Tyrosine 146 of Thymidylate Synthase Assists Proton Abstraction from the 5-Position of 2′-Deoxyuridine 5′-Monophosphate†

Yaoquan Liu,‡ Jeannie E. Barrett,§ Peter G. Schultz,§ and Daniel V. Santi*,‡

Departments of Biochemistry and Biophysics, and Department of Pharmaceutical Chemistry, University of California at San Francisco, San Francisco, California 94143-0448, and Department of Chemistry, University of California at Berkeley, Berkeley, California 94720

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ABSTRACT: Tyr 146 of TS has been proposed to assist in the removal of the proton from the 5-carbon of the pyrimidine in a steady-state intermediate [Hyatt, D. C., Maley, F., and Montfort, W. R. (1997) *Biochemistry 36,* 4585−4594]. We prepared a replacement set of mutations at position 146 of *L. casei* TS. The k_{cat} and k_{cat}/K_m values of 15 mutants studied were significantly lower than wild-type TS. There was no effect on the K_m of dUMP, and only moderate effects on the K_m of the cofactor. We concluded that Y146 is not directly involved in substrate binding, but contributes significantly to catalysis. We also examined the Y146 mutants as catalysts for cofactor-independent dehalogenation of BrdUMP, a reaction which simulates early steps of the normal pathway up to and including enzyme−nucleotide covalent adduct formation. Many mutants had activity comparable to the wild-type enzyme, and we concluded that the effects of Tyr 146 mutations occur after the initial covalent adduct is formed. A covalent steady-state intermediate-containing enzyme, dUMP, and cofactor accumulated with Tyr 146 mutants, and could be isolated by SDS−PAGE. The complex was kinetically competent as an intermediate in dTMP formation. Using Y146D and F, it was shown that removal of the C-5 proton from the covalent intermediate was defective. We conclude that in the wild-type enzyme Tyr 146 assists in proton removal from the covalent intermediate. Mutants containing fluorinated tyrosines at position 146 showed an inverse linear correlation of activity versus acidity, again indicating that the basicity of the phenolic oxygen plays an important catalytic role. Speculations of how the poorly basic phenol group might assist proton removal are made in which Tyr 146 acts as a proton conduit to N5 of the cofactor or as a cohort of a water molecule serving as the direct general base catalyst.

Thymidylate synthase (TS)[1] 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 (H_2-folate). TS has been extensively studied in terms of structure and mechanism. The kinetics of the TS reaction are well established, X-ray structures of free and bound enzyme forms have been determined, and several hundred mutants have been produced and studied to relate structure and function (*1*).

The salient features of the enzymatic reaction mechanism are depicted in Scheme 1. After formation of a reversible ternary complex, nucleophilic attack by the thiol of a conserved cysteine residue (Cys 198 in *L. casei* TS)[2] at C-6 of dUMP converts the 5-carbon to the enol **I**. This is followed by covalent bond formation between C-5 of dUMP and the one-carbon unit of CH_2H_4folate, which has been activated by formation of an iminium ion at N-5, to produce intermediate **II**. The C-5 proton of **II** is removed, followed by β-elimination of H_4folate to give the exocyclic methylene intermediate **III**. Hydride transfer from H_4folate to the exocyclic methylene intermediate **III** and β-elimination of the enzyme result in the products H_2folate and dTMP, and regenerate active enzyme. As indicated, general catalysts are believed to be required for proton transfers at O-4 and C-5 of the pyrimidine.

It seems accepted that water within a H-bond network involving Glu 60 is responsible for proton transfers at O-4 of dUMP (*2*−*4*). However, the identity of the base that abstracts the C-5 proton of **II** remains controversial. Water^{c1}, water^{c7}, and Tyr 146 have each been suggested as candidates for accepting the C-5 proton based on their proximity to C-5 in the TS−FdUMP−CH_2H_4folate and related ternary complexes (Figure 1, Table 1). Matthews et al. (*5*) favor water^{c1} (wat401 in *E. coli* TS) as the acceptor for the C-5 proton, whereas Fauman et al. (*6*) argue that water^{c7} is better positioned to serve as the acceptor; the issue of how a poorly basic water could drive the dissociation of the poorly acidic C-5 proton was not addressed. Hardy et al. (*7*) proposed a clever variation in which water served as a conduit to transfer

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* Correspondence should be addressed to this author.

‡ University of California at San Francisco.

§ University of California at Berkeley.

[1] Abbreviations: TS, thymidylate synthase; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dUMP, 2′-deoxyuridine 5′-monophosphate; BrdUMP, 5-bromo-2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; dTMP, 2′-deoxythymidine 5′-monophosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TES, 2-[*N*-[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; DTT, dithiothreitol.

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

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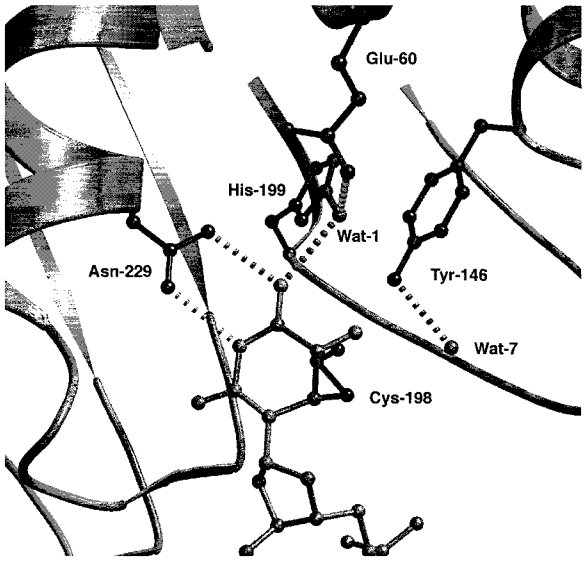

FIGURE 1: Active site region of the TS−FdUMP−H_4folate complex (*8*) showing conserved waters and side chains of TS in relation to the pyrimidine ring. Cofactor is not visible in the plane of projection.

Scheme 1: Salient Features of the Chemical Mechanism of TS

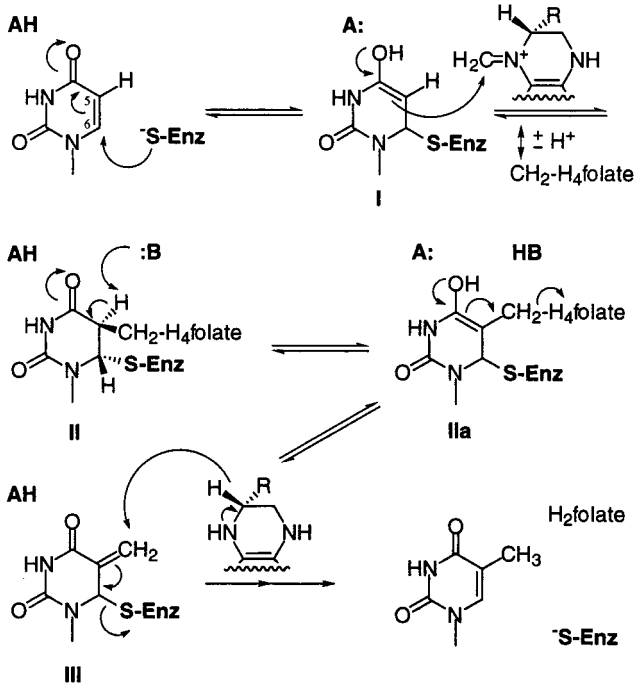

Table 1: Intra-atomic Distances (Å) from Structures of the TS−FdUMP−CH_2H_4folate Complex (*8*)

	H_2O^{C1}	H_2O^{C7}	Y146-O	N-5	N-10	5-H(F)	H199-N
H_2O^{C1}	−						
H_2O^{C7}	5.9	−					
Y146-O	4.0	2.9	−				
N-5	5.5	6.1	5.5	−			
N-10	5.5	7.8	7.9	2.8	−		
5-H(F)	3.7	3.5	3.2	3.2	5.0	−	
H199-N	2.7	6.4	3.6	7.5	8.1	5.0	−

the C-5 proton directly to N-5 of the cofactor in intermediate **II**; this mechanism provides a reasonable recipient for the C-5 proton, and also serves to enhance the leaving group ability of N-5 for subsequent C−N bond cleavage to **III**. Hyatt et al. (*8*) proposed that the phenolate anion of Tyr 146 may be the C-5 proton acceptor, and cited unpublished data that mutation of Tyr 146 to Phe results in loss of activity. However, they acknowledged a difficulty in explaining how Tyr 146 might be ionized to a phenolate in the absence of a nearby basic residue.

Because of its conservation in TS and proximity to the C-5 of dUMP, we felt that the possible involvement of Tyr in C-5 proton abstraction deserved further investigation. In the present work, we prepared a replacement set of 19 mutants of Tyr 146 of TS. We show that Tyr 146 is important for catalysis, but not for substrate binding. Most important, the mutants were defective in the specific step involving removal of the C-5 proton from intermediate **II**. We conclude that Tyr assists in abstracting the C-5 proton of **II**.

MATERIALS AND METHODS

Materials. *E. coli* strain χ2913recA (ΔthyA572, recA56) and plasmid pSCTS9 have been described (*9*, *10*). Oligonucleotide synthesis and DNA sequencing were performed at the UCSF Biomolecular Resource Center. (6*R*,*S*)-CH_2H_4-folate was a gift from SAPEC S.A. (Lugano, Switzerland). [5-^{3}H]dUMP (16 Ci/mmol), [6-^{3}H]dUMP (15 Ci/mmol), [2-^{14}C]dUMP (52 mCi/mmol), and [6-^{3}H]FdUMP (15 Ci/mmol) were from Moravek Biochemicals. 2-Fluoro-L-tyrosine and 3-fluoro-L-tyrosine were purchased from TCI America. 3,5-Difluoro-L-tyrosine was synthesized as previously reported (*11*). All other materials were the highest purity available from commercial sources and used without purification.

Mutagenesis. Cassette mutagenesis was performed using a synthetic *L. casei* TS gene in the plasmid pSCTS9(*Spe*I−*Bss*H II stuffer) which contains a noncoding sequence with a *Not*I site between the *Spe*I and *Bss*H II sites of the synthetic gene (*12*). Two different coding cassettes were ligated into *Spe*I/*Bss*H II-digested pSCTS9 (*Spe*I−*Bss*H II stuffer). The first contained the degenerate sequence (ACG)(N)(CG) at codon 146, and the second contained T(GT)(CG). Together, these two cassettes encode all amino acids except Tyr. The majority of the mutants were obtained by sequencing these two pools. Methods for obtaining pools of mutants representing all codons and for screening for complementation of TS-deficient *E. coli*, which assesses catalytically active TS, have been described (*13*). Y146W, -M, -H, and -K were made by ligating their individual oligonucleotide cassettes. *E. coli* strain χ2913recA (ΔthyA572, recA56) was used for plasmid preparation and protein expression.

Protein Purification. The mutant enzymes were purified using sequential chromatography on phosphocellulose and hydroxyapatite (*14*) and were >95% homogeneous as judged by Coomassie-stained SDS−PAGE; 25−100 mg of pure protein was obtained from 1 L of cell culture. Purified enzymes were desalted and concentrated using Amicon Centriprep-30 concentrators and stored in 10 mM potassium phosphate buffer, pH 7.4, and 0.1 mM EDTA at −80 °C.

Enzyme Assays. TS activity was monitored spectrophotometrically at 340 nm as described (*15*). The standard TES 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. All measurements were obtained using a Hewlett-Packard 8452 diode array spectrophotometer. Where

possible, initial velocities were determined by varying the concentration of one substrate in the presence of an excess ($>10 \times K_m$) of fixed concentration of the second substrate. Kinetic constants were determined by a nonlinear least-squares fit to the appropriate equations using the program Kaleidagraph 3.0.2 (Abelbeck Software, 1993) run on a Macintosh PowerPC. TS-catalyzed tritium release from [5-^{3}H]dUMP was monitored by the decrease in the ^{3}H/^{14}C ratio of [2-^{14}C,5-^{3}H]dUMP (*16*). Reaction mixtures contained 100 μM [2-^{14}C,5-^{3}H]dUMP (0.5 mCi of ^{3}H/mmol, 0.12 mCi of ^{14}C/mmol) and 500 μM CH_2H_4folate in standard TES assay buffer at 25 °C. Aliquots (50 μL) were assayed for tritium release. TS-catalyzed dehalogenation of BrdUMP was monitored spectrophotometrically by the decrease in absorbance which accompanies dehalogenation ($\Delta\epsilon_{285} = 5320$ M^{-1} cm^{-1}) (*17*). Reaction mixtures contained TES/DTT assay buffer (standard TES buffer containing 5 mM DTT instead of β-mercaptoethanol), 200 μM BrdUMP, and 5 μM enzyme.

SDS−PAGE of Enzyme−dUMP−CH_2H_4folate Complexes. Covalent ternary complexes were formed by incubating mixtures containing 4 μM Y146 mutants, 50 μM [5-^{3}H]-dUMP (3.75 Ci/mmol), and 1 mM CH_2H_4folate in standard TES assay buffer at ambient temperature. At various times, aliquots (10 μL) were denatured by boiling with 2× loading buffer for 5 min, then loaded on 12% SDS−PAGE with a 4% stacking gel, and electrophoresed as described (*16*). For quantitation of Y146 TS−[5-^{3}H]dUMP−CH_2H_4folate covalent complex, the radioactivity associated with the protein band at its optimum formation was compared with that of the wild-type TS−[6-^{3}H]FdUMP−CH_2H_4folate, which contains one FdUMP per TS monomer. The Coomassie-stained protein bands were excised; radioactivity was extracted by heating with 1× SDS−PAGE loading buffer at 50 °C and then counted. The kinetic parameters of the covalent complex were assessed by exposing the radioactive protein gel to Biomax film (Kodak) and scanning the autoradiogram with a densitometer. The decomposition rate of the covalent complex was obtained by adding a 100-fold excess of nonradioactive dUMP after the maximal formation of the complex, and then monitoring the first-order decrease of radioactivity associated with the complex. The apparent first-order rate constant of formation was calculated by dividing the initial rate of formation by the concentration of TS monomer.

TS Containing Fluorotyrosines at 146. A His_6 tag was placed at the N-terminus of the TS gene (plasmid pSCTS9) via PCR, and the gene was cloned into pAED4 T7 (*18*). Kunkle mutagenesis (*19*) was used to convert codon 146 to TAG, and incorporation of 2-fluoro-L-tyrosine, 3-fluoro-L-tyrosine, and 3,5-difluoro-L-tyrosine was accomplished by in vitro suppression of the TAG mutation with chemically aminoacylated suppressor $tRNA_{CUA}$ (*20*). The in vitro transcription−translation conditions were as described (*21*) with the following modifications: 2.2 M Tris−Glu, pH 7.4, replaced 2.2 M Tris−AcOH, and 2.8 M potassium Glu and 380 mM $Ca(OAc)_2$ replaced the KCA mix. Fluorinated tyrosine analogues were incorporated with suppression efficiencies of 40−70%. With un-acylated $tRNA_{CUA}$, less than 2% full-length TS was produced compared to in vitro expression of wild-type TS lacking the TAG codon. Mutant proteins were purified on His•Bind (Novagen) using standard procedures, except that the column was washed with 25 mM imidazole, 0.5 M NaCl, and 20 mM Tris−AcOH, pH 7.9, instead of the recommended wash buffer. Purified enzymes were concentrated (Amicon Centriprep-30) and stored in 10 mM K_2PO_4, pH 7.4, and 0.1 mM EDTA. Total protein was estimated by the Bradford method, and densitometry of SDS−PAGE was used to determine the percent of TS in mixtures. A 3 mL reaction produced about 1−2 μg of TS.

Table 2: Steady-State Kinetic Constants for dTMP Formation and BrdUMP Dehalogenation by Wild-Type TS and Y146 Mutants

TS	k_{cat}, s^{-1}	$k_{cat}/K_{m(THF)}$	$K_{m,\mu M}$ dUMP	$K_{m,\mu M}$ THF	k_{cat}, min^{-1} (BrdUMP)
wild-type	8.0	0.8	5.0	10	0.48
Y146H	8.3×10^{-2}	1.3×10^{-3}	7.4	65	0.041
Y146C	5.8×10^{-2}	6.7×10^{-4}	5.9	87	0.33
Y146M	3.6×10^{-2}	5.6×10^{-4}	4.1	64	0.16
Y146S	3.7×10^{-2}	5.7×10^{-4}	7.5	65	0.23
Y146T	3.2×10^{-2}	5.2×10^{-4}	5.1	62	0.39
Y146Q	4.2×10^{-2}	8.9×10^{-4}	3.0	47	0.017
Y146N	2.2×10^{-2}	1.6×10^{-4}	3.3	135	0.030
Y146W	1.1×10^{-2}	1.6×10^{-4}	6.1	70	0.10
Y146D	2.2×10^{-3}	1.0×10^{-5}	7.1	216	<0.006
Y146E	8.2×10^{-3}	2.5×10^{-4}	8.9	33	<0.006
Y146K	8.9×10^{-3}	6.6×10^{-5}	3.4	135	0.093
Y146R	4.3×10^{-3}	2.8×10^{-5}	7.7	152	0.25
Y146F	1.9×10^{-2}	6.0×10^{-5}	5.4	319	0.082
Y146L	3.8×10^{-2}	1.4×10^{-4}	3.2	279	0.72
Y146G	2.4×10^{-2}	4.2×10^{-4}	3.9	57	0.37

RESULTS AND DISCUSSION

We have prepared a complete replacement set of 19 mutations at position 146 of *L. casei* TS. Of these, 15 mutants were chosen and subjected to steady-state kinetic analysis (Table 2). The k_{cat}/K_m values of the mutants studied were 615−80 000-fold lower than wild-type TS, demonstrating the importance of Y146 to catalytic efficiency. Similarly, k_{cat} values were 100−4000-fold lowered, showing that one or more catalytic events were detrimentally affected. The mutations had no effect on the K_m of dUMP, which is not surprising since there are no direct interactions between Y146 and the nucleotide substrate. The mutants did show a 3−32-fold increase in the K_m of the cofactor. However, since some mutants showed little effect on the K_m of the cofactor, the tyrosine side chain is concluded to be permissive but noncontributory to cofactor binding, and the mutants with high K_m values (Y146F, -L, -D, -R, -N, and -K) probably interfere with cofactor binding. From these studies, we conclude that Y146 is not directly involved in substrate binding but contributes significantly to catalysis.

We also examined the cofactor-independent dehalogenation of BrdUMP by Y146 mutants to assess the role of Tyr 146 in early steps of the pathway. In this reaction, the catalytic thiol of TS adds to the 6-position and a proton adds to the 5-position of BrdUMP to form a 5-bromo-5,6-dihydropyrimidine adduct. Next, a thiol of the medium abstracts the 5-Br as bromonium ion, and the covalent adduct undergoes α,β-elimination to provide dUMP and TS. The dehalogenation reaction thus shares steps with the normal pathway up to and including formation of the initial enzyme−nucleotide covalent adduct. As shown in Table 2, Y146 mutants show a wide range of activities in the BrdUMP dehalogenation reaction. Mutants with negatively charged residues (i.e., Y146E, -D) display no detectable activity; some mutants show decreases of up to 30-fold whereas several

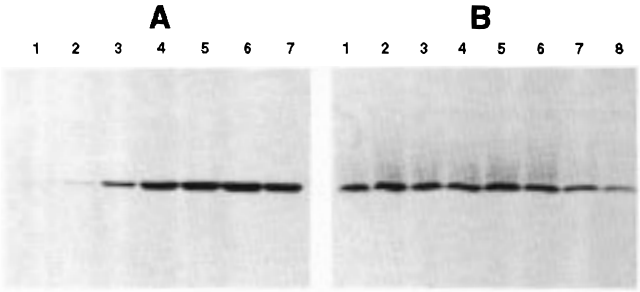

FIGURE 2: Autoradiogram of SDS−PAGE showing the covalent complexes containing Y146D/Y146F, dUMP, and CH_2H_4folate. (A) Y146D TS was incubated with [5-^{3}H]dUMP and CH_2H_4folate and denatured at 30 s (lane 1), 1 min (lane 2), 3 min (lane 3), 10 min (lane 4), 30 min (lane 5), 60 min (lane 6), and 120 min (lane 7). (B) Y146F TS was incubated with [5-^{3}H]dUMP and CH_2H_4folate and denatured at 10 s (lane 1), 20 s (lane 2), 30 s (lane 3), 45 s (lane 4), 1 min (lane 5), 2 min (lane 6), 5 min (lane 7), and 10 min (lane 8).

show equal to or greater activity than the wild-type enzyme. We conclude that Tyr 146 is not involved in steps leading to the initial covalent adduct of TS and nucleotide substrates, and that effects of mutations on dTMP reaction occur after that intermediate is formed.

Structural studies have led to the suggestion that Tyr 146 may play a role in abstraction of the C-5 proton from intermediate **II** (*8*). We reasoned that if Y146 was involved in proton abstraction from intermediate **II**, and if that step was hindered in Y146 mutants, there might be sufficient accumulation of **II** to allow its detection on SDS−PAGE. Our rationale was that if the breakdown of **II** is enzyme-catalyzed, denaturation of the enzyme should inhibit the breakdown and yield a chemically stable intermediate. Indeed, this rationale was the basis for experiments leading to isolation of intermediate **II** in mutants of Glu 60 (*22*).

We studied three Y146 mutants (Y146D, -F, and -R) for their ability to form covalent complexes with [5-^{3}H]dUMP with and without CH_2H_4folate. In the presence, but not in the absence, of CH_2H_4folate, all three gave radioactive bands on SDS−PAGE which migrated slightly slower than the free ezyme (Figure 2). The properties of the complex are consistent with structure **II** based on the expected chemistry, and the precedents of trapping intermediate **II** with Glu 60 mutants (*22*), and the analogous TS−FdUMP−CH_2H_4folate complex (*16*).

We chose TS Y146D and -F for more detailed studies: Y146D had the lowest turnover of the mutants examined and hence the greatest prospect for maximal accumulation of the intermediate, whereas mutation from Tyr to Phe would cause the least structural perturbation. As shown in Figure 2, formation of the ternary complex rapidly reached a steady-state level corresponding to about 15% and 5% of the total enzyme used for Y146D and Y146F, respectively. With the previously reported Glu 60 mutants, the corresponding levels of the intermediate approached only about 2% (*22*). Next, we measured the kinetics of covalent complex formation and disappearance; for the complex to qualify as a kinetically competent intermediate in dTMP formation, these rates must be equal to or higher than k_{cat}. Under near-saturating substrate concentrations, Y146D had an apparent rate constant of 0.0027 s^{-1} for covalent complex formation, and 0.0019 s^{-1} for decomposition. The k_{cat} for dTMP formation by Y146D is 0.0022 s^{-1}. For Y146F, the rates of complex formation (complete in 5 s) and decomposition (apparent rate constant about 0.05 s^{-1}) were too fast to measure accurately. These results are consistent with the isolated complexes being kinetically competent as intermediates for dTMP synthesis.

Table 3: Steady-State Kinetic Constants for dTMP Formation by His_6 Wild-Type TS and Mutants Containing Fluorinated Tyrosines at Position 146

	wt TS, His_6	2-F-Tyr	3-F-Tyr	3,5-di-F-Tyr
pK_a	10.0	9.3	8.8	7.0
K_m, dUMP, μM	2.4	2.8	1.4	1.5
K_m, THF, μM	36	15	23	34
k_{cat}, s^{-1}	12	2.3	1.8	0.25

We next measured the rate of 5-H release from [5-^{3}H]-dUMP for Y146D, -F, and -R. Here, the release would be equal to dTMP formation if proton abstraction from **II** is slow compared to subsequent steps in the pathway, or faster than dTMP formation if proton abstraction and exchange with solvent protons are rapid compared to subsequent steps in the pathway. Under similar reaction conditions, we found that the release of the 5-^{3}H from [5-^{3}H]dUMP occurred at essentially the identical rate as the rate of dTMP formation for all the three mutants examined, indicating that after proton abstraction from **II** to give **III** the steps leading to dTMP are comparatively rapid.

It is revealing to compare the properties of Y146 mutants to those of wild-type TS and E60 mutants. With wild-type TS, the rate of 5-H release from dUMP is likewise comparable in rate compared to subsequent steps in the reaction. However, with TS E60 mutants, abstraction and exchange with solvent protons are rapid, and the exchange reaction can be monitored independently of dTMP formation. Since exchange requires formation and reversal of intermediate **IIa**, the primary defect of E60 mutants was somewhere in the pathway after **IIa**. Since Y146D forms **II** as do E60 mutants, yet there is no exchange of the 5-H of dUMP, the primary defect must exist in converting **II** to **IIa**—the step involving proton abstraction of **II**. We therefore conclude that a primary role of Y146 in the TS reaction is to assist in proton abstraction of **II**.

We also prepared and examined the effects of mutants containing mono- and difluoro-substituted Tyr 146, thus varying the pK_a of the phenol from 10 to 7.0 (Table 3). We observed an inverse linear correlation between log [k_{cat}/K_m(CH_2H_4folate)] and the pK_a of the phenol with a slope α of 0.56 (Figure 3), indicating proton movement of the phenol hydroxyl during catalysis.

Before addressing how Y146 might assist in C-5 proton abstraction, it is informative to consider the proton transfers necessary to convert **II** to **III**. As indicated in Scheme 1, the C-5 proton removed from **II** is balanced by one placed at N-5 of H_4folate in **III**. However, the initial recipient of the proton must be N-5 of the cofactor in **IIa** since protonation is necessary for cleavage of the N-5−carbon bond to form H_4folate in **III**; the alternative that bond cleavage occurs with H_4folate anion as leaving group is unlikely. Since a direct transfer of proton from C-5 to N-5 of **IIa** would require an improbable four-membered transition state, the transfer must occur in a concerted or stepwise manner through a base serving as a proton conduit. As previously described, intra-atomic measurements made from

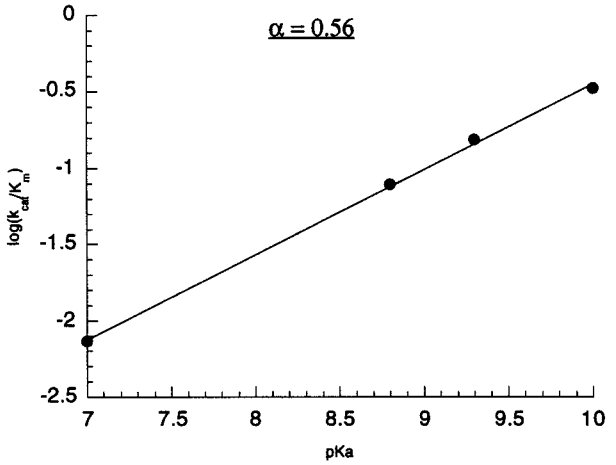

FIGURE 3: Plot of log [k_{cat}/K_m(CH_2H_4folate)] of wild-type TS and fluorinated Y146 mutants versus pK_a of tyrosine and fluorinated tyrosine.

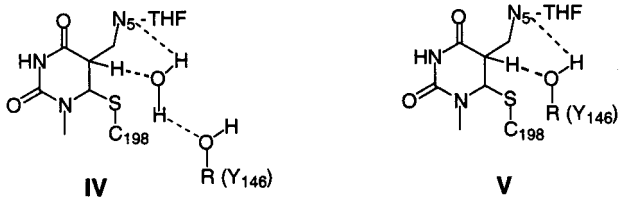

FIGURE 4: Proposed two possible models of hydrogen abstraction at C-5.

structures of the TS−FdUMP−CH_2H_4folate complex indicate that water[c1], water[c7], and the Tyr 146 phenol are sufficiently close to C-5 to act as a proton acceptor (Table 1), although none is sufficiently close to both the 5-H(F) and N-5 to act as a proton conduit without some movement. However, prior to proton abstraction, a conformational change occurs in **II** in which the initially formed equatorial 5-H is converted to the axial position; rearrangements of the nearby candidate bases to more appropriate positioning could certainly accompany this change.

It has been proposed that the phenolate of Tyr 146 may act directly to abstract the C-5 proton of **II** (*8*). Indeed, the correlation of log [k_{cat}/K_m(CH_2H_4folate)] with the pK_a of the Tyr 146 phenol suggests proton movement of the phenol hydroxyl during catalysis. Although lowering the pK_a of Tyr 146 would increase the concentration of phenolate, it concurrently decreases the basicity of the phenolate oxyanion, so experiments with the fluorotyrosine analogues neither support nor detract from this mechanism. Unassisted ionization of Tyr residues in proteins is, to our knowledge, without precedent. However, the only basic residue in proximity to the Tyr 146 phenol is His 199 (3.6 Å, Table 1), but it can be mutated with only minor impact on activity (*23*). More attractive possibilities of the mechanism are variants of a proposal made by Hardy et al. (*7*), in which a water molecule serves to concertedly remove the C-5 proton, and protonate N-5 of the cofactor. To explain the necessity of Tyr 146 in the context of this mechanism, we would propose that Tyr 146 serves to polarize and/or orient a water molecule, probably water[c1], to make it a more efficient general acid/base (Figure 4, IV). Alternatively, the phenol of Tyr 146 could itself serve as a conduit for proton transfer from C-5 to N-5 (Figure 4, V). The attractive feature about either of these mechanisms is that the ultimate recipient of the released proton is a nitrogen with pK_a ∼5 (*24*), so the poorly basic phenol hydroxyl or water molecule could reasonably serve a role as a proton conduit. Also, the protonated cofactor product would facilitate the subsequent conversion of **IIa** to **III**. Although we cannot distinguish these mechanisms, proton abstraction by water (Figure 4, **IV**) is attractive since it allows easy explanation of the low activity remaining upon mutation of Tyr 146 to nonpolar residues. These mechanisms are parsimonious, consistent with all data thus far available, and cause no disruption of the fundamentals of proton-transfer reactions.

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REFERENCES

1. Carreras, C. W., and Santi, D. V. (1995) *Annu. Rev. Biochem. 64*, 721−762.
2. Huang, W., and Santi, D. V. (1997) *Biochemistry 36*, 1869−1873.
3. Agarwalla, S., LaPorte, S., Liu, L., Finer-Moore, J., Stroud, R. M., and Santi, D. V. (1997) *Biochemistry 36*, 15909−15917.
4. Finer-Moore, J. S., Liu, L., Birdsall, D. L., Brem, R., Apfeld, J., Santi, D. V., and Stroud, R. M. (1998) *J. Mol. Biol. 276*, 113−129.
5. Matthews, D. A., Villafranca, J. E., Janson, C. A., Smith, W. W., Welsh, K., and Freer, S. (1990) *J. Mol. Biol. 214*, 937−948.
6. Faunan, E. B., Rutenber, E. E., Maley, G. F., Maley, F., and Stroud, R. M. (1994) *Biochemistry 33*, 1502−1511.
7. Hardy, L. W., Graves, K. L., and Nalivaika, E. (1995) *Biochemistry 34*, 8422−8432.
8. Hyatt, D. C., Maley, F., and Montfort, W. R. (1997) *Biochemistry 36*, 4585−4594.
9. Climie, S. C., Carreras, C. W., and Santi, D. V. (1992) *Biochemistry 31*, 6032−6038.
10. Climie, S. C., and Santi, D. V. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 633−637.
11. Thorson, J. S., Shin, I., Chapman, E., Stenberg, G., Mannervik, B., and Schultz, P. G. (1998) *J. Am. Chem. Soc. 120*, 451−452.
12. Climie, S., Ruiz-Perez, L., Gonzalez-Pacanowska, D., Prapunwattana, P., Cho, S.-W., Stroud, R., and Santi, D. V. (1990) *J. Biol. Chem. 265*, 18776−18779.
13. Huang, W., and Santi, D. V. (1994) *Anal. Biochem. 218*, 454−457.
14. Kealey, J. T., and Santi, D. V. (1992) *Protein Expression Purif. 3*, 380−385.
15. Pogolotti, A. L. J., Danenberg, P. V., and Santi, D. V. (1986) *J. Med. Chem. 29*, 478−482.
16. Carreras, C. W., Climie, S. C., and Santi, D. V. (1992) *Biochemistry 31*, 6038−6044.
17. Garett, C., Wataya, Y., and Santi, D. V. (1979) *Biochemistry 18*, 2798−2804.
18. Doering, S. D. (1992) Ph.D. Thesis, Massachusetts Institute of Technology.
19. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) *Methods Enzymol. 204*, 125−139.
20. Cload, S. T., Liu, D. R., Froland, W. A., and Schultz, P. G. (1996) *Chem. Biol. 3*, 1033−1038.
21. Ellman, J., Mendel, D., Anthony-Cahill, S., Noren, C., and Schultz, P. G. (1991) *Methods Enzymol. 202*, 301−337.
22. Huang, W., and Santi, D. V. (1994) *J. Biol. Chem. 269*, 31327−31329.
23. Dev, I. K., Yates, B. B., Atashi, J., and Dallas, W. S. (1989) *J. Biol. Chem. 264*, 19132−19137.
24. Kallen, R. G., and Jencks, W. P. (1966) *J. Biol. Chem. 241*, 5845−5850.

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