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Biochemistry

Asn^{177} in *Escherichia coli* thymidylate synthase is a major determinant of pyrimidine specificity

(catalytic mechanism/folate enzyme/site-directed mutagenesis/charge stabilization)

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ABSTRACT **The substrate preference of recombinant *Escherichia coli* thymidylate synthase (TS) has been altered from 2′-deoxyuridylate (dUMP) to 2′-deoxycytidylate (dCMP) by site-directed mutagenesis of the codon for Asn^{177}, which was changed to aspartic acid. The side-chain amide of Asn^{177} forms hydrogen bonds with O4 and N3 of dUMP bound to the crystalline enzyme [Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G. F., Hardy, L., Maley, F. & Stroud, R. M. (1990) *Biochemistry* 29, 6964–6977]. This Asn is invariant in all natural sequences for TS known. The values of k_{cat} for the mutant enzyme, TS(N177D), with dCMP and dUMP are, respectively, 0.09 and 0.002 times the value of k_{cat} of wild-type TS with dUMP as substrate. TS(N177D) turns over dCMP at 35 times its rate of dUMP turnover, whereas wild-type TS turns over dCMP at $<10^{-5}$ of its rate of dUMP turnover. Thus Asn^{177} is a major determinant of the pyrimidine nucleotide specificity of TS. The mutant enzyme, like wild-type TS, forms a covalent complex with 5-fluoro-dUMP in the presence of 5,10-methylenetetrahydrofolate. TS(N177D) also has a newly acquired ability to be transiently inactivated by dUMP. This time-dependent inactivation requires the presence of methylenetetrahydrofolate and may be due to the accumulation of the enzyme in the form of a catalytic intermediate. The likely mechanistic basis for discrimination by TS between dUMP and dCMP is their differing requirements for charge stabilization during covalent catalysis.**

Attempts to explain the exquisite specificity of enzymes began nearly a century ago (1). It is now recognized that many enzymes enhance reaction rates by utilizing potential binding energies to lower transition-state energies (2). Hence the structural complementarity which dictates substrate specificity should be most fully expressed in the interactions offered to a catalytic intermediate or transition state. Progress toward some understanding of the origins of substrate specificity is evidenced by the recent successes in altering the specificities of several enzymes by genetic engineering (3–5). This report describes a variant of *Escherichia coli* thymidylate synthase (TS) (EC 2.1.1.45) for which the pyrimidine specificity has been altered by site-directed mutagenesis.

TS catalyzes a key step in the *de novo* biosynthesis of dTMP, which is required for DNA synthesis. The enzyme catalyzes the transfer of a methylene group from methylenetetrahydrofolate (CH_2THF) to C5 of dUMP and reduction of the transferred one-carbon unit to a methyl group, utilizing a hydride equivalent provided by tetrahydrofolate. The products are dTMP and dihydrofolate (DHF). A wealth of mechanistic detail is available for TS, from studies with alternative substrates, mechanism-based inactivators, and model reactions (reviewed in refs. 6 and 7). The substrate analogue 5-fluoro-2′-deoxyuridylate (FdUMP), a metabolite of the anticancer agent 5-fluorouracil, has been particularly useful. This nucleotide forms a complex with TS and CH_2THF in which the nucleotide is covalently linked via its pyrimidine to both the folate cofactor and the enzyme. The complex closely mimics a probable catalytic intermediate.

X-ray diffraction studies of crystalline TSs from several species have yielded structural models of these enzymes (8–11). An active-site residue interacting directly with bound dUMP is Asn^{177} (Fig. 1), which is invariant in all known natural sequences for TS. A pair of hydrogen bonds links the side-chain amide of Asn^{177} to N3 and O4 of the pyrimidine; this implies but does not prove that Asn^{177} dictates the exclusive utilization of dUMP, and not dCMP, by TS. An enzyme which has the opposite substrate specificity is the bacteriophage T4 dCMP hydroxymethylase. This phage enzyme catalyzes a reaction analogous to that catalyzed by TS, probably via a similar mechanism (K. L. Graves, M. M. Butler, and L.W.H., unpublished work). The amino acid sequences of TS and the hydroxymethylase have many similarities, implying structural homology. However, the residue in the sequence of dCMP hydroxymethylase which corresponds to Asn^{177} is an aspartic acid. For this reason, we replaced Asn^{177} in *E. coli* TS with aspartic acid, and compared the properties of the mutant enzyme, TS(N177D), with those of wild-type TS [TS(wt)].

MATERIALS AND METHODS

Genetic Engineering. Plasmids were constructed by standard methods (12). pLHTS5 was constructed by ligation of (*i*) a 2011-base-pair (bp) *Bam*HI–*Pst* I fragment [the *Pvu* II–*Pst* I segment from pZ152 (13) with the *Pvu* II site converted to a *Bam*HI site] containing the filamentous phage IG sequence, the plasmid replication origin, and half of the *bla* gene and (*ii*) a 2283-bp *Pst* I–*Bam*HI fragment [obtained from pBTAH (14) by partial digestion with *Pst* I and complete digestion with *Bam*HI] containing *thyA* and the promoter-proximal half of *bla*. A circular heteroduplex form of pLHTS5, prepared with a gap of 282 bases in the Dam-methylated (antisense) strand exposing the codon for Asn^{177}, was mutagenized (15) using the synthetic oligodeoxynucleotide 5′-AGCTGGCGATATC-GAACGGC-3′. This primer, prepared at the University of Massachusetts Medical Center DNA Synthesis Facility, annealed to the sense strand within *thyA* and converted the Asn codon (AAC) to an Asp codon (GAT). pLHTS7 is the variant of pLHTS5 bearing the *thyA*(N177D) allele. DNA sequencing of the entire *thyA* gene in pLHTS7 confirmed that the desired primer-directed changes were the only mutations. Vectors for overproduction of TS(wt) and TS(N177D), pLHTS6 and pLHTS9, respectively, were created using pKC30 (14).

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Abbreviations: CH_2THF, 5,10-methylenetetrahydrofolate; DHF, dihydrofolate; FdUMP, 5-fluoro-2′-deoxyuridylate; TS, thymidylate synthase; TS(wt), wild-type TS; TS(N177D), $Asn^{177} \rightarrow$ Asp mutant of TS.

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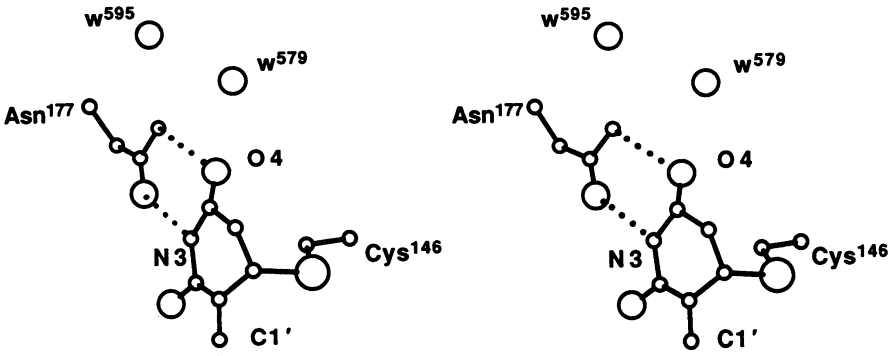

FIG. 1. This crossed-eyes stereo view of the pyrimidine ring of 2'-dUMP covalently bound to Cys^{146} in the active site of *E. coli* TS shows the hydrogen bonding (dotted lines) between the side chain of Asn^{177} and the N3 and O4 positions of the nucleotide. Also shown are two tightly bound waters (w79 and w595). The model is that of the ternary complex with 10-propargyl-5,8-dideazafolate (9).

[pLHTS6 is identical to pKTAH (14).] Minimal medium lacking thymidine was M9 glucose plus charcoal-treated Casamino acids.

Enzyme Preparation and Assays. TS(wt) was overproduced by nalidixic acid induction of pLHTS6 in *E. coli* AR120 (16). To prevent contamination with TS(wt), TS(N177D) was overproduced from pLHTS9 in *E. coli* RR1Δ*thyA* (17), which has a deletion of a portion of *thyA* including the codon for Asn^{177}. Control of pLHTS9 in RR1Δ*thyA* was maintained with pRK248cI(ts) (18). TS(wt) and TS(N177D) were purified by the procedure developed for TS(C50F) (19). This procedure yielded ≈100 mg of homogeneous enzyme per 100 g of cells. Enzyme concentrations, determined by the Bradford method (20), are stated in terms of the dimer unless noted otherwise, using a monomer molecular weight of 30,441 (21).

TS activity was routinely assayed spectrophotometrically (22) at 30°C in 0.1 M Tris·HCl, pH 7.0/30 mM $MgCl_2$/20 mM 2-mercaptoethanol (standard assay buffer). Mes/acetate and Mes/Tris (23) were used at pH 5.5 and 7.0, respectively, to examine the effect of pH upon the values of k_{cat} and K_m. Stock solutions of CH_2THF were prepared using DL-tetrahydrofolic acid (Sigma). The concentrations of CH_2THF reported here are the values corresponding to the L-(6*R*) isomer. Assays of TS activity by tritium release (24) employed 5[^{3}H]dUMP or 5[^{3}H]dCMP (Moravek Biochemicals, Brea, CA), diluted to a specific activity of 200–600 Bq·nmol^{-1} with unlabeled nucleotides. The tritium release and filter binding assays and the analysis of enzyme-nucleotide complexes by SDS/PAGE will be described elsewhere (K. L. Graves, M. M. Butler, and L.W.H., unpublished work). Binding of nucleotides to TS was monitored in some experiments by a centrifugal column method (25).

Preparation of Nucleotides. A sample of the nucleotide produced from the TS(N177D)-catalyzed reaction of dCMP with CH_2THF was purified by chromatography on Dowex-1 and converted to its sodium salt. The NMR spectra of this nucleotide and of dCMP, in deuterium oxide containing a trace of sodium (2,2,3,3-$^2H_4$)-3-trimethylsilylpropionate as a standard, were determined on a Varian 300-MHz NMR spectrometer by Frank Mari of the University of Massachusetts Medical Center NMR Laboratory.

RESULTS

Mutation of Asn^{177} to Asp. The Asn^{177} → Asp alteration in TS dramatically decreased the enzyme's rate constant for catalyzing dTMP synthesis (Table 1). Nonetheless, TS(N177D) expressed from the multicopy plasmid pLHTS7 provides enough dTMP to allow JM101Δ*thyA* cells to grow without exogenous thymidine at the same rate as they did in medium containing thymidine. Thus, reducing the TS activity to 0.1% of that produced by pLHTS5 does not starve *E. coli* for thymidylate, but a further five- to six-fold reduction does (unpublished data).

Alteration of Substrate Specificity. TS(wt) has no detectable activity with dCMP as substrate when assayed either by monitoring DHF production (Table 1) or by measuring the release of tritium from 5[^{3}H]dCMP (data not shown). Using these methods and milligram quantities of wild-type enzyme, we estimated the value of k_{cat} for dCMP to be $<10^{-5}$ sec^{-1}, $>10^5$-fold lower than the value for dUMP. TS(wt) does bind dCMP weakly; this nucleotide is a competitive inhibitor versus dUMP, with K_i = 7.5 mM. If dCMP is a substrate *at all* for TS(wt), the value of its K_m must also be 7.5 mM. Hence the value of k_{cat}/K_m for dCMP with TS(wt) can be no greater than 1.3×10^{-3} M^{-1}·sec^{-1}. This is 6×10^8-fold lower than the value for dUMP (7.8×10^5 M^{-1}·sec^{-1}).

TS(N177D) has activity on both dUMP and dCMP, with dCMP being a considerably more reactive substrate. In the tritium release assay (Fig. 2), the N177D variant of TS gives an initial velocity with dCMP which is 35-fold faster than that obtained with dUMP. Tritium release from either nucleotide is dependent on the presence of CH_2THF, which is converted to a compound with the characteristic absorbance spectrum of DHF (data not shown). The complete reaction of either dUMP or dCMP with excess CH_2THF, catalyzed by TS(N177D), produces an amount of DHF which is stoichiometric with nucleotide. Some of the spectrophotometric assays of TS(N177D) showed apparent anomalies, which are described below.

The formation of dTMP from dUMP and CH_2THF catalyzed *in vitro* by purified TS(N177D) was confirmed by HPLC (data not shown). The complementation of *thyA* indicates that TS(N177D) must also catalyze dTMP synthesis *in vivo*. The NMR spectrum of the nucleotide produced by TS(N177D) with dCMP as substrate confirmed it to be 5-methyl-dCMP. This spectrum contained a singlet at 2.03 ppm integrating to three hydrogens, which was the only significant difference from the spectrum of dCMP.

The initial velocity of TS(N177D)-catalyzed turnover with dCMP as substrate in the spectrophotometric assay was the same as that obtained in the tritium release assay (Fig. 3*B*). However, the spectrophotometric assay with dUMP re-

Table 1. Kinetic parameters for TS (wt and N177D) from spectrophotometric assays at pH 7

Allele and substrate	k_{cat}, sec^{-1}	K_m for nucleotide, μM	K_m for CH_2THF, μM
TS(wt)			
dUMP	3.5 ± 0.2*	4.5 ± 0.6†	9.8 ± 1.8*
dCMP	$<10^{-5}$	7500 (K_i)†	—
TS(N177D)			
dUMP	0.0075 ± 0.0003‡	71 ± 10‡	ND
dCMP	0.33 ± 0.03*	120 ± 27†	93 ± 16*

Values are means ± SEM determined by varying the concentration of one substrate at a fixed concentration of the second substrate. ND, not done.
*Determined at 0.5 mM nucleotide.
†Determined at 70 μM CH_2THF.
‡Determined at 210 μM CH_2THF.

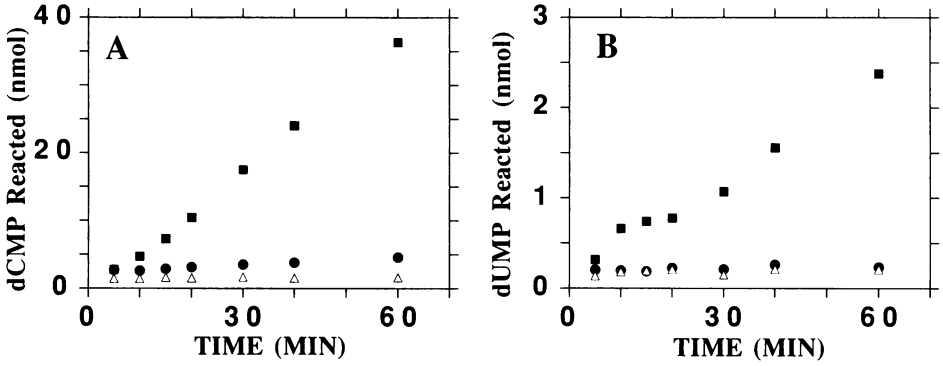

FIG. 2. Tritium release from 5[^{3}H]dCMP (*A*) and 5[^{3}H]dUMP (*B*) catalyzed by TS(N177D). Reaction mixtures contained, in standard assay buffer, 2 mM CH_2THF and either 0.5 mM dCMP and purified enzyme at 20 $\mu g \cdot ml^{-1}$ (0.33 μM dimer) (*A*) or 0.5 mM dUMP and enzyme at 50 $\mu g \cdot ml^{-1}$ (0.82 μM dimer) (*B*); these data are indicated by the squares. Circles represent data for reactions containing 0.2 mM FdUMP, and triangles, for reactions lacking enzyme.

vealed an unusual feature (Fig. 3*A*). The absorbance changes observed with this substrate were biphasic, with a rapid initial burst of increased absorbance, followed by a slower linear increase in absorbance. Values of the absorbance change at 340 nm, ΔA, as a function of time, t, were fitted to Eq. 1,

$$\Delta A = v_{ss}t + A_{burst}(1 - e^{-k_{inact}t}), \quad (1)$$

which corresponds to an exponential approach to a straight line. The velocity calculated from the linear steady-state phase for dUMP (v_{ss}) was equivalent to the velocity obtained from the tritium release assay with this nucleotide. The amplitude of the spectral burst (A_{burst}) was proportional to the enzyme concentration, with a ratio of 11,250 absorbance units (au)$\cdot M^{-1}$ TS(N177D) monomer. This value is comparable to the value for the molar absorbance changes observed with TS(N177D) upon complex formation with CH_2THF and FdUMP (12,800 au$\cdot M^{-1}$).

The values of k_{cat} and K_m for TS(wt) and TS(N177D) with both dUMP and dCMP as substrate in the spectrophotometric assay at pH 7.0 are shown in Table 1. (For dUMP, the "initial" velocities used to calculate these values were determined in the steady-state phase of the reactions.) Unlike the wild-type enzyme, the TS(N177D) has considerable activity on dCMP, with a k_{cat}/K_m ratio of 2.8×10^3 $M^{-1} \cdot sec^{-1}$. The k_{cat}/K_m ratio for TS(N177D) with dUMP is >1000-fold lower than that of TS(wt) with dUMP and is 28-fold lower than k_{cat}/K_m for TS(N177D) with dCMP as substrate.

The values of the kinetic parameters of TS(wt) and of TS(N177D) with their preferred nucleotides were also determined at pH 5.5 and pH 7.0, at saturating concentrations of CH_2THF, by using the tritium release assay. (The enzymes precipitated at pH $\leq$ 5.0.) At pH 7, the values were essentially identical to those obtained with the spectrophotometric assay. The values of k_{cat} and k_{cat}/K_m for TS(wt) with dUMP decreased 26% and 65%, respectively, as the pH was lowered from 7 to 5.5. For TS(N177D) with dCMP, the value of k_{cat} increased 69%, and the value of k_{cat}/K_m decreased 76%, as the pH was lowered from 7 to 5.5.

Inhibition of TS(N177D) by FdUMP and dUMP. Although TS(N177D) is more active on dCMP than on dUMP, the variant enzyme, like TS(wt), is inhibited by FdUMP (Fig. 2); the time-dependent inactivation process requires CH_2THF (data not shown). When TS(N177D) inactivated with 6[^{3}H]-FdUMP and CH_2THF is denatured with SDS and subjected to PAGE, the labeled nucleotide remains bound to the enzyme, indicating that the FdUMP is covalently bound to TS(N177D).

TS(N177D) is also inactivated transiently by dUMP (Fig. 3). Exposure of the enzyme to 0.5 mM dUMP leads to a time-dependent decrease in the rate of dCMP-dependent production of DHF (Fig. 3*B*) and to a 90% decrease in the rate of tritium release from 5[^{3}H]dCMP (data not shown). This time-dependent inactivation requires the additional presence of CH_2THF; no inactivation was caused by CH_2THF alone, nor by dCMP or dTMP. The value of the first-order rate constant for the loss of activity toward dCMP, monitored spectrophotometrically (Fig. 3*B*), was consistently about half the value of the first-order rate constant for the initial (burst) phase of absorbance changes seen with dUMP alone (Fig.

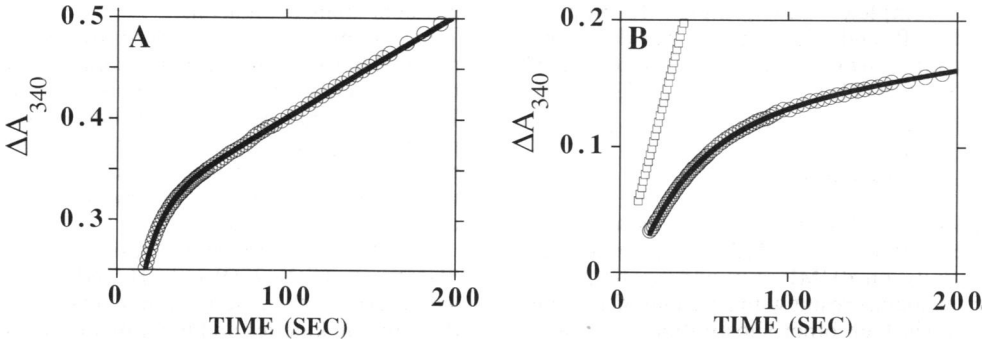

FIG. 3. Spectrophotometric assay for oxidation of CH_2THF catalyzed by TS(N177D), monitored at 340 nm. Each reaction mixture contained 0.4 mM CH_2THF and was initiated by addition of enzyme. Data have been corrected for the slow nonenzymatic rate of CH_2THF oxidation. (*A*) This reaction mixture contained 0.5 mM dUMP and 12 μM enzyme. The data (circles) were fitted (solid line) to Eq. 1, yielding a value of 0.075 sec^{-1} for k_{inact}. The amplitude of the transient burst (A_{burst}) was used to calculate the molar absorbance change due to formation of the inactivated enzyme complex (see text). (*B*) These reaction mixtures contained 0.5 mM dCMP, 2.4 μM enzyme, and either 0.5 mM dUMP (circles) or no dUMP (squares). The data for the former reaction were corrected for the absorbance changes observed due to the turnover of dUMP and fitted (solid line) to an equation similar to Eq. 1. The value of the rate constant for the first-order process is 0.029 sec^{-1}.

Table 2. Binding of nucleotides to TS (wt and N177D)

Allele and nucleotide	Bound nucleotide, pmol	
	− CH_2THF	+ CH_2THF
TS(wt)		
5[^{3}H]dUMP	18.2 ± 1.6	6.2 ± 0.6
6[^{3}H]FdUMP	16.7 ± 1.3	20.5 ± 3.2*
6[^{3}H]dUMP	14.1 ± 1.1	8.3 ± 1.2
TS(N177D)		
5[^{3}H]dUMP	3.8 ± 0.3	5.8 ± 0.5*
6[^{3}H]FdUMP	6.0 ± 0.4	34.3 ± 4.9*
6[^{3}H]dUMP	11.3 ± 2.0	44.8 ± 1.7*

Values are means ± SEM of two to nine determinations. Enzyme (3.85 μg, 126 pmol of monomer) was reacted with 0.2 mM nucleotide, with or without 2 mM CH_2THF, in 50 μl of the standard assay buffer for 10 min at 30°C and assayed for bound nucleotide by nitrocellulose filter binding. Specific activities of the nucleotides were 10–40 Bq·pmol^{-1}. Data have been corrected for small amounts of nucleotides retained in the absence of enzyme.
*These conditions maximally inhibit the indicated enzyme.

3A). The values of the rate constants for both of these first-order processes were independent of enzyme concentration but were dependent upon the initial concentration of dUMP (data not shown). After sufficient time for complete conversion of dUMP to dTMP, the N177D variant enzyme began to reactivate and eventually recovered full activity. The half-time for reactivation was ≈15 min, corresponding to a first-order rate constant of 8×10^{-4}·sec^{-1}.

The biphasic kinetics for absorbance changes at 340 nm with TS(N177D) and dUMP, and the inactivation by dUMP of the enzyme's ability to catalyze turnover with dCMP, suggested that TS(N177D) accumulates with dUMP, or a dUMP-derived intermediate, tightly bound. This hypothesis was tested in nitrocellulose filter binding assays using radiolabeled nucleotides (Table 2). The amounts of 6[^{3}H]dUMP and 6[^{3}H]FdUMP bound by TS(N177D) were increased significantly by CH_2THF. In contrast, addition of CH_2THF produced no significant increase of enzyme-bound tritium when 5[^{3}H]dUMP was reacted with TS(N177D). With TS(wt) and either 5[^{3}H]- or 6[^{3}H]dUMP, the enzyme-bound tritium was decreased by CH_2THF. The conditions of the binding reactions allowed TS(wt) to completely convert dUMP to dTMP, which binds more weakly than dUMP.

A complex between TS(N177D) and 6[^{3}H]dUMP, formed in the presence of CH_2THF, was isolable by the column centrifugation method (data not shown). After treatment of the complex with 4 M urea on ice for 10 min, which results in total denaturation of TS(wt), the amount of 6[^{3}H]dUMP remaining bound to TS(N177D) was 40–60% of that found in the nondenatured complex. The complex formed by TS(N177D), 6[^{3}H]dUMP, and CH_2THF was not detectable by autoradiography after SDS/PAGE, unlike those formed with 6[^{3}H]FdUMP, CH_2THF, and either TS(wt) or TS(N177D).

DISCUSSION

Replacement of Asn^{177} by Asp in *E. coli* TS produces an enzyme with novel activity on dCMP as substrate, and greatly reduced activity on dUMP. Liu and Santi (26) recently reported an analogous result with a variant of TS from *Lactobacillus casei*. Thirteen other substitutions of Asn^{177}, examined by suppression of an *am177* allele of *thyA* (27), decreased the activity of *E. coli* TS by at least 100-fold.

The first indication of how this invariant Asn in TS might interact with dUMP came from a molecular modeling exercise (8). In that exercise, docking of dUMP into the active site of crystalline TS suggested an interaction between O4 of dUMP and Asn^{229} of *L. casei* TS (corresponding to Asn^{177} in *E. coli* TS). Subsequent structures of actual nucleotide complexes of *E. coli* TS (9, 11) confirmed the initial guess and showed that the side-chain amide of Asn^{177} also interacts with N3 of dUMP (Fig. 1). The importance of the protonation state of N3 for binding of nucleotides to TS was inferred from studies with 4-thio-FdUMP (28).

The relative values of k_{cat}/K_m for dUMP and dCMP (Table 1) provides a comparison of the binding energy between TS and these substrates in the transition state for the first irreversible catalytic step. At physiological temperatures and nucleotide concentrations, this interaction of dUMP with TS(wt) is at least 11 kcal more favorable than that of dCMP. The origin of the substrate specificity is unlikely to lie in conformational differences between the unbound nucleotides, since the conformation of enzyme-bound dUMP more closely resembles the conformation of crystalline dCMP than it does the conformation of crystalline dUMP (L.W.H., unpublished observations). Likewise, the hydrogen bonds between the side-chain amide of Asn^{177} and N3 and O4 of dUMP alone are not sufficient to explain the discrimination of TS(wt) between dUMP and dCMP. Loss of two hydrogen bonds between uncharged heteroatoms would decrease the interaction energy by ≈3 kcal·mol^{-1} (29). In contrast, loss of a hydrogen bond or proton donor to a charged group on a substrate or intermediate could provide much more discrimination (29). We propose that the basis for the specificity of TS is mechanism-based and is due to the differing needs for charge stabilization in catalytic intermediates, on O4 of dUMP or on N3 of dCMP.

Matthews *et al.* (11) have proposed that Asn^{177} stabilizes, by hydrogen bonding, the partial negative charge developed on O4 of covalently bound dUMP during catalysis. In the analogous intermediates with dCMP, the negative charge would not be delocalized onto the exocyclic N4 but would instead be borne by N3. An amine anion at N3 would be considerably less stable than an O4 oxyanion. A hydrogen bond from the amide of Asn^{177} is, not surprisingly, insufficient to stabilize the amine anion in a dCMP intermediate.

We hypothesize that replacing Asn^{177} with Asp provides an acid that could stabilize amine anion intermediates by protonation (Fig. 4). [The reciprocal mutation in dCMP hydroxymethylase reverses the substrate specificity of that enzyme (K. L. Graves, M. M. Butler, and L.W.H., unpublished work).] This hypothesis is supported by the increase in k_{cat} for TS(N177D) as the pH decreases from 7 to 5.5, in contrast to the decrease in k_{cat} for TS(wt). The values of k_{cat}/K_m for the two enzymes decrease proportionately as the pH decreases from 7 to 5.5. Thus, the N177D mutation probably does not much affect formation of the initial enzyme–nucleotide complex; it principally affects subsequent covalent steps in catalysis.

In the mechanism proposed by Matthews *et al.* (11), removal of the proton from C5 coincides with the rupture of the bond connecting the methylene group to N5 of THF. Transfer of this proton, via a bound water, to O4 of the pyrimidine polarizes the exocyclic methylene carbon and activates it for hydride acceptance. The intermediate formed with dCMP could be activated by protonation of either N3 or an exocyclic N4 imine.

One of the intermediates resulting from the reaction of dUMP with TS(N177D) and CH_2THF may be too stable. This would explain the inactivation of TS(N177D) by dUMP, and the biphasic kinetics and low rate of turnover observed with dUMP. The CH_2THF-dependent binding of 6[^{3}H]dUMP by TS(N177D) (Table 2) suggests that inactivation by dUMP is due to the accumulation of a catalytic intermediate. We suggest that this intermediate contains dUMP covalently linked to both CH_2THF and enzyme (Fig. 4), by analogy with the covalent complex containing TS, FdUMP, and CH_2THF. Enzyme-bound tritium does not increase when TS(N177D)

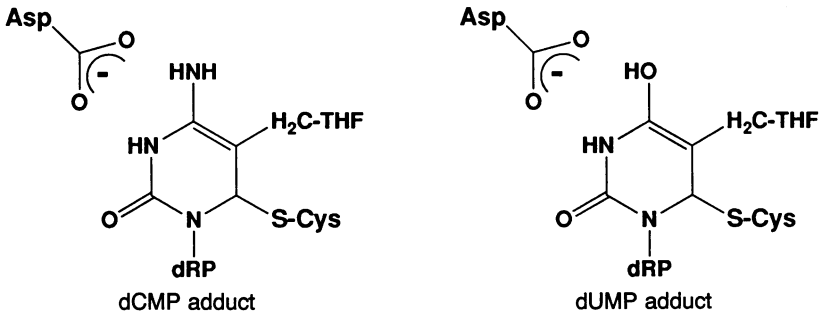

FIG. 4. Proposed structures for covalent intermediates formed during the reaction of dCMP (*Left*) or dUMP (*Right*) with CH_2THF, catalyzed by TS(N177D). dRP, 1'-(2'-deoxyribosyl 5'-monophosphate); sulfur atom of the cysteine residue is shown explicitly.

reacts with 5[^{3}H]dUMP (Table 2), so the enzyme-bound dUMP has already released the proton (or $^3H^+$) from C5. This intermediate is further along the reaction pathway than that which is mimicked by the FdUMP complex, since the fluorine on C5 cannot be released as F^+. The persistence of about half of the enzyme-bound dUMP after urea denaturation supports the idea that the nucleotide is bound covalently, although its loss after heating with SDS indicates that this complex is more labile than that formed with FdUMP.

A serious flaw in the consideration of dUMP-inactivated TS(N177D) as a long-lived catalytic intermediate is a kinetic incompetence of this species. If the inactivity of dUMP-inactivated enzyme toward dCMP is due simply to accumulation of a catalytic intermediate, the rate constant for reactivation should equal the rate constant for turnover of dUMP at steady state. In fact reactivation is ≈10 times slower than dUMP turnover. One conceivable explanation is negative cooperativity between the active sites of the TS dimer (see ref. 6).

We have not measured the intracellular levels of 5-methyl-dCMP produced by TS(N177D). Since dUMP effectively inhibits the turnover of dCMP, little 5-methyl-dCMP may accumulate. Any that does must be nontoxic, since *E. coli* cells making enough TS(N177D) to complement Δ*thyA* have no obvious negative phenotype. 5-Methylcytosine residues in DNA are mutagenic (30) and would block cleavage of the DNA by *Ava* II, *Bam*HI, and *Pst* I endonucleases. Neither prediction was observed. Even if 5-methyl-dCMP were made *in vivo*, it should not be a DNA precursor (31), although it can be *in vitro* (32). 5-Methyl-dCMP appears to be a poor substrate for the kinases of *E. coli* (31).

There is evidence that DNA (cytosine-5)-methyltransferases employ Michael adducts similar to the initial adduct in catalysis by TS (33–35). DNA methyltransferases may stabilize such adducts by protonating N3 of the acceptor cytosine residue. Alternatively, this methylation may be more simple, due to the different one-carbon donor used (*S*-adenosylmethionine), and may not require the stabilization of a long-lived intermediate.

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