Substitution of Glutamine for Glutamic Acid-58 in *Escherichia coli* Thymidylate Synthase Results in Pronounced Decreases in Catalytic Activity and Ligand Binding†

James W. Zapf,‡ Mary S. Weir,‡ Victoria Emerick,‡ J. E. Villafranca,§ and R. Bruce Dunlap*,‡

Department of Chemistry and Biochemistry, The University of South Carolina, Columbia, South Carolina 29208, and Agouron Pharmaceuticals, Inc., 3565 General Atomics Court, San Diego, California 92121

Received February 23, 1993; Revised Manuscript Received June 11, 1993®

ABSTRACT: The recent determination of the crystal structure of *Escherichia coli* thymidylate synthase (TS) [Matthews *et al.* (1989) *J. Mol. Biol. 205*, 449–454] has implicated the glutamic acid residue at position 58 in a mechanistic role which could involve the interaction of its γ-carboxyl side chain with the nucleotide substrate and/or the folate cofactor. The site-specific mutagenesis of Glu-58 to Gln-58 in *E. coli* TS provided the opportunity to explore its functional role in activity and binding. When profiled by the spectrophotometric and tritium release assays, the 370- and 760-fold decreases, respectively, in k_{cat} and the elevated K_m values for the Gln-58 mutant enzyme indicated a significant involvement of Glu-58 in substrate binding and turnover. The apparent dissociation constant for the covalent FdUMP–enzyme binary complex was 30 μM, which is 5-fold higher than that found for the wild-type enzyme, while the inhibitory ternary complex apparent dissociation constants for FdUMP and CH_2H_4folate for the Gln-58 enzyme were 10- and 60-fold higher, respectively, than those for the wild-type enzyme under saturating conditions. The extent of covalent FdUMP binding to the Gln-58 enzyme was reduced from 1.5 to 0.7 per dimer in the inhibitory ternary complex but only from 0.7 to 0.5 per dimer in the binary complex of the Gln-58 enzyme. The usual 2.1-fold enhancement of FdUMP binding to wild-type TS in the presence of CH_2H_4folate was not observed for the Gln-58 enzyme. The ^{19}F NMR spectrum of the enzyme–FdUMP binary complex formed with the Gln-58 enzyme was similar in chemical shifts but less intense than the spectrum of the same complex formed with the wild-type enzyme. The fluorine resonance ascribed to the enzyme:FdUMP:H_4folate complex was not only less intense but also more deshielded with the mutant enzyme than with the wild-type enzyme. Finally, a characteristic resonance at −12.5 ppm attributed to FdUMP that is covalently attached to enzyme and CH_2H_4folate in the inhibitory ternary complex was not observed with the mutant enzyme. These results indicated that the mutation greatly impaired folate binding but that nucleotide binding was less perturbed.

Thymidylate synthase (TS)[1] catalyzes the reductive methylation of 2′-deoxyuridine monophosphate (dUMP) to 2′-deoxythymidine monophosphate (dTMP) utilizing 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) as both reductant and source of the methyl group. This reaction is critically important in the cell as it provides the sole *de novo* source of dTMP for DNA synthesis. For this reason, the enzyme has been a target of chemotherapeutic antimetabolites such as 5-fluoro-2′-deoxyuridine (FdU) and 10-propargyl-5,8-dideazafolate (PDDF), both of which are inhibitors of TS (Duschinsky *et al.*, 1959; Jones, 1980).

Most of the studies on the binary and ternary complexes of thymidylate synthase have utilized the enzyme isolated from amethopterin-resistant *Lactobacillus casei* (Dunlap *et al.*, 1971). The first evidence that TS could form a *covalent* binary complex with FdUMP was provided by ^{19}F NMR (Lewis *et al.*, 1980). These results were confirmed through a nitrocellulose filter technique (Ahmed *et al.*, 1985) and by a trichloroacetic acid precipitation procedure (Moore *et al.*, 1984). The presence of folates has been shown to increase the binding of nucleotides to the enzyme (Lewis *et al.*, 1981; Santi & Danenberg, 1984). The inhibitory ternary complex consisting of FdUMP, CH_2H_4folate, and TS has been studied by a number of techniques such as gel electrophoresis, absorbance, fluorescence, and circular dichroic spectroscopy (Donato *et al.*, 1976). The ^{19}F NMR spectrum of the *L. casei* enzyme in the covalent inhibitory ternary complex (−12.5 ppm resonance) (Byrd *et al.*, 1977) was markedly different from that of the binary complex, which exhibited resonances at −1.5 and −34.5 ppm, which were assigned to the noncovalent and covalent FdUMP–enzyme complexes, respectively. More recently, Cisneros *et al.* (1990a) have characterized the inhibitory ternary complex through a TCA precipitation procedure, where it was shown that the presence of CH_2H_4-folate could enhance the covalent binding of FdUMP to TS some 2.5-fold over that seen for FdUMP alone.

In recent years, the easy isolation of *Escherichia coli* TS from recombinant bacterial sources has permitted the determination of a number of X-ray crystal structures of thymidylate synthase first in the apoenzyme form (Hardy *et al.*, 1987) and then in several ternary complex forms, such as enzyme complexed with dUMP and PDDF (Montfort *et al.*, 1990), with FdUMP, the biologically active form of FdU, and

† This research was supported by NIH Grant CA 15645 (R.B.D.) from the National Cancer Institute.

* Author to whom correspondence should be addressed.

‡ The University of South Carolina.

§ Agouron Pharmaceuticals.

® Abstract published in *Advance ACS Abstracts*, August 15, 1993.

[1] Abbreviations: dUMP, 2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; TS, thymidylate synthase; Glu-58, glutamic acid 58; Gln-58, glutamine 58; H_4folate, 5,6,7,8-tetrahydrofolate; CH_2H_4folate, 5,10-methylenetetrahydrofolate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; NMR, nuclear magnetic resonance; CFE, cell-free extract; PDDF, 10-propargyl-5,8-dideazafolate; DTT, dithiothreitol.

0006-2960/93/0432-9274$04.00/0

Chart I: First Proposed Role of Glu-58 in Which It Stabilizes the Protonation of the Nitrogen-10 of the CH_2H_4folate

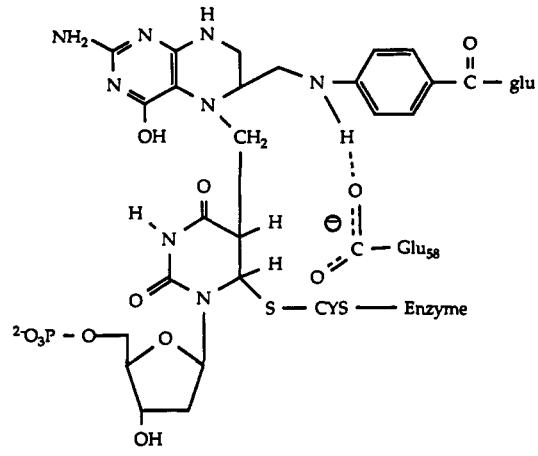
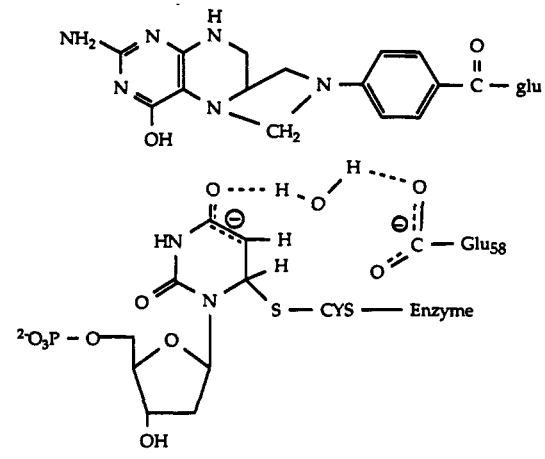


Chart II: Second Proposed Role of Glu-58 in Which It Stabilizes the Nucleotide Enol/Enolate Conversion via Hydrogen Bonding through a Water Molecule



PDDF (Matthews *et al.*, 1989), and with FdUMP and CH_2H_4-folate (Matthews *et al.*, 1990b). The X-ray crystal structure has implicated a number of amino acid residues in roles associated with nucleotide and folate binding, one of which is the glutamic acid residue at position 58 (Glu-58) (Matthews *et al.*, 1989). It is thought that Glu-58 may play two possible catalytic roles: (1) Through either direct or possibly solvent water molecule mediated interaction with nitrogen-10 of the folate cofactor, the side chain of Glu-58 is proposed to either facilitate the opening of the imidazolidine ring of CH_2H_4-folate or stabilize the resulting cationic iminium ion at nitrogen-5 of the coenzyme (Chart I) (Matthews *et al.*, 1990a,b). (2) Alternately, Glu-58 may stabilize a nucleotide enol/enolate conversion via hydrogen bonding through a structural solvent molecule to the C-4 oxo group of the nucleotide (Chart II) (Matthews *et al.*, 1990a,b). The enolate form of the nucleotide has been postulated as a reactive intermediate which attacks the methylene group of the cationic iminium ion, thus establishing a one-carbon bridge between C-5 of dUMP (or FdUMP) and nitrogen-5 of H_4folate [reviewed by Lewis *et al.* (1981)]. The suggestion of Matthews *et al.* (1990b) that Asn-177 plays a significant role in the stabilization of the enolate form of the bound nucleotide has been corroborated by Hardy and Nalivaika (1992) and Liu and Santi (1992), who also showed that replacement of this amino acid with an aspartate residue converted TS to a deoxycytidylate methylase. The objective of the present study was to further define the role of Glu-58 in substrate turnover and inhibitor binding by studying the properties of a mutant enzyme in which Glu-58 was altered to Gln-58 by site-specific mutagenesis.

MATERIALS AND METHODS

Materials. dUMP, dTMP, FdUMP, DTT, and 2-mercaptoethanol were purchased from Sigma. The radiolabeled [6-^{3}H]FdUMP and [5-^{3}H]dUMP were products of Moravek Biochemicals. Tris(hydroxymethyl)aminomethane (Tris) and potassium chloride were from Research Organics. PhastGel gradient gel 8–25%, PhastGel Blue R, PhastGel native buffer strips, Q-Sepharose fast flow, and Affi-Gel Blue resins were purchased from Pharmacia. (6*S*)-Tetrahydrofolate (H_4folate) was prepared by the enzymatic reduction of folate by *L. casei* dihydrofolate reductase, and (6*R*,*S*)H_4folate was prepared by the catalytic hydrogenation of folic acid as described by Hatefi *et al.* (1960). H_4folate was converted to CH_2H_4folate by the addition of a 25-fold molar excess of formaldehyde in 125 mM $NaHCO_3$, pH 7.0, containing 300 mM 2-mercaptoethanol to form a stock cofactor solution (1 mM CH_2H_4-folate). Folic acid was purchased from Calbiochem.

Site-Directed Mutagenesis. The Gln-58 mutant enzyme was created by the procedure described by Villafranca *et al.* (1983). Restriction fragments from the plasmid pCV29 containing the wild-type TS gene were cloned into bacteriophage M13mp8. Oligonucleotides designed to produce the desired mutations were used a primers on the single-stranded phage DNA. Mutations were confirmed by DNA sequencing.

Purification of E. coli Wild-Type and Mutant Thymidylate Synthases. Levels of thymidylate synthase were measured by the spectrophotometric and trichloroacetic acid precipitation assays (see below). Cell-free extract (CFE) was obtained by three consecutive 4-min sonication cycles (Heat Systems-Ultrasonics cell disruptor W-220-F) of 20 g of frozen Rue10 TS overexpressing *E. coli* cells suspended in 80 mL of 50 mM bis-Tris, 20 mM KCl, 20 mM 2-mercaptoethanol, and 1 mM EDTA, pH 6.5, followed by centrifugation at 15000*g* for 1 h. CFE was applied to 150 g of Affi-Gel Blue resin which had previously been washed with copious amounts of 50 mM bis-Tris, 20 mM KCl, 20 mM 2-mercaptoethanol, and 1 mM EDTA, pH 6.5 (Blue A), and aspirated to dampness. The cell-free extract and the resin were incubated in a 400-mL beaker for 30 min at 5 °C. The resin suspension was then quantitatively returned to the sintered glass funnel, and excess liquid was allowed to gravity filter through the resin. The resin was then successively washed with 4 × 200 mL of Blue A, 4 × 200 mL of 50 mM phosphate, 75 mM KCl, 20 mM 2-mercaptoethanol, and 1 mM EDTA, pH 7.4 (Blue B), 4 × 200 mL of 50 mM phosphate, 350 mM KCl, 20 mM 2-mercaptoethanol, and 1 mM EDTA, pH 7.4 (Blue C), and 1 × 250 mL of 50 mM phosphate, 1500 mM KCl, 20 mM 2-mercaptoethanol, and 1 mM EDTA, pH 7.4 (Blue D). TS-containing fractions were determined as described below, pooled, and dialyzed against 50 mM Tris, 20 mM KCl, 20 mM 2-mercaptoethanol, and 1 mM EDTA, pH 7.8 (QA buffer).

The dialyzed enzyme fraction was loaded onto a 2.5 × 6.0 cm Q-Sepharose column. This chromatographic step was performed with a Pharmacia FPLC system equipped with an LCC 500 liquid chromatography controller. Unbound proteins were washed away with 300 mL of QA buffer (4.0 mL/min), bound contaminating proteins were removed with a 100-mL gradient (2.0 mL/min) from 0% to 10% QB buffer (50 mM Tris, 1 M KCl, 20 mM 2-mercaptoethanol, and 1 mM EDTA,

pH 7.8), and, finally, TS was eluted as a major protein peak with a 360-mL linear gradient (1.0 mL/min) from 10% QB to 40% QB buffer. TS-containing fractions were determined according to the spectrophotometric assay (see below), pooled, and dialyzed against QA buffer.

Gel Electrophoretic Procedures. The purity of column fractions was assessed following electrophoresis of native and inhibitory ternary complexes of 20-fold concentrates of each column fraction exhibiting TS activity. Concentration of fraction aliquots was carried out with CentriconTM microconcentrators (Centricon 30, Amicon Corp.). For native TS lanes, 1 μL of 1 mg/mL TS was mixed with 2 μL of 0.1% bromophenol blue. Inhibitory ternary complexes were prepared for electrophoresis by incubating 1 μL of 1 mg/mL TS with 1 μL of 2.5 mM FdUMP and 1 μL of 1 mM CH_2H_4folate for 5 min. Samples were then subjected to electrophoresis on Pharmacia PhastGel gradient gel 8–25% utilizing PhastGel native buffer strips with a Phast electrophoresis system.

Protein Assays. The protein concentrations of column fractions and column pools were estimated by A_{280} values and further determined by the Bio-Rad dye binding microassay using bovine serum albumin as the standard (Bradford, 1976). Concentrations of homogeneous TS were measured using the extinction coefficient of 127 000 M^{-1} cm^{-1} at 280 nm for the *E. coli* enzyme.

Spectrophotometric Assay. Enzyme activity was determined by monitoring the increase in absorbance at 340 nm due to the formation of dihydrofolate for 30 s after the addition of the enzyme to the substrate pre-mix (Dunlap *et al.*, 1971). One unit of enzyme activity is defined as the production of 1 μmol of dTMP/min at 30 °C. To accurately determine the specific activity of the mutant enzyme at each step, the pools were concentrated with Amicon microconcentrators. Typically, 100 μL of a 1.0 μM wild-type TS stock solution or 100 μL of a 2.0 mM Gln-58 TS stock was added to 900 μL of pre-mix solution consisting of 120 mM Tris, 60 mM MES, 60 mM acetic acid [Morrison buffer (Ellis & Morrison, 1982)], pH 7.0, containing 75 mM $MgCl_2$, 100 μM CH_2H_4folate, and 100 μM dUMP. To determine K_m values for the wild-type enzyme, typical substrate concentrations were 2, 4, 8, 12, 16, and 20 μM dUMP and 10, 30, 60, 100, and 200 μM CH_2H_4-folate, while with Gln-58 TS typical substrate concentrations were 20, 40, 60, 80, 120, and 160 μM dUMP and 25, 75, 125, 225, and 400 μM CH_2H_4folate, and a matrix approach was employed. All spectrophotometric assays were carried out at 30 °C. Data were collected with a Hewlett-Packard 8450A UV–vis spectrophotometer and processed with an on-line computer.

Tritium Release Assay. TS activity constants and K_m values of both wild-type TS and Gln-58 TS were measured by the tritium release assay (Roberts, 1966). Typically, reaction mixtures contained 100 μM CH_2H_4folate and 1–50 μM [5-^{3}H]-dUMP in Morrison buffer and about 20 nM thymidylate synthase in a total volume of 300 μL. Reactions were initiated at 30 °C by the addition of enzyme. Seven 35-μL aliquots of the reaction mixture were withdrawn over the course of 1 min, and the reaction was quenched by delivery into Eppendorf tubes containing 25 μL of 25% TCA. One milliliter of 2.5% (w/v) acid-washed charcoal suspension in water was then added to each tube, vortexed, allowed to stand 10 min, and centrifuged at 14 000 rpm for 10 min. A Beckman LS-7500 liquid scintillation counter equipped with a data reduction package was then used to determine the counts in 700 μL of the supernatant. The rate was calculated by converting the number of counts into nanomoles of [^{3}H]H_2O produced/min. One unit of activity was defined as the amount of enzyme that produced 1 μmol of product/min at 30 °C. To determine K_m values by the tritium release assay for wild-type and Gln-58 enzymes, substrate levels employed were the same as described for K_m determination by the spectrophotometric assay.

Trichloroacetic Acid Precipitation Assay. The TCA assay was employed to determine maximum covalent FdUMP/TS binding ratios along with apparent K_d values for the inhibitory ternary complex formed from enzyme, FdUMP, and CH_2H_4-folate (Cisneros & Dunlap, 1990). The 0.5-mL reaction mixture, containing 0.1–100 nM [6-^{3}H]FdUMP (24 Ci/mmol), 1 nM TS, 50 μM CH_2H_4folate, and Morrison buffer, pH 7.0, was incubated at 37 °C for 30 min followed by quenching with the addition of 125 μL of 50% TCA. This solution was then centrifuged at 14 000 rpm for 2 min, decanted, washed four times with 10% TCA, dissolved in an ethanolic wash (0.2 N NaOH in 50% ethanol), and transferred to scintillation vials for subsequent counting. For the TCA assay, units are defined as nanomoles of covalently bound FdUMP (in this case, in the presence of CH_2H_4folate).

Binding Ratios. The covalent binary and ternary complex binding ratios were determined with the TCA assay. Reaction mixtures (0.5 mL) containing 0.2 μM of enzyme with 50 μM [6-^{3}H]FdUMP for the binary complex and with 20 μM [6-^{3}H]FdUMP and 200 μM CH_2H_4folate for the inhibitory ternary complex were incubated for 45 min at 37 °C. The covalent binding ratio was defined as nanomoles of FdUMP bound per nanomole of thymidylate synthase.

K_d Determination. The covalent binary and ternary complex dissociation constants, K_d values, were determined using the TCA assay procedure described above. For the covalent binary complex apparent K_d, a 0.5-mL reaction mixture containing 0.2 μM of either wild-type TS or Gln-58 mutant TS in Morrison buffer, pH 7.0, was incubated with [6-^{3}H]FdUMP (600 000 dpm/nmol) ranging in concentration from 5 to 100 μM (45 min at 37 °C). The inhibitory ternary complex apparent K_d values for both the wild-type and the mutant enzymes, using the same pH and incubation conditions as described above, were determined by alternately varying the folate and FdUMP concentrations. In all cases, 0.02 nM TS, 100 mg/mL BSA, and Morrison buffer, pH 7, were incubated in a total volume of 0.5 mL with either a varied nucleotide concentration [K_d-(FdUMP)] or a varied folate concentration [K_d(folate)]. For wild-type TS, the concentration of FdUMP ranged from 8 nM to 1 μM and the CH_2H_4folate concentration was held constant at 1 mM. For the mutant enzyme, the FdUMP concentrations ranged from 8 nM to 10 mM with a constant CH_2H_4folate concentration of 1 mM. The concentration of the folate ranged from 0.5 to 100 μM for the wild-type enzyme and from 0.5 μM to 1 mM for the mutant enzyme. The [6-^{3}H]-FdUMP (500 000 dpm/nmol) was held constant at 10 μM.

^{19}F NMR. Approximately 10 mg of enzyme in a 10–30-mL volume was dialyzed against NMR buffer (50 mM Tris, 200 mM KCl, 5 mM DTT, and 1 mM EDTA, pH 7.4) and concurrently concentrated in a ProDiCon negative pressure concentrator overnight. The enzyme samples (1.5 mL) were further concentrated to 0.3 mL, and buffer was exchanged into 40% deuterium oxide NMR buffer with the aid of an Amicon Centricon 30 microconcentrator. Binary complexes were prepared by incubating 400 μL of 400–500 μM enzyme with a 4-fold molar excess of FdUMP at 37 °C for 1 h prior to data acquisition. FdUMP was added as a 20 mM stock dissolved in NMR buffer minus DTT. H_4folate:FdUMP: enzyme complexes (pseudoternary complexes) were prepared by the addition of a 10-fold molar excess of (6*R*,*S*)-H_4folate

Table I: *E. coli* Thymidylate Synthase Purification

purifn step	total binding[a] (units)	total protein (mg)	sp binding[b] (units/mg)	vol (mL)	x-fold purifn	% recov
(a) wild-type TS						
1. CFE	3498	3180	1.2	74	1	100
2. Affi-Gel Blue	2205	298	7.4	830	6	56
3. Q-Sepharose	1968	80	24.6	35	20	52
(b) Gln-58 TS						
1. CFE	570[c]	1140	0.5	74	1	
2. Affi-Gel Blue	nd	321	nd	270	nd	
3. Q-Sepharose	760	72	10	60	21	

[a] Units are defined as nanomoles of covalently bound FdUMP. [b] Specific binding is defined as nanomoles of covalently bound FdUMP per milligram of total protein. [c] The binding assay consistently underestimated the number of binding units of the Gln-58 enzyme in the CFE.

to the latter binary complex solution. H_4folate was freshly prepared as a 30 mM stock in 100 mM Tris, 100 mM KCl, 100 mM $KHCO_3$, 10 mM DTT, and 1 mM EDTA, pH 7.4. Enzyme–FdUMP–CH_2H_4folate (inhibitory ternary complex) was prepared by the addition of a 25-fold molar excess of formaldehyde over H_4folate to the sample containing the H_4folate:FdUMP:enzyme complexes. Fluorine-19 NMR spectra were obtained at 188.28 MHz in the Fourier transform mode on a Bruker WP-200 equipped with a 5 mM fluorine probe. All data were obtained at 6 °C by employing the following acquisition parameters: 60° flip angle, 25 000-Hz spectral window, 0.164-s acquisition time, 0.24-s relaxation delay, and 8K data points. An exponential multiplication producing a line broadening of 15 Hz was applied to each free induction decay. Chemical shifts were externally referenced to 0.5 mM FdUMP in NMR buffer. Typically 60 000–80 000 transients were collected.

RESULTS AND DISCUSSION

Glu-58 is conserved in all of the TS sequences known (Perry *et al.*, 1990), suggesting the importance of this residue in enzyme function. Crystallographic studies of the *E. coli* wild-type enzyme complexed with FdUMP and PDDF have implicated two possible roles for Glu-58 in substrate binding and catalytic turnover (Matthews *et al.*, 1990). First, Glu-58 may stabilize a nucleotide enol/enolate conversion via hydrogen bonding through a structural water molecule to the C-4 oxo group of the nucleotide (Matthews *et al.*, 1990). A nucleotide enolate has been postulated as a reactive intermediate which leads to the formation of a covalent bond between the cofactor and the C-5 position of the nucleotide (Lewis *et al.*, 1981). Second, Glu-58 may stimulate the opening of the imidazolidine ring of the cofactor by functioning as a general acid catalyst (Matthews *et al.*, 1990). Such acid catalysis would assist bond breakage between the N-10 and C-11 of the cofactor by increasing the leaving group ability of the N-10 nitrogen (Benkovic, 1978; Santi *et al.*, 1987). The pK_a of the γ-carboxyl group of free glutamic acid is too low to serve as a proton donor at physiological pH, but alterations in the pK_a of amino acid residues occur often in proteins. The site-specific substitution of the glutamic acid residue 58 with a glutamine residue was designed to alter hydrogen bonding at amino acid 58 while minimizing structural perturbations.

Purification. As shown in Table I, the purification scheme for Gln-58 TS is very similar to that of the wild-type enzyme. The procedure used to purify wild-type and mutant TS consisted of two purification steps: Affi-Gel Blue and Q-Sepharose chromatography. TS-overexpressing *E. coli* host cells were disrupted by sonication. Three 4-min sonication cycles were found to yield the highest TS activity, where only 20% of the host cells remained intact after the third sonication cycle. Further sonication resulted in TS activity loss. The CFE was incubated with Affi-Gel Blue resin for 30 min, after which time the resin was washed batchwise in a sintered glass funnel. Contaminating proteins were eluted by washing the resin with Blue A and Blue B buffers. The majority of the TS was eluted by washing the resin with Blue C. *E. coli* TS was resolved from the remaining contaminating proteins by a Q-Sepharose column by a gradient elution from 10% QA to 40% QB. TS activity was eluted as a symmetric peak 60% into the gradient. Ninety percent of the loaded units were recovered from the column in the case of wild type. TS obtained after the Q-Sepharose step was judged to be homogeneous by native PAGE and FdUMP cofactor ternary complexes PAGE. Overall, this method yielded large amounts of pure enzyme (about 100 mg) in only two chromatographic steps.

Determination of Kinetic Parameters. The catalytic parameters for both the wild-type and the mutant enzyme are shown in Table II. The mutation had a more pronounced effect on K_m(dUMP) (18–26-fold) than on K_m(CH_2H_4folate) (2–6-fold). This result initially suggested that the mutation had a larger effect on nucleotide binding than on folate binding. The results of the less sensitive spectrophotometric assay showed that the k_{cat} of the Gln-58 enzyme was some 370-fold lower than for the wild-type enzyme, while the more sensitive tritium release assay indicated that the Gln-58 mutant TS was 760-fold less active than wild-type TS. The 2–3 order of magnitude drop in k_{cat} for the mutant compared to the wild-type enzyme showed the catalytic importance of Glu-58. The difference in k_{cat} for the mutant enzyme measured between the two assays was unexpected.

At this time, it is not clear whether the different decreases in k_{cat} measured by the spectrophotometric and tritium release assays reflect the limited sensitivity of the former assay, the operation of a tritium kinetic isotope effect in the latter assay, or some combination of both. The data from the spectrophotometric assay for the Gln-58 TS are valuable because the appearance of H_2folate, as measured at 340 nm, demonstrates that this mutant enzyme remains capable of dTMP synthesis. Such a conclusion cannot be reached solely on the basis of data from the tritium release assay. However, because of the low level of dTMP synthesis activity displayed by Gln-58 TS, the spectrophotometric assay was conducted near the limits of its sensitivity. Also, since relatively high concentrations of enzyme were necessary, perhaps challenging the validity of the steady-state assumption, these data are not as trustworthy as those from the tritium release assay. Thus, the difference in k_{cat} for the mutant enzyme may be attributed, in part, to the large relative error in measuring the catalytic activity of mutant enzyme with miniscule activity. This difficulty may have been compounded by slight differences in the specific activity of the mutant enzyme obtained from different preparations. It has been suggested that the difference in k_{cat} for the mutant enzyme observed with the two assays may be attributed, in part, to a greater kinetic isotope effect with the Gln-58 mutant than with the wild-type enzyme. If the rate-limiting step with respect to k_{cat} for the mutant enzyme decreased to a magnitude similar to the rate constant for the step where the C-5 proton is abstracted from dUMP, catalysis by the mutant enzyme would exhibit a primary isotope effect. Indeed, with wild-type TS when [5-^{3}H]dUMP is employed as a substrate, the rate of tritium release is some 80–85% that of H_2folate, producing an isotope effect consistent with

Table II: Catalytic Parameters of *E. coli* Wild-Type and Gln-58 Mutant Enzymes

assay	enzyme	sp activity [μmol/(min·mg)]	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})		K_m (μM)	
				dUMP	CH_2H_4folate	dUMP	CH_2H_4folate
^3H release	wild type	7.4 ± 0.3	3.75 ● 0.2	2.9	0.2	1.3 ± 0.3	18 ± 2
	Gln-58	0.009 ± 0.003	0.005 ± 0.002	1.5×10^{-4}	1.3×10^{-4}	34 ± 2	40 ± 17
spectral	wild type	7.2 ± 0.3	3.7 ± 0.2	1.2	0.06	3 ± 0.5	19 ± 1
	Gln-58	0.02 ● 0.005	0.010 ± 0.003	1.8×10^{-4}	9×10^{-5}	55 ● 5	111 ± 10

rehybridization at C-5 (Pogolotti & Santi, 1977). However, we observed no isotope effects directly from our data with the wild-type enzyme. This fact was ascribed to the low percentage of tritium (1%) label in the dUMP used. Thus, it was unlikely that the 2-fold difference in k_{cat} for the mutant enzyme measured by the two assays was solely due to directly observing an isotope effect. The difference may be due to the combination of error and the isotope effect.

$$\mathrm{TS} \underset{k_2}{\overset{k_1[\mathrm{dUMP}]}{\rightleftharpoons}} \mathrm{TS\text{–}dUMP} \underset{k_4}{\overset{k_3[\mathrm{fol}]}{\rightleftharpoons}}$$

$$\mathrm{TS\text{–}dUMP\text{–}CH_2H_4folate} \xrightarrow{k_5} \mathrm{dTMP} + \mathrm{H_2folate}$$

It is shown that at saturating concentrations of the folate k_{cat}/K_m(dUMP) equals the rate of association (k_1) of dUMP with wild-type enzyme (eq 1) (Santi *et al.*, 1990); however,

$$k_{cat}/K_m(\mathrm{dUMP}) = k_1 \quad (1)$$

this relationship may not be true with the Gln-58 mutant TS. The specificity constant k_{cat}/K_m(dUMP), as measured for the mutant enzyme, decreased roughly 8000-fold relative to that for the wild-type enzyme. If it is assumed that FdUMP binding is a good model for dUMP binding to TS (Pogolotti & Santi, 1977; Lewis & Dunlap, 1981; Santi & Danenberg, 1984), it follows that the 8000-fold decrease in k_{cat}/K_m(dUMP) measured for the mutant should be manifested as a 18000-fold decrease in the rate that the FdUMP binary complex forms with the Gln-58 mutant enzyme. The FdUMP binary complex with the wild-type enzyme is known to require minutes to form (unpublished results, M. S. Weir and R. B. Dunlap). Thus, if the rate that the binary complex forms was decreased by 18000-fold by the mutation, the FdUMP binary complex should take days to form with the mutant. However, we found that the FdUMP binary complex formed to the same extent with the mutant as with the wild-type enzyme within 60 min. Further, an 18000-fold decrease in k_1 should also be manifested in a large decrease in the stability of the FdUMP binary complex; however, we observed only a 5-fold decrease in stability (Table II). To address this discrepancy, we propose that the k_{cat}/K_m(dUMP) is described by eq 2

$$\frac{k_{cat}}{K_m(\mathrm{dUMP})} = \frac{k_1k_3k_5[\mathrm{CH_2H_4folate}]}{k_2(k_4 + k_5) + k_3k_5[\mathrm{CH_2H_4folate}]} \quad (2)$$

instead of eq 1. Equation 2 is the expression for k_{cat}/K_m(dUMP) at low concentrations of folate (Santi *et al.*, 1990). With the wild-type enzyme, saturating concentrations of folate reduce eq 2 to eq 1 assuming $k_2 \ll k_3$(folate). However, since the Glu-58 may catalyze the ring opening or stabilize the opened product of the imidazolidine ring of folate (k_3), we theorized that the rate at which the imidazolidine ring of folate was opened may have been decreased to such an extent that this step may have become rate-limiting or near-rate-limiting for the reaction catalyzed by the Gln-58 enzyme. This proposal is reasonable since the folate would be unable to bind completely to the active site of TS due to steric constraints imposed by the imidazolidine ring (Hardy *et al.*, 1987). Since k_3 may have decreased to such a large extent on the mutant enzyme, eq 2 would not reduce to eq 1 no matter how much of the folate cofactor was used to drive the reaction. Thus, k_{cat}/K_m(dUMP) would no longer be a measure of k_1; instead, it would be a mixture of rate constants that would have a marked dependence on the rate of folate binding (eq 2). Thus, a decreased k_{cat}/K_m(dUMP) would be consistent with the impaired binding of folate, as opposed to impaired nucleotide binding.

Table III: Binding Constants and Covalent Binding Ratios of Complexes Formed with *E. coli* Wild-Type and Gln-58 Mutant Enzymes

	enzyme	
	wild type	Gln-58
binary complex		
binding ratio	0.7 ± 0.2	0.5 ± 0.2
K_d (μM)	6 ± 1	30 ± 2
inhibitory ternary complex		
binding ratio	1.5 ± 0.2	0.7 ± 0.2
K_d (μM)		
FdUMP	0.04 ± 0.01	0.3 ± 0.1
CH_2H_4folate	0.3 ± 0.03	18 ± 3

Binding Ratio Determination. To further elucidate the role of residue 58 in substrate binding, the covalent binding parameters measured by the TCA assay were investigated (Table III). In the absence of CH_2H_4folate, the mutant enzyme bound FdUMP covalently to roughly the same extent as the wild-type enzyme, with binding ratios of 0.5 and 0.7, respectively. When CH_2H_4folate was added, however, there was a significant difference in the covalent inhibitory ternary binding ratios for the wild-type (1.5) and mutant (0.7) enzymes as measured with [6-^{3}H]FdUMP. The striking similarity in the covalent inhibitory ternary complex binding ratio (0.7) and the covalent binary complex binding ratio (0.5) for the Gln-58 mutant was much more than coincidental and dramatically illustrated the inability of CH_2H_4folate to enhance covalent nucleotide binding in the mutant enzyme.

Determination of Apparent Dissociation Constants. To determine intrinsic dissociation constants, K_d, the binding site concentration should be 10-fold lower than the determined dissociation constant (Connors, 1987). Since we used enzyme concentrations of 0.3 μM, our dissociation constants with values of 3.0 μM and below should be termed apparent dissociation constants. In this case where the binding site concentration was the same or higher than the measured K_d, the K_d value obtained depends not only on the affinity of the ligand for the binding site but also on the binding site concentration (Bennett *et al.*, 1985). When it is determined that an apparent K_d has been measured, it can be concluded that the intrinsic K_d is at a lower value than the apparent K_d. The K_d for the wild-type *E. coli* TS is estimated to be 1.6×10^{-9} M (Dev *et al.*, 1988). Thus, determining the intrinsic K_d from FdUMP titration experiments would have required the use of low enzyme concentrations (below 8×10^{-11} M) and very high specific

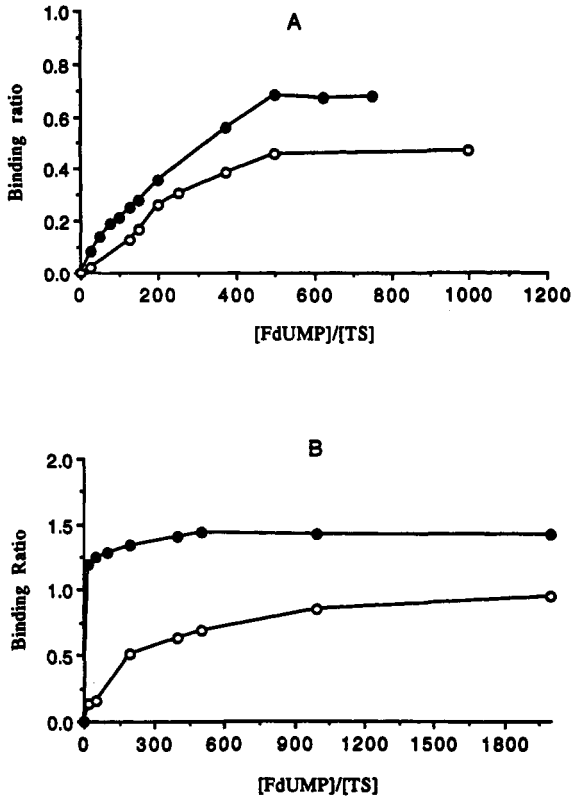


FIGURE 1: Saturation profiles of *E. coli* wild-type and Gln-58 TS in its covalent binary and inhibitory ternary complex. (A) Binary complex. (●) represents the wild-type enzyme, and (○) represents the Gln-58 enzyme. (B) Inhibitory ternary complex. (●) represents the wild-type enzyme, and (○) represents the Gln-58 enzyme.

activity [6-^{3}H]FdUMP. We found that binding experiments with Gln-58 mutant enzyme would have been prohibitively expensive under these conditions. The difficulty in determining the K_d of the ternary complex has been discussed in the literature (Galivan *et al.*, 1976). Thus, we chose conditions that allowed us to easily compare the mutant and wild-type enzymes.

The apparent dissociation constants, K_d, as determined by Scatchard analysis, for the covalent binary and inhibitory ternary complexes are shown in Table III. The covalent binary complex for the mutant was 5-fold less stable than the same complex for the wild-type enzyme as demonstrated by the difference in binary complex apparent K_d values. The decrease in the stability of the binary complex suggested that Glu-58 plays some role in the binding of the nucleotide in the absence of folate. The differences in apparent K_d values for the covalent inhibitory ternary complex, as measured by varying either nucleotide or folate, were 10- and 60-fold less than those measured for the wild type. Thus, this mutation diminished the stability of all complexes formed by this enzyme. The K_d is not only a measure of stability of the inhibitory ternary complex but also a measure of the ligand concentration needed for half-maximal binding. The 10-fold difference in nucleotide-varied inhibitory ternary complex K_d values indicated that 10 times higher concentration of FdUMP was needed to promote half-maximal binding at saturating levels of folate. On the other hand, the 60-fold difference seen for the folate-varied inhibitory ternary complex K_d demonstrated that a 60-fold higher concentration of folate was needed to induce half-maximal binding of FdUMP. Since both assays measure FdUMP binding, the higher folate-varied K_d suggested that folate binding and subsequent enhancement of nucleotide binding by the folate were more affected by the mutation.

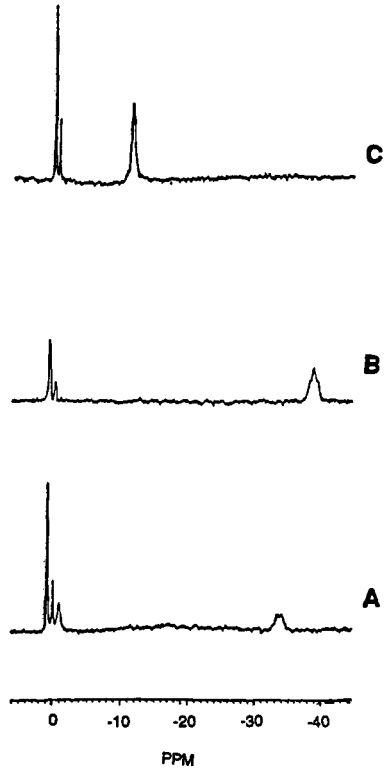


FIGURE 2: 188.8-MHz ^{19}F NMR spectra of *E. coli* wild-type thymidylate synthase FdUMP complexes. (A) FdUMP binary complex. (B) Pseudoternary complex of TS–FdUMP–H_4folate. (C) Inhibitory ternary complex of TS–FdUMP–CH_2H_4folate. All complexes were formed as described under Materials and Methods.

Figure 1 shows the saturation profile for wild-type and Gln-58 TS in both binary and ternary complexes. Both enzymes exhibited similar binary complex saturation profiles where saturation was achieved at 500-fold excess of FdUMP, although the extent of nucleotide binding to the mutant was decreased slightly. In contrast, the saturation profiles for the inhibitory ternary complex (FdUMP–CH_2H_4folate–TS) were markedly different for the wild-type and mutant enzymes. The wild-type enzyme was saturated at 10-fold excess of FdUMP over TS, while the mutant did not reach saturation until a 500-fold excess of FdUMP.

^{19}FNMR. ^{19}F NMR was used to assess the FdUMP binding to *E. coli* wild-type enzyme in the binary, pseudoternary, and inhibitory ternary complexes. The ^{19}F spectra of *E. coli* wild-type TS–FdUMP complexes were remarkably similar in appearance and chemical shift values to the corresponding ^{19}F spectra of the *L. casei* enzyme (Byrd *et al.*, 1977), which may reflect the high degree of homology that exists between these two enzymes. The ^{19}F NMR spectrum of TS titrated with a 4-fold excess of FdUMP is shown in Figure 2A. The *E. coli* FdUMP binary complex spectrum has the same characteristic resonances at −1.5 ppm, assigned to the noncovalent binary complex, and at −34.5 ppm, assigned to the covalent binary complex of *L. casei* TS. The similarities between the *L. casei* and *E. coli* TS–FdUMP binary complex ^{19}F NMR spectra showed that the *E. coli* enzyme, like the *L. casei* enzyme, bound FdUMP as an equilibrium mixture of covalent and noncovalent forms. Furthermore, the downfield narrow resonances at 0.0 and −0.7 ppm have been assigned to the 5′-phosphate isomer of FdUMP and the 3′-phosphate isomer of FdUMP, both free in solution, respectively (Lewis *et al.*, 1980). A 4-fold excess of FdUMP over enzyme gave strong resonances at −34.5 and 0.0 ppm. Excesses of FdUMP over enzyme of 5-fold or more resulted in an apparent

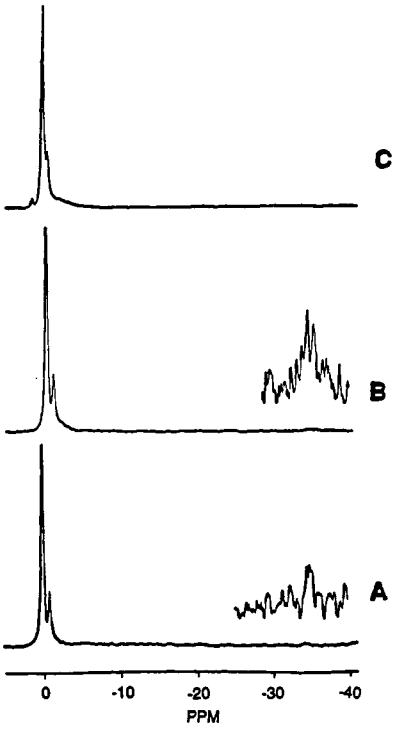


FIGURE 3: 188.8-MHz ^{19}F NMR spectra of *E. coli* Gln-58 thymidylate synthase FdUMP complexes. (A) FdUMP binary complex. (B) Pseudoternary complex of TS:FdUMP:H_4folate. (C) Mixture of covalent and noncovalent complexes of CH_2H_4folate TS and FdUMP. All complexes were formed as described under Materials and Methods.

resonance intensity loss at −34.5 ppm as a result of signal digitizer dynamic range limitations.

The binary complex sample was converted to pseudoternary complex by the addition of H_4folate (Figure 2B). As previously described for the *L. casei* enzyme, a resonance attributed to the covalent binary complex (−34.5 ppm) was shielded (−39.0 ppm) and increased in signal intensity. Moreover, the binary complex resonance at −1.5 ppm attributed to noncovalently bound FdUMP completely disappeared in the pseudoternary complex spectrum (Lewis *et al.*, 1981). The disappearance of a resonance attributed to noncovalently bound FdUMP (−1.5 ppm) as well as the upfield shift and increased intensity of the resonance assigned to the covalent bound FdUMP in the pseudoternary complex (Figure 2B) was consistent with the enhancement of covalent nucleotide binding by the folates (Byrd *et al.*, 1978; Lewis *et al.*, 1981).

The pseudoternary complex sample was converted to the inhibitory ternary complex by the addition of a 25-fold molar excess of CH_2O over H_4folate (Figure 2C). Again, similar to the *L. casei* enzyme, the resulting large symmetric resonance at −12.5 ppm of the inhibitory ternary complex has been ascribed to FdUMP covalently linked to both enzyme and CH_2H_4folate (Byrd *et al.*, 1978). ^{19}F NMR studies of the *L. casei* inhibitory ternary complex have indicated that the nucleotide is covalently bound to both enzyme and CH_2H_4-folate with the nucleotide and CH_2H_4folate arranged in a *trans*-diaxial configuration (Byrd *et al.*, 1978). Further, the single FdUMP inhibitory ternary complex ^{19}F resonance suggested that FdUMP is bound in an identical environment in both active sites.

Similar ^{19}F NMR experiments were used to assess binding of FdUMP to the mutant enzyme in all three complex forms (Figure 3). The binary complex, which was created by incubating a 4-fold molar excess of FdUMP with the enzyme (Figure 3A), exhibited resonances at 0.0 and −0.7 ppm which were attributed to free 5′-phosphate and 3′-phosphate isomers of FdUMP, respectively. Indicative of weakened FdUMP binary complex in the mutant enzyme, the ^{19}F NMR resonance at −34 ppm assigned to covalently bound FdUMP was less intense for the mutant enzyme than for the wild-type enzyme. Further, the resonance at −1.5 ppm assigned to the noncovalent binary complex was absent in the spectrum of the Gln-58 mutant enzyme–FdUMP binary complex. Thus, the mutation perturbed binding interactions between FdUMP and enzyme that are important for noncovalent binding of FdUMP, but this perturbation was not great enough to block covalent binding (Figure 1).

The addition of H_4folate to the Gln-58 mutant enzyme FdUMP binary complex resulted in the ^{19}F NMR spectrum shown in Figure 3B. As seen in the pseudoternary complex spectrum, the bound FdUMP resonance is at −34 ppm, and it is a weak resonance. Thus, no enhancement of intensity or upfield shift of the resonance was observed in the spectrum of the FdUMP:H_4folate:Gln-58 mutant TS. As can be seen in the spectra of the wild-type enzyme (Figure 2), addition of H_4folate to the binary complex (TS:FdUMP) increased the shielding by 5 ppm from −34 to −39 ppm and also increased the intensity of the binary complex resonance. In contrast, as can be seen in spectra of the Gln-58 mutant enzyme (Figure 3), addition of H_4folate did not increase the shielding or the intensity of the resonance representing the covalent binary complex. Increases in the wild-type binary complex resonance intensity are correlated with increased covalent binding of FdUMP (Lewis *et al.*, 1981). Further, this increased binding of FdUMP is attributed to energetically favorable bases stacking that occurs between cofactor and FdUMP (Matthews *et al.*, 1990a). Consistently, increased shielding of the fluorine chemical shift in the pseudoternary complex is also attributed to electronic interactions, such as base stacking, between the H_4folate and FdUMP (Lewis *et al.*, 1981). This base stacking may account, in part, for the increased stability of ternary complexes over binary complexes. The lack of H_4folate-induced increases in shielding and intensity of the fluorine chemical shift implied that Glu-58 is important for base-stacking interactions between FdUMP and H_4folate.

Furthermore, the addition of CH_2O to the mixture of FdUMP, H_4folate, and Gln-58 mutant enzyme yielded a spectrum (Figure 3C) that did not have a prominent resonance at −12.5 ppm, a resonance characteristic of the inhibitory ternary complexes, thus implying that little, if any, covalent inhibitory ternary complex existed under these conditions. Similarly, measurement of covalent binding to Gln-58 mutant enzyme by the TCA precipitation assay showed that CH_2H_4folate did not induce a large increase in the molar binding ratio for covalently bound FdUMP over that seen for the binary complex (Table III). With wild-type enzyme, forming the ternary complex by adding CH_2H_4folate enhanced FdUMP binding by 2.1-fold over that found for the FdUMP binary complex. More specifically, the covalent binding ratio for FdUMP was increased from 0.7 to 1.5 by adding CH_2H_4folate to the FdUMP binary complex. "Folate enhancement" of covalent binding of dUMP or FdUMP appears to be a general characteristic of TS isolated from any source (Cisneros *et al.*, 1988). Thus, Gln-58 mutant enzyme not only did not display folate-dependent enhancement of covalent FdUMP binding but also did not appear to form a typical covalent inhibitory ternary complex with FdUMP and CH_2H_4folate. However, since the Gln-58 mutant enzyme is capable of catalytic turnover, it must be able to bind

CH_2H_4folate covalently at least to a minimal extent. Therefore, we are currently verifying the covalent attachment of CH_2H_4folate with radiolabeled CH_2H_4folate. The NMR experiment may not have been sensitive enough to detect a weak ternary complex resonance, or the resonance at −12.5 ppm may have been absent due to exchange phenomena.

Conclusions. The experimental findings in this paper support the proposal that the primary role of the Glu-58 side chain is most probably the stimulation of the opening of the imidazolidine ring of the cofactor or the stabilization of the resulting cationic iminium version of the cofactor. The k_{cat} of the wild-type enzyme was at least 370-fold greater than that of the mutant, illustrating the functional importance of the Glu-58 side chain in catalysis. We sought to gain insight into the catalytic importance of Glu-58 by studying the inhibitory ternary complex (TS–FdUMP–CH_2H_4folate), which is a stable model for the covalent catalytic ternary complex (TS–dUMP–CH_2H_4folate). We evaluated complex formation by two methods: ^{19}F NMR and the trichloroacetic acid precipitation assay (Cisneros & Dunlap, 1990a,b). ^{19}F NMR can detect both covalent and noncovalent interactions between FdUMP and TS. The instability of the FdUMP complexes of the mutant enzyme made it difficult to acquire strong signal to noise ratios under the necessary conditions of the ^{19}F NMR experiments.

The TCA assay is a sensitive method to measure covalent binding of FdUMP to TS. Thus, information from the TCA assay not only was used to verify ^{19}F NMR results but also was used to measure binding stoichiometries and K_d values for the complexes. Binding of the inhibitor, FdUMP, in a binary complex was relatively unaffected by the mutation. FdUMP bound to mutant enzyme in a binary complex with a molar stoichiometry (0.5 mol of FdUMP/mol of TS) that was similar to that of the wild-type enzyme (0.7 mol of FdUMP/mol of TS) and with a dissociation constant, K_d that was 5-fold higher than that of the wild-type enzyme (30 μM compared to 6 μM for wild type). However, binding of FdUMP in a ternary complex (TS–FdUMP–CH_2H_4folate) was destabilized by the mutation. The molar stoichiometry of FdUMP binding in the ternary complex was 53% less in the mutant than in the wild-type enzyme, which suggested that FdUMP was only binding at one of the two active sites in the Gln-58 mutant enzyme. The K_d of the ternary complex for the mutant was 60-fold higher than the K_d for the wild-type enzyme. ^{19}F NMR and TCA precipitation assay analysis of complexes formed with Gln-58 mutant enzyme demonstrated that the γ-carboxylate of Glu-58 in wild-type enzyme was essential for forming a stable inhibitory ternary complex. Overall, the results indicated that the mutation greatly impaired CH_2H_4folate binding but that nucleotide binding was less perturbed.

ACKNOWLEDGMENT

We thank Dr. Ralph J. Cisneros, Dr. Jeffery O. Boles, and R. Todd Reilly for their helpful discussions. We thank Ron Garber for his help with the NMR experiments.

REFERENCES

Ahmed, F., Moore, M. A., & Dunlap, R. B. (1985) *Anal. Biochem. 145*, 151–159.

Bell, J. E., & Bell, E. T. (1989) in *Proteins and Enzymes*, Chapter 14, Prentice-Hall, Englewood Cliffs, NJ.

Benkovic, S. J. (1978) *Acc. Chem. Res. 11*, 8606–8613.

Bennett, J. P., & Yamamura, H. I. (1985) in *Neurotransmitter Receptor Binding* (Yamamura, H. I., Enna, S. J., & Juhar, M. H., Eds.) Chapter 3, Raven, New York.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Byrd, R. A., Dawson, W. H., Ellis, P. D., & Dunlap, R. B. (1977) *J. Am. Chem. Soc. 99*, 6139–6141.

Byrd, R. A., Dawson, W. H., Ellis, P. D., & Dunlap, R. B. (1978) *J. Am. Chem. Soc. 100*, 7478–7486.

Cisneros, R. J., & Dunlap, R. B. (1990a) *Biochim. Biophys. Acta 1039*, 149–156.

Cisneros, R. J., & Dunlap, R. B. (1990b) *Anal. Biochem. 186*, 202–208.

Connors, K. A. (1987) in *Binding Constants*, Wiley, New York.

Danenberg, P. V., & Danenberg, K. D. (1978) *Biochemistry 17*, 4018–4024.

Dev, I. K., Yates, B. B., Leong, J., & Dallas, W. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1472–1476.

Donato, H., Aull, J. L., Lyon, J. A., Reinsch, J. W., & Dunlap, R. B. (1976) *J. Biol. Chem. 251*, 1303–1310.

Dunlap, R. B., Harding, N. G. L., & Huennekens, F. M. (1971) *Biochemistry 10*, 88–97.

Duschinsky, R., Pleven, E., & Heidelberger, C. (1959) *J. Am. Chem. Soc. 81*, 4559–4560.

Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405–426.

Finer-Moore, J. S., Montfort, W. R., & Stroud, R. M. (1990) *Biochemistry 29*, 6977–6986.

Galivan, J. H., Maley, G. F., & Maley, F. (1976) *Biochemistry 15*, 356–362.

Hardy, L. W., & Nalivaika, E. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9725–9729.

Hardy, L. W., Finer-Moore, J. S., Montfort, W. R., Jones, M. O., Santi, D. V., & Stroud, R. M. (1987) *Science 235*, 448–455.

Hatefi, Y., Talbert, P. T., Osborn, M. J., & Huennekens, F. M. (1960) *Biochem. Prep. 2*, 89–92.

Jones, T. R. (1980) *Eur. J. Cancer 16*, 707–711.

Lewis, C. A., & Dunlap, R. B. (1981) in *Topics in Molecular Pharmacology* (Burgen, A. S. V., & Roberts, Eds.) pp 169–219, Elsevier/North Holland Biomedical Press, Amsterdam.

Lewis, C. A., Ellis, P. D., & Dunlap, R. B. (1980) *Biochemistry 19*, 116–123.

Lewis, C. A., Ellis, P. D., & Dunlap, R. B. (1981) *Biochemistry 20*, 2275–2285.

Liu, L., & Santi, D. V. (1992) *Biochemistry 31*, 5100–5104.

Matthews, D. A., Appelt, K., & Oatley, S. J. (1989) *J. Mol. Biol. 205*, 449–454.

Matthews, D. A., Appelt, K., Oatley, S. J., & Huong, Ng H. (1990a) *J. Mol. Biol. 214*, 923–936.

Matthews, D. A., Villafranca, J. E., Janson, C. A., Smith, W. W., Welsh, K., & Freer, S. (1990b) *J. Mol. Biol. 214*, 937–948.

Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G. F., Hardy, L., Maley, F., & Stroud, R. M. (1990) *Biochemistry 29*, 6964–6977.

Moore, M. A., Ahmed, F., & Dunlap, R. B. (1984) *Biochem. Biophys. Res. Commun. 124*, 37–43.

Pogolotti, A. L., Jr., & Santi, D. V. (1977) in *Bioorganic Chemistry: Enzyme Action*, Vol. 1, pp 277–311, Academic Press, New York.

Santi, D. V., & Danenberg, P. V. (1984) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.), Vol. 2, pp 345–398, Wiley, New York.

Santi, D. V., McHenry, C. S., Raines, R. T., & Ivanetich, K. M. (1987) *Biochemistry 26*, 8606–8613.

Santi, D. V., Pinter, K., Kealy, J., & Davisson, V. J. (1990) *J. Biol. Chem. 265*, 6770–6775.

Villafranca, J. E., Howell, E. E., Voet, D. H., Strobel, M. S., Ogden, R. C., Abelson, J. N., & Kraut, J. (1983) *Science 222*, 782–788.