A Structural Role for Glutamine 214 in Human Thymidylate Synthase[†]

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ABSTRACT: Studies of the crystal structures of thymidylate synthase (TS) have revealed that a kink is present in β-sheets that form the core of the enzyme. The β-kink is proposed to serve as a "hinge" during conformational changes that occur in the enzyme after ligand binding at the active site. A residue in one of the β-bulges that form the kink, glutamine at position 214 of human TS, is highly conserved in all TSs and is postulated to interact with nucleotide ligands that bind at the active site. To examine the role of this residue, glutamine at position 214 was replaced by residues that differ in volume, hydrophobicity, electrostatic charge, and hydrogen bonding potential. Genetic complementation studies utilizing a TS-deficient bacterial strain revealed that residues with large side chain volumes or that are prohibited in β-bulges created loss of function proteins. Kinetic studies indicated that residue hydrophobicity is not correlated with catalytic activity. Residues that are predicted to alter the charge at position 214 created enzymes with k_{cat}/K_m values at least 10^3 lower than those of the wild type. Kinetic and ligand binding studies indicated that residue 214 is involved in nucleotide binding; however, hydrogen bonding potential does not contribute significantly to nucleotide binding energy. The data are consistent with the hypothesis that residue 214 is involved in maintaining the enzyme in a conformation that facilitates nucleotide binding and catalysis.

Thymidylate synthase (TS;[1] EC 2.1.1.45) catalyzes the reaction in which dUMP is reductively methylated by 5,10-methylenetetrahydrofolate (CH_2H_4PteGlu) to form dTMP and 7,8-dihydrofolate (H_2PteGlu). Because TS catalyzes a critical reaction necessary for DNA replication, it has been an attractive target of chemotherapeutic agents. One agent, 5-fluorouracil, is metabolized in the cell to FdUMP which acts as a mechanism-based inhibitor of TS by forming a stable covalent complex with CH_2H_4PteGlu and the enzyme (*1*).

The reaction mechanism of TS has been extensively analyzed using substrate analogues, chemical modification, site-directed mutagenesis, and analysis of X-ray crystal structures of TS (*1*−*8*). These studies have implicated several amino acid residues in the TS reaction mechanism. Interestingly, mutagenesis studies indicate that TS is relatively tolerant of substitution, a result surprising in view of the high degree of homology among TS enzymes from widely divergent species (*9*).

Three-dimensional structural studies have revealed the presence of a discontinuity in the β-sheets forming the central core of the enzyme. The discontinuity is formed by bulges in β-sheets, which are stabilized by hydrogen bonding among the peptide backbones of highly conserved residues in the protein (*9*, *10*). It has been suggested that this region acts as a "hinge" during conformational changes in the protein. TS exhibits a pronounced conformational change upon binding of ligands at the active site, with the major effect being induced by the folate ligand (*11*). Comparison of unliganded TS with the ternary complex revealed that there is a shift of residues toward the active site in the ternary complex (*11*, *12*). The magnitude of the shift ranges from 0.3 Å for residues located near the bulge to 4 Å for the C-terminal residues (*11*).

One of the bulges comprising the discontinuity in the β-sheets is close to the active site. The residues that are located adjacent to and within this bulge—Tyr213, Gln214, Arg215, and Ser216 of human TS—are highly conserved among all TS enzymes examined to date (*9*). The residues that lie within the bulge are Gln214 and Arg215. Gln214 is implicated in hydrogen bonding and/or hydrophobic interactions with the pyrimidine base of the nucleotide (*4*, *5*). Recently, it has been suggested that glutamine at this relative position in TS isolated from *Lactobacillus casei* (Gln217) forms a hydrogen bond network with two residues at the active site, Asn229 and Ser219 (Figure 1) (*13*).

To analyze the role of Gln214 of human TS, a mutant protein was created, in which glutamine was replaced with a glutamate residue (J. W. Zapf et al., submitted for publication). A glutamate residue was selected because it is isosteric with respect to glutamine, but differs in hydrogen bonding and hydrophobic interactions. TS-deficient mammalian cell lines were transfected with expression vectors encoding Q214 and Q214E enzymes. Transfectants expressing the mutant protein exhibited an 8-fold greater resistance to FdUrd

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[1] Abbreviations: TS, thymidylate synthase (EC 2.1.1.45); CH_2H_4-PteGlu, 5,10-methylenetetrahydrofolate; H_2PteGlu, 7,8-dihydrofolate; dUMP, 2′-deoxyuridylate; dTMP, thymidylate; FdUMP, 5-fluoro-2′-deoxyuridylate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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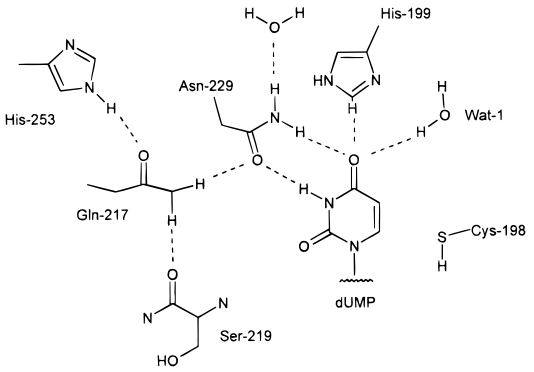

FIGURE 1: Proposed role of Gln217 in *L. casei* TS. Diagram showing the proposed hydrogen bond network between Asn229, Gln217 (corresponding to Gln214 of human TS), Ser219, His253, and 3-NH and 4-O of dUMP. The figure is adapted from Finer-Moore et al. (*13*).

than transfectants expressing the wild-type TS. This is likely due to the observation that the apparent K_d for FdUMP is significantly higher for Q214E TS than for Q214 TS. In addition, regulation of protein levels differed in cells expressing the mutant TS relative to cells expressing wild-type TS. These results suggested that glutamine at position 214 plays a significant role in TS function.

To further analyze the role of Gln214 of human TS, site-directed mutagenesis was utilized to replace glutamine with amino acids that alter side chain volume, charge, hydrophobicity, and hydrogen bonding character. The effects of substitution were determined by genetic complementation analysis, kinetic characterization, and ligand binding studies.

EXPERIMENTAL PROCEDURES

Materials. 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 (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, bovine serum albumin (BSA), 2-mercaptoethanol (2-ME), 4-chloronaphthol, 2,2′-azinodi(3-ethylbenzthiazoline-6-sulfonic acid), antibiotics, and salts 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 (*14*). Blue Sepharose CL-6B and Sephacryl S-200 HR were purchased from Pharmacia Biotech (Piscataway, NJ).

Bacterial Expression System. The *Escherichia coli* strain TX61 (*thyA*$^-$) and the plasmid pTS080, expressing wild-type human TS, were provided by W. S. Dallas (Wellcome Research Laboratories, Research Triangle Park, NC) and have been described previously (*15*, *16*). TX61 was created by transposon-mediated mutagenesis and lacks detectable TS activity (*15*). To characterize the antibiotic resistance of TX61, the bacteria were grown on LB agar plates containing either 50 μg/mL ampicillin, 50 μg/mL tetracycline, or 40 μg/mL kanamycin. The *thyA*$^-$ phenotype of TX61 was confirmed using a minimal medium described previously (*17*). The medium, at pH 7.0, was modified by decreasing the sodium and potassium salt concentrations by 50% and adding casein amino acids to a final concentration of 2%. TX61 was grown in the minimal medium in the presence or absence of 100 μg/mL thymidine to confirm that the bacterial strain is a thymidine auxotroph and that the casein amino acids do not contain thymidine.

Mutagenesis. Derivatives of pTS080 encoding mutations at position 214 of human TS were produced by in vitro site-directed mutagenesis using materials and following the protocol provided with the Transformer Site-Directed Mutagenesis Kit. Following mutagenesis, TX61 was transformed using plasmids containing wild-type or mutated TS cDNA using the $CaCl_2$−heat shock method of Maniatis et al. (*18*). Mutations were confirmed by dideoxy DNA sequencing of the entire *thyA* gene of pTS080. Sequences were compared to a previously published sequence of the *thyA* gene (*19*). Dideoxy DNA sequencing was performed by the dideoxy chain terminating method using modified T7 polymerase provided with the Sequenase kit. Sequencing reactions were performed as described in the Sequenase manual of Amersham Life Science (Cleveland, OH).

Growth Complementation Analysis. Transformants were incubated overnight at 37 °C in minimal medium supplemented with 100 μg/mL thymidine, 25 μg/mL kanamycin, and 10 μg/mL tetracycline. The cultures were diluted 1:100 into minimal medium with or without 100 mg/mL thymidine and supplemented with 25 mg/mL kanamycin and 10 mg/mL tetracycline. The resulting cultures were incubated at 37 °C and the optical densities at 600 nm measured over a 10 h period. TX61 transformants containing expression vectors lacking a TS insert were used as negative controls.

Enzyme-Linked Immunosorbent Assay (ELISA). Bacterial pellets (500 mg) were lysed in PBS containing 0.1% SDS at 100 °C for 10 min. After centrifugation at 18000*g* for 30 min at 4 °C, extract protein and BSA (total protein concentration, 2.5 mg/mL) were denatured at 90 °C for 10 min in the presence of 0.1% SDS and allowed to adsorb to 96-well microtiter plates (Costar, Cambridge, MA) overnight at 4 °C. Cross-reacting protein was detected as described previously (*20*) by utilizing the murine anti-human TS monoclonal antibody, D3B31 (1:100 dilution in PBS-T), as the primary antibody, goat anti-murine monoclonal antibody conjugated to horseradish peroxidase (1:100 dilution in PBS-T) as the secondary antibody, and 2,2′-azinodi(3-ethylbenzthiazoline-6-sulfonic acid) as the horseradish peroxidase substrate. TS was quantitated by measuring the sample absorbance at 410 nm using a Dynatech Model 650 Microtiter Plate Reader (Dynatech Laboratories, Alexandria, VA) and extrapolation of a linear plot relating absorbance at 410 nm and human TS concentration.

Western Blot Analysis. Cell protein (200 μg per lane) was separated by electrophoresis in 12% SDS−polyacrylamide gels and transferred in a buffer containing 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.

For immunodetection, standard procedures were employed utilizing the primary and secondary antibodies described for ELISA. TS was detected by utilizing the horseradish peroxidase substrate, 4-chloronaphthol.

Purification of Wild-Type and Mutant Enzymes. All purification steps were conducted at 4 °C. Purification buffers were filtered with 40 μm membranes (Gelman, Ann Arbor, MI) and degassed with He(g) for 45 min prior to the addition of 2-ME. Cell pellets of transformed bacteria (3−5 g) were suspended in buffer A (50 mM Tris, 1 mM EDTA, and 0.2% 2-ME 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 Sonifer 450 (Branson Ultrasonics, Danbury, CT). Cell debris was removed by centrifugation at 18000*g* at 4 °C for 30 min. Cell-free extract was loaded onto a Blue Sepharose CL-6B column; TS was eluted from the column with buffer B (buffer A containing 1 M KCl). Fractions containing TS were pooled and concentrated with Centriprep-30 membranes (Amicon, Beverly, MA), and loaded onto a Sephacryl S-200 gel column. Fractions containing TS were eluted with buffer A. Purified TS was analyzed by 12% SDS−PAGE for purity and stored in buffer A containing 15% glycerol at −20 °C.

Enzymatic Assays. Enzyme activity was measured spectrophotometrically by monitoring the absorbance change accompanying the conversion of CH_2H_4PteGlu to H_2folate (*21*) using a Shimadzu UV 1601 spectrophotometer equipped with a TCC 240A temperature-controlled cell holder (Shimadzu Corp., Columbia, MD). Measurements were carried out at pH 7.4 and 37 °C in Morrison buffer (*22*). One unit of enzyme activity is defined as the amount of enzyme required to synthesize 1 μmol of thymidylate per minute. For determination of kinetic constants for dUMP, initial velocities were measured by utilizing 40−400 nM purified TS, 0.15−1 mM CH_2H_4PteGlu, and dUMP ranging from 0.001 to 2 mM. For kinetic constants for CH_2H_4PteGlu, initial velocities were measured by utilizing 40−400 nM purified TS, 0.15−2 mM dUMP, and CH_2H_4PteGlu ranging from 0.002 to 2 mM. Kinetic constants were determined by fitting the data by nonlinear least-squares analysis to the hyberbolic form of the Michaelis−Menten rate equation using the program KaleidaGraph (version 2.1.2, Abelbeck Software, Reading, PA) on a Macintosh Quadra 650 computer.

pH Dependence of k_{cat}/K_m. The specificity constant (k_{cat}/K_m) for dUMP was measured in the pH range of 6.0−8.5 using Morrison buffer to maintain a constant ionic strength. Reactions were carried out and kinetic constants for dUMP were determined as described previously.

Equilibrium Dissociation Constants. To determine the dissociation constants for FdUMP binding to TS in the presence of CH_2H_4PteGlu, 1.0 nM TS was incubated for 6 h at 24 °C with 150 μM CH_2H_4PteGlu and varying concentrations of [6-^{3}H]FdUMP as described previously (*23*). The binding data were fit by nonlinear least-squares analysis to a hyperbolic equation using the program KaleidaGraph on a Macintosh Quadra 650 computer.

Stopped-Flow Fluorescence Spectroscopy. K_d values for dUMP and FdUMP binding to Q214, Q214A, Q214G, and Q214N enzymes were measured by stopped-flow fluorescence spectroscopy as described previously (*24*). Changes in fluorescence were measured with an Applied Photophysics SX.18MV Stopped-Flow Spectrometer (Applied Photophysics) at pH 7.4 and 20 °C. Final enzyme concentrations were 2 μM. Final concentrations of dUMP ranged from 5 to 200 μM, and final concentrations of FdUMP ranged from 25 to 100 μM. The rates of nucleotide binding (k_{on}) and release (k_{off}) were determined from a linear expression obtained by fitting the data to a linear least-squares analysis using the program KaleidaGraph on a Macintosh Quadra 650 computer. Dissociation constants were calculated as the ratio of k_{off} to k_{on}.

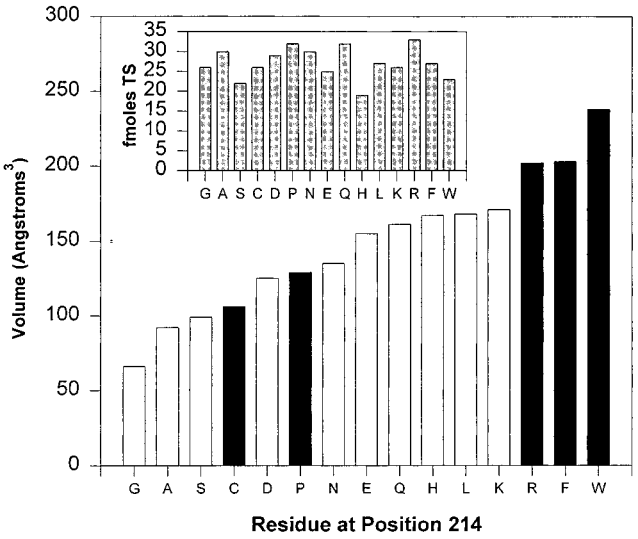

FIGURE 2: Relationship between residue volume at position 214 and TS function. Growth rates of TX61 transformed with expression vectors encoding human TSs with substitutions at position 214 were determined as described in Experimental Procedures. TSs that support the growth of a TS-deficient bacterial strain in the absence of thymidine are depicted by open bars; TSs that cannot support the growth of the thymidine auxotrophs are depicted by solid bars. Volumes for amino acid residues were excerpted from Chothia (*34*). The inset shows the levels of expression of TS in TX61 transformants. TS levels were determined by ELISA as described in Experimental Procedures. TS was quantitated by extrapolation of a linear plot relating absorbance at 410 nm and the concentration of human TS.

RESULTS

Growth Complementation Analysis. As an initial estimate of the effect of amino acid substitution at position 214 on growth complementation, the growth of TX61 transformants was examined in minimal medium in the presence and absence of thymidine. Cells transformed with cDNA encoding wild-type human TS served as a positive control, while transformants with plasmid containing no insert served as a negative control. All transformants were able to support bacterial growth in the presence of thymidine. In the absence of thymidine, all transformants supported bacterial growth except those expressing Q214C, Q214P, Q214R, Q214F, and Q214W enzymes (Figure 2). All transformants capable of growth in the absence of thymidine exhibited similar rates of growth.

ELISA. To ensure that the thymidine auxotrophy of a subset of transformants is not the result of low expression or rapid degradation of TS, the levels of TS in the transformants were measured. In all transformants, the levels of TS were 58−110% of the level of the wild-type enzyme

Table 1: Purification of Human TS from *E. coli* Strain TX61[a]

fraction	volume (mL)	protein (mg)	units (mM/min)	specific activity (units/mg)	percent recovery
cell-free extract	25	370	38	0.076	100
Blue Sepharose	10	28	21	0.59	55
Sephacryl S-200	3.5	10	14	1.1	38

[a] Human TS was purified as described in Experimental Procedures.

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

		dUMP		CH_2H_4PteGlu	
enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
Q214	3.1 ± 0.1	2.9 ± 0.2	1.1	6.8 ± 0.9	0.46
Q214A	1.7 ± 0.1	11 ± 1	0.16	11 ± 1	0.16
Q214C	<0.003	ND[b]	ND[b]	ND[b]	ND[b]
Q214D	0.078 ± 0.01	250 ± 17	3.2×10^{-4}	44 ± 4	1.8×10^{-3}
Q214E	0.27 ± 0.01	230 ± 20	1.2×10^{-3}	29 ± 2	0.010
Q214G	0.92 ± 0.11	24 ± 2	0.038	24 ± 3	0.038
Q214H	2.1 ± 0.3	29 ± 2	0.072	ND[b]	ND[b]
Q214K	0.027 ± 0.001	225 ± 17	1.2×10^{-4}	32 ± 1	8.4×10^{-4}
Q214L	0.21 ± 0.01	90 ± 7	2.3×10^{-3}	18 ± 1	0.012
Q214N	1.6 ± 0.1	7.4 ± 0.3	0.22	11 ± 1	0.15
Q214R	<0.003	ND[b]	ND[b]	ND[b]	ND[b]
Q214S	1.7 ± 0.3	34 ± 4	0.050	ND[b]	ND[b]
Q214W	<0.003	ND[b]	ND[b]	ND[b]	ND[b]

[a] Kinetic constants were determined by measuring the change in absorbance at 340 nm as described in Experimental Procedures. Concentrations of dUMP and CH_2H_4PteGlu and the method of data analysis are described in Experimental Procedures. The data represent the mean of three separate experiments. [b] Not determined.

(Figure 2 inset). Western analysis revealed that the monoclonal antibody used in the ELISA is monospecific for human TS (data not shown).

Expression and Purification of TS. The purification scheme developed for the recombinant enzymes is shown in Table 1. This scheme resulted in an 18-fold purification of wild-type and mutant enzymes with an average of 7 mg of TS recovered from the crude cell extract. The homogeneity of the enzymes was analyzed by SDS−PAGE as described in Experimental Procedures. A single band was observed for both wild-type and mutant enzymes with an approximate molecular mass of 36 kDa, consistent with the molecular mass of 35.7 kDa predicted by the nucleic acid sequence of the human TS cDNA (*19*).

Steady-State Kinetic Analysis. Purified TS enzymes were characterized by steady-state kinetic analysis. The kinetic constants for each enzyme are listed in Table 2. All mutants exhibited detectable activity except Q214C and Q214W with k_{cat} values of <0.003 s^{-1}. The specific activity of purified wild-type recombinant human TS was 1.1 units/mg, a result similar to that published previously (*25*). The k_{cat} of purified wild-type human TS was 3.1 s^{-1} which is similar to the published value of 2.5 s^{-1} for recombinant human TS (*26*). Mutant enzymes that are active by complementation analysis exhibited turnover numbers 1.5−115-fold lower than those of the wild-type enzyme. K_m values for dUMP and CH_2H_4-PteGlu for wild-type TS were determined to be 2.8 and 6.8 μM, respectively. These are similar to previously published K_m values of 3.4 μM for dUMP and 8.4 μM for CH_2H_4-PteGlu for recombinant human TS (*26*). For mutant enzymes that are active by growth complementation analysis, K_m values for dUMP are 4−86-fold higher than that of wild-type TS and 2−7-fold higher for CH_2H_4PteGlu. K_m values for Q214C, Q214R, and Q214W proteins were not determined due to activities which are undetectable at dUMP concentrations of 10 mM.

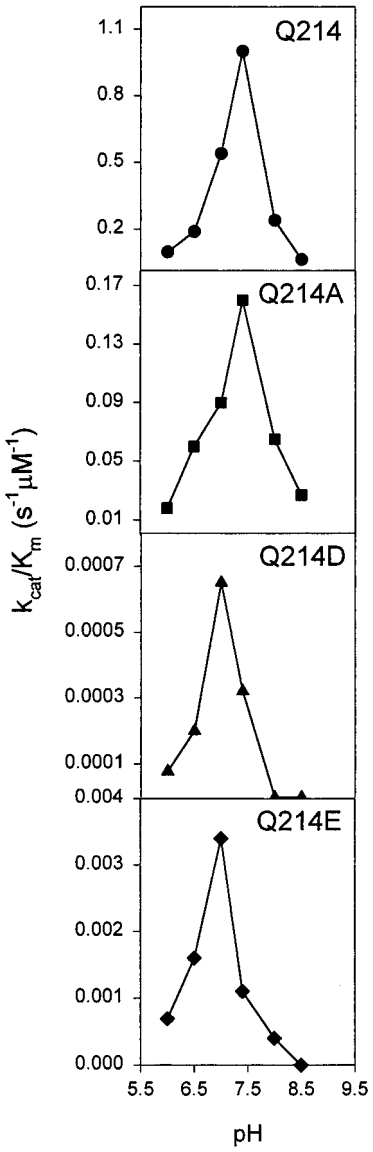

FIGURE 3: Dependence of k_{cat}/K_m on pH. k_{cat}/K_m for dUMP was measured for wild-type and mutant enzymes over the pH range of 6.0−8.5, as described in Experimental Procedures.

pH Dependence of k_{cat}/K_m. Kinetic constants (k_{cat} and K_m) were measured for Q214, Q214A, Q214D, and Q214E TSs over the pH range of 6−8.5. Figure 3 shows the relationship between k_{cat}/K_m and pH. Maximal k_{cat}/K_m values were observed for Q214 and Q214A enzymes at pH 7.4 and for Q214D and Q214E TSs at pH 7.0.

Equilibrium Dissociation Constants. Apparent dissociation constants (K_d) for FdUMP binding to a binary complex of TS and CH_2H_4PteGlu were determined by equilibrium binding studies. The data are shown in Table 3. The K_d for Q214 was determined to be 4.5×10^{-10} M, which is similar to that reported previously for wild-type human TS (*23*). The binding data for Q214 TS and the mutant enzymes suggested the existence of a single class of binding sites for FdUMP.

Table 3: Equilibrium Binding Constants for FdUMP for Wild-Type and Mutant TSs[a]

enzyme	K_d (nM)
Q214	0.45 ± 0.02
Q214A	6.8 ± 1.7
Q214G	7.6 ± 1.6
Q214H	3.0 ± 0.6
Q214K	ND[b]
Q214L	76 ± 3
Q214N	5.4 ± 0.2
Q214R	ND[b]
Q214S	21.9 ± 0.1
Q214W	ND[b]

[a] Purified TS (1 nM) was incubated with 150 μM CH_2H_4PteGlu and varying concentrations of [6-^{3}H]FdUMP as described in Experimental Procedures. Apparent K_d values were determined from nonlinear regression analyses of FdUMP binding data. The data represent the mean of three separate experiments. [b] Not detected.

Table 4: Kinetic and Thermodynamic Parameters for Nucleotide Binding to Human TS[a]

	dUMP			FdUMP		
enzyme	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)
Q214	6.7	16	2.5	4.0	12	3.0
Q214A	4.1	97	24	ND[b]	ND[b]	ND[b]
Q214G	4.1	120	29	4.9	160	33
Q214N	5.2	57	11	4.7	103	22

[a] The data represent the mean of two separate determinations. [b] Not determined.

Stopped-Flow Fluorescence Spectroscopy. Dissociation constants for the binding of dUMP and FdUMP to the wild-type and mutant enzymes are shown in Table 4. The k_{on} values for Q214 TS were determined to be 6.7 μM^{-1} s^{-1} for dUMP and 4.0 μM^{-1} s^{-1} for FdUMP. The values of k_{off} for Q214 TS were 16 s^{-1} for dUMP and 12 s^{-1} for FdUMP. Values of k_{on} for dUMP and FdUMP for the mutant enzymes were similar to the wild-type values, while k_{off} values were increased by 4−8-fold for dUMP and 9−13-fold for FdUMP, relative to those of the wild-type enzyme. The K_d value for dUMP for Q214 TS was 2.5 μM, which is 3-fold lower than that determined by equilibrium dialysis studies of recombinant human TS at pH 7.5 and 4 °C (*16*). The K_d for FdUMP was determined to be 3.0 μM for Q214 TS.

Correlation between Residue Hydrophobicity and Kinetic Parameters. The effects of side chain hydrophobicity on kinetic parameters were examined by using EISEN and GUY M hydrophobicity scales normalized according to Cornette et al. (*27*). A poor correlation was observed between side chain hydrophobicity and k_{cat}, K_m, and k_{cat}/K_m for dUMP ($r < 0.1$).

DISCUSSION

Studies using a TS-deficient mammalian cell line revealed that expression of human TS with glutamate at position 214 confers resistance to FdUrd and alters protein regulation through effects either on translation or on protein stability (Zapf et al., submitted). Ligand binding studies utilizing recombinant human TS enzymes purified from TX61 revealed that Q214E TS has a significantly lower affinity for FdUMP than Q214 TS. The decreased affinity of Q214E TS for FdUMP is consistent with the higher concentration of FdUrd that is required to inhibit the growth of cells expressing Q214E TS. These results suggested that glutamine at position 214 plays a role in both the structure and reaction mechanism of human TS.

A genetic complementation assay was utilized to estimate the effect of amino acid substitution at position 214 on enzyme function. When expressed at 6% of the total cell protein, all mutant proteins were capable of supporting the growth of a TS-deficient bacterial strain in the absence of thymidine, except Q214C, Q214P, Q214R, Q214F, and Q214W enzymes. Residues with the largest side chain volumes created loss of function mutants (Figure 2). Of the remaining residues that created enzymes with loss of function, proline is forbidden at position 1 in classical β-bulges (*28*). Glutamine at position 214 occupies position 1 of the β-bulge. The only residue associated with loss of TS function that is predicted to neither disrupt β-bulges nor introduce a large side chain volume is cysteine.

That the complementation assay identifies proteins that are defective was shown by kinetic studies. As observed in Table 2, the catalytic activity of three representative defective mutants was less than 0.2% of that of wild-type TS. All proteins identified as functional by the complementation assay exhibited catalytic activities of 1−68% of that of wild-type TS. It is interesting that expression of enzyme with as low as 1% of wild-type activity was associated with thymidine prototrophy. In previous investigations of *L. casei* TSs, the V316K mutant exhibited 2% of the activity of wild-type TS yet retained the ability to complement thymidine auxotrophy (*29*).

Kinetic analyses also indicated that an increase in side chain volume at position 214 had the largest effect on catalytic function. The presence of a large side chain at position 214 dramatically reduced or eliminated catalysis. At the opposite spectrum of side chain volume, glycine at position 214 created an enzyme which is able to complement growth of TS-deficient bacteria, but the k_{cat}/K_m is 30-fold lower, relative to that of wild-type TS. Glycine residues are found at position 1 (G1) in β-bulges; however, $\phi\psi$ conformational angles are different in G1-type and classical β-bulges (*28*). This may account for the altered kinetic properties of Q214G. The introduction of cysteine at position 214 created a mutant enzyme with no detectable catalytic activity. The loss of function cannot be due to the effects of a nucleophile at position 214, since the k_{cat} of Q214S is approximately 50% of that of Q214. We have determined that Q214C contains a disulfide bond (A. W. Williams, unpublished results); no disulfide linkages exist in the wild-type enzyme. It is likely that the disulfide bond alters the conformation of the β-bulge and that this structural alteration causes a loss of catalytic function. These data are consistent with the interpretation that the β-bulge is playing an essential role in catalysis.

The introduction of residues at position 214 that are predicted to be charged at neutral pH created enzymes that exhibit significantly lower k_{cat}/K_m values relative to that of wild-type TS. This is not unexpected since investigations of the crystal structures of TS from various sources indicated that electrostatic interactions play a prominent role in dUMP binding and in activation of Cys195 for nucleophilic attack on C-6 of the nucleotide (*4*, *5*, *30*). In addition, the introduction of charged residues in the vicinity of the TS

active site has been reported to exert pronounced effects on TS function (*8*, *31*, *32*). Thus, the low k_{cat}/K_m for TSs that introduce charged residues at position 214 may be a general phenomenon associated with charge perturbations at the active site, rather than specific to the role of glutamine at 214. Alternatively, introduction of a charged side chain at the β-bulge may create electrostatic interactions that alter the conformation of the protein. That electrostatic interactions are likely to contribute to the poor catalytic efficiency of Q214D and Q214E is supported by studies of the effect of pH on k_{cat}/K_m. Q214 and Q214A enzymes exhibited maximal k_{cat}/K_m values at pH 7.4; however, this maximum was shifted to lower pH values for Q214D and Q214E enzymes (Figure 3). In fact, these enzymes exhibited significant decreases in k_{cat}/K_m values as the pH was increased above 7.0. This suggests that ionization of the carboxyl side chain is associated with loss of catalytic activity.

Previous studies of the crystal structure of TS from *E. coli* indicated that glutamine at a position corresponding to position 214 in human TS plays a role in nucleotide binding, through hydrophobic and/or hydrogen bonding interactions. That glutamine is playing a role in nucleotide binding is supported by ligand binding studies utilizing dUMP and FdUMP. Stopped-flow fluorescence spectroscopy studies revealed that the representative mutant enzymes examined exhibited higher K_d values for nucleotide binding relative to that of Q214. For all mutant enzymes examined, the increase in K_d was due to an increase in the rate of nucleotide dissociation. These results suggested that position 214 is not involved in the initial binding of the nucleotide but is instead involved in "anchoring" the nucleotide ligand once it is bound. Consistent with ligand binding studies, kinetic studies indicated that the K_m values of mutant enzymes were increased more significantly for dUMP than for CH_2H_4PteGlu (Table 2). K_m values for CH_2H_4PteGlu were increased 2−7-fold, while K_m values for dUMP were increased 3−86-fold. This indicates that altering position 214 has a more profound effect on nucleotide binding than on binding of the folate. That position 214 is affecting nucleotide binding is also suggested by studies of the binding of FdUMP into a ternary complex. The apparent K_d values of all mutant enzymes were higher than that of wild-type TS, indicating a decreased affinity of the mutant enzymes for FdUMP. Graphical analysis indicated that wild-type and mutant enzymes exhibited a single class of binding sites that were saturated under the conditions of this study. In previous studies, Q214E TS exhibited two distinct binding sites with apparent K_{d1} and K_{d2} values 660- and 3600-fold greater than that of wild-type TS, respectively (Zapf et al., submitted). These results indicated that FdUMP binding activity of Q214E TS is greatly reduced. Furthermore, they suggested that a negatively charged residue at position 214 alters the interaction between the two subunits, producing a pronounced asymmetry in FdUMP binding. To address this possibility, the binding of FdUMP to the Q214D TS enzyme was examined. Unfortunately, the binding of FdUMP into a ternary complex with this mutant enzyme could not be detected by equilibrium binding studies.

Although crystallographic data indicated that glutamine at a position corresponding to 214 in human TS is involved in hydrophobic and/or hydrogen bonding interactions with the nucleotide, no correlation was observed between side chain hydrophobicity and k_{cat}/K_m for dUMP for the mutant enzymes. This observation is interesting in view of the postulated role of glutamine at this relative position in hydrophobic interactions with the nucleotide ligand (*4*). Analysis of kinetic constants of the mutant enzymes also suggested that hydrogen bonding does not play an essential role at position 214 in human TS. Using the equilibrium binding and kinetic data, it is possible to estimate the contribution of a side chain group from the free energy of substrate or inhibitor binding (*33*). These free energy changes are shown in Table 5. The free energy changes are calculated from K_d values for dUMP and FdUMP, K_d values for FdUMP binding into an inhibitory ternary complex, and k_{cat}/K_m for dUMP. A remarkably good correlation was observed among the ΔΔG values, regardless of the constant used for calculation. Comparison of ΔΔG values for Q214A and Q214N enzymes revealed that loss of hydrogen bonding interactions at position 214 is not associated with a significant difference in the changes in the energy of nucleotide binding. Furthermore, the data indicated that, at least for the three enzymes examined, hydrophobic interactions are not contributing significantly to nucleotide binding energy.

Table 5: Free Energy Changes Associated with Ligand Binding to Human TS[a]

enzyme	K_d(dUMP) ΔΔG (kcal/mol)	k_{cat}/K_m(dUMP) ΔΔG (kcal/mol)	K_d(FdUMP) ΔΔG (kcal/mol)	K_d(FdUMP−ITC) ΔΔG (kcal/mol)
Q214A	1.3	1.2	−	1.6
Q214N	0.86	0.99	1.2	1.4
Q214G	1.4	2.1	1.4	1.6

[a] ΔΔG values were calculated from K_d values for dUMP and FdUMP, K_d values for equilibrium binding for FdUMP into an inhibitory ternary complex, and k_{cat}/K_m values for dUMP (*33*).

Collectively, these data are consistent with the interpretation that glutamine at position 214 is playing a role in nucleotide binding, but this does not appear to be related to hydrophobic or hydrogen bonding interactions. It has been postulated that the β-bulge containing glutamine at this relative position is a component of a structurally critical region of TS involved in ligand-induced conformational changes (*11*). Thus, substitutions at position 214 may be altering nucleotide binding through effects on enzyme conformation. The data are consistent with position 214 exerting a structural role in TS function. In fact, stopped-flow fluorescence studies of the reaction mechanisms of Q214A, Q214N, and Q214G enzymes indicate that the mutant enzymes differ from Q214 TS in the isomerization between conformations presumed to reflect the open and closed forms of enzyme−substrate complexes (D. Steadman, unpublished results).

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