A Hydroxyl Group at Residue 216 Is Essential for Catalysis by Human Thymidylate Synthase†

Angela W. Williams,‡ R. Bruce Dunlap,‡ and Sondra H. Berger*,§

Departments of Chemistry & Biochemistry and Basic Pharmaceutical Sciences, University of South Carolina, Columbia, South Carolina 29208

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ABSTRACT: Structural analyses of bacterial thymidylate synthases (TSs) implicate a serine residue corresponding to Ser216 in human TS in hydrogen bond networks that are involved in binding of the nucleotide substrate, 2′-deoxyuridylate (dUMP), and that stabilize a β-bulge in the protein. Utilizing site-directed mutagenesis, 12 mutant proteins were created with substitutions at residue 216. DNA complementation studies utilizing a TS-negative bacterial strain revealed that only one mutant, Thr216 TS, supports the growth of the bacteria in the absence of thymidine. Kinetic characterization of the mutant proteins revealed that all TSs except Thr216 TS exhibited k_{cat}/K_ms for dUMP that are 10^3-10^4 times lower, relative to that of wild-type TS. In addition, Thr216 TS was the only mutant to bind the mechanism-based inhibitor, 5-fluoro-2′-deoxyuridylate (FdUMP), into a ternary complex. Ligand binding studies revealed that K_ds for dUMP binding to two defective mutants, Ala216 and Leu216 TSs, are 12−16-fold higher than that of wild-type TS. The data are consistent with the hypothesis that serine at this relative position is involved in dUMP binding; however, the data indicate that Ser216 has effects on catalysis, in addition to effects on dUMP binding. Catalysis is initiated by nucleophilic attack of the active site cysteine of TS on dUMP. The reaction rates of cysteine residues with the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) were slower for Ala216 TS than for wild-type TS.

Thymidylate synthase (TS;[1] EC 2.1.1.45) catalyzes the reductive methylation of dUMP by CH_2H_4folate to produce TMP and H_2folate. The enzymatic reaction provides the sole de novo source of TMP, an essential precursor required for DNA biosynthesis. During this oxidoreduction reaction, a methylene group is contributed by CH_2H_4folate to position 5 of dUMP (Figure 1) (*1*). dUMP is activated for substitution at position 5 by the addition of an enzyme nucleophile at C-6. Loss of the proton at position 5 of dUMP results in the formation of an exocyclic methylene, and subsequent elimination of H_4folate. The transfer of a hydride from H_4-folate to the exocyclic methylene results in its reduction to a methyl group and oxidation of H_4folate to H_2folate. H_2-folate then dissociates from the active site prior to TMP. The nucleophile was shown to be a cysteinyl residue of the enzyme by chemical labeling studies and by X-ray crystallographic studies of the liganded structure of *Escherichia coli* TS (*2*−*4*).

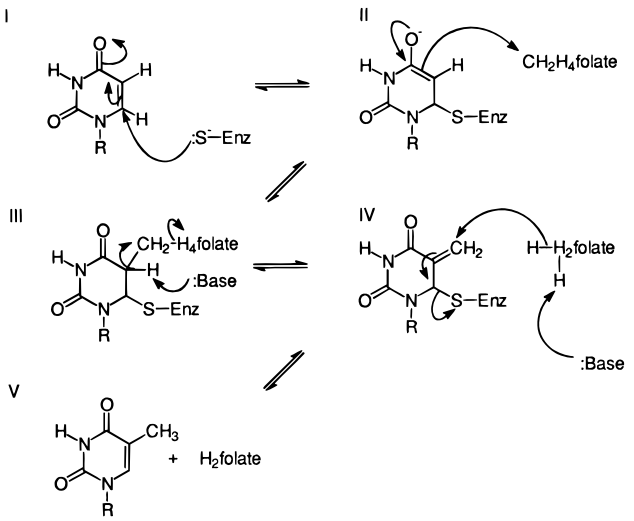

FIGURE 1: Key features of the reaction mechanism of TS.

TS catalyzes a reaction that is essential for DNA replication; thus, the enzyme has been an attractive target of chemotherapeutic agents. One such agent, 5-fluorouracil, is metabolized intracellularly to FdUMP, which acts as a mechanism-based inhibitor of TS by forming a stable covalent complex with CH_2H_4folate and the enzyme (*1*). A ternary complex is formed which is postulated to be analogous to the catalytic ternary complex (**III**, Figure 1); unlike the catalytic ternary complex, the inhibitory ternary complex is stable, with dissociation constants of 10^{-10} to 10^{-11} M being reported (*5*, *6*).

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* To whom correspondence should be addressed: Department of Basic Pharmaceutical Sciences, University of South Carolina, 700 Sumter St., Columbia, SC 29208. E-mail: berger@phar2.pharm.sc.edu.

‡ Department of Chemistry & Biochemistry.

§ Department of Basic Pharmaceutical Sciences.

[1] Abbreviations: TS, thymidylate synthase; CH_2H_4folate, 5,10-methylenetetrahydrofolate; H_2folate, 7,8-dihydrofolate; dUMP, 2′-deoxyuridylate; TMP, thymidylate; FdUMP, 5-fluoro-2′-deoxyuridylate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PDDF, 10-propargyl-5,8-dideazafolate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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Studies of the three-dimensional structures of native bacterial TSs (*7*) and, more recently, of native human TS (*8*) have shown that a series of β-bulges form a kink in three of the central β-sheets that form the dimer interface. It has been suggested that this region acts as a hinge during conformational changes in the protein that occur upon the binding of ligands at the active site (*9*). One of the bulges comprising the kink is close to the active site. The residues that are located within and adjacent to this bulge in human TS—Tyr213, Gln214, Arg215, and Ser216—are highly conserved among all TSs examined to date. On the basis of nucleotide sequence analysis, only one of 29 TSs from divergent species has a residue other than serine at a position corresponding to 216 in human TS (*1*). Studies of the crystal structures of bacterial TSs have led to the suggestions that the hydroxyl side chain of serine at this relative position is involved in hydrogen bond networks that contribute to the stability of the β-bulge and to the binding of the phosphate group of dUMP (*4*, *10*, *11*).

Characterization of a TS-deficient human colonic tumor cell line revealed that a base change is present at nucleotide 647 (*12*) in TS mRNA which predicts the substitution of Ser216 by a leucine residue in the protein (D. K. Hoganson et al., submitted for publication). To establish that the sole basis for the TS-negative phenotype in this cell line involved Ser216, a TS cDNA encoding the S216L mutation was created. Growth complementation analysis of transformants expressing Leu216 TS revealed that the mutant protein is unable to confer thymidine prototrophy. Since leucine at position 216 could create a defective protein due to either loss of hydrogen bonding potential or the introduction of a side chain with increased volume, additional substitutions were created at this position. This paper reports the biological properties of mutant human TSs in which 12 residues have been substituted for Ser216.

EXPERIMENTAL PROCEDURES

Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). The CLONTECH Transformer Site-Directed Mutagenesis Kit was from CLONTECH Laboratories (Palo Alto, CA). Oligonucleotide primers for site-directed mutagenesis and sequencing were purchased from the Oligonucleotide Synthesis Facility of the Institute for Biological Research and Technology (IBRT, University of South Carolina). The T7 Sequenase version 2.0 DNA sequencing kit was from Amersham Life Science (Cleveland, OH). [6-^{3}H]-5-Fluoro-2′-deoxyuridylate ([6-^{3}H]FdUMP, 15 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). FdUMP, dUMP, 10-propargyl-5,8-dideazafolic acid (PDDF), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), bovine serum albumin (BSA), 2-mercaptoethanol (2-ME), and antibiotics were obtained from Sigma (St. Louis, MO). Tris(hydroxymethyl)aminomethane (Tris) and ethylenediaminetetraacetic acid (EDTA) were obtained from Research Organics, Inc. (Cleveland, OH). (6*S*)-5,6,7,8-Tetrahydrofolic acid was prepared and converted to (6*R*)-5,10-methylenetetrahydrofolate (CH_2H_4PteGlu) as described previously (*13*). Blue Sepharose CL-6B and Sephacryl S-200 HR were purchased from Pharmacia Biotech (Piscataway, NJ).

Bacterial Strains, Plasmids, and Primers. Plasmid pTS080 is a bacterial expression vector containing a human TS cDNA insert under the control of the ϕ10 promoter for T7 RNA polymerase (*14*). The plasmid was kindly provided by W. Dallas (Glaxo-Wellcome Research Laboratories, Research Triangle Park, NC). Site-directed mutagenesis was conducted by utilizing *E. coli* strain BMH 71−18 mutS which is commercially packaged in the Transformer Site-Directed Mutagenesis Kit. A *thyA*-negative *E. coli* derivative of BL21(DE3), designated TX61, was obtained from W. Dallas. TX61 requires thymidine for growth, is resistant to kanamycin, and has no detectable TS activity (*15*). As mutagenic primers, 22−28 bp oligonucleotides were utilized, with appropriate nucleotide changes at positions complementary to residues 646−648 (codon 216) of human *thyA*. A 20 bp oligonucleotide that creates a G → C mutation at position 345 of human *thyA* was utilized as a primer for the selection of mutated DNAs. This mutation eliminates the unique *Ava*I site in pTS080.

Mutagenesis and DNA Sequencing. Site-directed mutagenesis was performed by utilizing the Transformer Site-Directed Mutagenesis Kit. The mutated TS cDNAs were analyzed by sequence analysis utilizing a T7 Sequenase version 2.0 DNA sequencing kit. All TS cDNAs were sequenced in their entirety.

Growth Complementation Analysis. TX61 was transformed with expression vectors containing wild-type or mutated TS cDNA. For complementation studies, TX61 transformants were grown in minimal medium (*16*), which was modified by reducing the concentrations of sodium and potassium salts by 50% and by adding 2% casein acid. The minimal medium was supplemented with 25 μg/mL kanamycin, 10 μg/mL tetracycline, and, if indicated, 100 μg/mL thymidine. Growth rates were determined by measuring the optical density at 600 nm over a 10 h period.

Enzyme-Linked Immunosorbent Assay (ELISA). Transformed bacteria were lysed in phosphate-buffered saline (PBS) containing 0.1% SDS at 100 °C for 10 min. After centrifugation at 18000*g* for 30 min at 4 °C, cell extracts and BSA (total protein concentration, 2.5 mg/mL) were allowed to adsorb to microtiter plate wells (Costar, Cambridge, MA) for 12 h at 4 °C. ELISAs were conducted utilizing D3B31 murine anti-human TS monoclonal antibody as the primary antibody (IBRT Monoclonal Antibody Facility), goat anti-murine monoclonal antibody conjugated to horseradish peroxidase as the secondary antibody, and the horseradish peroxidase substrate, 2,2′-azinodi(3-ethylbenzthiazoline-6-sulfonic acid), as described previously (*17*). Cross-reacting protein was quantitated by measuring the absorbance at 410 nm using a Dynatech Model 650 Microtiter Plate Reader (Dynatech Laboratories, Alexandria, VA).

Western Blot Analysis. Cell protein (20 μg of total protein per lane) was separated by electrophoresis in 12% SDS−polyacrylamide gels. The protein was transferred in 25 mM Tris-base, 192 mM glycine, and 20% methanol to Immobilon-PVD transfer membranes (Immobilon, Bedford, MA) with a Bio-Rad Transblot apparatus (Bio-Rad, Hercules, CA) at 4 °C. Cross-reacting protein was detected by utilizing standard procedures with the primary and secondary antibodies described for ELISAs and the horseradish peroxidase substrate, 4-chloronaphthol.

Purification of Thymidylate Synthase. Cell pellets of transformed bacteria (3.5−5 g) were suspended in buffer A (50 mM Tris-base, 0.2% 2-ME, and 1 mM EDTA at pH 7.4

and 4 °C) containing 20 μg/mL leupeptin, 50 μg/mL aprotinin, and 0.1 mM phenylmethanesulfonyl fluoride and sonicated using a Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT). Extracts were prepared by centrifugation at 18000*g* for 30 min. Purification buffers were filtered with 40 μm membranes (Gelman, Ann Arbor, MI) and degassed with He(g) for 45 min prior to addition of the reducing agent. Purification was conducted at 4 °C. Cell extracts were loaded onto a Blue Sepharose CL-6B column (Pharmacia). Fractions containing TS were eluted from the column using buffer B (buffer A containing 1 M KCl). Fractions were assayed for TS by a spectrophotometric activity assay. Fractions containing TS were pooled and concentrated with Centriprep-30 membranes (Amicon, Beverly, MA), and loaded onto a Sephacryl S-200 gel column (Pharmacia). Fractions containing TS were eluted with buffer A, and glycerol was added to a final concentration of 15%. Purified TS was analyzed by SDS−PAGE.

Protein Determination. The protein concentration of cell extracts was determined by utilizing the Bradford dye-binding protein assay (*18*), according to the manufacturer's protocol (Bio-Rad).

Spectrophotometric Assay of TS. Enzyme activity was measured at 37 °C by using a Shimadzu UV 1601 spectrophotometer equipped with a TCC 240A temperature-controlled cell holder (Shimadzu Corp., Columbia, MD). The purified enzyme (40−400 nM) was incubated in Morrison buffer (*19*) containing 1 mM dUMP and 150 μM (6*R*)-CH_2H_4folate. The absorbance change at 340 nm due to the conversion of CH_2H_4folate to H_2folate (ϵ_{340} = 6.4 mM^{-1} cm^{-1}) (*20*) was monitored for 1−10 min. One unit of enzyme activity is defined as the amount required to synthesize 1 μmol of thymidylate per minute under these conditions. Specific activity is expressed as units per milligram of protein. For determination of kinetic constants for wild-type and Thr216 TSs, initial velocities were measured by utilizing 40 nM purified TS, 1−100 μM dUMP, and 2−200 μM CH_2H_4folate. For determination of kinetic constants of TSs which are nonfunctional in complementation studies, initial velocities were measured using 400 nM purified TS, 0.1−2 mM dUMP, and 0.2−4 mM CH_2H_4folate. The data were fit to the hyperbolic form of the Michaelis−Menten rate equation using the program KaleidaGraph (version 2.1.2, Abelbeck Software, Reading, PA) by nonlinear least-squares analysis on a Macintosh Quadra 650 computer.

Ligand Binding Analyses. The equilibrium concentration of ternary complexes of TS with FdUMP was determined as described previously (*5*). Briefly, 1.0 nM TS was incubated for 6 h at 24 °C with 150 μM CH_2H_4folate and 0.3 nM to 20 μM [6-^{3}H]FdUMP. The binding data were fit to the Langmuir binding isotherm using the program KaleidaGraph and a Macintosh Quadra 650 computer. The binding of dUMP and PDDF to purified TSs was analyzed by stopped-flow fluorescence spectroscopy according to the method of Spencer et al. (*21*). Fluorescence was measured at pH 7.4 and 20 °C using an Applied Photophysics SX.18MV spectrophotometer (Applied Photophysics). Final enzyme concentrations were 2−5 μM. Final concentrations of dUMP ranged from 25 to 300 μM. The data were analyzed with a Macintosh Quadra 950 computer and fitted by nonlinear least-squares analysis using the program KaleidaGraph to equations describing single-exponential terms (*21*).

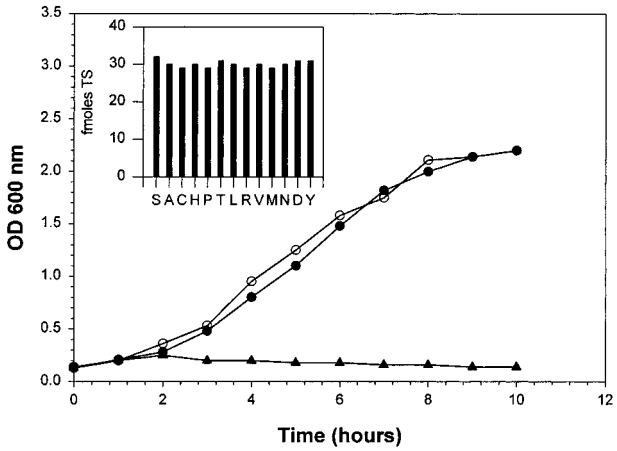

FIGURE 2: DNA complementation analysis. A TS-negative bacterial strain was transformed by expression vectors containing TS cDNAs that encode substitutions at position 216. The growth of transformed bacteria in the absence of thymidine was measured as described in Experimental Procedures. The growth of transformants expressing TS with serine (○), threonine (●), or alanine, cysteine, histidine, proline, leucine, arginine, valine, methionine, tyrosine, asparagine, and aspartate (▲) at position 216 is shown. In the inset, TS levels in extracts of the transfectants were determined by ELISA utilizing a murine anti-human monoclonal antibody that is monospecific for human TS as described in Experimental Procedures. Proteins are designated by the residue at position 216. TS was quantitated by extrapolation of a linear plot relating the absorbance at 410 nm and TS concentration.

Sulfhydryl Group Modification with 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB). Prior to incubation with DTNB, TS was dethiolated by gel filtration at 4 °C through a Sephadex G-50 column which was pre-equilibrated with 0.1 M potassium phosphate buffer at pH 7.4. The dethiolated enzyme (5 μM) was suspended in 0.5 mg/mL EDTA, 250 μM DTNB, and 0.1 M potassium phosphate buffer at pH 7.4 with and without 2% SDS as described by Ellman (*22*). The absorbance at 412 nm (ϵ = 13 600 M^{-1} cm^{-1}) at 20 °C was monitored by stopped-flow spectroscopy.

RESULTS

DNA Complementation Analysis. To investigate the effect of the mutations on TS function in cell growth, a TS-deficient bacterial strain, TX61, was transformed with plasmids containing wild-type and mutant human TS cDNAs. Transformants were screened in the presence or absence of thymidine to evaluate the effect of the substitution at position 216 on cell growth. All transformants grown in the presence of thymidine exhibited similar growth rates. Only those transformants containing TS cDNA encoding Ser216 (wild type) or Thr216 could grow in the absence of thymidine (Figure 2).

Monoclonal Antibody Analysis. It is possible that thymidine auxotrophy arises from either low expression or rapid degradation of mutant TS proteins. To ensure that thymidine auxotrophy results from a defective TS, cross-reacting protein was analyzed in transformant extracts. For these studies, a monoclonal antibody that is monospecific for human TS was utilized. The antibody was created against SDS-denatured human TS and cross-reacts with all human TSs that have been examined in the laboratory, including mutant TSs. By

Table 1: Equilibrium Binding of FdUMP into a Ternary Complex

enzyme	apparent K_d (nM)[a]	FdUMP bound per mole of enzyme
Ser216	0.45	2.0
Thr216	0.51	1.9

[a] Apparent dissociation constants (K_ds) were determined using 1.0 nM TS, 150 μM CH_2H_4folate, and 0.3 nM to 20 μM [6-^{3}H]FdUMP as described in Experimental Procedures.

using Western analysis, a single band of cross-reacting protein was observed in all mutant extracts, and this protein was similar in size (36 kDa) to the wild type (data not shown). By using ELISA, similar levels of cross-reacting protein were detected in extracts of transformants expressing mutant or wild-type TS (Figure 2 inset). No cross-reacting protein was detected in extracts of untransformed bacteria. The data suggested that TSs with residues at position 216 other than serine or threonine cannot confer thymidine prototrophy.

FdUMP Binding. FdUMP is a mechanism-based inhibitor of TS which has been utilized as a probe of the TS reaction mechanism (*1*). The binding of FdUMP into ternary complexes with the purified mutant and wild-type proteins was measured under equilibrium conditions established for wild-type human TS. No ternary complex formation was detected utilizing the mutant TSs identified as defective in TS catalysis. Thr216 TS exhibited an apparent K_d value of 0.51 nM for FdUMP binding into a ternary complex, with a stoichiometry of 1.9 mol of FdUMP bound per mole of dimeric enzyme (Table 1). The data are similar to those reported previously for the wild-type enzyme (*5*). These results indicated that proteins with residues at position 216 other than serine or threonine are defective in FdUMP binding into a ternary complex.

Kinetic Properties of Purified TSs. To confirm the DNA complementation studies and to obtain additional information regarding the role of Ser216 in human TS function, the kinetic properties of the enzymes purified from wild-type and mutant proteins were analyzed (Table 2). The complementation experiments suggested that all mutant TSs except Thr216 are defective in TMP production. As shown in Table 2, mutant proteins identified by DNA complementation as defective exhibited a k_{cat}/K_m for dUMP 10^3 to 10^4 times lower than that of the wild type. The k_{cat}/K_m for CH_2H_4folate for a representative mutant protein, Leu216 TS, was also significantly lower than that of the wild type. Consistent with DNA complementation studies, Thr216 TS exhibited a similar K_m for dUMP and CH_2H_4folate and a k_{cat} for dUMP 2-fold lower than that of the Ser216 mutant.

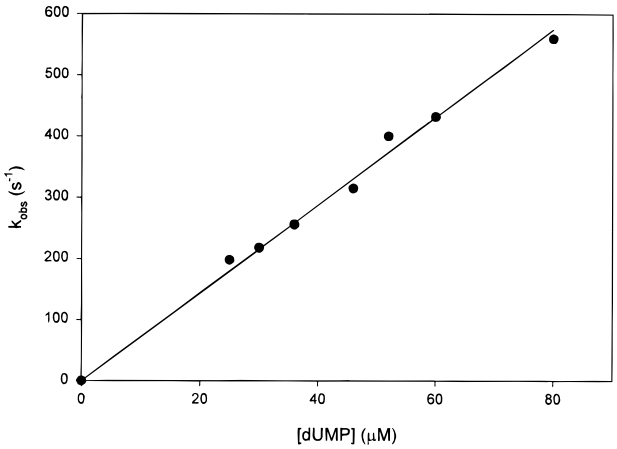

FIGURE 3: Kinetics of dUMP binding to wild-type TS. The binding of dUMP into a binary complex with wild-type (Ser216) TS was determined by stopped-flow fluorescence spectroscopy as described in Experimental Procedures. The relationship between k_{obs} and dUMP concentration is shown.

Ligand Binding. Binding of dUMP to human TS quenches the intrinsic fluorescence of the enzyme. The dissociation constant (K_d) for dUMP binding was determined by measuring the dependence of fluorescence intensity on nucleotide concentration (Figure 3 and Table 3). The data indicated that only one dUMP binding site per dimeric TS is available in the absence of folates. The K_d value for Thr216 TS for dUMP binding is 7-fold greater than that of the wild type due to a faster rate of dissociation (69 s^{-1}, Table 3). The K_d for binding of dUMP to two representative defective mutant TS proteins, Ala216 and Leu216 TS, was approximately 12−16-fold greater than that of the wild type, with similar k_{on}s for dUMP, while k_{off}s were approximately 8−10-fold higher (Table 3). The binding of PDDF to representative enzymes in a binary complex was also examined. PDDF is a structural analogue of CH_2H_4folate that assumes an orientation that is similar to the folate

Table 2: Kinetic Constants for Wild-Type and Mutant TSs[a]

		dUMP		(6*R*)-CH_2H_4folate	
enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
Ser216	3.1 ± 0.1 (3)	2.9 ± 0.2 (3)	1.1	4.9 ± 0.1 (3)	0.63
Thr216	1.6 ± 0.03 (3)	4.6 ± 0.2 (3)	0.34	4.5 ± 0.3 (3)	0.35
Cys216	0.07 ± 0.002 (3)	1.5×10^3	4.5×10^{-5}	ND[b]	ND[b]
Arg216	0.05 ± 0.001 (3)	280 ± 3 (3)	1.7×10^{-4}	ND[b]	ND[b]
Leu216	0.04 ± 0.001 (3)	238 ± 5 (3)	1.9×10^{-4}	273 ± 5 (3)	2.2×10^{-4} (2)
Met216	0.06 ± 0.003 (3)	219 ± 7 (3)	2.8×10^{-4}	ND[b]	ND[b]
Asp216	0.06 ± 0.003 (3)	153 ± 6 (3)	3.9×10^{-4}	ND[b]	ND[b]
Ala216	0.04 ± 0.001 (3)	214 ± 9 (3)	2.1×10^{-4}	ND[b]	ND[b]
His216	0.05 ± 0.002 (3)	166 ± 8 (3)	2.8×10^{-4}	ND[b]	ND[b]
Tyr216	0.05 ± 0.001 (3)	159 ± 7 (3)	2.9×10^{-4}	ND[b]	ND[b]
Asn216	0.05 ± 0.002 (3)	256 ± 9 (3)	1.8×10^{-4}	ND[b]	ND[b]
Val216	0.05 ± 0.001 (3)	263 ± 9 (3)	1.9×10^{-4}	ND[b]	ND[b]
Pro216	0.05 ± 0.001 (3)	244 ± 8 (3)	2.0×10^{-4}	ND[b]	ND[b]

[a] Kinetic constants were determined by measuring the absorbance change at 340 nm as described in Experimental Procedures. The number of separate determinations is shown in parentheses. [b] Not determined.

Table 3: Kinetically Derived Constants for dUMP Binding[a]

enzyme	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_d(dUMP)[b] (μM)
Ser216	6.7	16	2.5
Thr216	4.7	69	15
Ala216	4.7	138	29
Leu216	4.1	161	40

[a] The binding of dUMP into a binary complex was determined by stopped-flow fluorescence spectroscopy as described in Experimental Procedures. The data were generated from two separate experiments, utilizing five to seven concentrations of dUMP. The standard deviation of all parameter values is less than 10%. [b] Calculated by the ratio of k_{off} to k_{on}.

Table 4: Reaction of TS with DTNB[a]

enzyme	modified SH[b] (without SDS)	modified SH (with SDS)	k_{obs}[c] (without SDS)	k_{obs} (with SDS)
Ser216	9.6	10.0	0.629	0.631
Ala216	9.2	9.9	0.326	0.625

[a] Dethiolated TS with or without SDS was reacted with DTNB as described in Experimental Procedures. The rates of modification of sulfhydryl groups (SH) in the enzymes were determined by stopped-flow absorbance spectroscopy as described in Experimental Procedures. The data are the mean of three separate determinations. [b] Moles of SH groups modified per enzyme dimer. [c] Units of s^{-1}.

cosubstrate in ternary complexes of *E. coli* TS (*23*). The data indicated that the rate of PDDF binding to Ser216, Ala216, and Leu216 TSs is similar (data not shown). The results are consistent with the hypothesis that Ser216 is involved in stabilization of the phosphate group of the nucleotide by serving as a hydrogen bond donor; however, the data did not explain the nearly complete loss of activity of Ala216 and Leu216 TSs.

Titration with DTNB. A critical step in the reaction mechanism of TS is nucleophilic addition at C-6 of dUMP, which activates C-5 for addition of CH_2H_4folate (*1*). The enzyme nucleophile is a cysteine residue, which is highly reactive with sulfhydryl reagents such as DTNB (*22*). To address the possibility that substitution at position 216 alters the nucleophilicity of active site Cys195, the rate of enzyme modification by DTNB was examined. The rate of modification of the sulfhydryl groups of wild-type (Ser216) TS and Ala216 TS was determined by stopped-flow absorbance spectroscopy because the reaction occurred too rapidly for us to use conventional spectroscopic measurements. Human TS has five free sulfhydryl groups per monomeric enzyme (*12*). For native and SDS-denatured wild-type TS, 98−100% of the sulfhydryl groups were modified at an enzyme:DTNB ratio of 1:50 (Table 4). In the presence of SDS, 99% of the sulfhydryl groups of Ala216 TS were modified, and the rate of reaction was similar to that of wild-type TS. Interestingly, native Ala216 TS exhibited a rate of modification that is approximately 50% of that of wild-type TS and 50% of that of denatured Ala216 TS. The data indicated that the overall rate of reaction of cysteine residues is slower for Ala216 TS than for wild-type TS.

DISCUSSION

On the basis of the three-dimensional structure, the main chain carbonyl of Ser219 in *Lactobacillus casei* TS (corresponding to Ser216 in human TS) is postulated to stabilize a β-bulge in the enzyme through a hydrogen bond with the side chain of Gln217 (*10*). The β-bulge is one component of a kink in the β-sheet backbone of TSs that is postulated to serve as a hinge mechanism for ligand-induced conformational changes in the protein (*9*). Thus, it is possible that residues with differing side chain volumes at position 216 could exert effects on protein catalysis through steric interactions. The data obtained by DNA complementation studies and kinetic analysis are not consistent with a structural role for Ser216. Analysis of the relationship of k_{cat}/K_m and residue volume (*24*) at position 216 revealed that a poor correlation exists between these parameters, regardless of whether all proteins ($r = 0.22$) or only defective proteins ($r = 0.16$) are examined. Likewise, a poor correlation ($r < 0.1$) was observed between side chain hydrophobicity and k_{cat}/K_m for the defective proteins, utilizing the hydrophobicity scales of EISEN and GUY M, as normalized by Cornette et al. (*25*).

Crystal structures of TSs isolated from *E. coli* indicated that the hydroxyl group of Ser167 (corresponding to Ser216 of human TS) forms a hydrogen bond with an oxygen atom of the phosphate group of the nucleotide ligand (*4*, *11*). This hydrogen bond is one of seven hydrogen bonds that are thought to contribute to the binding of the phosphate group at the active site. DNA complementation studies of the mutant TSs indicated that the presence of a hydroxyl substituent at position 216 is essential for TS function, since threonine is the only substitution at position 216 that resulted in a protein that confers thymidine prototrophy in a *thyA*$^-$ bacterial strain. The results of the complementation studies were confirmed by kinetic analysis and FdUMP binding studies, in that Thr216 TS is the only mutant that exhibits catalytic activity and inhibitory ternary complex formation that are similar to those of wild-type TS. The data are consistent with the proposed role of a serine at this relative position as a hydrogen bond donor for nucleotide binding. It was surprising that Cys216 TS is nonfunctional as determined by DNA complementation studies and inactive as determined by kinetic analysis, since cysteine residues are hydrogen bond donors, albeit weak. Furthermore, TS expressed in the protozoan *Crithidia fasciculata* contains cysteine at this relative position (*26*). In preliminary studies, we observed that Cys216 TS, unlike wild-type TS, contains a disulfide bond. The mutant enzyme is reactivated in the presence of 250 mM dithiothreitol, exhibiting a k_{cat}/K_m for dUMP similar to that of Ser216 TS (A. Williams, unpublished results). Thus, the data, in total, are consistent with the hypothesis that serine at this relative position is involved in stabilization of the nucleotide by donating a hydrogen bond to an anionic oxygen atom of the phosphate group.

Investigation of ligand binding into binary complexes revealed that loss of hydrogen bonding potential results in a decrease in dUMP binding, with no detectable effect on the binding of PDDF. The higher K_d for dUMP binding to Ala216 and Leu216 TSs was due primarily to an increase in k_{off}. This observation is consistent with the proposed role of serine at this relative position in the stabilization of nucleotide binding. Of interest is a comparison of the effects of substitution by alanine or leucine at position 216 on K_d for dUMP and k_{cat}/K_m for dUMP. While the substitutions increased the K_d for dUMP binding into a binary complex by 12−16-fold, k_{cat}/K_m for dUMP was decreased by 10^4-fold. The effect of the substitutions on free energies of

Table 5: Free Energies of dUMP Binding to TS

enzyme	K_d[a] $\Delta\Delta G$ (kcal/mol)	k_{cat}/K_m[b] $\Delta\Delta G$ (kcal/mol)
Thr216	1.040	0.730
Ala216	1.420	5.250
Leu216	1.610	5.315

[a] Calculated from K_ds for dUMP binding into a binary complex, using the relationship $-1.98 \times 293\ K \times \ln[K_d(wt)/K_d(mut)]$. [b] Calculated from k_{cat}/K_m for dUMP, using the relationship $-1.98 \times 310\ K \times \ln[[k_{cat}/K_m(mut)]/[k_{cat}/K_m(wt)]]$.

FIGURE 4: Wire-frame representation of the active site of *E. coli* TS. Coordinates for the crystal structure of a monomer (PDB code 3tms; *30*) were obtained from the Brookhaven Protein Data Bank and visualized with the program *Rasmol*.

interaction is revealed by comparison of $\Delta\Delta G$ values for Ala216 and Leu216 TSs, relative to those of wild-type TS (Table 5). A significant difference is observed in $\Delta\Delta G$ values calculated from K_ds for dUMP and k_{cat}/K_ms for dUMP. The $\Delta\Delta G$ values for Thr216 TS calculated from these constants correlated reasonably well (Table 5). The data indicated that Ser216 has additional interactions that are essential for catalysis.

On the basis of the relatively close proximity (5.3 Å) of Ser167 (corresponding to Ser216) of *E. coli* TS to Cys146, the active site nucleophile (Figure 4), it is tempting to propose that the hydroxyl group at this relative position is involved in the activation of the active site cysteine by acting as a hydrogen bond acceptor. Analysis of the rates of modification of cysteine residues in wild-type and Ala216 TSs revealed that a difference exists in the rate of reactivity of the sulfhydryl groups of native Ala216 TS, relative to that of Ser216 TS. No apparent difference was observed in the rates of modification of the proteins denatured by SDS. This suggested that the observed differences between the enzymes are due to chemical interactions determined by secondary structure. Since alanine and serine are isosteric, it is less likely that the observed differences in rates of cysteine modification are due to differences in protein conformation. The studies are preliminary in that a difference is observed in the rates of modification of all cysteine residues, rather than in the active site cysteine. In previous studies of *L. casei* TS, the active site cysteine residues were observed to react the fastest, on the basis of an inverse correlation between modification with sulfhydryl reagents and loss of enzyme activity (*27*). The data are consistent with the interpretation that Ser216 is playing a more critical role in TS activity than nucleotide binding. Previous studies of the reactivity of the active site cysteine (Cys198) of *L. casei* TS with DTNB revealed that the rate of modification of this residue at low pH is faster than expected, on the basis of the pK_a of a sulfhydryl group (*28*). It was postulated that a general base is involved in activating Cys198 for nucleophilic attack to initiate catalysis. The data presented herein provide preliminary evidence that Ser216 is playing a role in activation of the active site cysteine in human TS, Cys195. Interestingly, Ser167 in *E. coli* TS is close (4.4 Å) to a histidine residue (His207) that is invariant in all TSs examined to date (*1*) (Figure 4). It is postulated that His207 forms a hydrogen bond with the O-3′ atom of the ribose of dUMP, which is an important determinant of ligand specificity of the enzyme (*4*, *9*). In recent studies in the laboratory, mutant human TSs were created in which the histidine residue corresponding to His207 (His256) was replaced by glutamine or leucine. The mutant enzymes had kinetic properties and a rate of DTNB modification of cysteine residues similar to those of Ala216 TS (A. Williams and K. Gamble, unpublished results).

Studies of the reaction mechanisms of mutant *L. casei* TSs have led to the suggestion that a general base is not necessary for activation of the active site cysteine for nucleophilic attack at C-6 of dUMP (*29*). It was suggested that water molecules in the active site cavity serve as acceptors of a proton from the active site cysteine. This implies a highly structured active site in which water molecules are strategically positioned to assist catalysis. Comparison of crystal structures of native human and bacterial TSs has revealed that a difference exists in the orientation of the active site cysteine (*8*). In human TS, a highly conserved loop containing the active site cysteine is rotated 180°, relative to its orientation in bacterial TSs. It will be of interest to determine whether the structural differences between human and bacterial TSs have an impact on the chemical interactions that are involved in the reactivity of the active site cysteine.

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