Asparagine 229 Mutants of Thymidylate Synthase Catalyze the Methylation of 3-Methyl-2′-deoxyuridine 5′-Monophosphate†

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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 2′-deoxyuridine 5′-monophosphate (dUMP). Asn 229 is not essential for substrate binding or catalysis [Liu, L., & Santi, D. V. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 8604−8608] but is a major determinant in substrate specificity [Liu, L., & Santi, D. V. (1993) *Biochemistry 32,* 9263−9267]. 3-Methyl-dUMP (3-MedUMP) is neither a substrate nor an inhibitor of wild type TS but is converted to 3-methyl 2′-deoxythymidine 5′-monophosphate by many TS Asn 229 mutants. Some of the Asn 229 mutants (N229C, -I, -M, -A, and -V) have k_{cat} values for 3-MedUMP methylation which are up to about 20% of that for wild type TS-catalyzed methylation of dUMP, and some mutants (N229C and -A) catalyze methylation of 3-MedUMP more efficiently than that of dUMP. Mutants with hydrophobic side chains tended to be more active in catalysis of methylation of 3-MedUMP than those with hydrophilic side chains. The ability of 3-MedUMP to serve as a substrate for Asn 229 mutants shows that the active form of dUMP involves the neutral pyrimidine base and that ionization of the 3-NH group does not occur in the course of catalysis. In contrast to the negligible binding of 3-MedUMP to wild type TS, both 3-MedUMP and dUMP showed similar K_m values with the Asn 229 mutants, suggesting similar binding affinities to the mutants. The X-ray crystal structure of the TS N229C−3-MedUMP complex showed that the side chain of Cys 229 was rotated away from the pyrimidine ring to allow placement of a water molecule and the 3-methyl group of 3-MedUMP in the active site. Our results suggest that the inability of 3-MedUMP to undergo methylation by wild type TS is due to its inability to bind to the enzyme, which in turn is simply a result of steric interference of the 3-methyl group with the side chain of Asn 229.

Thymidylate synthase (TS,[1] EC 2.1.1.45) catalyzes the reductive methylation of dUMP by 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) to give dTMP and 7,8-dihydrofolate. Much is known about the structure and function of TS [for review see Carreras and Santi (1995)]. Sequences of over 29 TSs from different sources have been determined, X-ray crystal structures of free and bound enzyme forms have been solved, and numerous mutants of the enzyme have been prepared and examined.

The interaction of the pyrimidine moiety of dUMP with TS has received considerable attention because of its relevance to inhibitor design, enzyme mechanism, and substrate specificity. The crystal structure of the TS−dUMP complex showed that the conserved Asn 229 of TS forms a cyclic H bond network between the enzyme and the 3-NH and 4-O of the pyrimidine ring of the substrate (Matthews et al., 1990; Montfort et al., 1990). It was originally believed that this network contributed to catalysis by assisting in concerted proton transfers to and from the 4-O at various stages of the catalytic cycle. However, it was subsequently shown that Asn 229 of TS could be changed to most other amino acids without large losses in binding or activity (Liu & Santi, 1993a). Although these experiments clearly demonstrated that Asn 229 and its H bonds to dUMP were not essential to binding or catalysis, they did not address the importance of the H bond partners, viz. the 3-NH and 4-O of dUMP.

It is not known whether the 3-NH of dUMP is important for binding or catalysis or what ionized form(s) of the pyrimidine binds and reacts with the enzyme during catalysis. An approach to such questions would be to investigate the enzymatic methylation of 3-MedUMP, where the H bond-donating ability and ionization of the 3-NH is prevented by alkylation. The effect of the 3-methyl group on other chemical properties of the pyrimidine ring is anticipated to be small, although the steric bulk of the methyl group could affect binding or reactivity with the enzyme. It has been reported that 3-MedUMP is not a substrate for TS (Holy &

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[1] Abbreviations: TS, thymidylate synthase; dUrd, 2′-deoxyuridine; 3-MedUrd, 3-methyl-2′-deoxyuridine; dUMP, 2′-deoxyuridine 5′-monophosphate; 3-MedUMP, 3-methyl-2′-deoxyuridine 5′-monophosphate; 3-MedThd, 3-methyl-2′-deoxythymidine; dTMP, 2′-deoxythymidine 5′-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; PLP, pyridoxal 5′-phosphate. Mutants are named as follows. TS N229C refers to the mutant TS containing Cys at position 229.

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

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Vortruba, 1974; Dzik et al., 1987). However, Asn 229 mutants are catalytically active, and the X-ray crystal structure of the TS N229C−dUMP complex revealed that the side chain of Cys 229 rotates away from the pyrimidine ring and leaves a vacancy adjacent to the 3-NH of dUMP (Finer-Moore et al., manuscript in preparation). It seemed possible that 3-MedUMP might bind to and possibly be a substrate for such mutants. In the present work, we describe the binding and methylation of 3-MedUMP by TS Asn 229 mutants.

MATERIALS AND METHODS

Materials, Mutagenesis, and Protein Purification. These were as described previously (Climie et al., 1990; Liu & Santi, 1993a).

Synthesis of 3-MedUMP. 3-MedUMP was prepared by the procedure of Brookes and Lawley (1962), by 3-N methylation of 2′-deoxycytidine with dimethyl sulfate and subsequent deamination in alkaline solution. The identity of the product, 3-methyldeoxyuridine (3-MedUrd), was confirmed by UV spectroscopy and ^{1}H-NMR (Tanabe et al., 1979). The 3-MedUrd was phosphorylated with phosphoryl chloride by the method of Sowa and Ouchi (1983), and the identity of the product 3-MedUMP was confirmed by UV spectroscopy and ^{1}H-NMR (Davies et al., 1974). 3-MedUMP was purified by anion exchange column chromatography on Dowex 1×8 (Cl^-) resin (1.6 × 30 cm column) (200−400 mesh, Fluka, Buchs, Switzerland) equilibrated with 80 mM ammonium formate (pH 4.6). The products were eluted with a linear gradient of 80 to 360 mM ammonium formate (pH 4.6). Two elution peaks were identified by UV spectroscopy, and each was lyophilized. The first peak was 3-MedUMP as characterized by UV spectroscopy (λ_{max} = 262 nm at pH 4) and ^{1}H- and ^{31}P-NMR. The second peak was 3-methyl-2′-deoxyuridine 3′-monophosphate, which was produced during the phosphorylation reaction.

Enzyme Assay. Assays for enzymatic methylation of dUMP were performed as described previously (Liu & Santi, 1993a). The assay for methylation of 3-MedUMP was performed in the standard TES buffer. Enzyme concentrations as high as 7 μM were used with reaction times up to 1 h to ensure detection of low activity for 3-MedUMP methylation. Initial velocities were determined by varying the concentration of CH_2H_4folate or 3-MedUMP in the presence of 800 μM 3-MedUMP or 300 μM CH_2H_4folate, respectively. Steady state kinetic parameters were obtained by a nonlinear least squares fit of the data to the relevant equations by using Kaleidagraph (Abelbeck Software, Reading, PA, 1989).

High-Performance Liquid Chromatography (HPLC) Analysis of Nucleotide Products. HPLC was performed on a Hewlett-Packard 1090 high-performance liquid chromatograph with a diode array detector and an Altex Ultrasphere IP column (4.6 mm × 25 cm); absorbance spectra were collected between 220 and 400 nm for all peaks. The reaction mixture contained 200 μM 3-MedUMP and 200 μM CH_2H_4folate plus either wild type TS or TS N229C in the standard TES buffer. After incubation, the protein was removed by filtration through a microfilterfuge tube (Rainin, Woburn, MA), and the reaction mixture was separated on HPLC by isocratic elution with 5 mM KH_2PO_4, 5 mM tetra-*n*-butylammonium sulfate (pH 7.0), and 5% acetonitrile at a flow rate of 1 mL/min. For analysis of ^{3}H-labeled reactants and products, fractions (1 mL) were collected and counted in 5 mL of Aquasol II.

Table 1: Crystallographic Statistics for the TS N229C−3-MedUMP Complex

space group	*P*6122
unit cell parameters (Å)	$a = 78.8$, $c = 231.1$
resolution (Å)	2.35
σ_{cutoff}	0.0
number of observations	61 608
number of unique reflections	16 918
% complete	87
$I/\sigma(I)$	5.0
$R_{merge} = \sum i[Ii - I_{mean}(i)] \times 100\%$, 1σ data	8.9

Table 2: Crystallographic Refinement Statistics for the TS N229C−3-MedUMP Complex

Parameters	
number of atoms	2663
number of solvent sites	51
B-factor model	restrained, isotopic
average *B*-factor (Å^2)	30
average *B*-factor for 3-MedUMP (Å^2)	64
Diffraction Agreement	
resolution used in refinement (Å)	7−2.5
number of reflections used in refinement	11 657
R_{crys}, 1σ cutoff (%)	20.5
Stereochemical Ideality	
bond lengths (Å)	0.006
bond angles (deg)	1.4
torsion angles (deg)	24.7

Crystallization. Crystals of the *L. casei* TS N229C−3-MedUMP complex were obtained as previously described for the *L. casei* TS−dUMP complex (Finer-Moore et al., 1993). Crystallization drops contained final concentrations of 3.5 mg/mL for mutant protein, 2.0 mM for 3-MedUMP, and 0.5−1% for saturated $(NH_4)_2SO_4$ in 20 mM potassium phosphate (pH 7.0).

X-ray Data Collection and Structure Solution. Data for the TS N229C−3-MedUMP complex were collected on an R-AxisIIC image plate detector system mounted on a Rigaku 18 kW generator run at 60 kV and 50 mA collimated to 0.3 mm using 1.0° wide frames in ω. The crystal to detector distance was set to 21.8 cm, and 2θ was set at 15°. Data were reduced using R-AxisIIC data reduction programs (Higashi, 1990; Sato et al., 1992). Crystallographic statistics are presented in Table 1.

The structure was solved by difference Fourier methods (Chambers & Stroud, 1977) and refined by energy minimization using XPLOR (Brunger et al., 1987). Manual adjustments to the structure were made using CHAIN (Sack, 1988). The ligand and the Cys 229 side chain were built into the initial $(F_o - F_c)\alpha_{calc}$ maps. Water molecules were chosen from the $(F_o - F_c)\alpha_{calc}$ maps after refinement of the protein structure. Individual, restrained *B*-factors were refined for all atoms (Table 2).

RESULTS

Product Identification. 3-MedTMP was identified as the product from the methylation of 3-MedUMP by several TS N229 mutants as follows. HPLC analysis (Figure 1) of the TS N229C-catalyzed reaction showed a new nucleotide product with a longer retention time (26.2 min) than 3-MedUMP (21.6 min). The λ_{max} for the new peak was

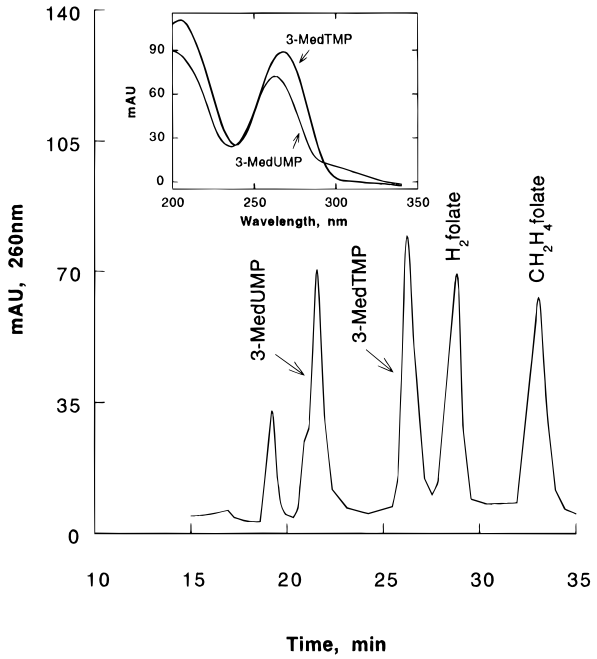

FIGURE 1: Partial HPLC profile of a reaction mixture of 3-MedUMP methylated by the TS N229C mutant. The inset shows the UV spectra of the HPLC peaks indicated.

shifted from 262 nm for 3-MedUMP to 266 nm which is identical to the reported λ_{max} for 3-MedThd (Tanabe et al., 1979) (Figure 1). When [6-^{3}H]CH_2H_4folate was used in the reaction of 3-MedUMP with the TS N229C mutant, the tritium of the cofactor was quantitatively transferred to 3-MedTMP. This experiment is analogous to that which established direct hydride transfer from CH_2H_4folate to dTMP in the normal TS reaction (Pastore & Friedkin, 1962). Further, using limiting 3-MedUMP as substrate, there was an increase in absorbance at 340 nm which corresponded to $\Delta\epsilon_{max}$ (340 nm) of 6590 M^{-1} cm^{-1}. This differential extinction coefficient is characteristic of the conversion of CH_2H_4folate to H_2folate [$\Delta\epsilon_{max}$ (340 nm) of 6400 M^{-1} cm^{-1}] (Wahba, 1961). Together, these data provide convincing evidence that TS N229C catalyzes the reaction of 3-MedUMP with CH_2H_4folate to produce 3-MedTMP and H_2-folate.

Interaction of 3-MedUMP with Wild Type TS. Using high concentrations of wild type TS (0.5 mg/mL) and 3-MedUMP (800 μM), no 3-MedTMP could be detected spectrophotometrically for up to 1 h. Assuming we could detect 10^{-4} unit/mL of TS activity (Carreras et al., 1992), the 3-MedUMP methylase activity of wild type TS was estimated to be less than 2×10^{-4} unit/mg of protein, which is equivalent to a k_{cat} of <0.0001 s^{-1}. Furthermore, when [6-^{3}H]CH_2H_4folate was incubated overnight with wild type TS and 3-MedUMP, the tritium remained with [6-^{3}H]CH_2H_4folate, and no detectable radioactive 3-MedTMP was formed. 3-MedUMP did not inhibit wild type TS at [3-MedUMP]/[dUMP] ratios of up to 34, which equates to a K_i for 3-MedUMP of >125 μM. Concentrations of 3-MedUMP of up to 3 mM did not displace PLP from the TS−PLP complex (3 μM TS and 10 μM PLP) (Liu & Santi, 1993b; Santi et al., 1993), equating to a K_d for 3-MedUMP of >250 μM.

Kinetic Parameters for 3-MedUMP with TS N229 Mutants. The steady state kinetic parameters for methylation of dUMP and 3-MedUMP by thirteen Asn 229 TS mutants are shown in Table 3. TS N229R, -D, -T, and -F showed no detectable

Table 3: Kinetic Constants for Methylation of 3-MedUMP and dUMP by Wild Type and N229 Mutants of *L. casei* TS[a]

	3-MedTMP formation			dTMP formation[b]		
229 residues	k_{cat} (s^{-1})	$K_{m,cofactor}$ (μM)	$K_{m,3\text{-MedUMP}}$ (μM)	k_{cat} (s^{-1})	$K_{m,cofactor}$ (μM)	$K_{m,dUMP}$ (μM)
N229 (wild type)	nd[c]	nd	nd	3.5	10	5
N229C	0.70	110	98	0.22	92	17
N229I	0.53	79	200	1.1	89	150
N229M	0.12	56	97	0.62	84	150
N229V	0.070	72	110	0.86	57	91
N229A	0.060	130	62	0.0015	nd	nd
N229L[d]	0.017	nd	nd	1.1	90	220
N229G[d]	0.0045	nd	nd	0.028	98	15
N229Q[d]	0.0033	nd	nd	0.017	38	11
N229S[d]	0.0016	nd	nd	0.0093	15	39
N229R	<0.001	nd	nd	<0.001	nd	nd
N229T	<0.001	nd	nd	0.077	66	104
N229F	<0.001	nd	nd	<0.001	nd	nd
N229D[d]	<0.001	nd	nd	0.0034	35	50

[a] The standard errors from a nonlinear least squares fit of the experiment data are $<20\%$ for all values. [b] The data for dTMP formation were from Table 1 of Liu and Santi (1993a). [c] Not detectable by spectrophotometric assay. [d] The k_{cat} value was estimated from an assay with 800 μM 3-MedUMP and 300 μM CH_2H_4folate.

3-MedTMP synthase activity ($k_{cat} < 10^{-3}$ s^{-1}). Nine of thirteen TS Asn 229 mutants showed detectable methylation of 3-MedUMP, with k_{cat} values spanning a 400-fold range. The lowest k_{cat} values ($1.6-4.5 \times 10^{-3}$ s^{-1}) were for TS N229S, -Q, and -G; intermediate k_{cat} values ($0.01-0.12$ s^{-1}) were found for TS N229L, -A, -V, and -M; and the highest k_{cat} values for 3-MedUMP methylation were for TS N229I and N229C ($0.5-0.7$ s^{-1}). The latter values correspond to 15−20% of the k_{cat} for dUMP methylation by wild type TS.

The k_{cat} values for TS N229A and TS N229C were 40- and 3-fold greater, respectively, for 3-MedUMP than for dUMP. The k_{cat} values for other mutants for 3-MedUMP were 2−10-fold lower than those for dUMP methylation. There is a clear trend between the ability to catalyze dUMP and 3-MedUMP methylation among the Asn 229 mutants; mutants with hydrophobic side chains were more active than those with hydrophilic residues for both substrates. The K_m values for CH_2H_4folate and 3-MedUMP varied by only 2−3-fold among the five most active mutants. The K_m values for both substrates were comparable for the methylation of 3-MedUMP and dUMP by these five mutants except for TS N229C, which exhibited a 6-fold increase in K_m for 3-MedUMP relative to dUMP (Table 3).

Crystal Structure of the TS N229C−3-MedUMP Complex. Weak density for the pyrimidine ring and the ribose hydroxyl group was visible in an initial $(F_o - F_c)\alpha_{calc}$ map phased with protein alone. The pyrimidine density was not well-resolved in the initial or final omit maps, suggesting thermal or statistical disorder. 3-MedUMP was fit to density by using the better-resolved ribose density to position the ribose ring, while the pyrimidine base in density was centered as well as possible. Neither positional refinement nor simulated annealing refinement (Brunger et al., 1987) significantly changed the position of 3-MedUMP deduced from the original difference map.

There was a translation of less than 1 Å of 3-MedUMP relative to dUMP in wild type TS which shifted the pyrimidine ring toward the long helix that forms part of the hydrophobic core in TS (Figure 2). The 3-methyl moiety was buried in a small cavity between the base of the long

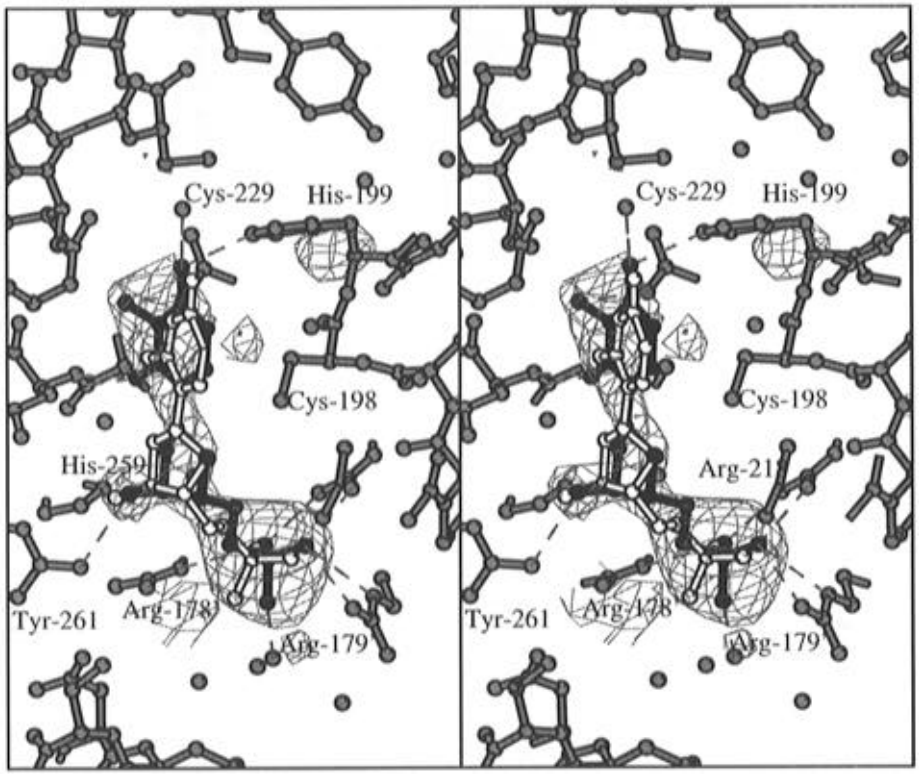

FIGURE 2: $(F_o - F_c)\alpha_{calc}$ omit map, calculated without coordinates for 3-MedUMP, showing the difference density for 3-MedUMP. 3-MedUMP is drawn with black bonds. Overlaid for camparison is dUMP from the TS N229C−dUMP complex, drawn with white bonds. Hydrogen bonds to 3-MedUMP are shown with dashed lines.

hydrophobic helix forming the core of the protein and the β-sheet interface. It was surrounded by backbone atoms of residues Ala 220, Asp 221, Gly 225, and Val 226 and the side chains Val 226 and Gln 217. The sulfhydryl group of Cys 229 was rotated out of the active site cavity, and an ordered water molecule occupied the space filled by the Asn 229 Nδ in the wild type enzyme. The 4-O of 3-MedUMP was hydrogen-bonded to this water molecule and possibly to an imidazole nitrogen of His 199. The 6-C of the pyrimidine base was well-oriented for Michael addition by the cysteine sulfhydryl and was ~3 Å from this atom. However, interatomic distances between 3-MedUMP and surrounding residues on the protein could not be determined very precisely because of the high temperature factors for 3-MedUMP pyrimidine and ribose moieties, ~75 Å^2. dUMP was not similarly shifted toward the hydrophobic helix in the TS N229C−dUMP complex. Rather, in this complex, the pyrimidine base was moved directly out from the active site cysteine and His 199, such that the distance from 6-C of dUMP to Cys 198 Sγ was 3.9 Å (compared to 3.2 Å in the wild type TS−dUMP complex) (Finer-Moore et al., manuscript in preparation) (Figure 2).

Most of the hydrogen bonds between protein and dUMP seen in the wild type TS−dUMP binary complex were also present in the TS N229C−3-MedUMP complex, including hydrogen bonds to the guanidinium groups of Arg 178′, Arg 179′, and Arg 218 and to Asp 221 NH, Ser 217 Oγ, His 256 Nϵ, and Tyr 261 Oη (Figure 2). However, the side chain of Arg 23 was disordered in the TS N229C−3-MedUMP complex.

DISCUSSION

The objective of the present work was to assess the importance of the 3-NH of dUMP to TS binding and catalysis. In binary and ternary complexes of TS with dUMP, the conserved Asn 229 forms a H bond network with the 3-NH and 4-O of the substrate. This network is important to substrate specificity in that the side chain of Asn 229 can "exclude" adjacent groups that interact unfavorably, such as the cytosine ring of dCMP (Liu & Santi, 1993b). However, mutagenesis studies indicate that the network *per se* is not essential for catalysis and that it does not provide net binding energy for the TS−dUMP interaction. Nevertheless, such studies did not address the issue of whether the 3-NH of dUMP is in some way important to the intrinsic binding or reactivity of the substrate. One approach to test for the importance of the 3-NH of dUMP was to examine the properties of 3-MedUMP as a substrate for TS and TS mutants. The *N*-methyl group precludes ionization at the 3-N of the pyrimidine and perturbations of reactivity which result from electronic effects of ionization; except for steric and H-bonding effects, 3-MedUMP should have similar properties to the completely un-ionized form of dUMP. However, since 3-MedUMP does not bind to wild type TS, one cannot assess whether the 3-MedUMP−TS complex has the intrinsic ability to undergo the catalytic reaction.

On the basis of other data, we suspected that Asn 229 mutants might accept 3-MedUMP as a substrate. First, TS Asn 229 mutants which do not have the ability to H bond with the 3-NH of dUMP bind to and catalyze methylation of dUMP. Second, X-ray structural analysis of a binary complex of one of these mutants with dUMP, the TS N229C−dUMP complex, indicates that the 229 side chain rotates away from the pyrimidine ring and produces a vacancy adjacent to the 3-NH which is partially filled by a water molecule. Thus, it was reasonable to suspect that Asn 229 mutants might bind to 3-MedUMP and permit its evaluation as a substrate.

Indeed, nine of the thirteen TS Asn 229 mutants examined catalyzed the methylation of 3-MedUMP to form 3-MedTMP (Table 3). The steady state kinetic parameters for methylation of 3-MedUMP by the active Asn 229 mutants were generally similar to those for their methylation of dUMP. As in the normal reaction, many of the Asn 229 mutants most active in methylation of 3-MedUMP have extended hydrophobic side chains. This may reflect the ability of the side chain at position 229 to rotate away from the pyrimidine and/or create a favorable reaction environment for a hydrophobic 3-methyl group of 3-MedUMP in the absence of the normal H bond network. TS N229C is one of the most active and most studied Asn 229 mutants. This mutant catalyzes the methylation of 3-MedUMP with a k_{cat} value 3-fold higher than that for dUMP. Thus, when the steric repulsion of Asn 229 is relieved by mutagenesis, many of the resultant enzymes can catalyze methylation of 3-MedUMP as effectively as they catalyze methylation of dUMP.

The aforementioned observations clearly show that the proton at 3-N of dUMP is not essential for TS catalysis. Nevertheless, as part of the H bond network with Asn 229 in the wild type enzyme, the 3-NH may contribute to some rate enhancement. Also, although the H bond network formed between Asn 229 and 3-NH and 4-O of dUMP contributes little to net binding energy (Liu & Santi, 1993b), it is clearly effective at discrimination between the normal substrate dUMP and congeners such as 3-MedUMP.

To gain further insight on the binding of 3-MedUMP to TS, the X-ray structure of the TS N229C−3-MedUMP binary complex was solved and compared to those of the TS−dUMP and TS N229C−dUMP binary complexes. First, most of the hydrogen bonds seen in the TS−dUMP binary complex are also present in the TS N229C−3-MedUMP complex. One exception is the side chain of Arg 23, which is H-bonded to the phosphate of dUMP in the TS−dUMP complex but disordered in the TS-3-MedUMP complex. This may partially account for the 6-fold lower K_m for dUMP relative to 3-MedUMP in TS N229C. Second, the distance between 6-C of the pyrimidine ring and the thiol of Cys 198 is ~3 Å in the TS N229C−3-MedUMP complex compared with 3.9 Å in the TS N229C−dUMP structure (Finer-Moore et al., unpublished). The shortened distance between the reactive atoms may account for the 3-fold higher k_{cat} for methylation of 3-MedUMP than for methylation of dUMP, assuming these distances persisted in their ternary complexes with the cofactor. Third, the side chain of Cys 229 was rotated out of the active site cavity in the TS N229C−3-MedUMP complex, and an ordered water molecule occupied the space normally filled by Asn 229 Nδ in the wild type enzyme. This water molecule is H-bonded to the 4-O of 3-MedUMP in the complex and may serve as a general acid−base catalyst for proton transfer reactions at the 4-O of 3-MedUMP (Liu & Santi, 1993a). The binding specificity of wild type TS for dUMP versus 3-MedUMP may thus be simply explained by a steric intolerance which does not allow concurrent occupancy of the side chain of Asn 229 and the 3-methyl group; relief of this steric effect, as in the TS Asn 229 mutants, allows the protein to bind 3-MedUMP in a configuration similar to that of dUMP in the normal binary complex.

One of the questions we addressed in this study was whether partial or complete ionization of the 3-NH was important during catalysis. 3-MedUMP cannot undergo any

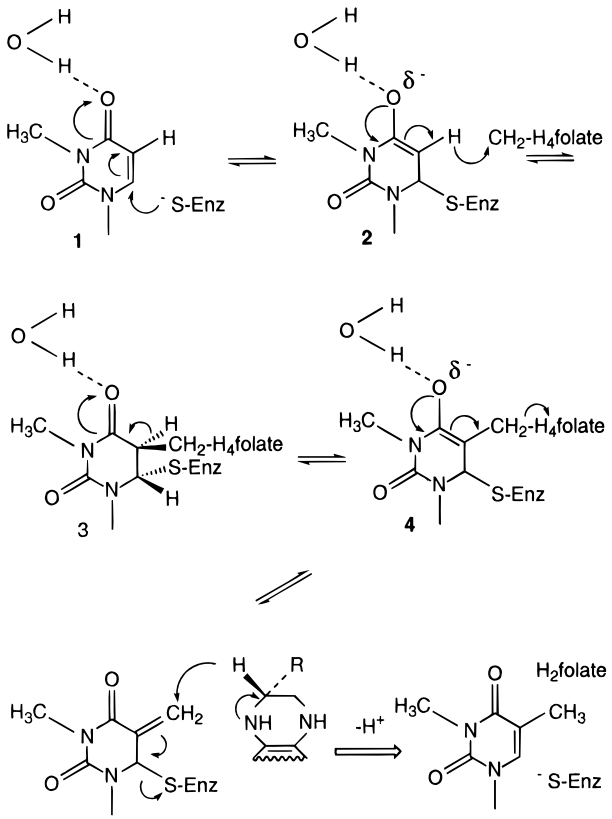

FIGURE 3: Proposed basic features of the catalytic mechanism of 3-MedTMP formation by TS Asn 229 mutant enzymes.

such ionization, so it may be considered a stable congener of the neutral form of the 4-keto tautomer of dUMP. The fact that TS N229C catalyzes methylation of 3-MedUMP faster than methylation of dUMP shows that ionization of the 3-NH of dUMP is neither necessary nor advantageous for the mutants. However, k_{cat} for methylation of dUMP for TS N229C is still some 10-fold lower than that for wild type TS, and it is possible that minor differences in mechanism occur between wild type and mutant enzymes. It is possible that partial ionization of the 3-NH occurs via the Asn 229 H bond network and plays some rate-enhancing role with the wild type enzyme and normal substrate. Nevertheless, the results presented here clearly show that the H bond-donating ability of the 3-NH of dUMP which occurs with the normal substrate and wild type enzyme is not an important feature of catalysis.

The results described here also allow some speculation on the role of the 4-O of dUMP as a H bond acceptor of the carboxamide side chain of Asn 229. Since Asn 229 mutants unable to form a H bond to the O-4 of dUMP catalyze methylation of dUMP and 3-MedUMP, the Asn 229 itself is not essential. However, as noted above, in the N229C−3-MedUMP complex, an ordered water molecule replaces the Asn 229 in formation of a H bond to the O-4 of the pyrimidine. It is possible that this water molecule preserves an essential function as a general acid−base catalyst in proton transfers at O-4 (Figure 3). If so, this observation adds to a growing body of evidence that the important general acid−base catalysts of the TS reactions are served simply by ordered water molecules.

Methylation of 3-MedUMP catalyzed by TS Asn 229 mutants may serve as the foundation for further or new structure−reactivity studies of the TS reaction. Additional

modifications at the 3-position of dUMP with different electronic properties at the sites of reaction, i.e. 4-O, 5-C, and 6-C, may offer a series of substrates for TS Asn 229 mutants. In this manner, after steric tolerance of 3-substituents is allowed for, it may be possible to identify some features of transition state structure during catalysis which cannot be accessed in any other manner.

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