

An Essential Role for Water in an Enzyme Reaction Mechanism: The Crystal Structure of the Thymidylate Synthase Mutant E58Q^{†,‡}

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ABSTRACT: A water-mediated hydrogen bond network coordinated by glutamate 60(58) appears to play an important role in the thymidylate synthase (TS) reaction mechanism. We have addressed the role of glutamate 60(58) in the TS reaction by cocrystallizing the *Escherichia coli* TS mutant E60(58)Q with dUMP and the cofactor analog CB3717 and have determined the X-ray crystal structure to 2.5 Å resolution with a final *R* factor of 15.2% (*R*_{free} = 24.0%). Using difference Fourier analysis, we analyzed directly the changes that occur between the wild-type and mutant structures. The structure of the mutant enzyme suggests that E60(58) is not required to properly position the ligands in the active site and that the coordinated hydrogen bond network has been disrupted in the mutant, providing an atomic resolution explanation for the impairment of the TS reaction by the E60(58)Q mutant and confirming the proposal that E60(58) coordinates this conserved hydrogen bond network. The structure also provides insight into the role of specific waters in the active site which have been suggested to be important in the TS reaction. Finally, the structure shows a unique conformation for the cofactor analog, CB3717, which has implications for structure-based drug design and sheds light on the controversy surrounding the previously observed enzymatic nonidentity between the chemically identical monomers of the TS dimer.

Thymidylate synthase (TS;¹ EC 2.1.1.45) catalyzes the reductive methylation of the substrate, deoxyuridine monophosphate (dUMP), using the cofactor, 5,10-methylenetetrahydrofolate (mTHF), to create the product, deoxythymidine monophosphate (dTMP), as a step in the sole *de novo* pathway for the synthesis of dTMP. Since dTMP is required for cellular DNA synthesis, TS is the target for anti-cancer drugs such as the currently used chemotherapeutic agents 5-FU and ZD1694 (Jackman *et al.*, 1991, 1993). The importance of TS as a chemotherapeutic target makes understanding the atomic details of the TS catalytic mechanism a critical part of the rational inhibitor design process. This understanding will also likely provide insight into the workings of the catalytic machinery of enzymes.

The TS reaction mechanism has been heavily investigated, and the major steps of the reaction have been mapped [Figure 1; see Carreras and Santi (1995) and Stroud and Finer-Moore (1993) for review]. Most biochemical research on the TS mechanism has been performed using TS from *Escherichia coli* and *Lactobacillus casei*; therefore, *L. casei* residue numbers will be listed followed by the *E. coli* numbers in parentheses.² The most important insight into the role of active site amino acids in the TS mechanism is provided by the high-resolution crystal structures of TS from various species complexed with substrates or substrate analogs [Hardy *et al.*, 1987 (*L. casei*); Matthews *et al.*, 1990 (*E. coli*);

Montfort *et al.*, 1990 (*E. coli*); Perry *et al.*, 1990 (*E. coli*); Fauman *et al.*, 1994 (*E. coli*); Finer-Moore *et al.*, 1994 (bacteriophage T₄); Schiffer *et al.*, 1995 (human)]. These atomic resolution views of the TS active site have redirected the use of site-directed mutagenesis to analysis of key residues in the active site. Although a number of highly conserved residues of TS have been extensively mutated, few substitutions abolish activity, presumably due to the “plastic” nature of the TS active site (Perry *et al.*, 1990; Michaels *et al.*, 1990; Climie *et al.*, 1990, 1992).

On the basis of crystallographic structural data (Montfort *et al.*, 1990; Finer-Moore *et al.*, 1990; Matthews *et al.*, 1990a,b) and the absolute conservation (Carreras & Santi, 1995; Perry *et al.*, 1990) of the glutamate at position 60 [E60(58)], it was proposed that E60(58) coordinates a charge-stabilizing hydrogen bond network in the TS active site and thus plays a key role in the catalytic mechanism. E60(58) has been the subject of several site-directed mutagenesis studies assessing its role in the TS mechanism at the biochemical level. Zapf and co-workers (1993), analyzing the E60(58)Q mutant, proposed that the role of E60(58) in the TS reaction is either promotion of ring opening of mTHF upon ternary complex formation or stabilization of the cationic iminium intermediate of the cofactor (II, Figure 1). Two additional groups (Hardy *et al.*, 1995; Huang & Santi, 1994) made the observation that mutations of E60(58) alter the rate-limiting step of the reaction so that dissolution of the covalent ternary complex between dUMP, mTHF, and TS became rate-limiting (IV, Figure 1) but differ in their interpretation of this result. Huang and Santi (1994) propose that E60(58) stabilizes a hydrogen bond network that

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[‡] Crystallographic coordinates have been submitted to the Brookhaven Protein Data Bank under 1ZPR [E60(58)Q] and 1KCE (WT-TS).

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¹ Abbreviations: TS, thymidylate synthase; dUMP, 2'-deoxyuridine 5'-monophosphate; mTHF, 5,10-methylenetetrahydrofolate; dTMP, thymidine 5'-monophosphate; DHF, dihydrofolate; CB3717, 10-propargyl-5,8-dideazafofolate.

² The amino acid sequence numbering used is that of the *L. casei* enzyme so as to be consistent with our previous publications. The *E. coli* sequence number is listed in parentheses. In addition, residues from the “second” monomer which enter into the discussion of the “first” monomer are indicated with a prime, e.g., R179(127)′.

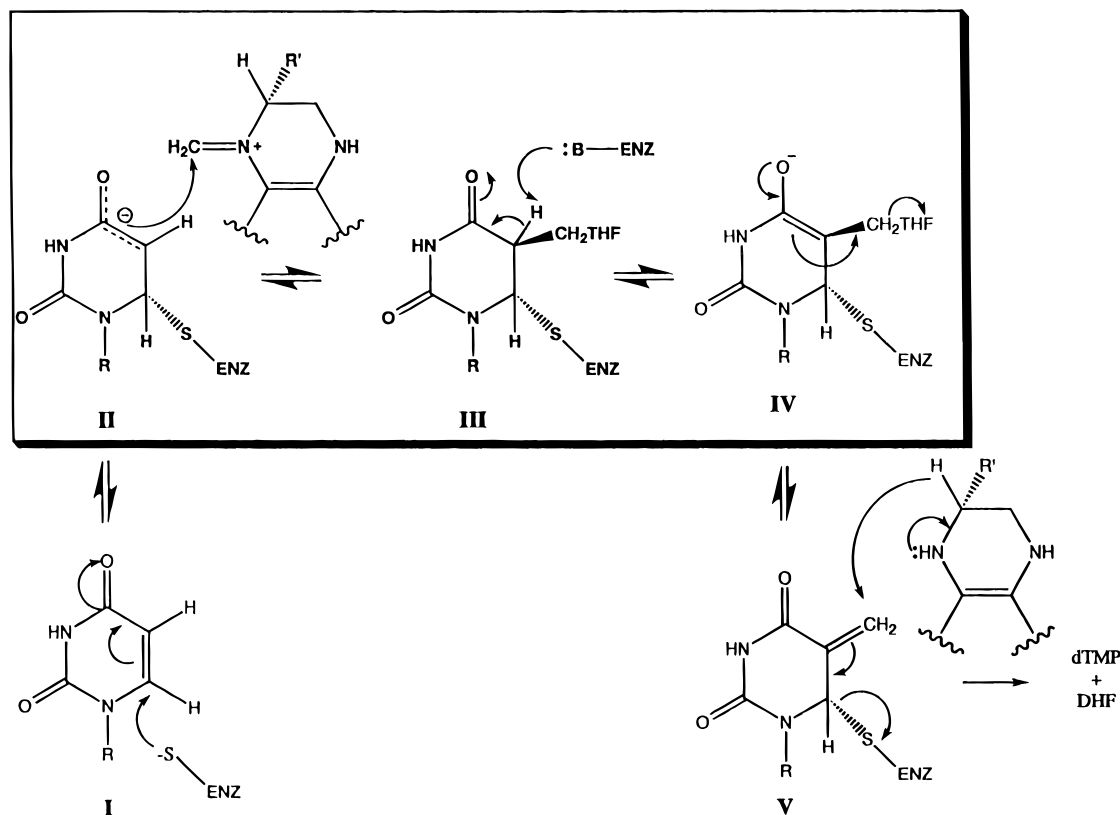


FIGURE 1: Proposed chemical mechanism and intermediates in the TS reaction. A box is drawn around intermediates which are proposed to be affected by E60(58)Q.

promotes proton transfer reactions at the substrate and cofactor and properly orients both ligands. Hardy *et al.* (1995) suggest that E60(58) acts *via* electrostatic repulsion of O4 of dUMP to complete the methylene transfer reaction.

To assess the role of E60(58) in the TS reaction mechanism at atomic resolution, we have determined the crystal structure of the *E. coli* TS mutant E60(58)Q cocrystallized with the substrate, dUMP, and the cofactor analog, CB3717, at 2.5 Å resolution. The conservative and isosteric glutamate to glutamine mutation at position 60(58) was chosen to allow observation of changes caused as a result of affecting only the functional group of the residue. The results support the notion that E60(58) coordinates a hydrogen bond network in which water plays an important role and illustrate the subtle changes in water position and hydrogen-bonding contacts that have drastic effects on the chemical reaction. In addition, the structure reveals an orientation for the ligands which sheds light on the nonequivalent enzymatic activity that has been observed between the two monomers of the dimer [reviewed in Carreras and Santi (1995)].

MATERIALS AND METHODS

Site-Directed Mutagenesis

A glutamate to glutamine point mutation at position 58 of *E. coli* TS was created by the method of Kunkel *et al.* (1987) using the synthetic DNA oligonucleotide 5'-CACAG-CAGCTGATGGATG-3' in the ThyA gene (Belfort *et al.*, 1983) which had been cloned into Bluescript (Stratagene). Clones were first selected by screening the resulting plasmids for the loss of a *NlaIII* restriction site; positive clones were subsequently confirmed by DNA sequence analysis (UCSF, BRC Sequencing Facility).

Purification of Thymidylate Synthase

Approximately 5 g of ThyA-strain χ 2913recA (Climie *et al.*, 1992) cells overexpressing E60(58)Q or wild-type TS (WT-TS) were obtained from 3 L of liquid LB culture grown for 18 h at 37 °C. TS was isolated as described by Maley and Maley (1988), and protein yields were similar for both E60(58)Q and wild-type TS [30 mg of TS/(g of cells)]. The purity of the isolated proteins was greater than 98% on the basis of Coomassie-stained SDS-PAGE gels.

Enzymatic Activity Assays of TS

The enzymatic activity of E60(58)Q and wild-type TS was assayed by monitoring the change in absorbance at 340 nm for the formation of H₂-folate (Wahba & Friedkin, 1961). The specific activity of purified E60(58)Q and wild-type TS was similar to that observed previously (Zapf *et al.*, 1993); however, it should be noted that Hardy *et al.* (1995) demonstrated that the change in absorbance at 340 nm does not reflect product formation for the E60(58)Q mutant but represents a stable chromophore. Product formation occurs at a rate 5-fold lower than that indicated by the spectrophotometric assay. Purified protein was stored as an 85% NH₄(SO₄)₂ slurry at -20 °C.

Crystallization

For crystallization, purified E60(58)Q TS and WT-TS were dialyzed against 20 mM KPO₄ (pH 7.5), 0.1 mM EDTA, and 1 mM DTT. E60(58)Q TS and WT-TS were cocrystallized with the folate analog CB3717 and dUMP as described previously (Montfort *et al.*, 1990). Large, single crystals grew over a 3 week period, and 2 days before data

Table 1: Data Collection and Refinement^a

	E58Q	WT-TS
space group	$P6_3$	$P6_3$
unit cell dimensions	$a = b = 127.2 \text{ \AA}$, $c = 68.2 \text{ \AA}$	$a = b = 126.8 \text{ \AA}$, $c = 67.8 \text{ \AA}$
R_{merge}	8.5% (26.5)	9.7% (26.1)
$\langle I/\sigma(I) \rangle$	7.4 (2.8)	7.3 (2.4)
completeness (50–2.5 \AA)	93.8% (84.6)	90.0% (69.3)
observations	200 624	221 970
unique reflections	26 346	37 807
refinement resolution	7.0–2.5 \AA	7.0–1.95 \AA
R factor	15.2% (20.9)	18.9% (28.2)
R_{free}	24.0% (29.6)	23.7% (34.6)
rms deviation from ideal		
geometry		
bond lengths	0.014 \AA	0.008 \AA
bond angles	3.20°	1.61°
dihedral angles	25.81°	25.54°
improper angles	1.37°	1.30°
Ramachandran outliers	none	none

^a Data shown in parentheses are those for the highest-resolution bin determined.

collection, β -mercaptoethanol was added to the wells to a final concentration of 1 mM to ensure the protein was under reducing conditions. For E60(58)Q TS, two data sets were collected from separate hexagonal rod crystals 0.25 mm in diameter and 0.40 mm long. For wild-type TS, a single data set was collected from a hexagonal rod 0.3 mm in diameter and 0.5 mm long.

Data Collection

X-ray diffraction data were collected on an R-Axis IIC imaging plate with a Rigaku RU-200 rotating anode generator operating at 15 kW (50 mA and 300 kV) fitted with a Cu anode ($\lambda = 1.5418 \text{ \AA}$). The crystal-to-detector distance was 90 mm. Exposures of 20 min/(deg of oscillation range) were used throughout the data collection, and 90 [E60(58)Q] or 75 (WT-TS) degrees of data were recorded for each crystal. The diffraction data to 2.3 \AA (E60(58)Q) or to 1.95 \AA (WT-TS) resolution were indexed, integrated, scaled, and merged in the hexagonal space group $P6_3$ with the following unit cell dimensions using the HKL software package (Otwinowski, 1990): $a = b = 127.2 \text{ \AA}$ and $c = 68.09 \text{ \AA}$. A summary of the data-processing statistics is presented in Table 1. For the E60(58)Q crystals, a total of 200 624 observations were integrated, scaled, and merged, yielding 26 346 unique reflections between 50 and 2.3 \AA [$R_{\text{merge(I)}} = 8.5\%$ with average redundancy of 7.6]. For the WT-TS crystal, a total of 221 970 observations were integrated, scaled, and merged, yielding 37 807 unique reflections between 50 and 1.95 \AA [$R_{\text{symm(I)}} = 9.7\%$ with average redundancy of 5.8].

Structure Solution

E60(58)Q. The E60(58)Q TS structure was solved by molecular replacement using two monomers of the *E. coli* TS product ternary complex with ligands and waters removed (Fauman *et al.*, 1994) superimposed onto the *E. coli* ternary complex (Montfort *et al.*, 1990) as the search model. Rigid-body refinement using the program X-PLOR (Brünger, 1992) gave an initial R factor of 31%. Ligands and waters were located from $F_o - F_c$ difference maps (Chambers & Stroud, 1977) during successive rounds of simulated annealing and

positional and B factor refinement in X-PLOR (Brünger, 1992) followed by manual inspection of electron density maps using the program CHAIN (Sack, 1988). The criteria for water addition were difference density greater than 3σ in $F_o - F_c$ maps and located within 3.5 \AA of a hydrogen bond mate. The final R factor is 15.3% to 2.5 \AA . The R_{free} is 24.0%. The E60(58)Q structure has been submitted to the Brookhaven Protein Data Bank as entry 1ZPR.

WT-TS. In order to directly compare mutant and wild-type diffraction data, it is important that the diffraction data be collected on a similar instrument and scaled and refined using equivalent software. Since the original TS–dUMP–CB3717 structure (Montfort *et al.*, 1990) was solved using data from a multiwire detector and was not scaled and refined using the software used here, we recollected data from TS–dUMP–CB3717 crystals and re-solved the structure by isomorphous molecular replacement using a dimer of the *E. coli* ternary complex (Montfort *et al.*, 1990), including waters and ligands, as the starting model. Successive rounds of positional and B factor refinement in X-PLOR (Brünger, 1992) followed by manual inspection of electron density maps using the program CHAIN (Sack, 1988) resulted in a solution which was nearly identical to the starting model, but has better geometry, and includes the N-terminal modification of *E. coli* TS (Fauman *et al.*, 1994). The final R factor is 18.9% to 1.95 \AA . The R_{free} is 23.7%. The re-solved structure has been submitted to the Brookhaven Protein Data Bank as entry 1KCE.

RESULTS

In order to address the role of E60(58) in the TS reaction at atomic resolution, the TS mutant E60(58)Q was created using oligonucleotide-directed mutagenesis. TS isolated from a ThyA[−] strain overexpressing E60(58)Q TS showed activity similar to that previously observed (Zapf *et al.*, 1993). Purified E60(58)Q TS was cocrystallized with the substrate, dUMP, and a quinazoline-based antifolate inhibitor, CB3717. Diffraction data were collected, and the structure was solved by molecular replacement. The E60(58)Q model was refined at 2.5 \AA to a final R factor of 15.2% ($R_{\text{free}} = 24.0\%$). The refined model has good geometry (Table 1), no Ramachandran outliers, and a $2F_o - F_c$ electron density map contoured at 1.0σ is well-defined for most of the molecule. As previously noted (Montfort *et al.*, 1990), this crystal form contains one TS dimer in the asymmetric unit, and each monomer in the dimer is related by an approximate noncrystallographic two-fold ($\kappa = 178.5^\circ$). In the TS dimer, each monomer contributes two phosphate binding arginine residues to the active site of the other monomer. To describe their participation from the second monomer, these residues are referred to as R178-(126)' and R179(127)'. In this study, the orientations of the ligands in the two active sites differ significantly; therefore, each monomer will be considered independently and compared to the corresponding monomer in the re-refined structure of wild-type *E. coli* TS in complex with dUMP and CB3717.

E60(58)Q Compared to Wild-Type *E. coli* TS Monomer 1

Monomer 1 [chain A in PDB entry 1KCE for wild type; chain A in PDB entry 1ZPR for E60(58)Q] of the E60(58)Q TS structure was compared to monomer 1 of the

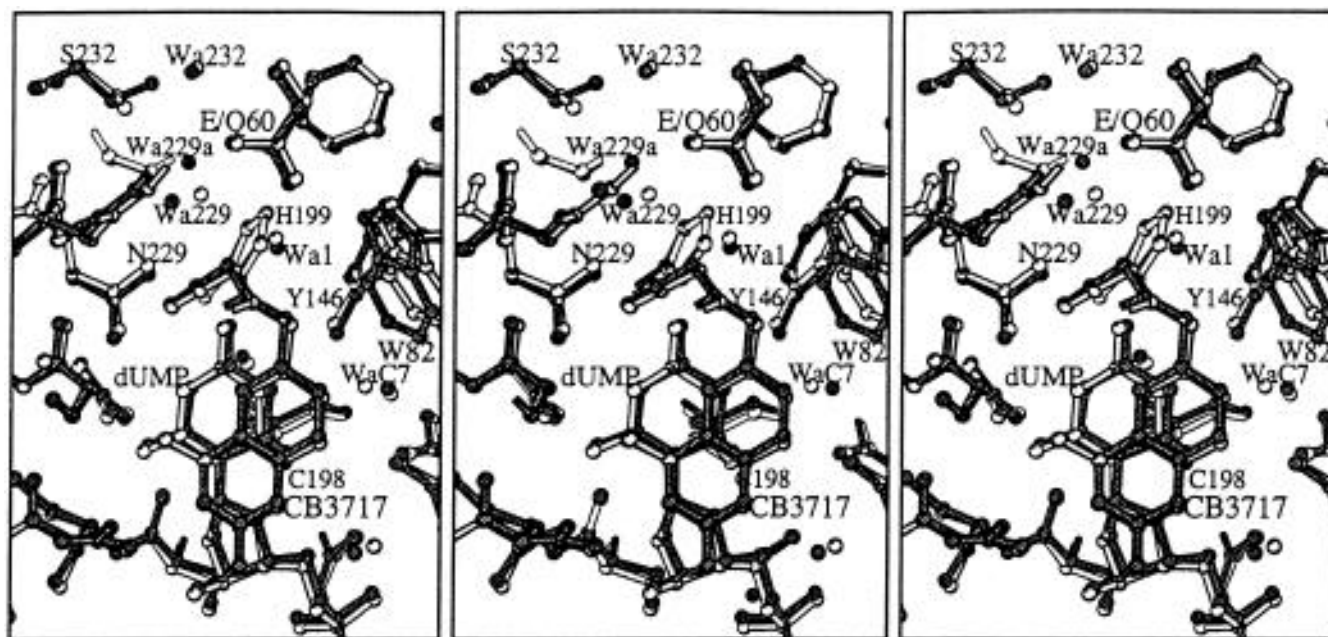


FIGURE 2: Stereo representation of the superimposition of the active sites of monomer 1 of wild-type *E. coli* TS (black) and E60(58)Q TS (white). This figure was produced using the program Molscript (Kraulis, 1991).

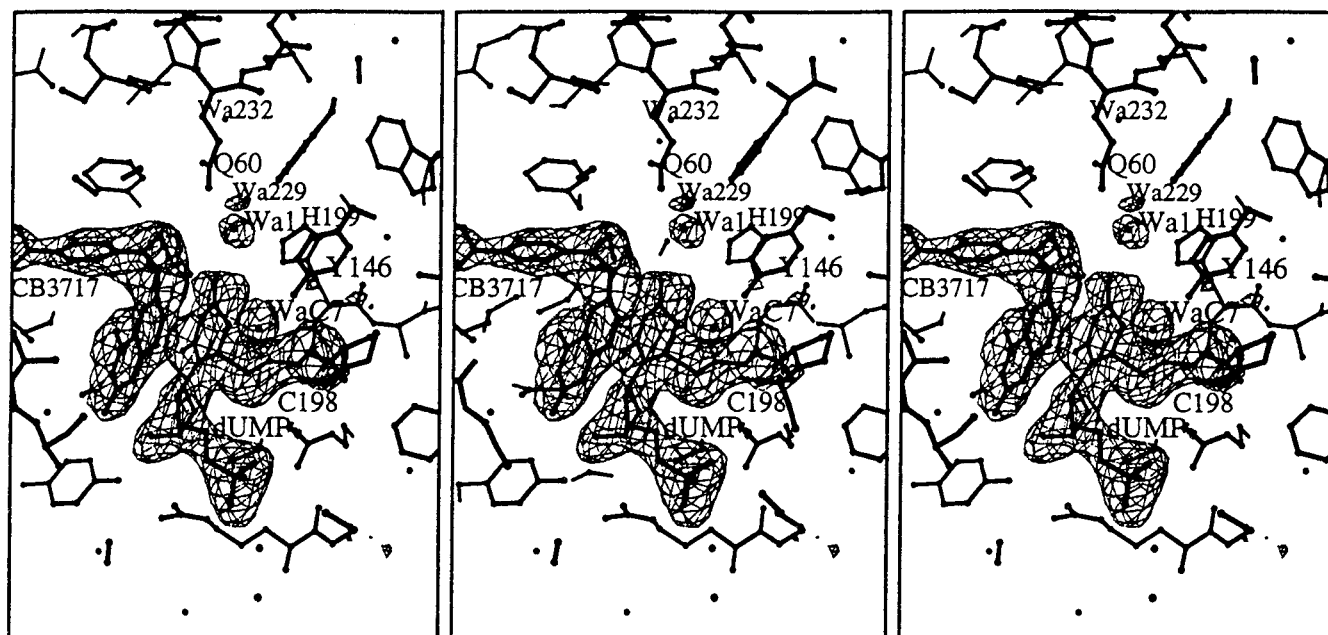


FIGURE 3: Stereo representation of a refined $F_o - F_c$ omit map of the active site of monomer 1 contoured at 3.0σ . This map was calculated after C198(146), dUMP, CB3717, water¹, water²²⁹, and water^{C7} were omitted from the PDB file and the coordinates subjected to 10 cycles of positional refinement. This figure was produced using the program Molscript (Kraulis, 1991).

re-refined structure of wild-type *E. coli* TS cocrystallized with the substrate dUMP and the cofactor analog CB3717 using the program GEM (Fauman *et al.*, 1994). After superimposition of monomer 1 from the two structures, the rms deviation of C α atoms is 0.286 Å, and the rms deviation of all protein atoms is 0.753 Å. The rms deviation for all dUMP atoms in monomer 1 is 0.461 Å, and the rms deviation for all atoms in CB3717 is 0.689 Å. In this monomer, the average *B* factor for the wild-type structure is 17.97 Å² and for E60(58)O is 18.59 Å².

Comparison of Active Site 1. As shown in Figure 2, the overall arrangement of the active site residues in E60(58)Q TS is almost identical to that of the wild-type structure. As observed in the wild-type ternary complex structure (Mont-

fort *et al.*, 1990), a covalent bond is formed between the Sy of C198(146) and C6 of dUMP (Figure 3). The main differences in residue positions between the two structures are small. The side chains of W82(80), Y146(94), F228-(176), and S232(180) exhibit the most dramatic movements (Figure 2). The side chain of W82(80) rotates by 10° around the Cβ–Cγ bond out of the active site as compared to that of the wild-type structure. The side chain of Y146(94) moves out of the active site by 0.36 Å. The side chain of F228(176) translates out of the active site by 0.8 Å. The S232(180)Oγ flips to a second rotamer 140° away. Major differences occur in the position of water molecules³ whose location is strongly conserved in several other TS structures. Water^{229a} is no longer constrained in the structure and not

Table 2: Comparison of Contacts in Active Site 1

interaction	distance (Å)	
	wild type	E60(58)Q
phosphate		
R218(166)NH1-PO4	2.66	2.83
R218(166)NH2-PO4	2.71	2.75
R218(166)NH2-PO4	3.35	3.38
R178(126)Nε-PO4	3.04	3.03
R178(126)NH1-PO4	3.32	3.21
R179(127)Nε-PO4	3.02	3.44
R179(127)NH1-PO4	2.77	2.98
R23(21)Nε-PO4	2.87	3.99
S219(167)Oγ-PO4	2.84	2.97
ribose		
R23(21)NH1-OR5	3.16	3.11
R178(126)NH1-OR5	3.49	2.94
Y261(209)Oζ-OR3	2.77	3.03
H259(207)Nε-OR3	2.84	2.83
S219(167)Oγ-OR3	4.20	4.34
uridine		
N229(177)Oδ-N3	2.98	2.95
N229(177)Nδ-O4	3.14	3.25
water ¹ -O4	2.89	3.25
D221(169)N-O2	3.15	3.05
Q217(165)Nε2-O2	3.13	3.17
Y146(94)Oη-C5	3.86	3.87
water ^{C7} -C5	3.91	3.32
pterin ring		
G225(173)N-OA4	3.02	3.01
A315(263)O-NA2	2.89	2.83
D221(169)Oδ-N3	2.70	2.81
water ³¹⁵ -CB3717 N1	2.89	3.29
water ²²¹ -CB3717 NA2	3.11	3.10
E60(58)Q		
water ²²⁹ -E60(58)QO/Nε	2.95	3.58
W82(80)Nε-E60(58)QOε	2.85	2.95
water ²³² -E60(58)QOε/Nε	2.74	2.90
water ¹ -E60(58)QOε	2.89	2.78
other important interactions		
N229(177)Nδ-water ²²⁹	2.91	2.74
water ¹ -H199(147)Nε	2.83	2.90
water ^{C7} -Y146(94)Oη	2.67	2.82
A315(263)O-water ³¹⁵	2.89	3.75
W85(83)Nε-I316(264)OT	3.17	2.93
water ^{C7} -A196(144)O	2.90	3.44
water ²²¹ -A315(263)N	3.17	2.82
water ³¹⁵ -I316(264)OT	3.00	2.84
water ^{229a} -water ²²⁹	3.01	NP ^a
water ^{229a} -S232(180)Oγ	2.79	NP
water ²³² -I57(55)O	2.83	2.83
water ²³² -water ^{229a}	2.69	NP
water ²²⁹ -H199(147)Nε2	4.55	3.02
water ²²⁹ -water ¹	3.67	2.98
water ²³² -S232(180)Oγ	2.69	2.62

^a NP = not present.

defined by crystallographic means. Water²²⁹ is moved 1.75 Å, water¹ 0.34 Å, and water^{C7} 0.75 Å.

A comparison of the intramolecular contacts in the active site is shown in Table 2; several important differences among these contacts are observed between the two structures in the active site. The distance between water²²⁹ and either the Nε [E60(58)Q] or the Oε (WT) of residue 60 increases from 2.90 Å in the wild-type to 3.58 Å in the mutant structure.

³ Waters are numbered according to the number or name of the residue to which it is closest with the exception of water¹ and water^{C7} which were described in Fauman *et al.* (1994). If more than one water is close to a single residue, the waters are further designated with letters. For example, there are two waters around N229; the first is designated water²²⁹ and the second water^{229a}.

The distance from water^{C7} to C5 of dUMP decreases from 3.91 Å in the wild-type structure to 3.32 Å in the mutant structure. In addition, the contacts of water^{C7} with the protein have changed. In the wild-type structure, water^{C7} makes a 2.67 Å hydrogen bond with Y146(94) and a 2.90 Å hydrogen bond with the main chain oxygen of A196(144), but in the E60(58)Q structure, these hydrogen bonds have been weakened to a 2.82 Å hydrogen bond with Y146(94) and a 3.44 Å contact with the main chain oxygen of A196(144). Most importantly, the distance between water¹ and O4 of dUMP increases from 2.89 Å in the wild-type to 3.25 Å in the E60(58)Q structure, weakening the hydrogen bond between water¹ and dUMP and creating a new hydrogen bond between water¹ and water²²⁹.

A relative comparison of *B* factors provides insight into the relative disorder of one structure *versus* another (Table 3). The overall *B* factors for the wild-type and mutant structures are similar: 17.97 Å² for the wild-type and 18.59 Å² for the mutant. In order to make a relative comparison of the thermal motion between the two active sites and to avoid the problems with a direct comparison of *B* factors, the *B* factors of the atoms in the active site were normalized to the *B* factors of the J helix [residues 225(173) to 245(193)], the innermost part of TS, to determine a relative disorder for each active site. Comparison of the normalized *B* factors for atoms in the active sites reveals that the mutant active site (average *B* = 17.38 Å²; relative disorder = 1.17) is more disordered than the wild-type (average *B* = 12.79 Å², relative disorder = 0.93) active site. This 25% increase in relative disorder may also contribute to the reduction of catalytic activity of the mutant enzyme.

E60(58)Q Disrupts the Extensive Hydrogen-Bonding Network Necessary for Proper TS Function. In a structure solved to 2.5 Å, hydrogen atoms are not discretely observed; however, with careful analysis, the hydrogen bond contacts can be inferred. An important means of elucidating the differences between two homologous structures is the calculation of an unbiased difference map. To directly compare the atomic differences between the wild-type and E60(58)Q structures and aid in the deduction of the hydrogen bond networks, newly collected diffraction data from wild-type TS cocrystallized with dUMP and CB3717 were scaled with diffraction data from E60(58)Q TS, also cocrystallized with dUMP and CB3717, and an $F_{o[E60(58)Q]} - F_{o(wild\ type)}$ difference map was calculated with phases from the re-refined wild-type TS model using XtalView (McRee, 1993). Shown in Figure 4 is the difference density around residue 60(58) contoured at 3.5σ along with the overlapped structures. Several important observations are made from this map. The side chain oxygen of serine 232(180) has rotated to a new rotamer 140° away from the wild-type, and difference density for the elemental change from Oε to Nε at the position of the E60(58)Q mutation is visible. There is WT difference density for both waters around N229(177) and E60(58)Q difference density for the single water which connects N229(177) and E60(58). As expected, the manner in which Q60(58) interacts with the hydrogen bond network is different than that in the wild-type structure. It is striking that, although the manner in which water²³² interacts with the protein is now different (Figure 5), its position is nearly identical with that of the wild-type (Figure 2).

Figure 5A shows the inferred wild-type hydrogen bond network interacting with dUMP. In this hydrogen bond

Table 3: Average B factors (\AA^2) for Specific Groups of Atoms

group	WT-TS		E60(58)Q	
	monomer 1	monomer 2	monomer 1	monomer 2
all C α 's	16.02	21.64	16.28	21.87
all protein atoms	17.91	23.69	18.06	23.71
all atoms of the J helix	13.74	19.12	14.80	21.44
all atoms of dUMP	15.21	18.57	30.09	39.58
all atoms of CB3717	23.13	30.52	44.74	62.38
all protein atoms <12 \AA from O4 of dUMP	12.79	18.26	17.38	23.03
all waters (number)	35.52 (241)		32.33 (178)	

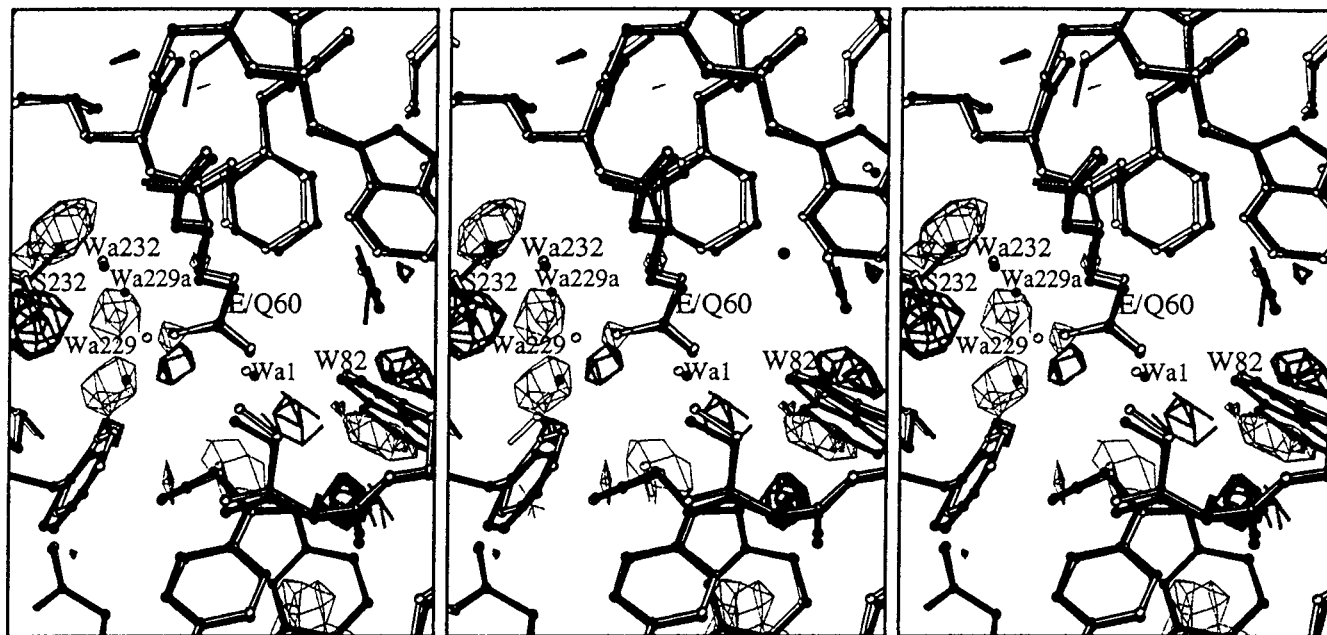


FIGURE 4: Stereo representation of an $F_{E60(58)Q} - F_{wild\ type}$ difference map around residue 60(58) contoured at 3.5σ . The map was calculated using phases from the wild-type model. Wild-type *E. coli* TS is shown in black, and E60(58)Q TS is shown in white. This figure was produced using Molscript (Kraulis, 1991).

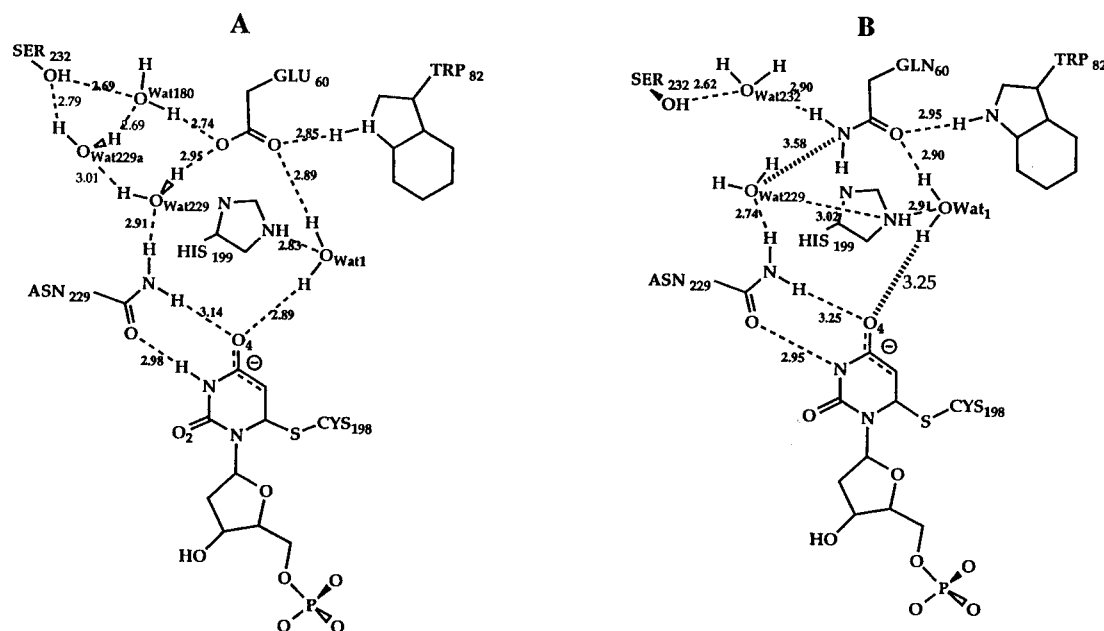


FIGURE 5: Schematic representation of the hydrogen bond network interacting with dUMP in the active site of monomer 1 of wild-type (A) and E60(58)Q (B) TS. Thick dashed lines emphasize the differences between the two hydrogen bond networks.

network, which is identical to that described from this laboratory earlier (Finer-Moore *et al.*, 1990), an intricate web of hydrogen bonds is set up so that water¹ completes the electrostatic connection between TS and O4 of dUMP and

stabilizes a negative charge which develops during transition states of the reaction. In the E60(58)Q structure, this hydrogen bond network is disrupted so that the developing charge on O4 of dUMP cannot be stabilized as well as in

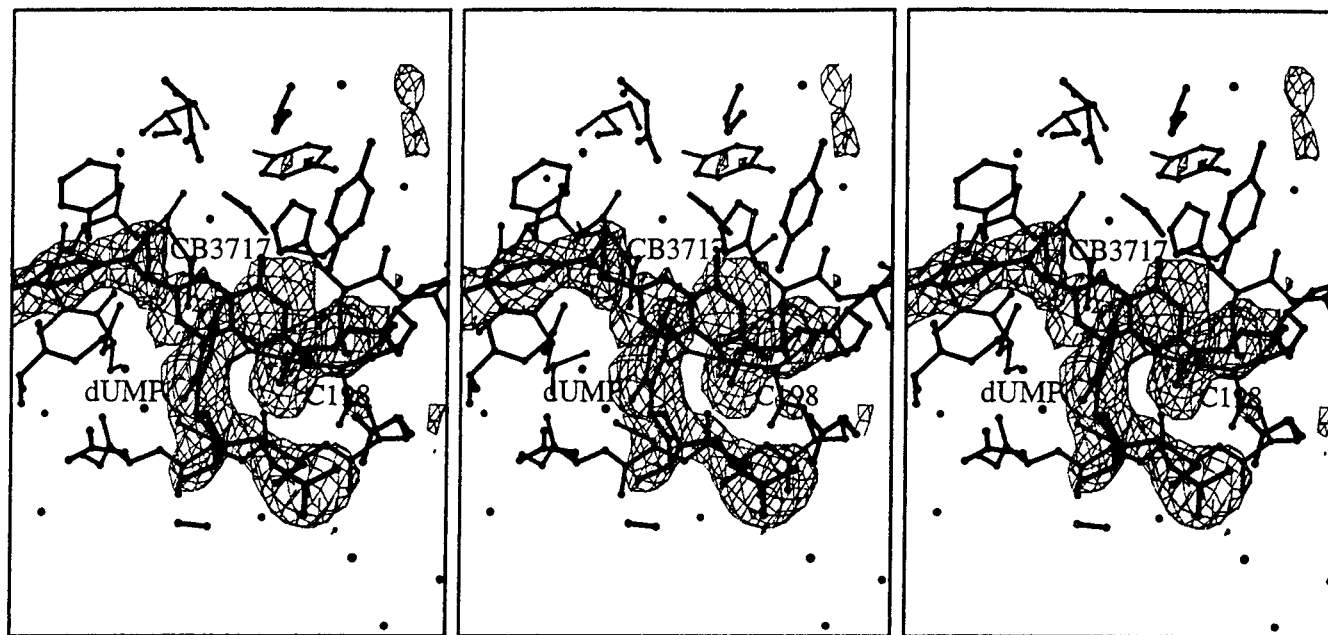


FIGURE 6: Stereo representation of a refined $F_o - F_c$ omit map for the active site of monomer 2 contoured at 2.5σ . This map was calculated after C198(146), dUMP, and CB3717 were omitted from the model and the remaining coordinates subjected to 10 cycles of positional refinement. This figure was produced using the program Molscript (Kraulis, 1991).

the wild-type structure (Figure 5B). The relationship between TS, water¹, water²²⁹, and dUMP has changed, and the network is missing two important connections: one between E60(58)Q and water²²⁹ and, most critical, the hydrogen bond between water¹ and O4 of dUMP. Thus, the E60(58)Q mutation changes the architecture of the active site in two critical ways. First, the mutant active site becomes more disordered relative to the rest of the protein, and second, the electrostatic connection between TS and dUMP is disturbed.

Comparison to Wild-Type *E. coli* TS Monomer 2

Monomer 2 of the E60(58)Q TS structure [chain B in PDB entry 1KCE for the wild type; chain B in PDB entry 1ZPR for E60(58)Q] was compared to monomer 2 of the resolved structure of wild-type *E. coli* TS cocrystallized with the substrate dUMP and the cofactor analog CB3717, using the program GEM (Fauman *et al.*, 1994). The two crystal structures superimpose with an rms deviation for C α atoms of 0.35 Å, and an rms deviation for all protein atoms of 0.85 Å. The rms deviation for all atoms in dUMP in monomer 2 is 1.11 Å, but the most dramatic difference based on the previous superposition is seen in the rms deviation for all atoms in CB3717, which is 3.31 Å. The average *B* factor for all atoms in the wild-type structure is 23.69 Å² and for E60(58)Q is 23.71 Å² (Table 3).

Comparison of Active Site 2. As shown in Figures 6–8, the arrangement of the ligands and the molecular contacts they make in the active site of the E60(58)Q monomer 2 are dramatically different than the orientation of ligands previously observed for TS, dUMP, and CB3717 (Montfort *et al.*, 1990). Although both dUMP and CB3717 are found with good density in the active site of monomer 2 (Figure 7), the ligands are not oriented in the productive orientation. As a result, C198(146) does not make a covalent bond with C6 of dUMP. Both the pyrimidine ring of dUMP and the quinazoline ring of CB3717 are oriented differently from the WT complex and monomer 1 of E60(58)Q and have a

completely new set of hydrogen-bonding contacts described schematically in Figure 8.

Contacts with dUMP. The uridine ring of dUMP is planar in active site 2 since no covalent bond is made with C198(146). The pyrimidine also translates away from the S γ of C198(146) by 1 Å, mostly due to a small change in the conformation of the ribose. O2 of dUMP now makes a 2.62 Å hydrogen bond with the amide nitrogen of D221(169) and a hydrogen bond with the amide nitrogen of Q217(165). O4 of dUMP no longer makes a hydrogen bond with water¹. The hydrogen bonds between O3 of the ribose and both Y261(209) and H259(207) have been maintained. The uridine ring now makes an edge-on hydrophobic interaction with CB3717 rather than the face-to-face stacking previously observed but makes no specific interactions with waters. The ribose OR1 however gains a specific 2.98 Å hydrogen bond with a new water. The interactions of the protein with the phosphate moiety of dUMP have also changed slightly. In the wild-type structure, the phosphate makes contacts with R23(21), R178(126)', R179(127)', R218(166), and S219(167). In the mutant structure, the phosphate moiety makes contacts with R178(126)', R179(127)', R218(166), and S219(167) but not with R23(21) (3.97 Å).

Contacts with CB3717. The conformation of CB3717 in the second active site of the E60(58)Q mutant structure is completely different from both the wild-type conformation (Figures 6 and 7) and the previously observed "oxidized" site observed in crystals under nonreducing conditions (Montfort *et al.*, 1990). Instead of forming a hydrophobic stacking interaction with the dUMP, the CB3717 has rotated around the N10–C9 bond by 108°, and the entire molecule has translated so that the majority of contacts made by the quinazoline moiety are hydrogen bonds to the protein (Figures 6 and 8). N3 of the quinazoline ring makes a 2.81 Å hydrogen bond with the O ζ of Y146(94). NA2 makes a strong 2.66 Å hydrogen bond with the main chain carbonyl oxygen of A196(144). OA4 of the quinazoline ring makes

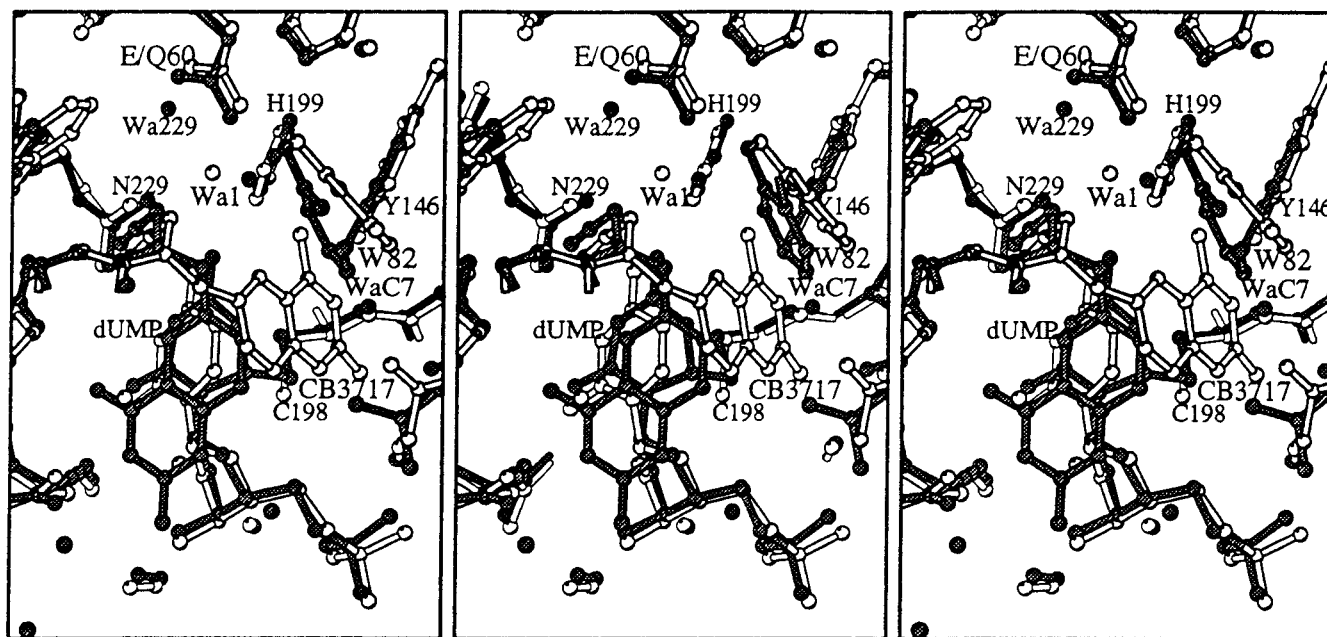


FIGURE 7: Stereo representation of the superimposition of the active sites of monomer 2 of wild-type *E. coli* TS (black) and E60(58)Q TS (white). The dramatically different conformation for the quinazoline moiety of CB3717 is readily apparent. This figure was produced using the program Molscript (Kraulis, 1991).

two hydrogen bonds: a 3.03 Å hydrogen bond with Y146(94) and a 3.24 Å hydrogen bond with water¹. In addition, the CB3717 makes hydrophobic contacts with dUMP and the side chains of W82(80), A231(179), C198(146), L195(143), W85(83), G225(173), and F228(176).

Conserved Waters Interacting with the Ligands. In the second active site of the wild-type TS structure, two conserved waters (water¹ and water²²⁹) make bridging contacts between the ligands and TS. In the mutant structure, water²²⁹ either has been displaced or has become too disordered to be resolved by crystallography. In addition, the temperature factors of the active site and the ligands are significantly increased compared to those of the wild-type structure (Table 3). The arrangement of the ligands and hydrogen bond network in monomer 2 of E60(58)Q TS directly shows that proper orientation of the ligands and the hydrogen bond network is necessary for covalent bond formation between TS and dUMP.

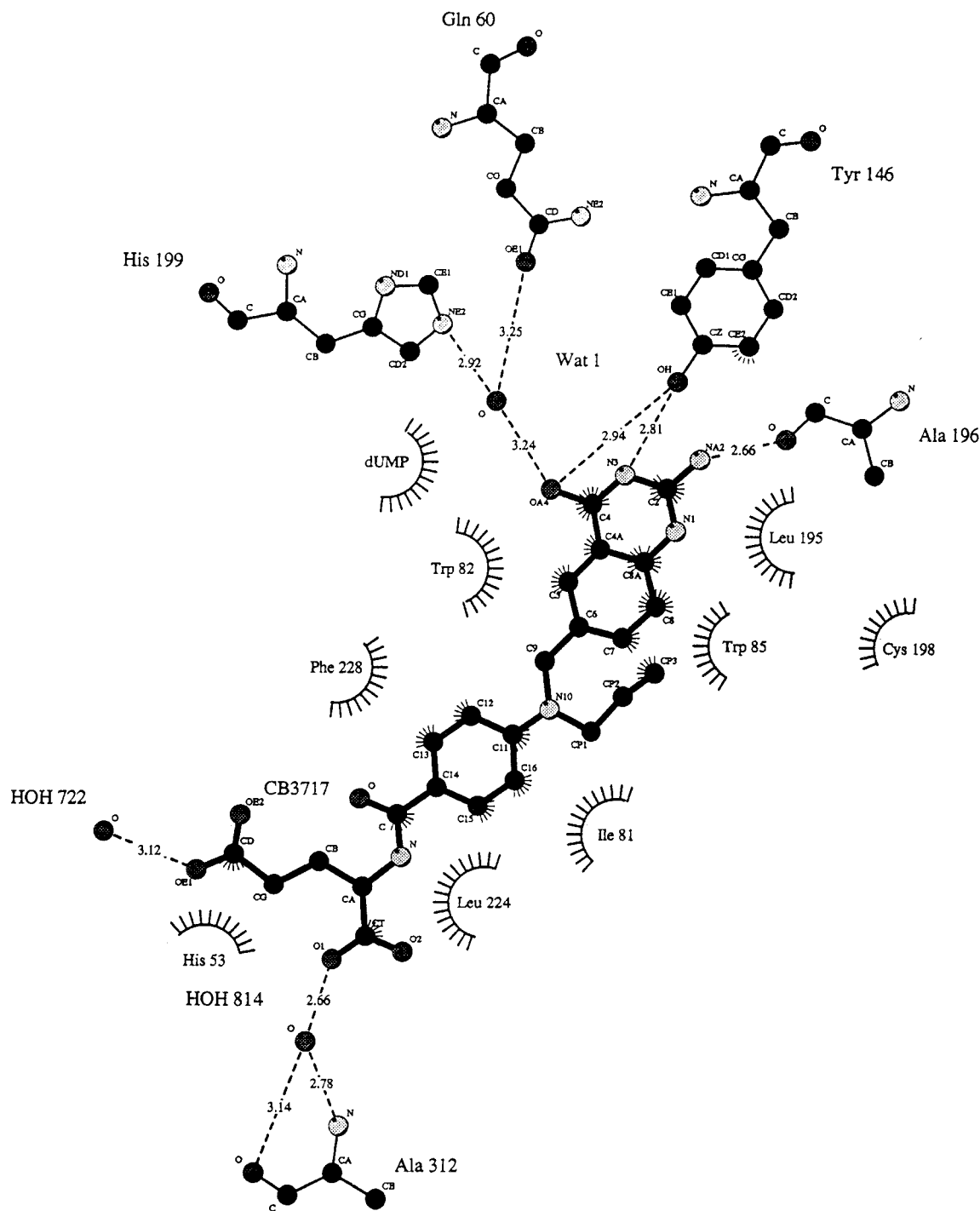
DISCUSSION

We have investigated the role of E60(58) in the TS reaction by determining the X-ray crystal structure of the purified mutant protein complexed with dUMP and the cofactor analog CB3717. This was accomplished by creating the site-directed mutant E60(58)Q in *E. coli* TS, overexpressing the mutant protein in a Thy⁻ background, and isolating the mutant protein to near homogeneity. This complex was isomorphous with wild-type crystals (Table 1; Montfort *et al.*, 1990). Data sets from two E60(58)Q crystals were collected and merged ($R_{\text{merge}} = 8.5\%$) to yield a data set that was 93.8% complete to 2.3 Å. The E60(58)Q structure was solved by molecular replacement with the refinement package XPLOR (Brünger, 1992) using a full dimer of the product complex structure without ligands (Fauman *et al.*, 1994) as the starting model. The final structure, refined to 2.5 Å, has an R factor of 15.3% ($R_{\text{free}} = 24.0\%$) and good geometry (Table 1). Similarly, data for

wild-type *E. coli* TS cocrystallized with dUMP and CB3717 were collected, and the structure was refined at 1.95 Å resolution with an R factor of 18.9% ($R_{\text{free}} = 23.7\%$).

Effect of the E60(58) Mutation on the TS Structure. Overall, the effects of the E60(58)Q mutation on the geometric structure of the TS dimer are small (Figures 1 and 2). Since glutamine is isosteric with glutamate, this result is not surprising. Comparison of the C α positions for each monomer independently revealed very small differences compared to the wild-type structure (0.29 Å for monomer 1, 0.35 Å for monomer 2), and a difference Fourier analysis showed that differences between the wild-type and E60(58)Q structures were localized to the active sites. The average B factors for the two separate monomers were similar, 18.6 Å² for E60(58)Q versus 18.0 Å² for wild-type monomer 1 and 23.7 Å² for E60(58)Q versus 23.7 Å² for wild-type monomer 2. An rms deviation comparison of the B factors between monomer 1 and 2 of E60(58)Q showed an rms deviation of 10.41 Å²; the same comparison for the wild-type structure gave an rms deviation of 10.14 Å², indicating that the difference in the degree of mobility between the two monomers is the same as observed previously in the wild-type structure (Montfort *et al.*, 1990). In addition, the E60(58)Q mutation has resulted in a general "relaxing" of the active site. The overall thermal factors in monomer 1 for E60(58)Q are similar to that of the wild-type protein (17.97 versus 18.59 Å²); however, a comparison of the thermal factors for just the active site residues and ligands revealed that the mutant active site is about 25% more disordered than the wild-type active site (Table 3).

Using Crystal Structures To Garner Information about the TS Reaction. Investigation of a dynamic process such as an enzyme reaction using X-ray crystallography relies on the assumption that the structure determined represents an intermediate along the reaction pathway. In our investigation of the E60(58)Q mutant, we have used the cofactor-based inhibitor CB3717 which was shown biochemically (Pogolotti



Key

- | | |
|------------------------------|---|
| Ligand bond | His 53 Non-ligand residues involved in hydrophobic contact(s) |
| Non-ligand bond | Corresponding atoms involved in hydrophobic contact(s) |
| Hydrogen bond and its length | |

FIGURE 8: Schematic diagram (Wallace *et al.*, 1995; McDonald & Thornton, 1994) of the hydrogen bonds and nonspecific hydrophobic interactions made between E60(58)Q TS and CB3717 in the active site of monomer 2. Radial spokes of hydrophobic packing pairs point toward one another.

et al., 1986) to halt the TS reaction at a covalent intermediate (II, Figure 1). To investigate how well CB3717 could mimic mTHF and its participation in the reaction at the atomic level, Finer-Moore *et al.* (1990) performed a detailed study which showed that the wild-type TS–dUMP–CB3717 structure

(Montfort *et al.*, 1990) served as an accurate model for structural analysis of the TS mechanism. Matthews *et al.* (1990b) compared crystal structures of TS complexed with either CB3717–FdUMP or mTHF–FdUMP and found little difference between the structures with the exception that the

propynyl group of CB3717 displaced a water which may participate in the hydrogen bond network. Fauman *et al.* (1994) suggested that large conformational changes of TS probably do not play a role in the reaction once ligands are bound in an orientation that leads to product formation. Thus, we have used CB3717 to mimic the role of mTHF in the TS reaction and have interpreted the effects of the E60(58)Q mutation on various steps in the TS reaction using the directly observed structural changes between the E60(58)Q and wild-type active sites.

What Role Does E60(58) Play in the TS Mechanism? Structural analyses of wild-type TS along with biochemical analyses of E60(58) mutants from various species have resulted in several different proposals for the role of E60(58) in the TS reaction. These include (1) opening of the cofactor methylene ring, (2) proper orientation of the ligands, or (3) affecting the stabilization of an intermediate in the reaction pathway (Hardy *et al.*, 1995; Huang & Santi, 1994; Matthews *et al.*, 1990b; Zapf *et al.*, 1993; Finer-Moore *et al.*, 1990). By analyzing the crystal structure of the E60(58)Q mutant complexed with dUMP and the cofactor analog CB3717, we can address the latter two of these three proposals. Since CB3717 is an open-ring analog of the natural cofactor mTHF, we cannot analyze the role of E60(58) in ring opening; however, with the exception of the study by Zapf and co-workers (1993), the biochemical evidence thus far suggests that the role of E60(58) in ring opening is minor (Hardy *et al.*, 1995; Huang & Santi, 1994). In addition, the differences in K_m for the ligands observed by Zapf and co-workers (1993) were not observed by either Huang and Santi (1994) or Hardy *et al.* (1995).

Role of E60(58) in the Formation of the Covalent Ternary Complex. The crystal structure of the E60(58)Q mutant in ternary complex with dUMP and the cofactor analog CB3717 represents intermediate II (Figure 1) in the TS reaction. This structure sheds light on the contribution of E60(58) to the enzymatic reaction in both orienting the ligands and stabilizing ionic intermediate II as the reaction proceeds to intermediate III (Figure 1). As shown in Figure 2 and Table 2, the general scheme of ligand contacts with monomer 1 of E60(58)Q TS is conserved, as is the overall arrangement of the ligands, suggesting that E60(58) is not essential for proper ligand positioning, but the new orientation of ligands in monomer 2 suggests that it may play some role. It appears that the most important role of E60(58) occurs after the ligands are in a productive orientation. As shown in Figure 5, the conserved network of water-mediated hydrogen bonds present in the wild-type TS structure has been disrupted. Water^{229a} is missing, and two important contacts between TS and the ligands have been distorted. First, the hydrogen bond between EQ60(58)Q and water²²⁹ is missing since the water has moved from 2.95 to 3.58 Å away from residue 60(58), diminishing the strength of the interaction. A second important missing contact is caused by an increase in the distance between water¹ and O4 of dUMP from 2.89 to 3.25 Å, reducing the strength of the hydrogen bond between water¹ and O4 of dUMP. These results support recent biochemical evidence which showed that E60(58) mutants cause accumulation of intermediate IV in the reaction with mTHF, and dUMP (Figure 1), as well as greatly slow the formation of the covalent ternary complex between FdUMP, mTHF and TS compared to the wild-type (intermediates III and IV, Figure 1; Huang & Santi, 1994; Hardy *et al.*, 1995).

The small changes observed in atomic position may also contribute to the disruption of the enzymatic reaction, but perhaps the most important contribution to the disruption of the TS reaction are the change in the electrostatic properties of the water-mediated hydrogen bond network and the connections it makes with the ligands.

Role of Water in the Formation of the Covalent Ternary Complex. Our results strongly support the proposal that, in wild-type *E. coli* TS, E60(58) works by stabilizing an ionic intermediate (II, Figure 1) via the water-mediated hydrogen bond network to reduce the transition state energy needed to reach intermediate III (Figure 1) in the enzyme reaction. In the structure of E60(58)Q, water^{229a} is lost, and three absolutely conserved waters (water¹, water²²⁹, and water^{C7}) have all shifted in position relative to the protein and ligands. The contribution of water¹ and water²²⁹ to the concerted hydrogen bond network in the wild-type structure and the absence of a similar role for these two waters in the E60(58)Q structure strongly support the importance of this hydrogen bond network in allowing proper enzyme function. The decrease in the strengths of the interaction between water¹ and O4 of dUMP and the interaction between water²²⁹ and E60(58)Q suggest that mutation from E60(58) to Q60(58) causes the movement of these two waters, which weakens stabilization of ionic intermediate II (Figure 1). The observation that water^{C7} moves from 3.91 Å (wild type) to 3.32 Å from C5 of dUMP also suggests that the negative charge developing at O4 is poorly stabilized, resulting in C5 being partially negatively charged, causing an attraction of water^{C7} toward C5. Thus, our observations provide a molecular explanation for the observation that mutants of E60(58) slow the formation of the covalent ternary complex of FdUMP and mTHF (Huang & Santi, 1994; Hardy *et al.*, 1995), suggesting that the E60(58)Q mutation raises the activation energy required for formation of intermediate III by disrupting the water-mediated hydrogen bond network, especially the interaction between water¹ and O4 of dUMP.

Role of E60(58) in the Dissolution of the Ternary Product Complex. Since the transition from the covalent ternary complex (III, Figure 1) is thought to proceed by base abstraction of the C5 proton of dUMP, resulting in another intermediate (IV, Figure 1) in which O4 of dUMP is charged, it is reasonable to speculate that the role of E60(58) in the transition from intermediate IV (Figure 1) to intermediate V (Figure 1) in the reaction is similar to that in the transition from intermediate II to III (Figure 1), to coordinate the hydrogen-bonding network so that the charged intermediate is stabilized and the energy of the transition state is lowered. In the case of E60(58)Q, it is likely that water²²⁹ and water¹ are not in position to properly stabilize charged intermediate IV (Figure 1); these changes in water position probably also prevent the lowering of the transition state energy between intermediate IV and intermediate V (Figure 1). These speculations are grounded in recently observed biochemical data (Hardy *et al.*, 1995; Huang & Santi, 1994). Both groups observed a 20–40-fold increase in the ratio of C5 hydrogen release to product formation for E60(58)Q relative to that of the wild-type enzyme which performs the reaction nearly perfectly, releasing one hydrogen per molecule of product formed. In addition, Huang and Santi (1994) observed a covalent intermediate consisting of TS, dUMP, and cofactor in E60(58) mutants which, in certain cases, could represent up to 2% of the reaction components at steady state. Thus,

E60(58)Q disrupts the charge-stabilizing hydrogen bond network so that the forward reaction (conversion of intermediate IV to V) is slowed to such an extent that the reverse reaction (conversion of intermediate IV to III) effectively competes, allowing the observation of the covalent intermediate and explaining the observation of the increase in the ^3H release ratio relative to product formation. It seems likely, therefore, that E60(58) serves to properly orient water at this final stage of the reaction, and it may be that the proximity of the localized charge on intermediate IV to the disrupted hydrogen bond network slows the progress of the TS reaction more during the dissolution of the covalent ternary complex than during the formation of the covalent ternary complex wherein a delocalized charge exists on intermediate II.

Role of Water^{C7} as a Base in the TS Reaction. Several proposals have been made concerning the identity of the base responsible for removal of the C5 hydrogen from dUMP in the dissolution of the covalent ternary complex (intermediates III and IV, Figure 1) in the TS reaction. Water^{C7} was proposed to be the specific base responsible for abstracting the C5 hydrogen (Fauman *et al.*, 1994). Additionally, N5 of the cofactor mTHF was proposed to be a general base that mediates its effects *via* a water (Hardy *et al.*, 1995). Our results show that water^{C7} has moved from its wild-type position 3.91 Å from C5 of dUMP to 3.32 Å away in the E60(58)Q structure which may be caused by attraction of water^{C7} by the poorly stabilized negative charge which has developed on O4 of dUMP as a result of the movement of water¹. In addition, the contacts water^{C7} makes with TS are weakened (Table 2), and the orientation of water^{C7} in the mutant structure is likely different than that in the wild-type structure. These observations may explain the previously observed decrease in the rate of deprotonation of C5 of dUMP (Huang & Santi, 1994; Hardy *et al.*, 1995). Thus, the changes in the position of water^{C7} are consistent with water^{C7} being the specific base in the TS reaction. The proposal of Hardy *et al.* (1995) that the general base in the reaction is the N5 of the cofactor is also consistent with our results if N5 mediates its effects *via* water^{C7}.

Effect of E60(58)Q on Monomer 2: Implications for the Enzymatic Nonidentity of the TS Monomers. In the wild-type structure, a covalent bond is observed between S γ of C198(146) and C6 of dUMP in active site 2. In the structure of E60(58)Q, active site 2 has no covalent bond between C198(146) and dUMP, and the positioning of the ligands and their contacts with TS are dramatically different. The quinazoline moiety of CB3717 has rotated about the C9–N10 bond so that it makes completely new contacts with TS (Figure 8). This new position for CB3717 is different than the “oxidized site” previously observed (Montfort *et al.*, 1990), occupying part of the catalytically functional dUMP site and making hydrogen bonds with residues that are proposed to be critical in the TS reaction. In addition, the water structure in the active site is almost completely different than that previously observed (Montfort *et al.*, 1990; Fauman *et al.*, 1994). In the active site of monomer 2, water¹, hydrogen-bonded to O ϵ of Q60(58), is still present but is much farther from the dUMP (3.83 Å), and water²²⁹ either has been displaced or is too disordered to observe crystallographically. The quinazoline ring system of CB3717 occludes the position of wild-type water^{C7}. The absence of the covalent bond in monomer 2 of the E60(58)Q structure is supported by the results of Zapf and co-workers (1993),

who showed that E60(58)Q TS formed a covalent complex with labeled FdUMP exactly half as well as wild-type, and Hardy *et al.* (1995), who demonstrated that E60(58)Q TS formed the covalent FdUMP complex less well than the wild type. The absence of a covalent bond between C6 and TS in the second active site of E60(58)Q shows directly that properly oriented ligands are necessary for covalent bond formation in the initial stages of the TS reaction.

The alternative conformation observed for the quinazoline also has implications for the physiological function of the enzyme. The newly observed conformations for the ligands in the second active site may represent a new conformational intermediate for the enzyme. The first active site represents a transition state analog (step II, Figure 1). However, the second active site appears to be trapped prior to proper orientation of the ligands. This observation suggests that E60(58) may play a role in proper ligand orientation, but the productive orientation of ligands in monomer 1 suggests that E60(58) is not essential for proper ligand orientation. The new ligand orientation has also been observed with *L. casei* TS crystals into which mTHF was soaked (Birdsall *et al.*, 1996). We observe strikingly different conformations of ligands for monomer 1 and monomer 2 which are consistent with early work providing evidence for asymmetry in the TS active site (Leary *et al.*, 1975; Galivan *et al.*, 1975; Danenberg & Danenberg, 1979) as well as provide a molecular explanation for recent data that showed that one competent active site retains full enzymatic activity (Maley *et al.*, 1995). The ligand position in monomer 2 may be caused by the existence of a productive orientation of ligands in monomer 1 and perhaps represents a prereaction storage site into which the cofactor is sequestered until it is used by the enzyme (Birdsall *et al.*, 1996). The occupancy of this storage site by the cofactor analog is increased in the E60(58)Q mutant.

Observation of a New Conformation for CB3717: Implications for Drug Design. The observation of a new conformation for the ligands in the second active site has important implications for rational drug design, since a new region of the available conformational space within the active site has been delineated. In this conformation, CB3717 makes specific hydrogen-bonding contacts with Y146(94) and H199(147), two residues which play an important role in the TS reaction (Carreras & Santi, 1995; Stroud & Finer-Moore, 1993). The disruption of the contacts of these two critical residues thus may represent a new manner by which to approach the inhibition of TS. Exploitation of the newly observed contacts along with conformation-based searches of the remaining active site volume may result in new leads for TS inhibitors.

CONCLUSIONS

The structure of the E60(58)Q mutant of *E. coli* TS refined to 2.5 Å resolution sheds light not only on the role of E60(58) in the general TS reaction mechanism but also on the role of the conserved waters involved in the concerted hydrogen bond network which were implicated in the TS mechanism. We have demonstrated that E60(58) is not required for proper positioning of the ligands and coordinates the hydrogen bond network which connects three conserved waters with the ligands. The positions, orientations, and polarities of these waters are clearly important, and we have

gained insight into their role in the TS reaction mechanism. In addition, we have presented a new conformation for the ligands in the TS active site which has profound implications both in the enzymatic nonidentity of the TS monomers and for the structure-based design of novel TS inhibitors.

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