Aspartate 221 of Thymidylate Synthase Is Involved in Folate Cofactor Binding and in Catalysis†

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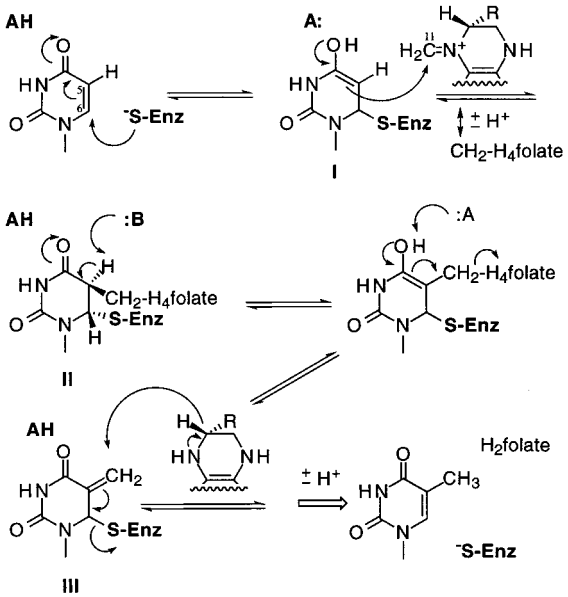
Received February 3, 1998; Revised Manuscript Received April 24, 1998

ABSTRACT: Structural studies indicate that Asp 221 of *Lactobacillus casei* thymidylate synthase forms a hydrogen bond network with the 2-amino and 3-imino groups of the folate [Matthews, D. A. (1990) *J. Mol. Biol. 214*, 937−948; Finer-Moore, J. S. (1990) *Biochemistry 29*, 6977−6986] that has been proposed to participate in catalysis. We prepared a complete replacement set of 19 mutants at position 221 of *L. casei* thymidylate synthase. Of these, the only one with sufficient activity to complement growth of a thymidylate synthase-deficient host was the Cys mutant. To further elucidate the function of the Asp 221 side chain, seven thymidylate synthase 221 mutants were studied in detail with regard to catalysis of dTMP formation and of thymidylate synthase partial reactions. Most of the mutants bound the nucleotide substrate dUMP with only moderate loss of binding affinity, indicating that the Asp side chain does not contribute to dUMP binding. Most of the mutants catalyzed the cofactor-independent dehalogenation of 5-bromodUMP; hence, the Asp side chain of TS is not essential for addition of the catalytic Cys residue to the nucleotide substrate. Mutants showed decreased affinity for the folate cofactor, but those with side chains capable of hydrogen bond formation were less severely affected. Some of the mutants were capable of forming covalent thymidylate synthase-5-fluorodUMP−methylenetetrahydrofolate complex; hence, the Asp side chain is not essential for steps leading to the covalent complex. We conclude that the hydrogen bond network between Asp 221 and the folate cofactor contributes to cofactor binding and a catalytic step after formation of the covalent ternary complex intermediate.

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_2folate). Much is known about the structure and mechanism of TS. 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 (for a recent review, see ref *1*).

The salient features of the enzymatic reaction mechanism are depicted in Scheme 1 (*1*). After formation of a reversible ternary complex, nucleophilic attack by a thiol (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 (C-11) of CH_2H_4-folate, which has been activated by formation of an iminium ion at N-5, to produce intermediate **II**. The C-5 proton of **II** is abstracted, followed by β-elimination of H_4folate to give the exocyclic methylene intermediate **III**. Hydride transfer from noncovalently bound H_4folate to the exocyclic methylene intermediate **III** and β-elimination of the enzyme result in the products H_2folate and dTMP, and regenerate

Scheme 1: Salient Features of the Chemical Mechanism of TS



†This work was supported by USPHS CA-14394 and a fellowship to G.C. from MURST (University of Modena).

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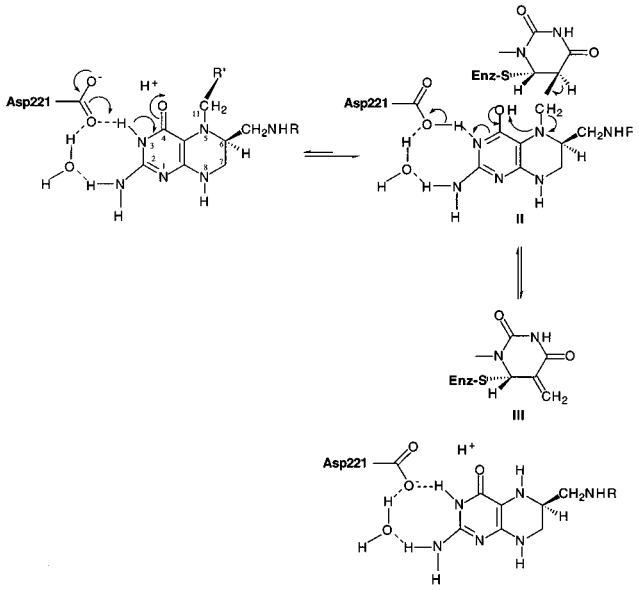
[1] Abbreviations: TS, thymidylate synthase; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dUMP, 2′-deoxyuridine 5′-monophosphate; BrdUMP, 5-bromo-2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; dTMP, 2′-deoxythymidine 5′-monophosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TES, 2-[*N*-[tris(hydroxymethyl)methyl]amino] ethanesulfonic acid; PLP, pyridoxal 5′-phosphate; DTT, dithiothreitol. TS mutants are named by suffixing the letter code of the mutant residue to D221; thus, D221C refers to the mutant containing Cys at position 221.

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

S0006-2960(98)00277-3 CCC: $15.00
Published on Web 06/05/1998

Scheme 2: Proposed Role of Asp 221 in Stabilization of the Enolate Form of the Cofactor Pterin and Breakdown of the Covalent Steady-State Intermediate



active enzyme. An important tool in understanding the mechanism of TS has been the mechanism-based inhibitor FdUMP which, through a mechanism analogous to that in Scheme 1, forms a stable analogue of the covalent intermediate **II**.

Currently, the least understood features of this reaction are those involving the cofactor. Structural studies have shown that, as depicted in Scheme 2, the side chain of the completely conserved Asp 221 is hydrogen bonded to the 3-NH and, through a bridging water, to the exocyclic 2-NH_2 of the pteridine ring of the cofactor *(2, 3)*; the peptide backbone NH of Asp 221 is also hydrogen-bonded to the 2-oxo group of the nucleotide substrate, dUMP. In view of the hydrogen-bond network with the cofactor, one might expect that mutants of Asp 221 would show decreased affinity for CH_2H_4folate. In addition, the hydrogen bond between Asp 221 and the 3-NH of CH_2H_4folate has been proposed to assist the protonation of N-5 of the cofactor moiety of covalent intermediate **II**, and thus facilitate the leaving group ability of H_4folate *(2)*. If this proposal is correct, mutants of TS Asp 221 should be defective in the breakdown of the ternary covalent intermediate, **III**.

In a previous study, Michaels et al. *(4)* showed that of 13 Asp 221 mutants of *Escherichia coli* TS studied, only D221C weakly supported growth of TS-deficient cells. In the present work, we studied a replacement set of 19 mutants of Asp 221 of TS to obtain information about the function of this residue. From detailed studies of seven of these mutants, we conclude that the side chain of Asp 221 is important in cofactor binding and in the breakdown of the ternary covalent intermediate.

MATERIALS AND METHODS

Materials. *E. coli* strain χ2913recA (ΔthyA572, recA56) *(5)* and plasmids pSCTS-D221A, pSCTS-D221N, pSCTS-D221R, and pSCTS-D221F have been described *(6)*. (6R)-CH_2H_4folate was a gift from SAPEC S. A. (Lugano, Switzerland). [5-^{3}H]dUMP (15.9 Ci/mmol) and [2-^{14}C]-dUMP (56 mCi/mmol) were from Moravek Biochemicals. All other materials were the highest purity available from commercial sources and were used without purification.

Mutagenesis. Cassette mutagenesis was performed using a synthetic *L. casei* TS gene in the plasmid pSCTS9 (*Not* I stuffer) which contains a noncoding sequence with a *Not*I site between the *Bgl*II and *Avr*II sites of the synthetic gene *(6)*. Two different coding cassettes were ligated into *Bgl*II/*Avr*II digested pSCTS9 (*Not* I stuffer). The first contained the degenerate sequence, G(GAT)A at codon 221, and the second contained (ACT)(N)(GC). Together, these two cassettes encode all amino acids except Asp and Ala; D221A was previously prepared *(6)*. 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 *(7)*. D221S was also obtained by ligating an oligonucleotide cassette with the sequence, TCT, at codon 221 into *Bgl*II/*Avr*II digested pSCTS9 (*Not*I stuffer).

Protein Purification. The mutant enzymes (D221A, N, R, F, C, E, and S) were purified using sequential chromatography on phosphocellulose and hydroxyapatite *(8)* and were >95% homogeneous as judged by Coomassie-stained SDS−PAGE. Five to 25 mg of pure protein was obtained from 1 L of cell culture. Purified enzymes were 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 *(9)*. 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.

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 *(10)*. Reaction mixtures contained 10 μM D221A or D221N, 0.53 mM [2-^{14}C,5-^{3}H]dUMP (8.9 mCi ^{3}H/mmol, 0.223 mCi ^{14}C/mmol), and 0.51 mM (6R,6S)-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 at 22 °C by the decrease in absorbance which accompanies dehalogenation ($\Delta\epsilon_{285} = 5320$ M^{-1} cm^{-1}) *(11)*. Reaction mixtures (750 μL) contained TES/DTT assay buffer (standard TES buffer containing 5 mM DTT instead of β-mercaptoethanol), 10 to 210 μM BrdUMP, and 4 μM enzyme. One unit of activity is the amount of enzyme that debrominates 1 μmol of BrdUMP per minute in a 1 mL reaction mixture.

All measurements were obtained using a Hewlett-Packard 8452 diode array spectrophotometer. Kinetic and thermodynamic 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 Macintosh PowerPC.

Determination of Dissociation Constants by Displacement of PLP from TS−PLP Complexes. The K_ds of TS−PLP complexes were determined by titration of enzyme with PLP and monitoring the absorbance at 330 nm *(12)*. Solutions containing complexes formed from 10 or 30 μM PLP and 3 μM enzyme in 50 mM TES/KOH, 1 mM EDTA, and 50 μM DTT, pH 7.4, were titrated with varying concentrations

Table 1: Equilibrium and Kinetic Constants of Wild-Type and D221 Mutants of *L. casei* TS[a]

		dTMP formation			BrdUMP dehalogenation		binding		
mutant	compl. *a*	k_{cat} (sec^{-1})	$K_{m,dUMP}$ (μM)	$K_{m,cofactor}$ (μM)	k_{cat} (sec^{-1})	$K_{m,BrdUMP}$ (μM)	$K_{d,\ PLP}$ (μM)	$K_{d,dUMP}$ (μM)	$K_{d,cofactor}$[b] (μM)
Asp221 (wild type)	+	5.0	5.0	10	0.1	5.7	0.3	0.4	stoich[c]
Asp221C	+	0.14	15.0	140	0.02	25	0.7	2.0[d]	stoich[c]
Asp221S	−	0.02	6.3	240	0.05	27	1.9	1.7	6.5
Asp221E	−	0.016	11.0	1000	0.004	46	5.1	12[d]	140
Asp221A	−	0.0006[e]			0.005	20	5.3	5.6	500
Asp221N	−	0.0006[e]			0.15	45	1.8	7.5	
Asp221F	−	nd[f]			nd	nd	0.9	200	
Asp221R	−	nd[f]			nd	nd	0.3	nd	

[a] Standard errors of nonlinear least-square plots were <10% of the values. [b] Apparent K_d determined by SDS−PAGE. [c] Stoichiometric binding; for wild-type TS, the reported K_d is 10^{-11} M *(17)*. [d] Only one PLP per TS dimer is displaced. [e] k_{cat} by tritium release assay. [f] Not detectable by spectrophotometric assay.

of dUMP or FdUMP, and the decrease in absorbance was monitored to obtain K_d values for the enzyme−nucleotide complexes.

Ultraviolet Difference Spectroscopy of the TS−FdUMP−CH_2H_4folate Complex. The ternary TS−FdUMP−(6R)-CH_2H_4folate complex was analyzed spectrophotometrically *(10, 13)*. For titrations with FdUMP, reaction mixtures (750 μL) containing 5.5 μM D221A or D221N and 75, 150, or 300 μM (6R)-CH_2H_4folate, respectively, in the TES/DTT assay buffer, were titrated with up to 560 μM FdUMP. For titrations with the cofactor, reaction mixtures (750 μL) containing 4.2 μM D221A or D221N and 100 μM FdUMP were titrated with up to 265 μM (6R)-CH_2H_4folate.

SDS−PAGE of Enzyme−FdUMP−CH_2H_4folate Complexes. Covalent ternary complexes were formed by incubating mixtures containing 1−3 μM D221 mutants, 8.5 mM FdUMP, and up to 6.6 mM (6R)-CH_2H_4folate in TES/DTT buffer at ambient temperature. After 2 h, aliquots (15 μL) were denatured, loaded on 12% SDS−PAGE (0.1 × 19 cm) with a 5% stacking gel *(14)* and electrophoresed for 4 h at 20 mA. The Coomassie Brilliant Blue stained gels were dried on Whatman 3MM paper, using a gel dryer at 80 °C under vacuum for 1 h. Digital images of the gels were obtained using a fotodyne camera interfaced to a Macintosh PowerPC, and quantified using NIH Image Program 1.61. The ratio of bound/total enzyme was plotted versus (6R)-CH_2H_4folate concentration to determinate the apparent K_d for (6R)-CH_2H_4folate in the ternary complex.

RESULTS

Complementation of TS-Deficient E. coli. In the present work, we tested two libraries which together contained all *L. casei* TS mutants of Asp 221 except for Ala for their ability to complement the growth of TS deficient *E. coli*. *L. casei* TS D221A does not complement growth (D. V. Santi, unpublished). In a library containing Glu, Gly, and Val at position 221, there were no surviving colonies in the absence of Thy. In a library containing the other 15 mutants, 6% were positive in the complementation assay; DNA sequencing of active mutants revealed only the codon for Cys at position 221.

dTMP Formation. In vitro assays for dTMP formation were performed spectrophotometrically or by the more sensitive assay for tritium release from [5-^{3}H]dUMP. Table 1 shows the activities and/or steady-state kinetic parameters for seven Asp 221 mutants chosen for further study (D221A, C, E, F, N, R, and S). D221A, F, N, and R (10 μM) were inactive by the spectrophotometric assay. In the more sensitive tritium release assay, D221A and N (10 μM) showed specific activities of ca. 6×10^{-4} U/mg, at least 10^4-fold lower than wild-type TS.

Dissociation Constants for dUMP and FdUMP. All seven mutant TSs studied (D221A, C, E, F, N, R, and S) formed complexes with PLP, showing the characteristic absorbance maximum at 328 nm *(12)*. The mutant enzyme−PLP complexes had K_d values in the low micromolar range (Table 1), and extinction coefficients similar to wild-type TS (data not shown). With exception of D221R, titration of enzyme−PLP complexes with dUMP or FdUMP and monitoring the loss of absorbance at 328 permitted calculation of K_d values for the enzyme−nucleotide binary complexes. For D221C and E, saturating dUMP only displaced ∼50% of the bound PLP, indicating that in the binary complex the nucleotide binds only to a single subunit of TS. The K_d values for dUMP for the D221C, E, A, N, and S mutants were from 4- to 30-fold higher than wild-type TS. For D221F the K_d was about 400-fold higher than wild-type TS, and binding was undetectable for D221R at up to 3.5 mM dUMP. With FdUMP as titrant, K_d values of 72 μM for D221A and 20 μM for D221N were obtained; other mutants were not examined for FdUMP binding.

Dehalogenation of BrdUMP. Wild-type TS and the D221A, N, C, E, and S mutants catalyzed the thiol-dependent dehalogenation of BrdUMP to produce dUMP (Table 1) *(11)*. No debromination of BrdUMP was detectable with up to 7 μM D221R or D221F after as long as 1 h. D221N and D221S had a similar k_{cat} values and 5- to 8-fold higher K_m values for debromination compared to wild-type TS. D221C had a 5-fold lower k_{cat} and a ∼4-fold higher K_m than did wild-type TS. The remaining mutants, D221A and E, showed ∼20-fold lower k_{cat} and ∼3 to 8-fold higher K_m values than did wild-type TS.

Covalent Complexes of Mutants with FdUMP and CH_2H_4folate. TS, FdUMP, and CH_2H_4folate interact to form a covalent complex analogous to steady-state intermediate **II** of the TS reaction (Scheme 1). As with wild-type TS *(15)*, D221A, C, E, and S form a ternary complex with FdUMP and CH_2H_4folate which shows a characteristic absorbance increase at 330 nm. No ternary complex formation was detected with D221N.

Covalent complex formation was also monitored by SDS−PAGE. When 3.5 μM D221A, C, E, and S plus 8.5 mM FdUMP were incubated with up to 2 mM (6R)-CH_2H_4folate, a cofactor-dependent protein band was observed just above

the band for the unbound 35 kDa subunit. The new band represents TS subunits covalently bound to FdUMP and (6R)-CH_2H_4folate. By plotting the intensity of the bands versus cofactor concentration, we determined the apparent K_d values for (6R)-CH_2H_4folate given in Table 1. It has previously been shown that these values are products of the equilibrium constants for noncovalent binding and covalent complex formation within the ternary complex *(10)*.

DISCUSSION

Structural studies have implicated functional roles for the conserved Asp 221 of *L. casei* TS *(2, 3)*. The side chain of Asp 221 is hydrogen bonded to the 3-NH and, through a bridging water, to the exocyclic 2-NH_2 of the pteridine ring of the cofactor (Scheme 2). This hydrogen bond network has been proposed to enhance protonation of N-5 of the cofactor and thus facilitate conversion of covalent intermediate **II** to intermediate **III** (Scheme 2) *(2)*. The objective of the present work was to ascertain whether the conserved Asp 221 of TS was indeed important in the TS reaction, and if so, where in the pathway it served its role(s).

To determine the necessity for Asp 221 of TS, we prepared a complete replacement set of 19 mutants at position 221 and assessed their abilities to support the growth of a TS-deficient strain of *E. coli*. It has previously been shown that this assay detects TS mutants with catalytic activity as low as 1% of the wild-type enzyme *(16)*. As previously reported for 13 *E. coli* Asp 221 mutants *(4)*, only D221C provided catalytically active enzyme by the complementation assay. We concluded that Asp 221 was important, but not essential for activity.

We chose seven mutants of TS Asp 221 for detailed studies—the noncomplementing Ala, Arg, Asn, Glu, Phe, and Ser mutants, and the complementing Cys mutant. Our objective was to define the specific effects of modifying the side chain of Asp 221 on TS function, and in turn obtain insight into the role of the native Asp side chain.

We first studied the activities and steady-state kinetic parameters of the selected Asp 221 mutants on dTMP formation (Table 1). The mutants which did not contain potential strong hydrogen bond donor/acceptor groups in their side chains (D221A, N, F, and R) showed negligible activity ($\geq$10000-fold lower k_{cat} than wild-type TS). The three mutants with potential hydrogen bond donor/acceptor groups at position 221 (D221C, E, and S) showed far less striking reductions in enzymatic activity. Expectedly, the D221E mutant, which contains the glutamate homologue at residue 221, retained activity; however, k_{cat} was reduced $\sim$300-fold and the K_m values for dUMP and folate were increased 3- and 100-fold, respectively, compared to wild-type TS. D221S showed a 250-fold decrease in k_{cat} compared to wild-type, a similar K_m for dUMP, and a 25-fold increase in K_m for the cofactor. D221C, the only mutant active in the complementation assay, showed only a 35-fold decrease in k_{cat}, a K_m for dUMP within 3-fold of the wild-type enzyme, and a 14-fold decrease in the K_m for cofactor. The catalytic efficiency ($k_{cat}/K_{m,cofactor}$) for D221C was some 12-fold greater than D221S and 62-fold greater than D221E. These results suggest that the side chain of residue 221 functions as a proton donor/acceptor for both folate binding and catalysis.

We next examined the ability of the Asp 221 mutants to catalyze several partial reactions of TS which, together, define the reaction pathway up to and including the formation of covalent intermediate II. It was hoped that such studies would identify specific defects in the pathway of the mutants, and thus assist in identification of specific roles of Asp 221. The partial reactions included substrate binding, reaction with the essential enzyme catalytic Cys thiol, and formation of the FdUMP analogue of steady-state intermediate II.

The first partial reaction studied was the ability of Asp 221 mutants to bind dUMP. Although the side chain of Asp 221 is not directly involved in nucleotide binding, the peptide chain NH of residue 221 is hydrogen bonded to the 2-oxo moiety of dUMP *(3)*. We speculated that such hydrogen bonding could (i) contribute to dUMP binding, (ii) enhance polarization of the pyrimidine for reaction with the sulfhydryl nucleophile, or (iii) stabilize the orientation of the pyrimidine within the active site. We found that D221C, N, and S bound dUMP only $\sim$4-fold poorer than wild-type, D221A and E bound dUMP $\sim$17-fold poorer than wild-type, D221F bound dUMP very poorly (500-fold lower K_d than wild-type), and D221R did not bind dUMP (Table 1). Since good binding of dUMP was observed with three of the mutants studied, the side chain of Asp 221 per se cannot contribute significantly to nucleotide binding. However, since some mutations (e.g., F and R) caused significant loss in binding affinity for dUMP, secondary effects of some side chains can confer detrimental effects—perhaps on backbone hydrogen bonding to dUMP which in turn affects dUMP binding or orientation.

The second partial reaction examined was the ability of the Asp 221 mutants to catalyze the dehalogenation of BrdUMP, a cofactor-independent partial reaction of TS which requires competency in nucleotide binding and the addition/elimination reactions of the Cys nucleophile of TS to the 6-position of the nucleotide substrate *(11)*. The K_m values of Asp 221 mutants for dehalogenation were decreased by 3- to 7-fold, in general agreement with the effects of the mutants on nucleotide binding. For BrdUMP dehalogenation, D221N and D221S had k_{cat} values similar to wild-type TS, demonstrating that the carboxylate side chain of Asp 221 was not essential for covalent bond formation of the nucleotide with Cys 198. However, the k_{cat} values of the remaining mutants were decreased from 5- to over 20-fold, indicating that mutant 221 side chains may secondarily affect the reaction of Cys 198 with the nucleotide, perhaps through perturbation of the peptide backbone interaction with the 2-oxo group of dUMP.

The third partial reaction examined was the ability of the mutants that bound dUMP (D221A, C, E, N, and S) to form TS−FdUMP−CH_2H_4folate covalent complexes. It is been well established that TS and many TS mutants react with FdUMP and CH_2H_4folate to form a stable, covalent analogue of steady-state intermediate II (Scheme 1). Formation of such covalent complexes shows that a mutant enzyme is capable of (i) binding nucleotide and cofactor, (ii) forming a covalent adduct with FdUMP, and (iii) forming a carbon−carbon bond between C-5 of the FdUMP adduct and the one carbon unit of the CH_2H_4folate. This study was intended to ascertain which of these steps might be impaired in Asp 221 mutants.

The covalent complex formed with the wild-type enzyme has an apparent K_d for CH_2H_4folate of $\sim 10^{-11}$ M, which reflects the products of the equilibrium constants for noncovalent binding, and covalent complex formation within the

ternary complex *(17)*. With D221N, no covalent complex was detectable by either UV difference spectroscopy or SDS−PAGE. D221N catalyzed the dehalogenation of BrdUMP, so this mutant is capable of forming the essential thiol adduct with nucleotides; also, the mutant must bind cofactor to some extent since it catalyzes dTMP formation. We therefore conclude that a defect in the D221N mutant is in the formation of the covalent adduct with CH_2H_4folate. With mutants D221A and E, the covalent complex formed, but only at very high folate concentrations. Indeed, monitoring covalent complex formation as a function of cofactor concentration gave apparent K_d values for CH_2H_4folate exceeding 10^{-5} M, 10^6-fold higher than wild-type enzyme. D221S formed a covalent complex with a low, but measurable K_d for CH_2H_4folate. With D221C, the covalent complex was formed at concentrations of cofactor which, under conditions used, were stoichiometric with the enzyme (i.e., apparent $K_d < 1\ \mu$M).

The primary conclusions of this study are as follows. First, the side chain of Asp 221 contributes little, if any, to the binding of dUMP or to the subsequent thiol addition to the nucleotide. Several mutants were nearly as effective as wild-type enzyme in dUMP binding and BrdUMP dehalogenation. However, mutations can secondarily affect these reactions, perhaps by perturbation of optimal backbone hydrogen bonding to the 2-oxo group of dUMP. Second, the hydrogen bond(s) formed between Asp 221 and CH_2H_4folate clearly contribute to cofactor binding. All of the mutants studied showed increases in both the K_m and apparent K_d values for the cofactor. However, the mutants that were least affected are those which are potentially capable of hydrogen bonding to the cofactor (D221C and S). Third, since Asp 221 clearly contributes to catalysis of dTMP formation, it may be involved in assisting a step after intermediate II which, as proposed from structural studies *(2)*, may involve the conversion of intermediate II to III. Fourth, the functions of Asp 221 on folate binding and catalysis are attributable to its participation in a hydrogen bond network to the cofactor, as surmised from structural studies *(2, 3)*. Those mutants with 221 side chains potentially capable of forming hydrogen bonds (e.g., Cys, Ser) with the cofactor were the most effective enzymes. Further, of these, Cys is the more effective hydrogen bond donor, and is also the most effective mutant catalyst. Since the Cys and Ser mutants can donate/accept only a single hydrogen bond, we assume that one rather than two hydrogen bonds from Asp 221 to cofactor is crucial for catalysis. We are seeking evidence for this hypothesis from structural studies of these mutants.

ACKNOWLEDGMENT

We thank Patricia Greene and Gary Liu for helpful discussions, and Kathryn Ivanetich for help with the manuscript. We also thank Jia Zhao, Ming Yu, and Maria Elena Diaz for their excellent technical assistance.

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BI9802770