviations: TS, thymidylate synthase; dUMP, 2′-deoxyuridine-5′-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate, CB3717, 10-propargyl-5,8-dideazafolate; dTMP, deoxythymidine-5′-monophosphate; dCMP, 2′-deoxycytidine 5′-monophosphate; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5'-phosphate; F_o, measured structure factor amplitude.

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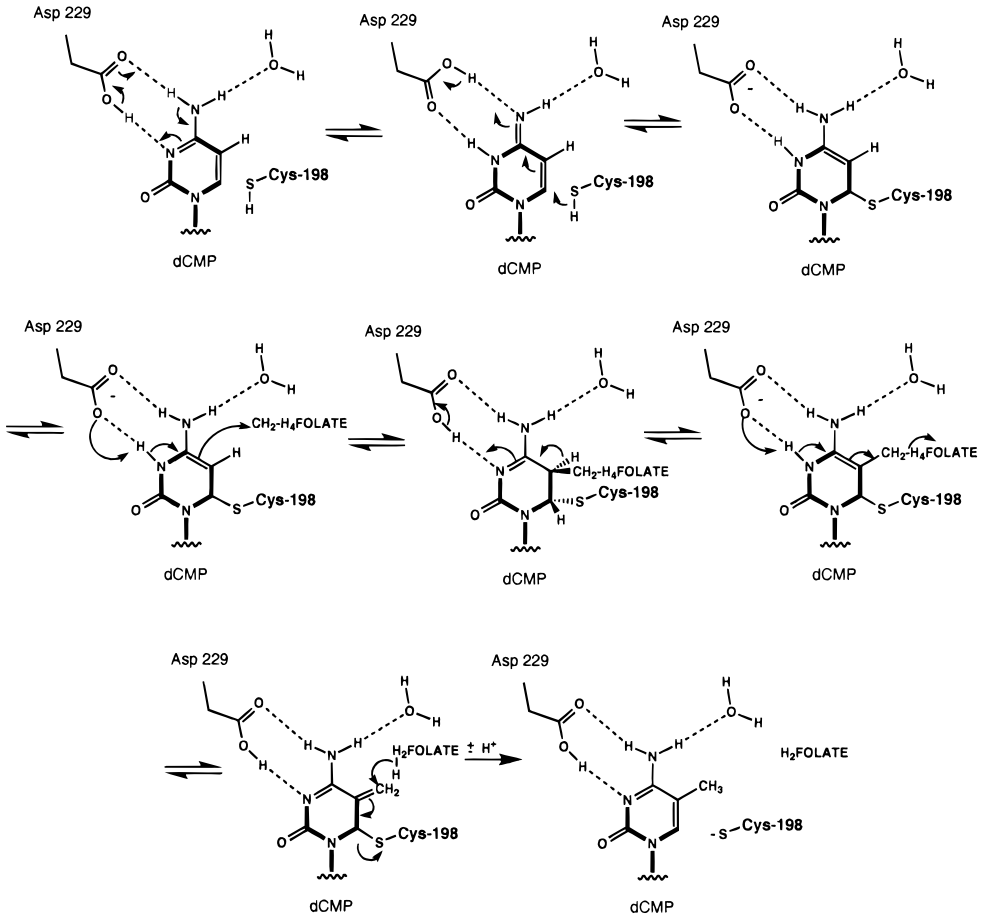

FIGURE 1: Proposed mechanism of dCMP methylation catalyzed by the TS N229D mutant series. The oxygens of TS N229D are each hydrogen bonded to dCMP in the binary TS N229D−dCMP complex. One carboxyl oxygen of Asp 229 can act as a base for the hydrogen of the 4-NH_2 setting up the imino tautomer. Concomitantly, the other ϵ oxygen of Asp 229 can act as an acid to facilitate proton transfer to 3-N. This protonation step may shift the equilibrium toward the normally disfavored imino tautomeric form of dCMP, thus activating the dCMP C6 for covalent addition by the Cys-198 sulfhydryl.

Since His 199 is not essential for TS catalysis and may be detrimental to TS N229D-catalyzed methylation of dCMP, we reasoned that mutations at the 199 position of TS N229D might allow closer approach of cytosine to the normal uracil binding site and thus enhance methylation of dCMP. Here, we describe the preparation, purification, characterization and the crystal structures of double mutations of *L. casei* TS where replacements at His 199 have been combined with N229D. We show that in TS N229D/H199A the activity of dCMP methylation is significantly enhanced, and the substrate specificity (k_{cat}/K_m) for dCMP versus dUMP is increased by a factor of $>10^{11}$. Finally, structural determinations of the TS N229D/H199A/dCMP and dUMP complexes confirmed that replacement of His 199 alleviates steric displacement of the pyrimidine from the position most suitable for catalysis.

MATERIALS AND METHODS

Materials. Restriction enzymes and other DNA modifying enzymes were purchased from New England Biolabs Inc. (Beverly, MA) or Life Technologies Inc. 6(R)-CH_2H_4folate was a generous gift from Eprova AG Research Institute (Basel, Switzerland). [2-^{14}C] and [5-^{3}H]dUMP were purchased from Moravek Biochemicals Inc. (Brea, CA). All other reagents were the purest grade commercially available.

Mutagenesis and Protein Purification. Plasmids encoding single mutants of TS N229D (*10*) and H199X (where X = G, A, V, N, P, R) (*13*, *15*, *16*) were employed to construct six double mutants. DNA from each of the pSCTSH199X plasmids encoding the H199X mutants was digested with *Apa*I and *Bgl*II. The resulting 360 bp fragment, harboring the codon for amino acid residue 199, was separated by agarose gel electrophoresis and purified. Likewise, pSCTSN229D was digested with *Apa*I and *Bgl*II, and the large fragment containing the codon for N229D was purified from agarose gel. Each of the H199X fragments was ligated with the N229D large fragment, and the ligation mixtures were transformed into *Escherichia coli* χ2913 recA (*15*, *16*). Plasmid DNA was isolated and the mutations were confirmed by automated DNA sequencing.

Protein Purification. Mutant enzymes were expressed in *E. coli* χ2913 recA, a thymidine auxotroph, in a medium supplemented with thymidine. Enzymes were purified by sequential phosphocellulose and hydroxyapatite chromatography (*17*) to apparent homogeneity. The purification was monitored using 12% SDS−PAGE on a Pharmacia Phast Gel apparatus. The purified enzymes were concentrated and desalted using Amicon Centriprep-30 concentrators and stored at −80 °C.

dCMP Methylation Assay. The standard assay buffer contained 50 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM ethylenediaminetetraacetic acid (EDTA), and 75 mM β-mercaptoethanol. Unless otherwise specified, the assay (1 mL) for dCMP methylase activity contained 200 μM CH_2H_4folate and 200 μM dCMP. Reactions were

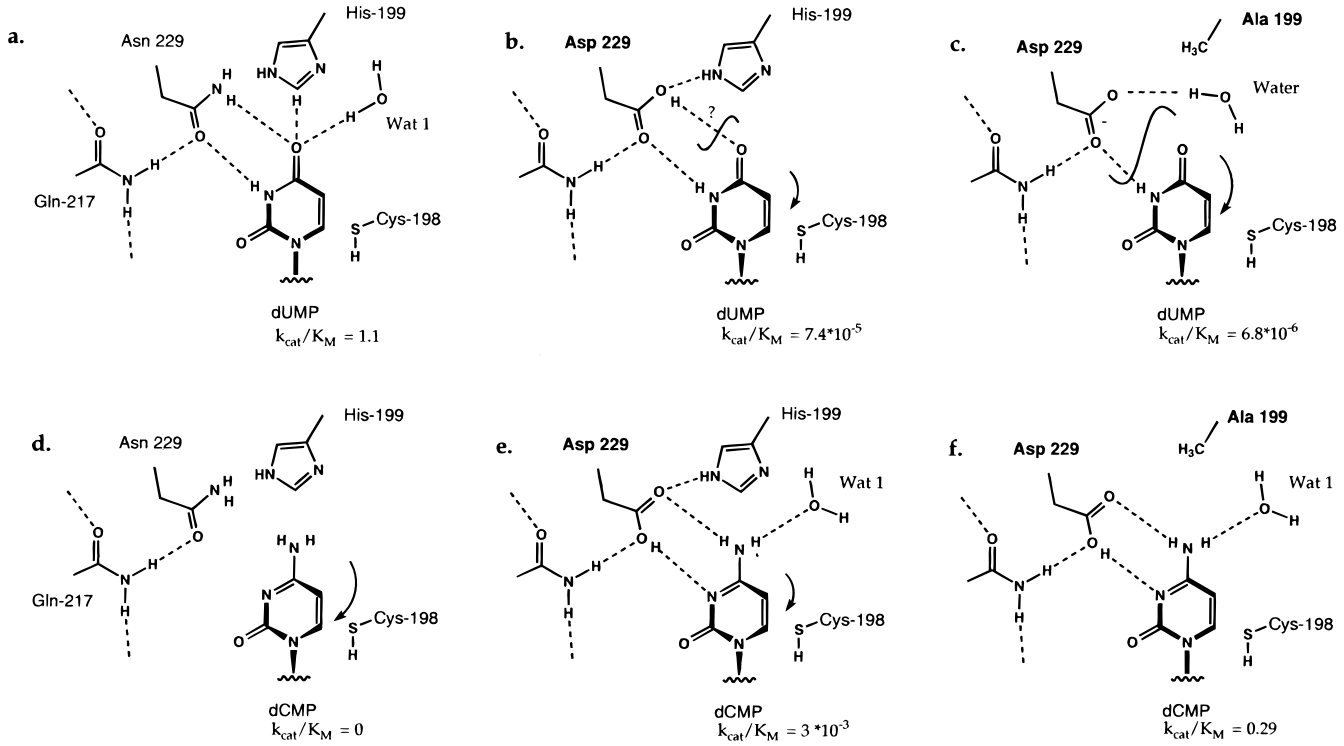

FIGURE 2: Schematic drawing of the interactions between dUMP (top row) and dCMP (bottom row) with the wild-type enzyme (left), TS N229D (center), and TS H199A/N229D (right). dUMP is moved out of the productive substrate binding site in TS N229D, and more so in TS H199A/N229D, as indicated by the curved arrows and by the use of tapered bonds that indicate relative twist out of the wild-type plane of the uridine ring. The progressive removal of dUMP from its binding site is in concert with the loss of the hydrogen bond between 4-O of dUMP and the C-H of H199, and the highly conserved water, Wat 1. dUMP is furthest from the productive site in the double mutant TS H199A/N229D accompanied by loss of the hydrogen bonds around H199. The alternate pyrimidine, dCMP, moves progressively in toward the productive binding site, reached in TS H199A/N229D (bottom right). The dCMP interaction with Wat 1 is restored in TS N229D, and in TS H199A/N229D. The values of k_{cat}/K_m for the nucleotides are given below the schematic structures. Mutated residues are printed in bold.

monitored by the increase in absorbance at 340 nm associated with the formation of dihydrofolate ($\Delta\epsilon = 6400\ M^{-1}\ cm^{-1}$) (18). For kinetic measurements, initial velocities were determined at various concentrations of dCMP in the presence of 200 μM CH_2H_4folate or at different concentrations of CH_2H_4folate in the presence of 200 μM dCMP. For the TS H199N/N229D mutant enzyme where the K_m for CH_2H_4folate was high, 400 μM CH_2H_4folate was used. 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, 1989).

Tritium Release and dTMP Formation Assays. TS-catalyzed exchange of the 5-hydrogen of dUMP was monitored by measuring the decrease in the $^3H/^{14}C$ ratio of [2-^{14}C, 5-^{3}H] dUMP (*19*). The reaction mixture contained 600 μM 6(*R*)-CH_2H_4folate and 0.1−3.0 mM [2-^{14}C, 5-^{3}H] dUMP along with 5−13 μM enzyme in the standard TES assay buffer. Aliquots of the reaction mixtures (20 μL) were added to 1 mL of methanol, evaporated to dryness under a heat lamp, dissolved in methanol, and evaporated again. Residues were dissolved in 1 mL of water and counted for ^{3}H and ^{14}C in 8 mL of Bio-Safe II scintillation cocktail, (Research Products International Corp., Mt. Prospect, IL) in a Beckman LS 3801 scintillation counter (*20*).

To verify that the tritium release reflected dTMP formation, product formation was monitored by HPLC on aliquots from the above reaction mixtures. HPLC was performed on a Hewlett-Packard 1090 HPLC equipped with a diode array detector. Samples were applied to a Beckman Ultrasphere IP column and eluted isocratically with a buffer containing 5 mM KH_2PO_4, 5 mM tetrabutyl ammonium hydrogen sulfate, and 4% acetonitrile (pH 7.0). Non-radioactive dTMP carrier was added to the samples prior to loading to the column. The progress of the reaction was quantitated by counting ^{14}C in the substrate and product peaks.

Determination of K_d. Dissociation constants for dCMP and dUMP were determined by displacement of pyridoxal phosphate (PLP) from the TS−PLP complex as described (*8*, *21*). Briefly, about 3 μM protein was titrated with increasing concentrations of up to 20 μM PLP. The increase in absorbance at 328 nm due to thiohemiacetal formation between PLP and enzyme was used to determine the K_d values for binding of PLP to TS. The nucleotide was then added to a preformed TS−PLP complex and K_d values were estimated by monitoring the decrease in absorbance at 328 nm resulting from displacement of PLP from the enzyme.

Crystallizations. Crystals were grown by vapor diffusion of drops containing 3.5−6.0 mg/mL protein and 2−20 mM dUMP or dCMP, in 40 mM potassium phosphate (pH 7.0), 1 mM DTT, and 1−2% ammonium sulfate (percent saturation). Drops were suspended over phosphate buffer, DTT, and EDTA. Crystals were isomorphous with either the large or small unit cell forms of the *L. casei* TS apoenzyme (*5*). dCMP was shown not to deaminate under crystallization conditions (*12*).

X-ray Data Collection. X-ray data were collected on an R-axis IIC image plate detector system mounted on a Rigaku 18 kW generator using frames 1.0−2.0° wide in ω. Data were reduced using R-axis II data reduction programs (*22*,

Table 1: Crystallographic Data Statistics

complex	cell axes (Å)	resolution (Å)	σ cutoff	no. of unique reflections	% complete	$I/\sigma(i)$	R(%)
H199A:dCMP	78.9, 242.4	2.5	1	13 786	89.7	5.4	11.7[c]
HA/ND:dC[a]	78.7, 230.4	2.4	0	12 157	72.7	7.7	9.0[d]
HA/ND:dU[b]	78.3, 241.1	2.4	0	16 408	91.1	18.1	8.0[d]

[a] HA/ND:dC is the H199A/N229D:dCMP complex. [b] HA/ND:dU is the H199A/N229D:dUMP complex. [c] $R_{mer} = \{[\sum_{hkl} \sum_i |I_{av} - I_i|]/\sum_{hkl}\sum_i I_i\} \times 100$. [d] $R_{sym} = \{\sum_{hkl} (\sum_i [(I_{av} - I_i)/\sigma(I_i)^2]/\sum_i [I_{av}/\sigma(I_i)^2]\}^{1/2} \times 100$.

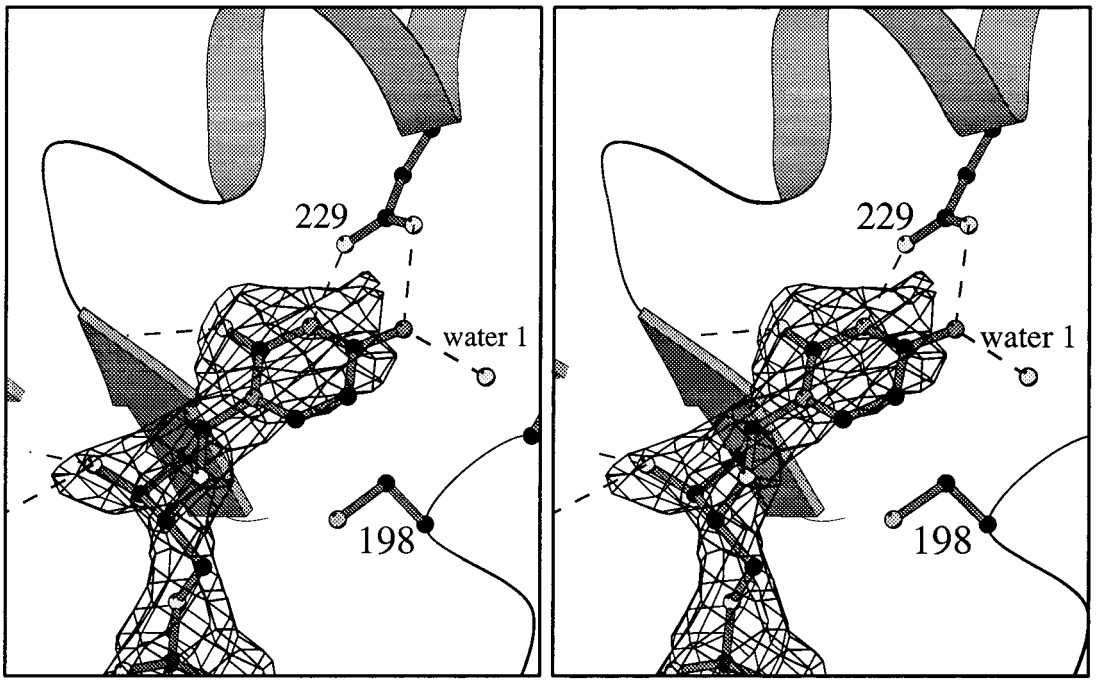

FIGURE 3: F_oF_c omit map contoured at 2.5σ for the binary complex of dCMP with TS H199A/N229D shows continuous difference density for the nonstandard nucleotide, dCMP.

Table 2: Crystallographic Refinement Statisitics

	H199A:dCMP	H199A/N229D:dCMP	H199A/N229D:dUMP
parameters			
no. of atoms	2661	2630	2656
no. of waters	54	25	48
diffraction agreement			
resolution (Å)	7−2.5	7−2.4	7−2.4
$I/\sigma(i)$ cutoff	0	0	0
R_{cryst} (%)	20.9	18.8	20.5
R_{free} (%)	30.7	28.6	29.6
RMS deviation from stereochemical ideality			
bond length (Å)	0.014	0.013	0.010
bond angles (deg)	3.41	3.05	1.66
torsion angles (deg)	24.5	24.8	25.1
av B (Å^2)	20.2	29.8	31.3
av B, ligand (Å^2)	39.5	30.6	54.7
av B, side chain 229 (Å^2)	12.6	11.5	18.7

23) and DENZO/Scalepack (*24*). Data collection statistics are summarized in Table 1.

Structures and Refinements. Structures were solved in difference Fourier syntheses initially between the measured structure factor amplitudes (F_o) for the mutant complex and the measured structure factor amplitudes (F_o) for the wild-type enzyme (*25*) with reference to the structure of the *L. casei* TS−dUMP complex (*5*). Structures were refined in cycles by fitting to electron density maps calculated using either coeffecients ($2|F_o| - |F_c|$), or ($|F_o| - |F_c|$) and phases calculated from the coordinates, using the density fitting program Chain (*26*) (Figure 3). Automated refinement was by molecular mechanics coupled to restrained least squares using XPLOR (*27*). Refinement statistics are summarized in Table 2.

RESULTS

Binding of dUMP and dCMP to Mutants

Table 3 shows the K_d values of dUMP and dCMP for their binary complexes with wild-type TS and mutants studied here as determined by competition with pyridoxal-5′-phosphate (PLP) (*21*). The K_d values for PLP binding to all the double mutants were below 10 μM, similar to that for TS N229D, and 3−10 fold higher than for wild-type TS. As previously reported, wild-type TS binds dUMP about 500-fold tighter than dCMP; mutation of Asn 229 to Asp resulted

Table 3: Dissociation Constants for Binding of PLP and Nucleotides to *L. casei* TS and Mutants[a]

TS	K_d (μM)			K_d^{dUMP}/K_d^{dCMP}
	PLP	dCMP	dUMP	
wild-type[b]	0.8	160	0.4	0.002
N229D[b]	3.9	2.8	2.7	1
H199G/N229D	8.4	4.0	21.7	5
H199A/N229D	3.5	0.8	40.6	51
H199V/N229D	4.3	3.0	92.7	31
H199N/N229D	4.2	1	70.4	71
H199P/N229D	5.6	0.4	9.8	22
H199R/N229D	2.6	>250	18.2	<0.07

[a] K_d for PLP was measured spectrophotometrically at 328 nm. The K_d values for the nucleotides were estimated by measuring the displacement of bound PLP as described in Materials and Methods. The standard errors for nonlinear least squares fit of the experimental data are <20% for all values. [b] Values for wild-type and N229D TS were from Liu and Santi (8).

Table 4: Steady-State Kinetic Parameters for Methylation of dCMP by *L. casei* TS[a]

TS	k_{cat} (s^{-1})	K_m (μM)		k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	
		dCMP	CH_2H_4folate	dCMP	CH_2H_4folate
wild-type	nd[p]	–	–	–	–
N229D[b]	0.45	150	170	0.003	0.003
H199G/N229D	2.54	12	31	0.22	0.08
H199A/N229D	1.76	6	29	0.29	0.06
H199V/N229D	1.93	13	13	0.15	0.15
H199N/N229D	0.33	8	97	0.04	0.003
H199P/N229D	nd	–	–	–	–
H199R/N229D	nd	–	–	–	–

[a] Kinetic parameters were measured spectrophotometrically as described in Materials and Methods. The standard errors for nonlinear least square fit of the experimental data are <20% for all values. Wild-type TS has a k_{cat} value of 8 s^{-1} and K_m values of 7 and 18 μM, for dUMP and CH_2H_4folate, respectively, for its reaction with dUMP. [b] Values for TS N229D were taken from Liu and Santi (10). nd[p] represents no detectable activity by the spectrophotometric assay (k_{cat} < 0.001 s^{-1}).

in a small increase in K_d for dUMP and a large decrease in K_d for dCMP, resulting in equal affinities for the two nucleotides. Mutation of His 199 of TS N229D to neutral residues G, A, V, N, or P showed little effect on or slightly decreased K_d values for dCMP (up to 7-fold) but had increased K_d values for dUMP (up to 93-fold). The binding specificity for dCMP increased in these mutants, and in the case of TS H199N/N229D gave a mutant with 35 000-fold more specificity for dCMP than wild-type enzyme. Introduction of the large, positively charged Arg residue at position 199 of TS N229D gave a mutant which bound to dCMP very poorly.

Kinetics of dCMP Methylation

Table 4 gives the steady-state parameters for dCMP methylation of the mutants studied here. As previously reported (*10*), wild-type TS does not catalyze detectable methylation of dCMP, but mutation of Asn 229 provides a good catalyst for dCMP methylation; the k_{cat} of TS N229D with dCMP is some 20-fold lower than wild-type TS with dUMP, the K_m for both substrates are 20-fold higher, and the k_{cat}/K_m, or "specificity constant" is about 330-fold lower.

When TS N229D was further mutated to Pro and Arg at position 199, no dCMP methylase activity could be detected (k_{cat} < 0.001 s^{-1}). As shown elsewhere, TS H199R/N229D binds dCMP very poorly or aberrantly, which may be the cause of its lack of catalytic properties; in contrast, the inactive TS H199P/N229D binds well to both substrates yet does not catalyze dCMP methylation. The other four double mutants—N229D with G, A, V, or N at position 199—all catalyze dCMP methylation. It is noted that under conditions of high substrate and enzyme concentrations (3 mM dCMP, 0.5 mM CH_2H_4 folate, and 3 μM enzyme), the single mutant TS H199A showed no detectable dCMP methylase activity. Of the mutants active in dCMP methylation, TS H199N/N229D had a slightly decreased k_{cat} compared to TS N229D, but significantly reduced substrate K_m values. In the remaining three mutants, the introduction of a mutation at position 199 of TS N229D led to significant enhancements in catalytic properties. All showed higher k_{cat} values (up to 5-fold), lower K_m values for both substrates (up to 25-fold), and higher catalytic efficiencies (up to 100-fold). Catalytic efficiencies of dCMP methylation by the most active double mutants (H199G, A, V/N229D) were comparable to those for wild-type TS toward its normal substrate dUMP. For example, the $k_{cat}/K_m^{nucleotide}$ for TS H199A/N229D was only 4-fold less with dCMP than it was for wild-type enzyme with dUMP.

Kinetics of dTMP Formation

Of the six TS H199X/N229D mutant enzymes studied, only TS H199P/N229D catalyzed the formation of dTMP at a rate detectable by the spectrophotometric analysis (k_{cat} ca. 1.5×10^{-2} s^{-1}). However, all of the six double mutants catalyzed dTMP formation when the product was monitored by HPLC after prolonged incubation. The more sensitive tritium release assay was therefore employed to obtain the kinetic parameters for the TS double mutants for their reaction with dUMP. This assay monitors the enzyme catalyzed release of ^3H from [2-^{14}C, 5-^3H] dUMP upon methylation. The tritium release assay was used to obtain kinetic parameters for dTMP formation by the two mutants, TS H199A/N229D and TS H199V/N229D which were most efficient dCMP methylases. Introduction of the H199 mutations in TS N229D results in opposite effects for dUMP methylation as compared to dCMP methylation; that is, there are large decreases in k_{cat} for dUMP methylation, and significant increases in the k_{cat} for dCMP. In general, the K_m effects were similar to effects on binding of these nucleotides. These mutants showed extremely low k_{cat} (ca. 10^{-3} s^{-1}) and high K_m (300−500 μM) values toward dUMP (Table 5). The k_{cat} for dTMP formation by each of these enzymes was approximately 10^{-3} s^{-1}.

Selected samples from the tritium exchange reaction were also subjected to HPLC to verify that tritium release corresponded to dTMP formed. For TS H199A/N229D and TS H199V/N229D, the catalytic rates measured by tritium release were similar to those obtained from HPLC assay of the ^{14}C-labeled product (data not shown).

Substrate Discrimination by TS H199X/N229D Double Mutants

The substrate specificity, or ability of an enzyme to discriminate among two or more substrates can be measured by comparing catalytic efficiencies, or k_{cat}/K_m (*28*). Table 5 shows the kinetic parameters for dUMP methylation by wild-type TS, TS N229D, TS H199A/N229D and TS H199V/N229D and compares their specificity for methylation of dUMP versus dCMP. As reported (*10*), dCMP

Table 5: Steady-State Kinetic Parameters for Methylation of dUMP and Nucleotide Discrimination by *L. casei* TS[a]

TS mutant	kinetic parameters for dUMP			k_{cat}/K_m^{dCMP} [b] / k_{cat}/K_m^{dUMP}
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	
wild-type[c]	8	7	1.1	–
N229D[d]	3.7×10^{-3}	50	7.4×10^{-5}	0.4×10^2
H199A/N229D	1.9×10^{-3}	280	6.8×10^{-6}	0.4×10^5
H199V/N229D	0.7×10^{-3}	470	1.5×10^{-6}	1×10^5

[a] dUMP methylation activity was estimated for TS H199A/N229D and H199V/N229D employing the tritium release assay, and 5 and 13 μM enzymes were used for H199A/N229D and H199V/N229D mutants, respectively. The standard errors for nonlinear least squares fit of the experimental data are <20% for all values. [b] k_{cat}/K_m values for dCMP were taken from Table 4. [c] Kinetic parameters for the wild-type enzyme were monitored by the spectrophotometric assay. [d] Values for TS N229D were estimated by the spectrophotometric assay and were taken from Liu and Santi (10).

methylation by wild-type TS is undetectable, but the N229D mutation shows a 40-fold higher specificity for dCMP than dUMP methylation. Further introduction of an Ala or Val mutation at position 199 of TS N229D results in an additional 1000−2500-fold increase in specificity over TS N229D, providing catalysts which have ~100,000-fold higher specificity for methylation of dCMP versus dUMP.

TS H199A-dCMP Structure

In the structure of wild-type TS bound to dCMP, the pyrimidine base was positioned outside the normal pyrimidine binding site and did not hydrogen bond to Asn 229 (Figure 4, white structure) (*12*). While it seemed probable that reorientation of the asparagine carboxamide group was not favorable, and that dCMP was excluded from the dUMP binding site by incompatible hydrogen bonding properties of asparagine, it also seemed possible that a steric clash between His 199 and dCMP interfered with dCMP binding in the wild-type enzyme. The crystal structure of the single mutant TS H199A−dCMP complex was determined to assess whether loss of the His 199 side chain allowed dCMP to bind to TS in a similar orientation as dUMP in wild-type TS (*12*). This structure shows that the cytosine ring is dislodged from the active site with a single hydrogen bond of 2.8 Å between Asn 229 Oδ 2 and the 4-NH_2 of dCMP (Figure 4, dark grey structure). The dCMP 3-N and the Asn 229 Nδ 1 are not within hydrogen bonding distance of each other at 3.3 Å apart. The dCMP C-6 is, however, at a similar distance and angle, 4.0 Å and 106°, from the Cys-198 Sγ to that observed in the TS N229D−dCMP complex. The average crystallographic *B*-factors for the H199A−dCMP structure are 20.2 Å^2 for the complex, 39.5 Å^2 for the dCMP, and 12.6 Å^2 for Asn 229 (Table 2). Thus, the Asn 229 carboxamide does not reorient to hydrogen bond to dCMP, and the lack of hydrogen bonding excludes dCMP from the dUMP binding site in the H199A mutant. Only when Asn 229 is changed to an aspartic acid, allowing hydrogen bonding to dCMP to occur, does steric interference of His 199 become a factor in dCMP orientation.

TS H199A/N229D−dCMP Structure

The crystal structure of the TS H199A/N229D−dCMP complex shows that both the hydrogen bonds between dCMP and Asp 229 are made and are similar to the wild-type TS−dUMP complex (Figures 2f and 5). Asp 229 Oδ 1 is 2.8 Å from dCMP 3-N (presumably Oδ 1 is protonated, thereby acting as hydrogen bond donor), and Asp 229 Oδ 2 is also 2.8 Å from dCMP 4-NH_2. dCMP 4-NH_2 has moved significantly back toward the wall of the active site cavity relative to its position in the TS N229D−dCMP complex. The orientation of the dCMP is essentially identical to the orientation of dUMP in wild-type TS−dUMP, as seen when the TS−dUMP and H199A/N229D−dCMP complexes are overlapped (Figure 5). The average crystallographic *B*-factors for H199A/N229D−dCMP are: 29.8 Å^2 for the complex, 30.6 Å^2 for the dCMP, and 11.5 Å^2 for Asp 229 (Table 2). When comparing the wild-type TS−dUMP and TS H199A/N229D−dCMP complexes with *B*-factors included, no significant difference in position at greater than a 2σ level is observed between dUMP and dCMP (29). The distance between dCMP C6 and Cys-198 Sγ is 3.3 Å and the attack angle C5−C6−Sγ is 88°, which is the same as in wild-type TS−dUMP.

TS H199A/N229D−dUMP Structure

In the crystal structure of the TS H199A/N229D−dUMP complex (Figure 6, dark grey structure), dUMP is more disordered than dCMP in wild-type TS−dCMP, even though the K_d for dUMP binding to the double mutant is 4-fold lower than for dCMP binding toTS. The average crystallographic *B*-factors (thermal) for TS H199A/N229D−dUMP are 31.3 Å^2 for the complex, 54.7 Å^2 for the dUMP, and 18.7 Å^2 for Asp 229 (Table 2). The density for the uridine ring suggests that it is in a range of orientations, with changes in the deoxyribose conformation pivoting the pyrimidine ring at N1 which results in this spectrum of orientations (data not shown). This result reflects weak or absent hydrogen bonding of dUMP 4-O and 3-N to Asp 229, since both 4-O and 3-N have *B*-factors greater than 60 Å^2. The side chain of Asp 229 is itself well-ordered and has the same orientation as in the wild-type TS−dUMP complex (Figure 6, white structure) and in the single mutant, TS N229D−dUMP complex (Figure 6, light grey structure). In this double mutant complex, Asp 229 Oδ 2, which hydrogen bonds to His 199 Nϵ2 in the single mutant TS N229D, is 3.2 Å from ordered water occupying the space immediately adjacent to the space vacated by the His 199 side chain (Figure 6, dark grey structure).

DISCUSSION

We have previously shown that mutation of Asn 229 of TS to Asp results in a remarkable change in substrate specificity of the enzyme (*10*). The changes include both appearance of a previously undetectable dCMP methylase activity, and greater than a 10^4-fold reduction in k_{cat}/K_m of dUMP methylase activity. TS N229D shows a 40-fold preference for methylation of dCMP versus dUMP. In the present work, we have used a structure−function approach to guide mutagenesis of TS N229D toward an enzyme even more specific for methylation of dCMP.

Crystallographic studies suggested that His 199 may play a role in determining the orientation of nucleotide substrates within the active site which, in turn, could affect substrate activity and specificity (*12*). In the *L. casei* TS binary complex with dUMP, dUMP 4-O is within H-bonding distance of the imidazole CH of His 199. In the *E. coli* ternary complex of TS with dUMP and the folate analog 10-propargyl-5,8-dideazafolate (CB3717) (*6*), His-199 (146

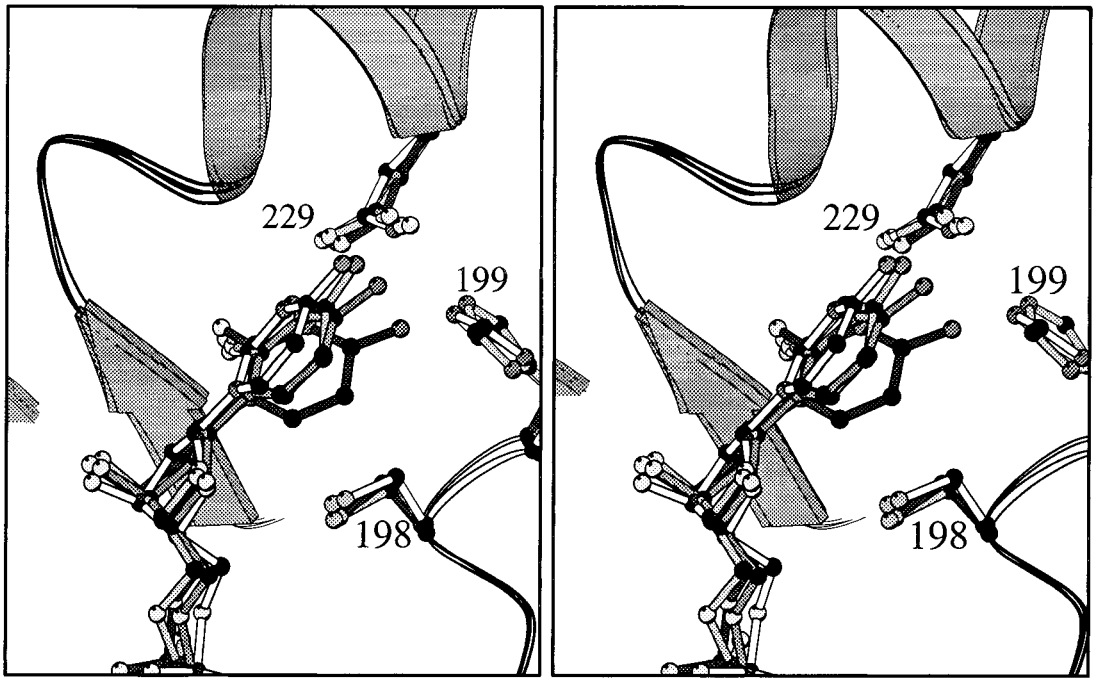

FIGURE 4: Superposition of the binary complexes of dCMP with wild-type TS (white), TS N229D (light grey), TS H199A (dark grey), and TS H199A/N229D (black) shows the gradual fall of dCMP into the nucleotide binding pocket with combined active site mutations. By regenerating the hydrogen bonds which fix the pyrimidine base within the active site, the enzyme methylation activity increases.

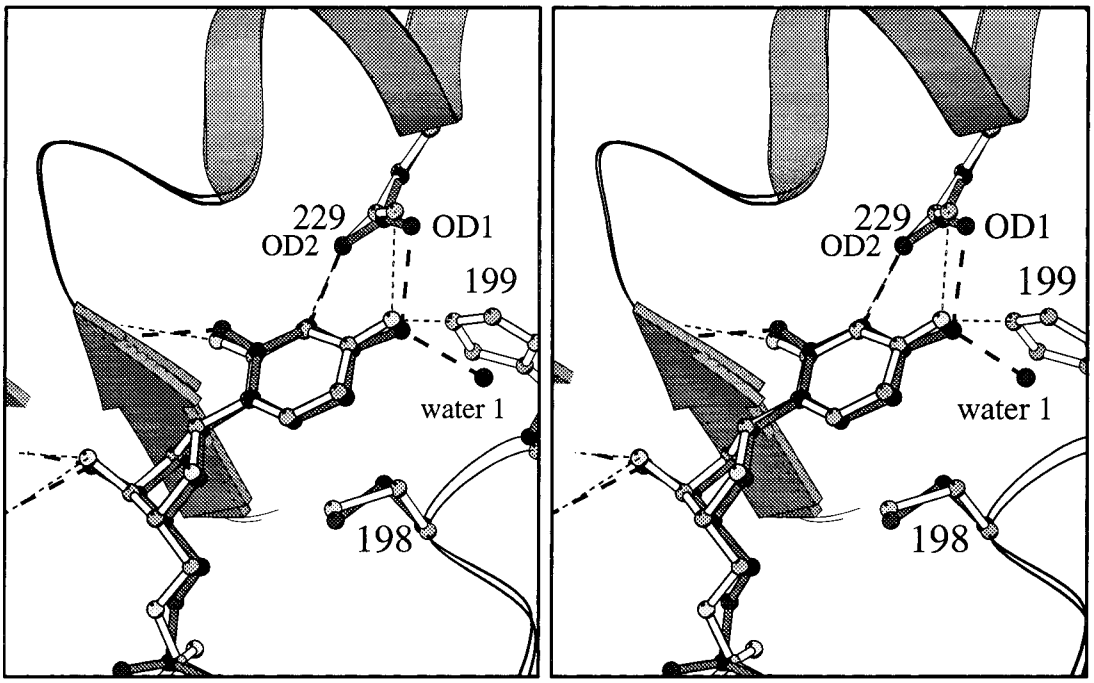

FIGURE 5: Superposition of the binary complexes, wild-type TS−dUMP and TS H199A/N229D−dCMP, shows the simliar hydrogen bond networks between the pyrimidine base and side chains of residues 229 and 199 and the ribose ring with the protein backbone. dCMP in the double-mutant, TS H199A/N229D (black), falls into the same position and orientation as dUMP with the wild-type TS (white) with respect to the catalytic Cys-198.

in *E. coli* TS) is indirectly hydrogen bonded to 4-O of dUMP via a conserved water (Wat 1 in Figures 2a,f and 3) which may serve as a general catalyst in proton transfer reactions at 4-O. Thus, although His 199 is not essential for catalysis (*13*, *14*), it may contribute to orienting the pyrimidine of dUMP in a position optimal for binding and catalysis.

In the TS N229D−dUMP complex, the 4-O of dUMP is 4 Å from His 199, and the pyrimidine is displaced from its "normal" binding pocket (Figure 6, light grey structure). Here, the disturbance in nucleotide orientation must be caused by the substitution of Asn by Asp, resulting in a moderate loss in binding and greatly reduced activity. In the TS N229D−dCMP complex, steric collision of 4-NH_2 of cytosine with the imidazole of His 199 causes displacement of the cytosine moiety from the position normally occupied by uracil in the wild-type TS−dUMP complex (Figure 4, light grey structure). Whereas His 199 may contribute to binding and catalytic efficiency in wild-type TS, it is too far from the 4-O to contribute in TS N229D (Figure 6, light grey structure), and it is involved in a steric clash with the 4-NH_2 of dCMP in TS N229D (Figure 4, light grey structure).

If this "steric clash" hypothesis is correct, one would predict that substitution of His 199 in TS N229D by residues

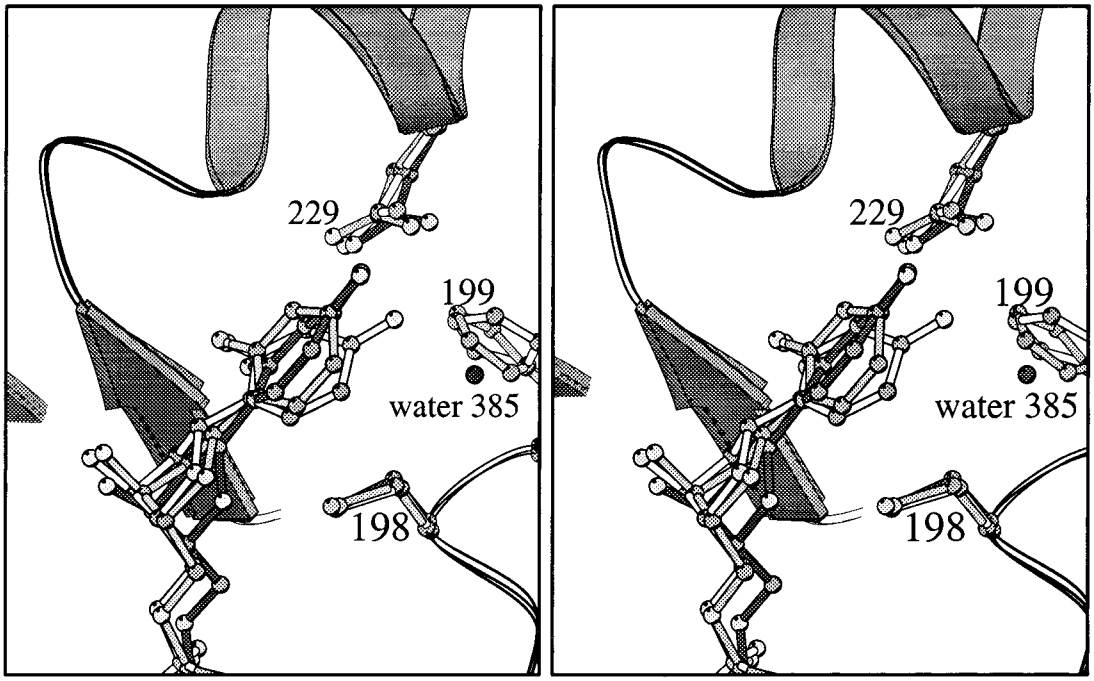

FIGURE 6: Superposition of the binary complexes of dUMP with wild-type TS (white), TS N229D (light grey), and TS H199A/N229D (dark grey) shows the nucleotide ejection from the active site. With progessive mutations in the active site, dUMP moves out of the nucleotide binding pocket. dUMP has weakened interactions with the protein as observed with lost hydrogen bonds and a water (numbered 385) satisfying an Asp 229 hydrogen bond.

which do not cause conflicts with dCMP in the "optimal" site of pyrimidine binding would (a) increase the catalytic efficiency of dCMP methylation and (b) have little effect on the catalytic efficiency of dUMP methylation compared to TS N229D; overall, this would increase the specificity for methylation of dCMP *versus* dUMP in mutants with smaller side chains at residue 199 in TS N229D. To test this hypothesis, we prepared six mutants of TS N229D in which the imidazole of His 199 was replaced by side chains which are small (Gly, Ala), hydrophobic (Val), polar-uncharged (Asn), and large and positively charged (Arg); we also prepared the H199P/N229D mutant because TS H199P was highly active in dUMP methylation (unpublished data). We found that TS N229D containing proline, or the positively charged arginine at position 199 no longer catalyzed dCMP methylation; therefore we focused on the remaining four His 199 mutants of TS N229D: His 199 to Gly, Ala, Val, and Asn.

Mutation of His 199 in TS N229D to neutral residues G, A, V, and N generally led to increased k_{cat} values and decreased K_m values for dCMP methylation, resulting in significantly enhanced specificity, as measured by k_{cat}/K_m. Binding of dCMP to TS N229D to form binary complexes was not affected by mutation of His 199, so the K_m decreases are attributed to events unique to the ternary complexes with cofactor. Catalytic efficiencies of dCMP methylation by the most active double mutants, N229D/H199G, A and V, were comparable to those for wild-type TS toward its normal substrate dUMP.

Changes in transition state stabilization energy caused by the individual mutations, N229D, H199A, H199V, and the combinations, H199A/N229D and H199V/N229D, are calculated to be 5.69, 1.59, 1.53, 7.1, and 7.99 kcal/mol, respectively. The sum of the free energy changes caused by the individual mutations N229D and H199X are close to those observed for the free energy changes for H199X/N229D double mutants. This indicates that His 199 and Asn 229 do not interact functionally in the TS-catalyzed reaction. Likewise, in the crystal structure of the free enzyme, no apparent structural interactions between these two residues are observed.

In contrast to the results for dCMP methylation, mutation of His 199 of TS N229D to the small neutral residues G, A, V, and N generally led to moderately increased K_m values and decreased k_{cat} values for dUMP methylation, together resulting in significantly reduced dUMP k_{cat}/K_m values. Here, K_d values for the binary TS−dUMP complexes increased moderately as did K_m. The crystal structure of the double mutant complex shows that dUMP is more disordered than in the TS N229D−dUMP structure, and positioned further from the wild-type binding site (Figure 6, dark grey structure). The detrimental effect on dUMP binding upon mutating His 199 results from replacing the imidazole, a hydrogen bond partner of Asp 229 in the single mutant, with an ordered water solvating the Asp 229 carboxyl and partially blocking the normal binding site for the pyrimidine base of dUMP. Since dUMP 4-O cannot hydrogen bond to Asp 229, displacement of this water of solvation is energetically costly and K_m for dUMP increases. The increase in K_m for dUMP contributes to the enhanced specificity for dCMP methylation.

The substrate specificity, or ability of an enzyme to discriminate among two or more substrates, can be measured by comparing the catalytic efficiencies, or k_{cat}/K_m values, of the substrates (*30*). For the two-substrate reaction of interest here, the relevant k_{cat}/K_m for specificity comparisons is that determined for the variable nucleotide substrate, under conditions of saturating cofactor. Methylation of dCMP by wild-type TS is undetectable, so it is impossible to assign an exact value for k_{cat}/K_m. However, if as previously determined, we could detect a rate of at least 10^{-4} μmol/min/mL by the spectrophotometric assay under conditions used here (20), and use the measured K_d of dCMP to wild-type TS as an approximation of K_m, we can estimate that

k_{cat}/K_m for dCMP with wild-type TS is less than 10^{-6} s^{-1} μM^{-1} ; using $k_{cat}/K_m = 1$ s^{-1} μM^{-1} for dUMP methylation, the specificity of wild-type TS for dUMP versus dCMP must be greater than 10^6. The TS N229D mutation shows a 40-fold higher specificity for methylation of dCMP *versus* dUMP (*10*). Further introduction of an Ala or Val mutation at position 199 of TS N229D results in an additional 1000−2500-fold increase in specificity over TS N229D, providing catalysts which have ∼100 000-fold higher specificity for methylation of dCMP versus dUMP. Thus, compared to wild-type TS, the change in specificity for dCMP versus dUMP in these mutants is at least 10^{11}.

The results and arguments from the steady state kinetic experiments are in accord with the hypothesis that steric displacement of the pyrimidine ring of dCMP by His 199 is detrimental to catalytic efficiency of dCMP methylation by TS N229D, and that substitution of His 199 by other residues can relieve this detrimental effect. To test this hypothesis, the structure of the dCMP complex with TS H199A/N229D was determined (Figures 3 and 4, black structure). The crystal structure shows that, as in the TS N229D−dCMP complex, the complex retains both the hydrogen bonds from the Asp 229 to the cytosine ring of dCMP. However, there is no steric clash between the 4-NH_2 and residue at position 199, the orientation of the dCMP is essentially identical to the orientation of dUMP in wild-type TS−dUMP, and the distance between C-6 of dCMP and Cys-198 is as in wild-type TS−dUMP (Figure 5). Thus, in going from TS N229D−dCMP to TS H199A/N229D−dCMP, there is repositioning of the pyrimidine ring in the active site to an orientation more favorable for catalysis.

The efficacy of conversion of binary enzyme−nucleotide complexes to products can be compared by k_{cat}/K_m values for CH_2H_4folate under conditions of saturating nucleotide; these parameters reflect the energy barrier in going from binary complex to transition state. In the TS N229D−dCMP complex, the distance between Cys-198 Sγ and C6 is 4.0 Å, which is 0.6 Å further than the optimal distance of 3.4 Å in the TS−dUMP complex (*5*) and too large for Michael addition at C6 of dUMP with optimal efficiency (Figure 1); the attack angle is 108°, which is close to the ideal angle of 90° found in the TS−dUMP complex. The TS H199A/N229D−dCMP structure reverts to the optimal proximity and orientation of the sulfhydryl and C6 observed in the wild-type TS−dUMP complex (Figure 5). The change in the distance between dCMP C6 and the Sγ of Cys-198 of 0.6 Å on going from TS N229D to TS H199A/N229D is accompanied by an increase of 20-fold in k_{cat}/K_m for CH_2H_4-folate or ∼1.5 kcal/mol in the transition state energy. Thus, a deviation of only 0.5 Å from the idealized position in the ground state may be a contributing factor in achieving optimal enzyme activity.

In summary, by tuning the orientation and alignment of dCMP bound to the active site of TS, we have engineered a dCMP methylase which shows high activity for a reaction that cannot be catalyzed by wild-type TS. Two mutations in wild-type TS, i.e. Asn 229 to Asp and His199 to Ala, resulted in a change of 10^{11} in the substrate specificity of the enzyme for dCMP *versus* dUMP. Structural comparisons of wild-type and mutant enzymes confirmed (i) the appropriate alignment of the catalytic Asp 229 with the 3N and 4-NH_2 of dCMP, (ii) the relief of steric clash between residue 199 and the 4-NH_2 of dCMP, and (iii) the appropriate proximity and orientation of the catalytic sulfhydryl with C6 of the substrate.

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