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Studies on glutamate-60 and an active site hydrogen-bond network of thymidylate synthese

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Studies on Glutamate-60 and an Active Site Hydrogen-bond Network of Thymidylate Synthase

by

Weidong Huang

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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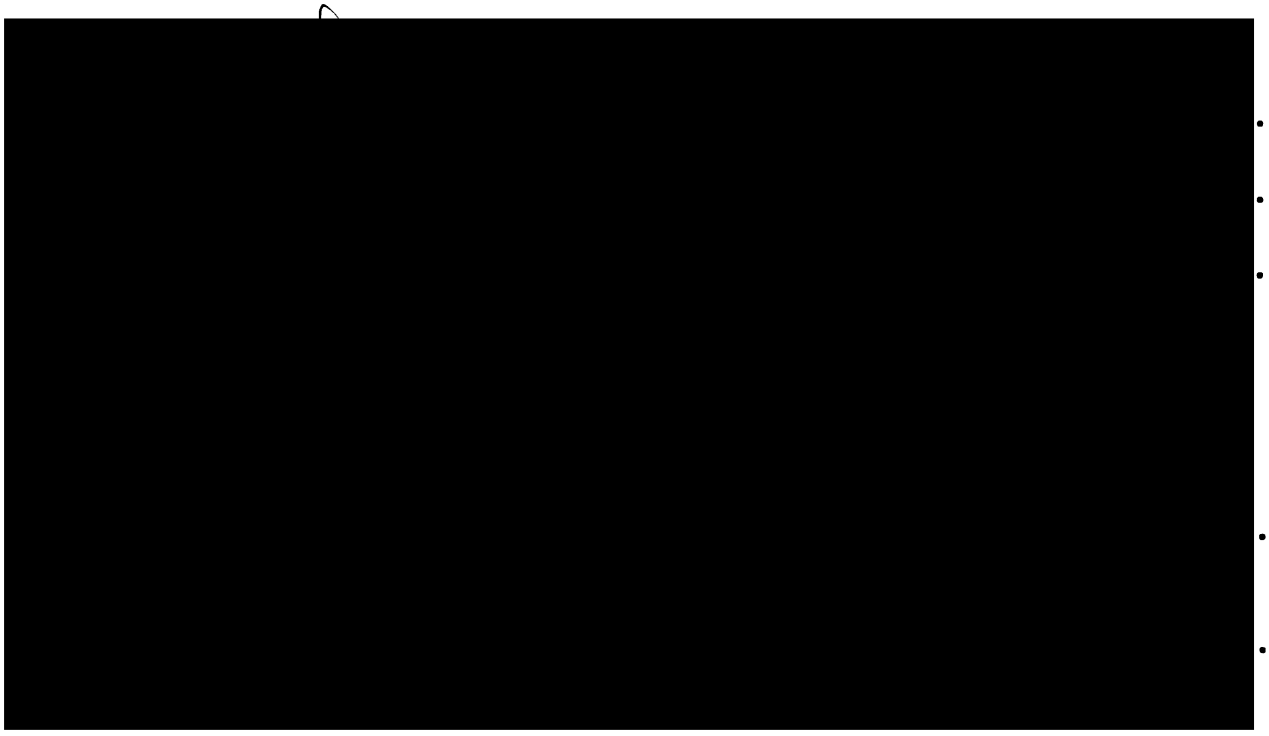

DEDICATION

To my parents, Tian and Fengjuan,

for always having faith in me and giving me continuos support and encouragement

and

To my wife, Xiaoping, and daughter, Jessica,

for unlimited love and joy that made my graduate study at UCSF a memorable experience

PREFACE AND ACKNOWLEDGMENTS

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ABSTRACT

Studies on Glutamate-60 and an Active-site Hydrogen-bond Network of Thymidylate Synthase

by

Weidong Huang

Thymidylate synthase (TS, EC2.1.1.45) catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) by 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) to give 2'-deoxythymidine-5'-monophosphate (dTMP) and dihydrofolate. TS is an important chemotherapeutic target because it represents the sole *de novo* pathway for synthesis of dTMP, an essential DNA precursor. Selective inhibition of the enzyme leads to blockage of DNA synthesis and death of the targeted cells. This thesis focuses on catalytic roles of glutamate-60 of TS and an active site hydrogen-bond network that includes glutamate-60, histidine-199, asparagine-229, ordered water molecules and the pyrimidine ring of dUMP. Chapter I gives a brief introduction to catalytic mechanism and structure of TS with emphasis on issues related to glutamate-60 and the hydrogen-bond network. Chapter II describes a new approach in identification of biologically active mutants by mutagenesis using degenerate codons that exclude the wild-type amino acid (Huang, W., and Santi, D. V. (1994) *Analytical Biochemistry 218*, 454-457). Chapter III describes the isolation of a covalent steady-state intermediate containing glutamate-60 mutant TS, dUMP and CH_2H_4folate (Huang, W., and Santi, D. V. (1994) *The Journal of Biological Chemistry 269,* 31327-31329). Chapter IV describes the complete replacement set mutagenesis of glutamate-60, the effects of mutations on various steps in TS reaction pathway and activation of catalytic activity of the mutant TSs by exogenous carboxylic acids. Chapter V describes the investigation of the hydrogen-bond network residues by enzymatic dehalogenation of 5-Bromo- and 5-

Iodo-2'-deoxyuridylate and shows that the network residues are not necessary for some proton transfer reactions of TS. Chapter VI provides a detailed picture of energetic interactions among the network residues and indicates that synergism is involved in the function of the network residues. Chapter VII describes several crystal structures of free and bound forms of glutamate-60 mutant enzymes and discusses the structural basis of the mutational effects.

Norman J. Oppenheimer

Date: 8/28/96

Norman J. Oppenheimer, Ph.D.
Professor of Pharmaceutical Chemistry
Chairman of the dissertation committee

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LIST OF PUBLICATIONS AND MANUSCRIPTS GENERATED IN THIS THESIS

Publications:

1. Huang, W., & Santi, D. V. (1994). Identification of Catalytically active Mutants by Combinatorial Cassette Mutagenesis. *Analytical Biochemistry*, 218, 454-457.
2. Huang, W., & Santi, D. V. (1994). Isolation of a covalent steady-state intermediate in glutamate 60 mutants of thymidylate synthase. *J. Biol. Chem.*, 269 (50), 31327-9.

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TABLES

FIGURES

c) E60Q-dUMP d) E60Q-FdUMP e) WT LCTS-dUMP-CB3717.

Figure 7.5: TS reaction scheme with intermediates labeled by roman numerals.

CHAPTER I: INTRODUCTION

Glutamate-60 and an active site hydrogen-bond network of thymidylate synthase

This thesis focuses on the catalytic roles of Glu-60[1] and a hydrogen-bond network in the active site of thymidylate synthase (TS[2], EC 2.1.1.45). TS has been extensively studied and the understanding of the catalytic mechanism and structure of the enzyme has significantly advanced over recent years (for reviews, see Carreras & Santi, 1995; Ivanetich & Santi, 1992; Bruice & Santi, 1991; Pogolotti & Santi, 1977). I will not exhaustively review here all features of the catalytic mechanism of TS, but I will instead cover only enough points to put the issues of my thesis work on Glu-60 and the hydrogen-bond network into proper framework. I will also concentrate on citing only those references that are crucial to the theme of this thesis.

I. Thymidylate Synthase

Thymidylate synthase catalyzes the reductive methylation of dUMP by CH_2H_4folate to produce dTMP and H_2folate. The cofactor CH_2H_4folate serves dual functions of one-carbon carrier and reductant and is regenerated by the sequential action of dihydrofolate reductase and serine trans-hydroxymethylase. TS is an

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[1] The amino acid numbering used is that of *Lactobacillus casei* TS.

[2] Abbreviations used: TS, thymidylate synthase; DHFR, dihydrofolate reductase; dUMP, 2'-deoxyuridine-5'-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dTMP, thymidine-5'-monophosphate; H_2folate, 7,8-dihydrofolate; CB3717, 10-propargyl-5,8-dideazafolate; FdUMP, 5-flouro-2'-deoxyuridine-5'-monophosphate; BrdUMP, 5-bromo-2'-deoxyuridine-5'-monophosphate; IdUMP, 5-iodo-2'-deoxyuridine-5'-monophosphate.

important chemotherapeutic target because it provides the sole intracellular *de novo* source of dTMP, an essential DNA precursor. Inhibition of the enzyme blocks DNA synthesis, leading to death of the target cells. In addition, studies of TS serve as paradigm for the mechanism and inhibition of related enzymes that catalyze one carbon transfer to pyrimidines, e.g., dUMP and dCMP hydroxymethylase and the RNA uracil and DNA cytosine methyltransferases (Santi *et al.*, 1978, Ivanetich & Santi, 1992; Carreras & Santi, 1995).

In most organisms, TS is a dimer of identical subunits of 30-35 kilodaltons each. In protozoa, TS is a bifunctional protein with DHFR on the same polypeptide chain (Ivanetich & Santi, 1990). Primary sequences of TS from some 29 different organisms are now available, and alignment of these sequences shows that TS is one of the most conserved enzymes (Carreras & Santi, 1995).

The catalytic mechanism of TS is well established (Figure 1.1) (Santi & Danenberg, 1984; Carreras & Santi, 1995). After formation of a reversible ternary complex of TS, dUMP and CH_2H_4folate, nucleophilic attack by the thiol of Cys-198 at C-6 of dUMP converts the 5-carbon to a nucleophilic enol, intermediate **II**. Formation of this binary covalent intermediate serves to activate C-5 of dUMP for condensation with the one carbon unit of CH_2H_4folate. This results in a covalent ternary complex, intermediate **III**, as a steady-state intermediate in which Cys-198 of the enzyme is covalently attached to C-6 of dUMP and the one carbon unit of the cofactor is covalently attached to C-5 of dUMP. In the reaction pathway leading to dTMP formation, it is proposed that the 5-H of intermediate **III** is removed as a proton, followed by β-elimination of H_4folate and hydride transfer to give the products, dTMP and H_2folate. Although our knowledge of the catalytic mechanism has advanced substantially over the years, the elucidation of several important aspects of the chemical mechanism of TS requires further investigation. For example, covalent bond changes are believed to be facilitated by general acid-base catalyzed proton

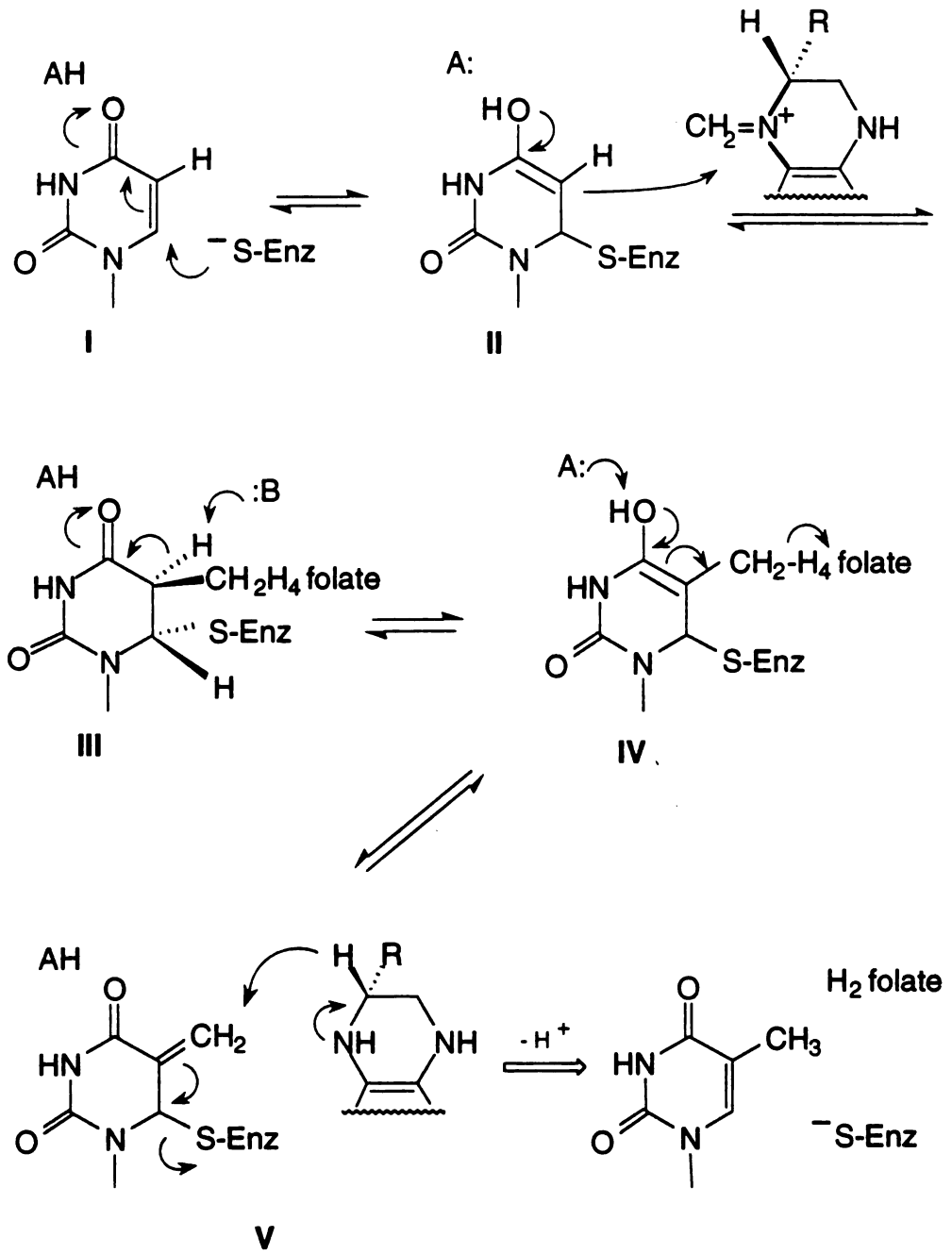


Figure 1.1: Schematic diagram of the chemical mechanism of TS.

transfers at O-4 and C-5 of the heterocycle (Santi & Danenberg, 1984), however, no amino acid residue in the vicinity of the pyrimidine ring of dUMP has been defined as a general acid/base catalyst for proton transfers. It is also unknown how TS facilitates the proton-transfer reactions that occur at the poorly acidic/basic site of the pyrimidine heterocycle. Steps up to and including the formation of the covalent ternary complex, intermediate III, are well understood. However, steps in the conversion of this covalent intermediate to products are basically hypothetical. The elucidation of the important role in the structure and function of TS of the ordered water molecules present in the active site remains a challenging question in understanding the catalytic mechanism of the enzyme.

The first crystal structure of TS, *L. casei* TS bound to inorganic phosphate (Hardy *et al.*, 1987), revealed structural details of the ligand binding site and showed that TS is a symmetric dimer of structurally similar or identical subunits. Since then several crystal structures from different organisms, including *E. coli* (Perry *et al.*, 1990; Matthews *et al.*, 1990b), T4 phage (Finer-Moore *et al.*, 1994), *Leishmania major* (Knighton *et al.*, 1994) and human (Schiffer *et al.*, 1991) have been solved and these have shown striking structural homology among these enzymes. Crystal structures of TS bound to various ligands, including structures of binary complexes of TS with nucleotides (Finer-Moore *et al.*, 1993), folate analogs (Kamb *et al.*, 1992b), ternary complexes with nucleotides and folates (Montfort *et al.*, 1990; Matthews *et al.*, 1990a; Kamb *et al.*, 1992a; Fauman *et al.*, 1994) as well as complexes with novel TS inhibitors (Schoichet *et al.*, 1993; Webber *et al.*, 1993; Reich *et al.*, 1992; Eckstein *et al.*, 1994), have also been reported. These structures provided important information about binding of the substrate and cofactor to TS, they showed conformational changes during catalysis, and they revealed key steps in the multi-step reaction of TS. These structures have also served as starting points for mutagenesis studies.

A large number of TS mutants have been made and characterized in recent years in and attempt to understand the structure-function relationships of TS. Most mutants were generated in *L. casei* TS (Climie *et al.*, 1990) and *E. coli* TS (Michaels *et al.*, 1990). Mutant TSs from T4 phage (LaPat-Polasko *et al.*, 1990), mouse (Zhang *et al.*, 1990) and human (Hughey *et al.*, 1993) have also been described. Multiple substitutions, so-called "replacement sets" of amino acids in which the wild-type residue is replaced by all 19 other natural amino acids, for a particular residue of TS have been used to study the structure-function role of the chosen residue. Multiple substitutions of *E. coli* TS are made by introduction of an amber stop codon at target sites in the gene (Michaels *et al.*, 1990; Kim *et al.*, 1992). The *L. casei* TS gene was designed and chemically synthesized to contain 35 unique restriction sites (Climie & Santi, 1990). "Replacement sets" of mutant TSs are then created by the replacement of restriction fragments of the synthetic gene with fragments containing oligonucleotide mixtures encoding all 20 amino acids at targeted positions (Climie *et al.*, 1990; Wells *et al.*, 1985). Some complete replacement sets of mutant TSs generated by this approach, e.g., that of Val-316 (Climie *et al.*, 1992), Asn-229 (Liu & Santi, 1993a) and Glu-60 (this thesis), have been extensively studied. In addition, a number of residues that interact with dUMP or CH_2H_4folate and residues believed to be important in structure and function of TS have been mutated and characterized (for review, see Carreras & Santi, 1995). The results of these mutagenesis studies have greatly contributed to the current understanding of structure-function of TS.

Previously knowledge of TS, together with recent advances in the ability to overexpress TS in heterogeneous hosts, crystal structures of TS bound to various ligands and characterizations of numerous TS mutants, have greatly enriched our understanding of catalytic mechanism of the enzyme. However, further structure-function studies are needed to understand the important molecular details of TS reaction that are currently unknown or poorly defined, e.g., important roles of ordered

water molecules in the active site, proton transfer reactions at O-4 and C-5 of dUMP and the conversion of the covalent intermediate to products.

II. Glutamate-60 of TS

Glutamate-60 is completely conserved in all known TS species (for sequence alignment, see Carreras & Santi, 1995). In a ternary complex of TS, dUMP and cofactor analog CB3717, Glu-60 makes hydrogen bonds to conserved residue Trp-82 and three ordered water molecules in the active site, which in turn form hydrogen bonds with other conserved residues and the pyrimidine ring of the substrate dUMP (Figure 1.2) (Finer-Moore *et al.*, 1990). Thus, Glu-60 appears to coordinate the extensive hydrogen bond network in the active site.

Replacement set mutagenesis of Glu-60 of *L. casei* TS (see Chapter II) and *E. coli.* TS (Zapf *et al.*, 1993; Hardy *et al.*, 1995) results in mutant TSs which have no detectable ability to synthesize dTMP except in the case of E60D TS where dTMP synthesis is reduced 125-fold. The extreme loss of catalytic activity in TMP synthesis is interesting considering the tolerance of the enzyme toward amino acid substitutions of many residues that are highly conserved and/or important for structure or function of TS (Santi *et al.*, 1990; Climie *et al.*, 1990; Kim *et al.*, 1992). The tolerance of such residues toward mutation has been explained as a "structural plasticity" of the enzyme or a compensation effect in which the loss of function of one residue can be accommodated by a more efficient utilization of the others that contribute to the same function (Perry *et al.*, 1990; Climie *et al.*, 1990). The high sensitivity of Glu-60 toward mutation indicates that Glu-60 probably represents a true "essential" residue of TS.

Several catalytic roles of Glu-60 have been proposed based on crystallographic studies of wild-type TS and/or kinetic studies of Glu-60 mutant enzymes. (1) Glu-60 may be important in the protonation of N-10 of the cofactor to

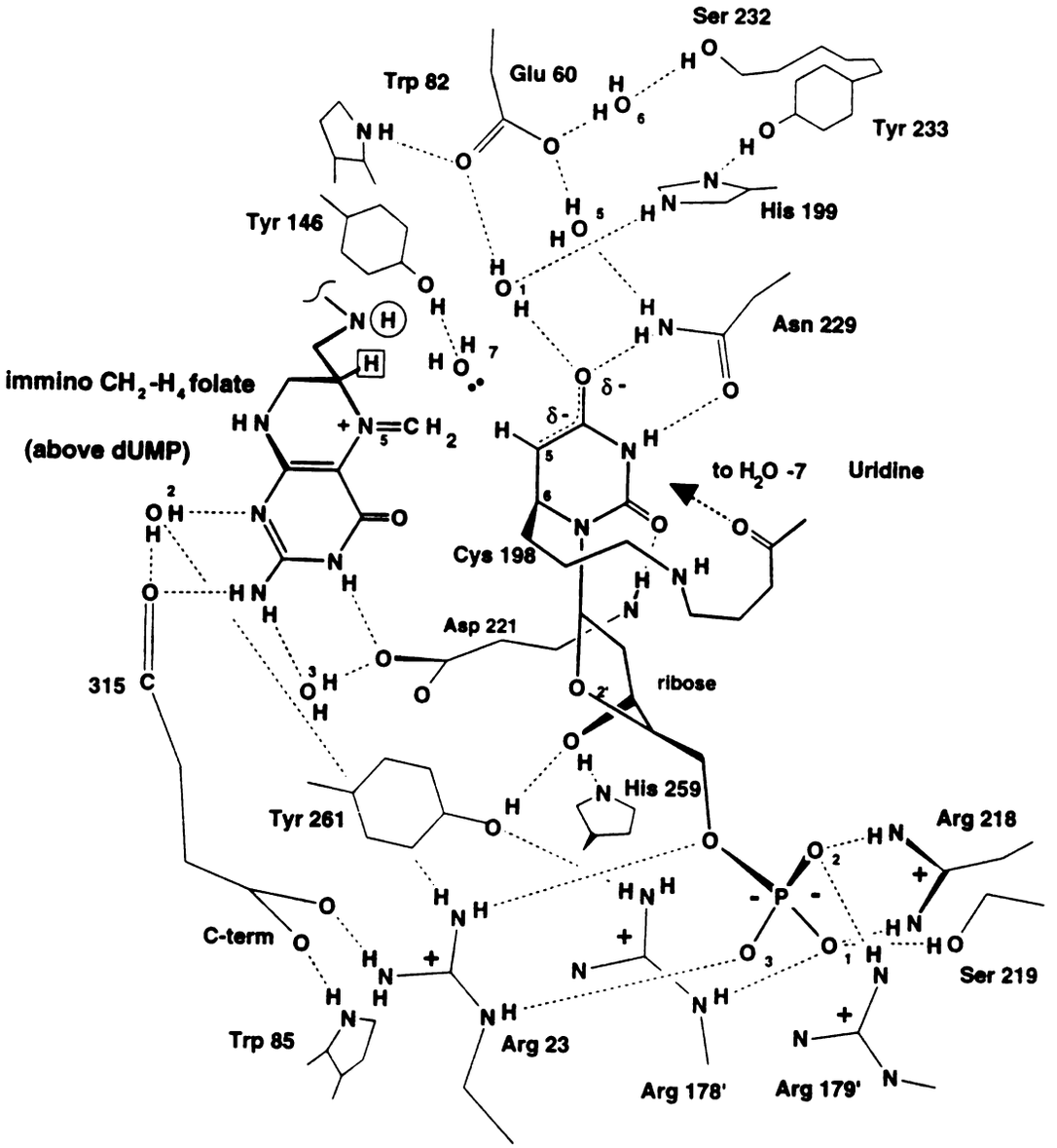


Figure 1.2: Schematic diagram of hydrogen-bond interactions in the active site of TS (Finer-Moore *et al.*, 1990).

promote iminium ion formation (Matthews *et al.*, 1990b; Matthews *et al.*, 1990a). In ternary complex of *E coli.* TS, FdUMP and CH_2H_4folate (Matthews *et al.*, 1990b), the carboxyl side-chain of Glu-60 is 4 Å from N-10 of CH_2H_4folate and is thus proposed to stabilize protonation of the cofactor's N-10 prior to cleavage of the C-11-N10 bond either directly or through a solvent molecule. Since Glu-60 is buried in a conserved hydrophobic cluster when substrate and cofactor bind to the active site, it may have an elevated pK_a value and could also be the source of the proton delivered to N-10. The protonation of N-10 and the concomitant cleavage of the C-11-N10 bond leads to formation of the N-5 iminium ion. The N-5 iminium ion is believed to be the reactive species of the cofactor based on kinetic studies of the hydrolysis of CH_2H_4folate (Kallen & Jencks, 1966). The results of reduction of CH_2H_4folate with KBH_4 to yield 5-methyl-CH_2H_4folate (Gupta & Huennekens, 1967) and the crystal structure of V136Am TS bound to FdUMP and 5-(hydroxymethyl)-THF (Perry *et al*, 1993) also implicate the N-5 iminium ion as the reactive species. (2) Glu-60 is proposed to stabilize the incipient negative charge of O-4 of dUMP (enolate intermediate) via an ordered water molecule that is directly hydrogen bonded to O-4 of dUMP (Water 1 in Figure 1.2) (Matthews *et al.*, 1990b; Finer-Moore *et al.*, 1990). The enolate form of dUMP has been suggested as a reactive intermediate that attacks the methylene group of the reactive N-5 iminium ion, resulting in condensation of substrate and cofactor via a one-carbon bridge between C-5 of dUMP and N-5 of H_4folate. (3) Glu-60 may assist the water-mediated abstraction of the proton from C-5 of dUMP, which is essential for the initiation of the breakdown of the catalytic covalent intermediate (Figure 1.1) (Matthews *et al.*, 1990b). In the covalent inhibitor complex of TS, FdUMP and CH_2H_4folate, water 1 makes van der Waals' contact with the nucleotide's fluorine atom and is directly hydrogen-bonded to Glu-60, His-199 and O-4 of FdUMP (Figure 1.2). Mutagenesis studies have shown that His-199 is not essential for catalysis (Climie & Santi, 1990; Michaels *et al.*,

1990), thus Glu-60 seems to be the key residue in positioning the water molecule. (4) Glu-60 may be important in binding of CH_2H_4folate. Binding of CH_2H_4folate is greatly impaired in the E60Q mutant of *E. coli* TS as shown by a TCA precipitation assay and by 19F NMR (Zapf *et al.*, 1993). (5) Glu-60 may be important for the conversion of intermediate **IV** to products. E60A and E60L mutants of *L. casei* TS have been shown to have a major defect in conversion of the intermediate **IV** to product (Chapter III). (6) Recently, E60D and E60Q mutants of *E. coli* TS and of bacteriophage T4 dCMP hydroxymethylase have been shown to cause uncoupling of the early and late steps of catalysis (Hardy *et al.*, 1995). It has been proposed that the primary role of Glu-60 is to accelerate bond cleavage between N-5 of tetrahydrofolate and to facilitate methylene transfer in the covalent ternary intermediate **III**.

III. The active site hydrogen-bond network

Crystallographic studies have shown an extensive hydrogen-bond network surrounding the pyrimidine ring of the substrate dUMP (Figure 1.2) (Montfort *et al.*, 1990; Finer-Moore *et al.*, 1990). The components of the network that are in close proximity to the substrate include the side chains of Glu-60, Asn-229, His-199, ordered water molecules, and NH-3 and O-4 of the pyrimidine ring of dUMP. This thesis focuses on these components of the hydrogen-bond network, although the network can be extended to other components that are further away from dUMP (Figure 1.2), e.g., Trp-82 which has been shown not essential for catalysis by mutagenesis study (Kealey *et al.*, 1995).

Based on the crystal structure of wild-type TS, His-199 was proposed as a candidate general base to assist removal of the C-5 proton (Hardy *et al.*, 1987). However, subsequent mutagenesis studies on His-199 have shown that this residue is not essential for catalysis (Climie & Santi, 1990; Michaels *et al.*, 1990; Climie *et al.*,

1990; LaPat-Polasko *et al.*, 1990; Dev *et al.*, 1989). The pH-rate profiles of His-199 mutants of *E. coli* TS are bell-shaped curves showing decreased TS activity at high pH values in contrast to the sigmoidal curve observed with wild-type TS (Dev *et al.*, 1989). This data led to the suggestion that His-199 stabilizes the acidic form of an important catalytic group.

Asn-229 is the only amino acid residue in the active site that makes direct hydrogen-bond interactions with O-4 and NH-3 of the heterocycle (Montfort *et al.*, 1990; Matthews *et al.*, 1990a; Finer-Moore *et al.*, 1993). Mutagenesis studies on Asn-229 mutant TSs show that the residue is not essential for catalysis (Michaels *et al.*, 1990; Liu & Santi, 1993a). Asn-229 mutants of *L. casei* TS show elevated affinity for dCMP in contrast to a very low affinity of wild-type TS for dCMP. Asn-229 is therefore proposed to provide specificity in the binding of dUMP by excluding dCMP from the active site (Liu & Santi, 1993b). The N229D TS reverses the enzyme's specificity from dUMP to dCMP and accordingly converts TS into a dCMP methylase (Liu & Santi, 1992; Hardy *et al.*, 1992) , whereas, an analogous Asp to Asn mutation in dCMP hydroxymethylase converts the enzyme to a dUMP hydroxymethylase (Graves *et al.*, 1992).

Structural water has long been recognized as influencing structure, function and stability of protein. Water is generally excluded from the apolar interior of globular proteins, yet some water molecules are found to be isolated from the bulk solvent and hydrogen-bonded to polar or charged groups in highly conserved positions in homologous proteins (Baker & Hubbard, 1984). However, the role of water molecules is uncertain or unknown in the majority of structure-function studies. Structurally ordered water molecules are generally observed by X-ray diffraction studies with protein crystals, but the positions of individual water molecules can to be identified only if the structure is solved at high enough resolution (< 2.5 Å). The highly refined crystal structures of L-Arabininose-binding protein have illustrated that

ordered water molecules can contribute directly to substrate specificity and affinity of the protein (Quiocho *et al.*, 1989). The crystal structure of the product complex of TS (1.83 Å) suggests that the enzyme uses a bound water molecule to disfavor binding of the product nucleotide (Fauman *et al.*, 1994). Crystal structure data can now be complemented by NMR studies in solution which show that water molecules observed in crystal structures are usually conserved in solution (Clore *et al.*, 1990; Forman-Kay *et al.*, 1991). Recently, a hydrolytic water molecule in trypsin was revealed by time-resolved Laue crystallography (Singer *et al.*, 1993). Site-directed mutagenesis is sometime useful in studies of structurally ordered water molecules. For example, residues that hydrogen-bond to internal water molecules are altered to study structural and functional roles of the internal water molecules in rat cytochrome C (Luntz *et al.*, 1989) and basic pancreatic trypsin inhibitor (Berndt *et al.*, 1993).

The two water molecules, water 1 and 6 (Figure 1.2) of the hydrogen-bond network are conserved in various TS crystal structures (Finer-Moore *et al.*, 1990; Finer-Moore *et al.*, 1993; Matthews *et al.*, 1990b; Matthews *et al.*, 1990a). In particular, water 1, which is hydrogen-bonded to Glu-60, His-199 and O-4 of the pyrimidine ring of dUMP, is highly ordered and assumed to be critical in the TS mechanism. Matthews *et al* proposed a mechanism for the breakdown of intermediate **III** based on the covalent inhibitory complex of *E. coli* TS, FdUMP and CH_2H_4folate, which involves proton abstraction from C-5 of dUMP to water 1 (Matthews *et al.*, 1990b). It has been further speculated that the protonated water 1 facilitates the next chemical step in the TS reaction, i. e, the hydride transfer from H_4folate to the =CH2 of intermediate **IV** (Figure 1.2), by protonating O-4 of the substrate. Since no group of the protein seems to be close enough to O-4 and C-5 to act directly as a general acid/base catalyst to facilitate proton transfer reactions (Finer-Moore *et al.*, 1990), water 1 may assist in mediating proton transfer reactions.

In addition to the lack of amino acid residues in the close vicinity of the pyrimidine ring of dUMP, it is puzzling how the O-4, which has a pK_a value less than -3.4, is protonated. Liu and Santi have suggested that water 1 is important in assistng both the proton transfer to the poorly basic O-4 and a proton abstraction from the weakly acidic C-5 of dUMP (Liu & Santi, 1993a). To rationalize enzyme-catalyzed proton abstraction from carbon acids, Gerlt *et al.* have pointed out that proton transfer to carbonyl oxygen can be assisted by week acids if an "enolic intermediate" is formed (Gerlt & Kozarich, 1991; Gerlt & Gassman, 1992; Gerlt & Gassman, 1993). The "enolic intermediate" is described as very strong hydrogen bond complex of the enol and the conjugate base of general acid catalyst (Gerlt & Gassman, 1993). A well-known example of very strong hydrogen bond is FHF- ion, in which the distance between fluorine atoms is 2.26 Å and the hydrogen bond energy is 37 kcal/mol (Hibbert & Emsley, 1990; Emsley, 1980). Gerlt *et al.* have suggested that the "enolic intermediate" (very strong hydrogen bond) can be formed and proton transfer to carbon oxygen can be assisted by a general acid if the pK_a between the resultant enol and the general acid is near 0 and the proton transfer is late in the transition-state(Gerlt & Kozarich, 1991; Gerlt & Gassman, 1992). The pK_a of the coresponding enol intermediate in the TS reaction, intermediate II, is estimated to be at least 12. Water (pK_a =15.7) can be a candidate for electrophilic catalyst to assist the formation of the "enolic intermediate" and proton transfer to O-4 of dUMP. The pK_a of water 1 can be fine-turned, through its hydrogen-bond interactions to Glu-60 and His-199, to match the pK_a of the enol intermediate in the TS reaction.

The enol intermediate at O-4 of the pyrimidine of dUMP could also be critical to the α-proton abstraction at the weekly acidic CH-5 of intermediate III and the subsequent β-elimination reaction. Gerlt *et.al.* have proposed a concerted general acid-base catalysis for α-proton abstraction form carbon acid, in which the pK_a of the α-proton can be reduced some 15 pK_a units by protonation of the carbonyl oxygen of

a carbon acid, and the proton is concretely abstracted by a general base (Gerlt & Kozarich, 1991). Although no residue in the TS active site seems suitable to serve as a general base to assist the proton abstraction, another ordered water molecule, water 7, has been proposed to be in the appropriate position to accept the CH-5 proton because of its close vicinity to C-5 in crystal structure (Fauman *et al.*, 1994).

IV. My thesis work on Glu-60 and the hydrogen bond network.

A study of the role of Glu-60 in the TS mechanism was chosen as the major part of this thesis project. By the time I began my thesis project, Glu-60 appeared to be critical for the TS reaction; however, there was little experimental evidence for the specific catalytic role of this residue. First, Glu-60 is a completely conserved residue in all known species, suggesting important catalytic role. Second, Glu-60 is involved in the extensive hydrogen bond network in the active site which has been proposed to be critical to TS mechanism. Third, my preliminary experiments during my lab rotation showed that mutation of Glu-60 results in pronounced decrease in catalytic activity. In contrast, TS is tolerant to mutations of most conserved residues of the enzyme, including the hydrogen bond network residues His-199 and Asn-229. Furthermore, study of Glu-60 seemed to be a good starting point to investigate the catalytic role of the hydrogen bond network because the apparent "coordinating" role of the residue for the hydrogen bond network.

Chapter II describes a simple approach that I have developed to improve the "replacement set" mutagenesis which involves replacement of one (or more) amino acid(s) of a protein by all other 19 natural amino acids (Huang & Santi, 1994a). In the classical "replacement set" mutagenesis, a degenerate codon (N)(N)(G+C) is used in cassette mutagenesis to encode all 20 natural amino acids at the target mutation site. However, the wild-type enzyme will always be identified as catalytically active in a screening process and in most cases can only be distinguished from

active mutants by DNA sequencing. Sequencing of background wild-type enzyme represents wasted effort in the identification of active mutants. The new approach described in this chapter involves the exclusion of the wild-type codon through the synthesis of two or three oligonucleotide mixtures. This exclusion greatly saves time and expense by elimination the sequencing of background wild-type plasmids. If a complementation assay or screening system is available, one of the major utilities of the current approach is to effectively assess whether any catalytically active mutation exists at a given residue. If no catalytically active subtitutions are obtained in any of the oligonucleotide mixtures, an amino acid residue can be rapidly identified as essential for catalysis without DNA sequencing. In the cases where there are catalytically active substitutions for a given residue, all positive colonies selected by screening represent active mutations, and the subsequent DNA sequencing is productive. I have successfully used the wild-type exclusion approach to identify catalytically active mutants of *L. casei* TS.

Chapter III describes isolation and characterization of a covalent steady-state intermediate in Glu-60 mutant enzymes (Huang & Santi, 1994b). The E60A and E60L mutants of *L casei* TS catalyzed tritium exchange from [5-^{3}H]dUMP for solvent protons faster than dTMP formation, indicating accumulation of a steady-state intermediate and a change in partitioning of the intermediate. A covalent complex consisting of the mutant TS, dUMP and CH_2H_4folate was isolated on SDS-PAGE, and shown to be chemically and kinetically competent to form dTMP. These results provide proof of the formation of a covalent steady-state intermediate in the TS reaction pathway. This also represents one of the few reports in which a mutation of an enzyme results in accumulation and isolation of a stable, normal steady-state intermediate.

Chapter IV describes a complete replacement set of Glu-60 mutants of *L. casei* TS. Complete and partial TS reactions were used to dissect the reaction pathway

and define the defects caused by the mutations. The results show that Glu-60 is critical to the TS reaction. Mutation of Glu-60 did not greatly affect substrate or cofactor binding, but greatly impaired both the formation and breakdown of the covalent intermediate **IV** with the greater effect occurring during the conversion of **IV** to products. Thus, TS Glu-60 mutants show differential effects on various steps in TS reaction pathway. Because of the change in partitioning of **IV**, there is a buildup of the steady-state intermediate **IV** which can be isolated under denaturing conditions. The ability to separate the TS reaction pathway into components may allow experimental approaches that further assign the role of Glu-60 in catalysis.

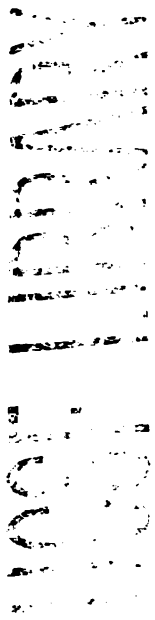
The restoration of catalytic activity of the mutant enzymes by exogenous carboxylic acids was also investigated. It appears that exogenous carboxylates served as true surrogates for the Glu-60 side chain in Glu-60 mutants. This work (manuscript in preparation) represents the most extensive mutagenesis and kinetics studies of Glu-60 of TS so far.

Chapter V describes investigation of three active site general catalysts — Glu60, His199 and Asn229 — by enzymatic dehalogenation of BrdUMP and IdUMP. The single E60L, H199A, and N229V mutants and the E60L/H199A/N229V triple mutant were constructed and subjected to kinetic studies of dehalogenation and dTMP formation reactions. The CH_2H_4folate-independent dehalogenation reaction served as an analog of the early events of normal TS reaction. This work (manuscript submitted for publication) indicates addition and elimination reactions involving the 5,6-bond of pyrimidine substrates do not require general acid/base catalysis or, alternatively, the water molecules in the TS active site serve this role. The function(s) of the triad of general catalyst resides elsewhere in the reaction pathway leading to dTMP synthesis.

Chapter VI describes an energetic study on functional interactions among residues of the hydrogen-bond network in TS active site. The mutational effects of

the single E60D, H199A, N229V and the corresponding double and triple mutant enzymes were analyzed. Nonadditive mutational effects of the multiple mutants on the dissociation constant of dUMP, the catalytic constant of dTMP formation and the rate of association of dUMP with free enzyme demonstrate the existence of functional interactions among the network residues. Pairwise interaction free energies between E60, H199, N229 and their dependency on the third residue were quantified and analyzed using multimutant cycles. This work (manuscript in preparation) provides a detailed picture of the energetic interactions among the network residues and indicates that synergism is a common underlying mechanism by which the network residues functions.

Chapter VI describes the X-ray crystallographic structures of TS E60D and E60Q and their complexes with dUMP, and dUMP and CH_2H_4folate or folate analog CB1737. The crystallographic study was performed by Drs. David L. Birdsall and Janet S. Finer-Moore in the Stroud lab at UCSF. I provided the mutant enzymes and crystals for this study and aided in the interpretation of the structures. This work (manuscript in preparation) provides insight into the structural basis of the mutational effects of the Glu-60 mutants.



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CHAPTER II

Identification of Biologically Active Mutants by Combinatorial Cassette Mutagenesis: Exclusion of Wild-type Codon from Degenerate Codons[1]

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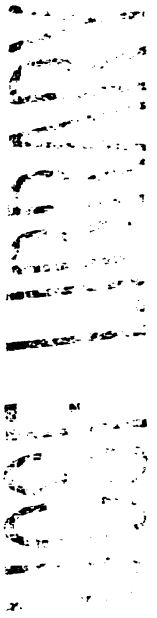

Running Title: Combinatorial cassette mutagenesis

Subject category: DNA recombinant techniques and nucleic acids

[1] This work was supported by Public Health Service Grant CA-14394 from the National Institutes of Health.

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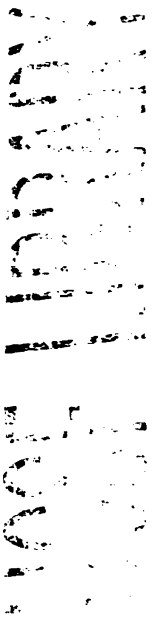
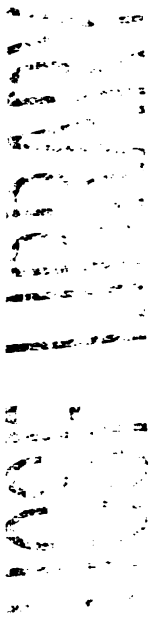
ABSTRACT

A degenerate codon (N)(N)(G+C) is often used in cassette mutagenesis to encode all 20 natural amino acids at the target mutation site. However, the presence of the wild-type codon in the degenerate codon presents some inconvenience in screening and identification of catalytically active mutants. The wild-type enzyme will always be identified as catalytically active in a screening process and in most cases can only be distinguished from active mutants by DNA sequencing. Sequencing of background wild-type enzyme represents wasted effort in the identification of active mutants. This paper describes a simple approach for exclusion of the wild-type codon in degenerate codons through the synthesis of two or three oligonucleotide mixtures. The minimum number of individual colonies required to achieve a high degree of certainty of including all possible codons for screening of catalytic activity can be estimated using statistical procedure. The use of degenerate codons that exclude the wild-type amino acid facilitates the screening process and saves time and expense in DNA sequencing.



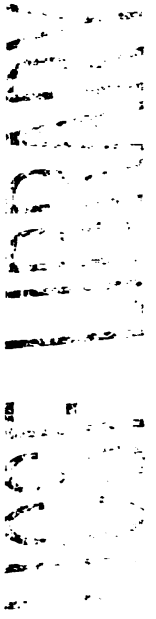
"Replacement set" mutagenesis involves replacement of one (or more) amino acid(s) of a protein by all other 19 natural amino acids. The objective is to ascertain whether substitutions for the target amino acids are catalytically active, and if so, to identify what the active mutant residues are. We have used replacement set mutagenesis to study structure-function relationships of *Lactobacillus casei* thymidylate synthase (TS, EC 2.1.1.45) (1-3). A double stranded synthetic oligonucleotide which contains a degenerate codon at the target site is used to replace an appropriate fragment of the synthetic TS gene by cassette mutagenesis. The degenerate codon, (N)(N)(G+C), provides a mixture of 32 codons which encode all 20 amino acids, including the wild-type (4-6). The plasmid mixture is transformed into Thy$^-$ *Escherichia coli,* and catalytically active mutants are selected by genetic complementation in Thy$^-$ media. However, the wild-type residue that is also contained within the mixture presents some inconvenience for the screening of active mutants. The wild-type enzyme will always be identified as active by the screening, and cause uncertainty in estimating the number of active mutants within a replacement set. In most cases, the wild type enzyme can only be distinguished from active mutants by DNA sequencing, which represents wasted effort in the identification of active mutants. Even when there are no catalytically active mutants in a replacement set, it is necessary to sequence a sufficient number of positive colonies to insure that they are all wild-type.



It would be advantageous to construct degenerate codons that provide all amino acids *except* the wild-type at any position. In the present work, we describe a simple approach which permits the exclusion of the wild type amino acid from degenerate codons through the synthesis of two or three oligonucleotide mixtures. The additional expense of oligonucleotide synthesis is in most cases outweighed by the savings in time and expense in screening and DNA sequencing.

METHODS

Mixture design. The strategy is to make oligonucleotide mixtures that code for all natural amino acids *except* the wild-type at the target position. We have developed an approach in which two oligonucleotide mixtures provide replacement sets for each of 15 amino acids, and three oligonucleotide mixtures provide replacement sets for the remaining 5 amino acids. Oligonucleotide mixtures for each of the 20 amino acids are shown in Table 1.



(A) The two mixtures required by each of fifteen amino acids (A, D, E, F, G, H, M, N, P, Q, R, T, V, W, and Y) are described as follows. In the first mixture, the first position of the target codon contains all bases except the base(s) found in the wild-type codon; the second position has a mixture of four bases, and the third has G+C. The second oligonucleotide mixture is designed to code for the amino acids not present in the first mixture. As shown in Figure 1, amino acids in the same quadrant(s) as the wild-type are to be included in the second mixture. The first position in the second mixture has the base (s) of the wild-type codon, and the wild type codon is excluded by proper design of mixtures of bases at either the second or third position of the target codon (see Table 1 and Figure 1). If the second base for the wild-type codon is unique among codons of amino acids that the second mixture encodes, the second position excludes that base,and the third position contains the minimum number of bases needed to encode the remaining amino acids. If the second base of the wild-type codon shares the same base with the codons of other amino acids, the second position of the second mixture contains four bases, and the third position has the minimum number of bases necessary to exclude the wild-type but encodes other amino acids.

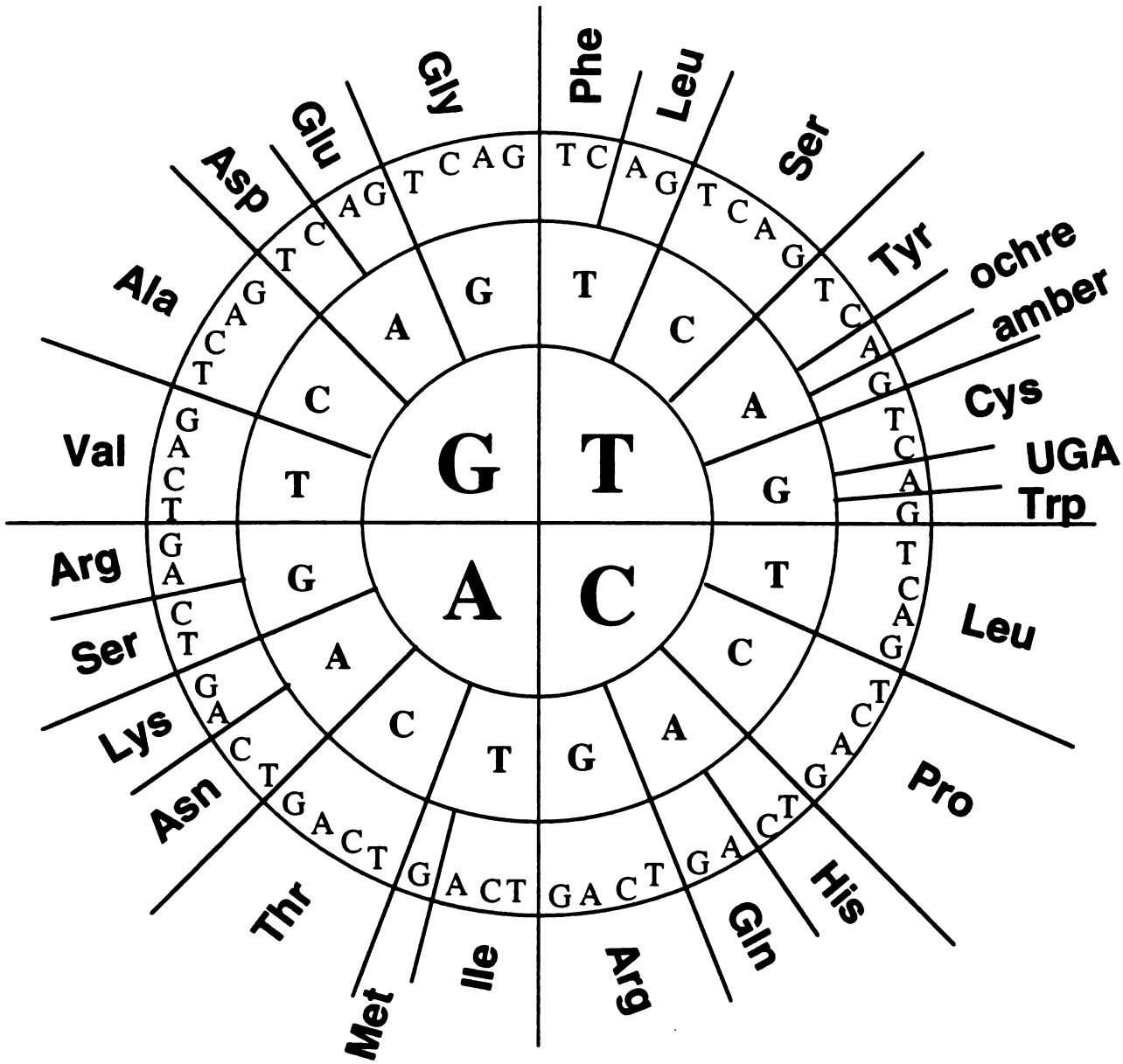


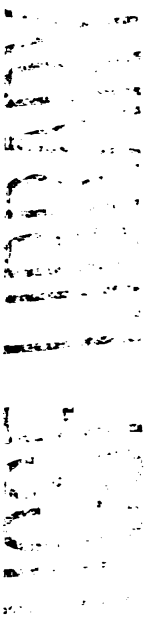
Figure 2.1: The genetic code. Names of amino acids and chain termination codons are on the periphery of the circle. The first base of the codon is identified in the center ring, the second base of the codon is in the middle ring, and the third base(s) of the codon is in the outer ring of the circle. (Reprinted, by permission of the publisher, from A. Ellington and J. M. Cherry, in Current Protocols in Molecular Biology (Ausubel, F. M., et al., Eds.) Supplement 12, Copyright © 1993, John Wiley & Sons, Inc.)

Table 2.1: Design of oligonucleotide mixtures for exclusion of wild-type amino acid in combinatorial cassette mutagenesis

Wild Type Amino Acid	Degenerate codons of Oligonucleotide Mixtures	Number of Codons	Colonies required for screening with 99% certainty*
Ala (A)	Mixture No. 1 : (ACT)(ACGT)(GC)	24	109
	Mixture No. 2 : (G)(AGT)(GC)	6	26
Arg (R)	Mixture No. 1 : (GT)(ACGT)(GC)	16	72
	Mixture No. 2 : (AC)(ACT)(GC)	12	53
Asn (N)	Mixture No. 1 : (CGT)(ACGT)(GC)	24	109
	Mixture No. 2 : (A)(ACT)(AG)	6	26
Asp (D)	Mixture No. 1 : (ACT)(ACGT)(GC)	24	109
	Mixture No. 2 : (G)(ACGT)(G)	4	17
Cys (C)	Mixture No. 1 : (ACG)(ACGT)(GC)	24	109
	Mixture No. 2 : (T)(AT)(C)	2	7
	Oligonucleotide No. 3 : (T)(G)(G)	1	1
Gln (Q)	Mixture No. 1 : (AGT)(ACGT)(GC)	24	109
	Mixture No. 2 : (C)(AC)(C)	2	7
Glu (E)	Mixture No. 1 : (ACT)(ACGT)(GC)	24	109
	Mixture No. 2 : (G)(ACGT)(C)	4	17
Gly (G)	Mixture No. 1 : (ACT)(ACGT)(GC)	24	109
	Mixture No. 2 : (G)(ACT)(GC)	6	26
His (H)	Mixture No. 1 : (AGT)(ACGT)(GC)	24	109
	Mixture No. 2 : (C)(AC)(G)	2	7
Ile (I)	Mixture No. 1 : (CGT)(ACGT)(GC)	24	109
	Mixture No. 2 : (A)(AC)(GC)	4	17
	Oligonucleotide No. 3 : (A)(T)(G)	1	1
Leu (L)	Mixture No. 1 : (GA)(ACGT)(GC)	16	72
	Mixture No. 2 : (TC)(ACG)(GC)	12	53
	Oligonucleotide No. 3 : (T)(T)(C)	1	1
Lys (K)	Mixture No. 1 : (CGT)(ACGT)(GC)	24	109
	Mixture No. 2 : (A)(ACT)(C)	3	12
	Oligonucleotide No. 3 : (A)(T)(G)	1	1
Met (M)	Mixture No. 1 : (CGT)(ACGT)(GC)	24	109
	Mixture No. 2 : (A)(ACT)(AC)	6	26
Phe (F)	Mixture No. 1 : (ACG)(ACGT)(GC)	24	109
	Mixture No. 2 : (T)(AG)(GC)	4	17
Pro (P)	Mixture No. 1 : (AGT)(ACGT)(GC)	24	109
	Mixture No. 2 : (C)(A)(GC)	2	7
Ser (S)	Mixture No. 1 : (CG)(ACGT)(GC)	16	72
	Mixture No. 2 : (T)(AGT)(GC)	6	25
	Mixture No. 3 : (A)(ACT)(GC)	6	25
Thr (T)	Mixture No. 1 : (CGT)(ACGT)(GC)	24	109
	Mixture No. 2 : (A)(AT)(GC)	4	17
Trp (W)	Mixture No. 1 : (ACG)(ACGT)(GC)	24	109
	Mixture No. 2 : (T)(ATG)(C)	3	12
Tyr (Y)	Mixture No. 1 : (ACG)(ACGT)(GC)	24	109
	Mixture No. 2 : (T)(GT)(GC)	4	17
Val (V)	Mixture No. 1 : (ACT)(ACGT)(GC)	24	109
	Mixture No. 2 : (G)(ACG)(GC)	6	25

*Calculated according to equation 1, with assumptions described in the text.

(B) The three mixtures required by each of the remaining five amino acids (C, I, K, L and S) are described as follows. For the replacement of Cys, Ile, and Lys, two oligonucleotide mixtures can be designed as described above in (**A**) for the first and the second mixtures. However, it is not possible to both exclude these amino acids and include Trp and Met in the two mixtures because Trp and Met each has only one possible codon; Met shares the same first base with Ile and Lys, while Trp shares the same first base with Cys (see Figure 1). To complete the replacement of Ile/Lys, a single oligonucleotide coding for Met is required. To complete the replacement of Cys, a single oligonucleotide coding for Trp is prepared.



For the replacement of Ser and Leu which have two possible bases at the first position of their codons, the first position of the first mixture excludes these two bases. The second position is a mixture of four bases and the third position is G+C. Two more mixtures are required for the replacement of Ser. Each of the two mixtures contains one of the two first bases of the wild-type, the second positions of the two mixtures have three bases but not the wild type base, and the third position contains G+C.

For the replacement of Leu, the first position of the second mixture excludes the two bases of the wild-type, the second position contains three bases but not the base of the wild type, and the third position has G+C (see Figure 1). Since it is not possible to include Phe in the two mixtures designed above for the exclusion of Leu, an additional oligonucleotide encoding Phe is required.

Mutagenesis of Glu 60 of TS. The general procedure of cassette mutagenesis has been described previously (1). The *Bcl* I / *Pst* I fragment of pSCTS13 was replaced by a "stuffer" oligonucleotide 5' GATCAGCGGCCGCAGGCCTGACGGTGATACC

AATATTCGCTTCCTGCCTGCA-3' to give pSCTS13-stuffer. A unique *Stu I* site is introduced in the stuffer, which reduces the size of the synthetic oligonucleotide cassette from 56 bp (*Bcl* I/*Pst* I) to 32 bp (*Bcl* I /*Stu* I). The stuffer does not code for TS and has a unique *Not* I site for restriction selection of recombinants (6). Two oligonucleotide mixtures were designed to replace Glu 60 with all amino acids except the wild type Glu (Table 1). The first mixture has (ACT)(ACTG) (GC) at position 60 and encodes all amino acids except Gly, Ala, Asp, Val and Glu. The second oligonucleotide mixture has (G)(ACGT)(C) at position 60 and encodes Gly, Ala, Asp, and Val. The two oligonucleotide mixture cassettes were individually ligated into the *Bcl* I/ *Stu* I large fragment of pSCTS13-stuffer. DNA from the ligation reaction was purified using Magic Clean-up (Promaga) and restricted by *Not* I to selectively eliminate the parent plasmid pSCTS13-stuffer. The plasmids from the ligation mixtures were used to transform Thy$^-$ *E. coli* χ2913 rec A cells which were then plated directly on minimal agar lacking thymidine. Catalytically active TS mutants were selected by screening for the ability of transformed Thy$^-$ *E. coli* χ2913 rec A cells to grow on minimal agar lacking thymidine.

Probability. A sufficient number of colonies should be screened for catalytic activity to obtain all active mutants with a high degree of certainty. Table 1 gives the minimum number of colonies required to be screened for each of the oligonucleotide mixtures to obtain 99% certainty of having all codons represented. The number of colonies required is given by

$$N=[\ln(1-P)]/[\ln((n-1)/n)] \qquad (1)$$

where N is the number of colonies, n is the number of possible codons at target position in an oligonucleotide mixture and P is the probability that every codon in the mixture is sampled for screening at least once. The calculation is based on the assumptions that (1) all four bases are incorporated with equal frequency during

synthesis, (2) all members of oligonucleotide mixtures are ligated into plasmids with equal probability, (3) all plasmids are transformed into appropriate cell strains and propagate with equal frequency, (4) the effect of codon usage preference is negligible. To obtain all active mutants encode by an oligonucleotide mixture with 99% certainty, all the active colonies selected from screening of the minimum number of colonies given in Table 1 require DNA sequencing. However, it is advisable to screen a larger number of colonies, e.g. two to five times (or more) of the minimum number given in Table 1, because the assumptions that the calculation is based on may not be correct in every case.

RESULTS AND DISCUSSION

We have successfully used the wild-type exclusion approach to identify catalytically active Glu 60 mutants of *Lactobacillus casei* thymidylate synthase (TS). TS catalyzes the conversion of dUMP and 5,10-methylentetrahydrofolate (CH_2H_4folate) to dTMP and 7,8-dihydrofolate (H_2folate). Glu 60 is completely conserved in all known TS (7), and coordinates a hydrogen bond network with ordered water molecules and other conserved residues in the active site of the enzyme (8,9).

To ascertain the important role of Glu 60 in TS, the current combinatorial cassette mutagenesis approach that excludes the wild-type amino acid was used to obtain a pool of Glu 60 mutants which represents a complete replacement set at position 60 of TS (described in Methods). A sufficient number of colonies were screened for complementation to obtain all active mutants with a high degree of certainty. The minimum number of colonies required to be screened for the first oligonucleotide

mixture with 99% certainty is 109 (Table 1). Over 2,000 colonies were screened and no catalytically active mutants were found by complementation (< 1% of the catalytic activity of the wild-type enzyme (1)). The minimum number of colonies required to be screened for the second oligonucleotide mixture with 99% certainty is 17 (Table 1). Thirty-five colonies were screened for complementation and three colonies were positive. All the three positive colonies were identified as E60D mutant by DNA sequencing. E60D mutant was purified and TS activity was assayed spectrophotometrically at 25 °C (10). E60D mutant has a specific activity of 0.061 unit/mg (specific activity of the wild type enzyme is 7-8 unit/mg). One unit of TS activity catalyzes the formation of 1 μmol of dTMP per minute in a 1-ml reaction mixture. All other Glu 60 mutants encoded by the second oligonucleotide mixture were also identified by DNA sequencing, and none of them was catalytically active by complementation or had spectrophotometrically measurable TS activity. These results show that the approach described in this paper can be used to effectively select and identify catalytically active mutants. The savings in time and expense of screening and DNA sequencing are enormous.

Provided that a complementation assay or screening system is available, one of the major utilities of the current approach is to effectively assess whether any mutation at a given residue is catalytically active. If no substitutions result in catalytically active mutants, an amino acid residue can be rapidly identified as essential for catalysis without DNA sequencing. In the cases where there are catalytically active substitutions for a given residue, all positive colonies selected by screening represent active mutations, and the subsequent DNA sequencing is productive. Although not yet experimentally tested, the approach described herein should be adaptable to combinatorial mutagenesis at multiple amino acid residues.

ACKNOWLEDGMENTS

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CHAPTER III

Isolation of a Covalent Steady-State Intermediate in Glutamate 60 Mutants of Thymidylate Synthase¶

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Running Title: Covalent Steady-State Intermediate of TS

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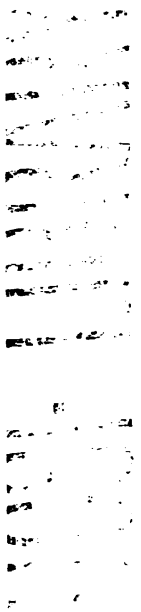
ABSTRACT

Glutamate 60 of thymidylate synthase coordinates a hydrogen bond network important in proton transfer reactions to and from the substrate dUMP. The E60A and E60L mutants of *Lactobacillus casei* thymidylate synthase catalyzed tritium exchange from [5-^{3}H]dUMP for solvent protons faster than dTMP formation, indicating accumulation of a steady-state intermediate and a change in partitioning of the intermediate. A covalent complex consisting of E60A or E60L thymidylate synthase, dUMP and the cofactor CH_2H_4folate was isolated on SDS-PAGE, and shown to be chemically and kinetically competent to form dTMP. These results provide proof of the formation of a covalent steady-state intermediate in the reaction pathway of thymidylate synthase, and demonstrate that the rate-determining step in the mutants occurs during conversion of the covalent intermediate to dTMP.



Thymidylate synthase (TS[3], EC2.1.1.45) catalyzes the reductive methylation of dUMP by CH_2H_4folate to give dTMP and H_2folate. In recent years, much insight has been gained about the structure and mechanism of this enzyme. Sequences from over 20 sources have revealed that TS is among the most conserved of known enzymes (1,2), and X-ray crystal structures of several free and bound forms of the enzyme have pointed to key residues involved in substrate binding and catalysis (3, 4, 5, 6). We have been particularly interested in correlations of structure-function relationships of TS as probed by the consequences of mutagenesis. In the present work, we show that Glu 60 mutants of TS catalyze formation of an isolable, covalent steady-state intermediate. This represents one of the few reports in which a mutation of an enzyme results in accumulation and isolation of a stable, normal steady-state intermediate (7,8).

The currently accepted minimal mechanism of TS is depicted in Figure 3.1. After Michael addition of Cys 198 [4] to C-6 of dUMP, CH_2H_4folate condenses with C-5 of dUMP to give the covalent ternary complex **III** as a steady-state intermediate; here, Cys 198 of the enzyme is covalently attached to C-6 of dUMP and the one carbon unit of the cofactor to C-5 of dUMP. Intermediate **III** is directly analogous to the much studied ternary complex formed between TS, FdUMP and CH_2H_4folate (9, 10). In the reaction pathway leading to dTMP, it is proposed that the 5-H of intermediate **III** is removed as a proton, followed by β-elimination of H_4folate and hydride transfer to give the products, dTMP and H_2folate. As indicated, covalent bond changes are believed to be facilitated by water mediated general acid-base catalyzed proton transfers at O-4 and C-5 of the heterocycle (9). A complex containing TS, dUMP

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[3] Abbreviations used : TS, thymidylate synthase; dUMP, 2'-deoxyuridine-5'-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dTMP, thymidine-5'-monophosphate; H_2folate, 7,8-dihydrofolate; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate.

[4] The amino acid numbering used is that of *Lactobacillus casei* TS.

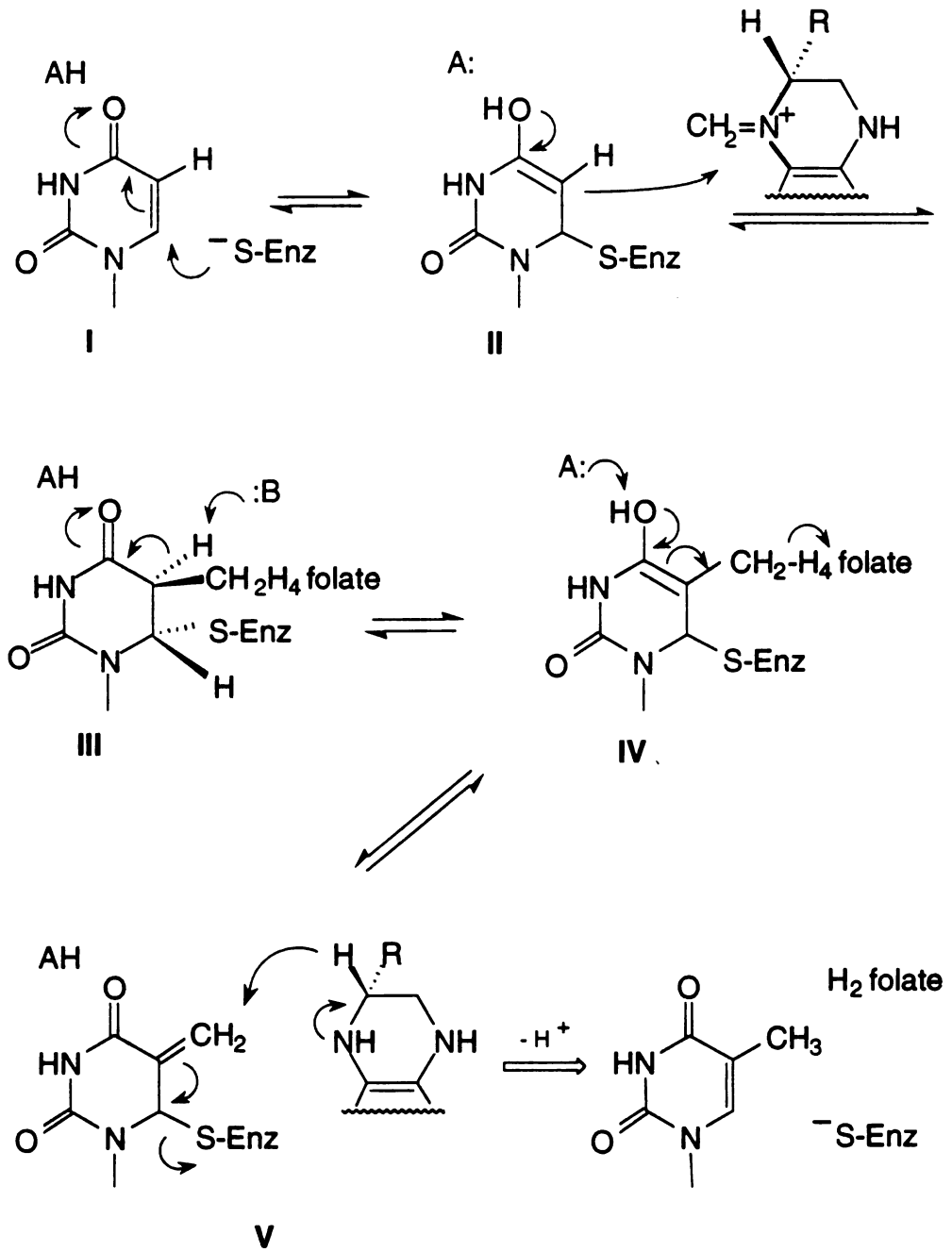

Figure 3.1: The currently accepted minimal mechanism of TS.

and CH_2H_4folate which is presumed to be **III** is isolable by rapid acid quenching of ongoing TS reactions (11); however, the putative intermediate is formed and processed too rapidly to allow convenient study, and we were unable to isolate it on SDS-PAGE.

Crystallographic studies have shown that the completely conserved Glu 60 of TS is involved in an extensive hydrogen bond network that includes several conserved side chains of the enzyme, ordered water molecules and the pyrimidine ring of the substrate (**Figure 3.2**) (5,12). Mutation of Glu 60 leads to large losses in catalytic activity (13,14), and it has been proposed that this residue plays a role in stabilizing the incipient negative charge at O-4 of dUMP (12), or aids in the opening of the imidazolidine ring of CH_2H_4folate (4). We show here that mutation of Glu 60 to Ala or Leu affects the partitioning of the putative steady-state intermediate **III**, and thereby allows its isolation.

MATERIALS AND METHODS

Mutagenesis and protein purification —The E60A and E60L mutants were constructed by cassette mutagenesis of the *L. casei* TS synthetic gene in plasmid pSCTS13 (15). The 56 bp *Bcl* I / *Pst* I fragment of pSCTS13 was replaced by a "stuffer" oligonucleotide

5'-GATCAGCGGCCGC AGGCCTGCA CGGTGATACCAATATTCGCTTCCTGCCTGCA-3'

Bcl I *Not I* *Stu I* *Pst I*

to give pSCTS13-stuffer. The stuffer was obtained using an 58-nt self-priming oligonucleotide which was filled using T4 DNA polymerase and digested by *Bcl I*

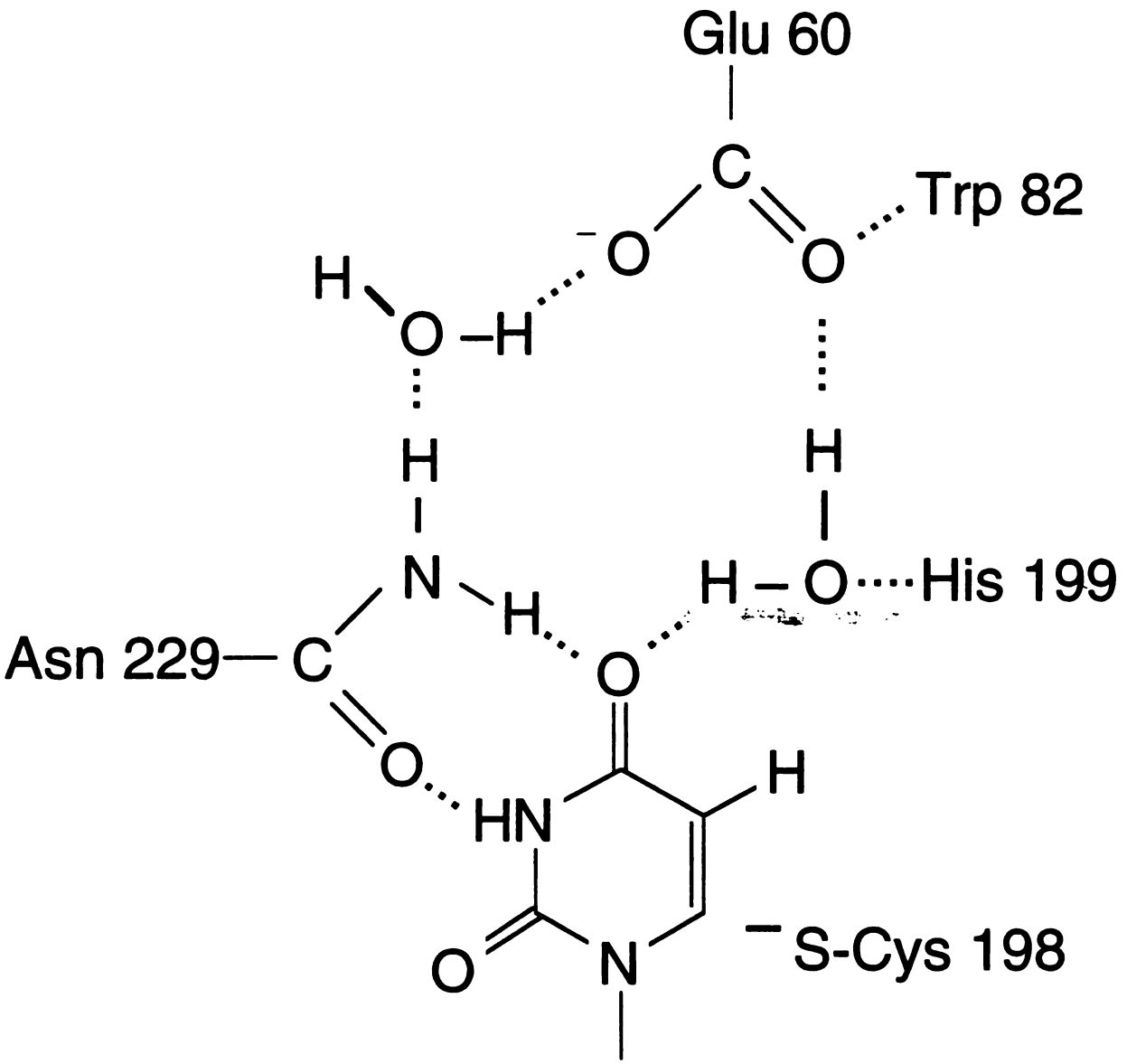

Figure 3.2: Schematic diagram of the hydrogen bond interactions among the conserved amino acid residues, ordered water molecules and the pyrimidine ring of the substrate dUMP (5,12).

and *Pst* I to generate cohesive ends (16). The *Stu* I/*Pst* I component of the "stuffer" retains the wild-type TS sequence; the *Bcl* I/*Stu* I component does not encode TS and has a unique *Not* I site for restriction purification (17). The unique *Stu* I site was introduced into pSCTS13-stuffer to reduce the size of the synthetic oligonucleotide cassette needed for mutagenesis from 56 bp (*Bcl* I/*Pst* I) in the original synthetic TS gene to 32 bp (*Bcl* I/*Stu* I). A mutagenic oligonucleotide duplex cassette containing 5'-GATCAAAAGCGCG (or CTG)CTGCTGTGGTT-3' was inserted into the *Bcl* I and *Stu* I sites of pSCTS13-stuffer; the bases underlined change codon 60 from Glu to Ala or Leu. The mutant enzymes were purified to homogeneity as previously described (18).

Enzyme Assays — TS activity was monitored spectrophotometrically at 340 nm (19). The standard assay buffer for dTMP formation contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA and 75 mM β-mercaptoethanol. TS activity of E60A or E60L mutants, which was too low to be measured spectrophotometrically, was monitored by HPLC analysis. HPLC was performed using a Rainin HPLC equipped with a Hewlett Packard 1040A diode array detector (20). Isocratic separation of dUMP and dTMP was accomplished on an Alex Ultrasphere IP column using 5 mM KH_2PO_4, pH 7.0, 5 mM tetra-n-butylammonium sulfate, and 2.5% (v/v) acetonitrile as the eluant with a flow rate of 1 mL/min. Retention volumes for dUMP and dTMP were 15 and 26 mL, respectively. For analysis of radioactive reactants and products, dUMP and dTMP UV markers were added to samples prior to chromatography.

TS-catalyzed tritium release from [5-^{3}H]dUMP was monitored by the decrease in $^3H/^{14}C$ ratio of [2-^{14}C, 5-^{3}H]dUMP (21). Reaction mixtures contained 0.5 to 3 μM TS, 200 μM [2-^{14}C, 5-^{3}H]dUMP (20 mCi ^{3}H/mmol, 4 mCi ^{14}C/mmol), and 400 μM of (6*R, S*) CH_2H_4folate in standard TES assay buffer at 25° C. Aliquots (20 μL) were assayed for tritium release as described (20). The exchange of tritium from [5-^{3}H]dUMP for solvent

protons was monitored by analysis of the $^{3}H/^{14}C$ ratio of [2-^{14}C, 5-^{3}H]dUMP isolated by HPLC .

SDS-PAGE of the covalent intermediate — Covalent ternary complexes were formed by incubating a mixture containing (a) 4.5 μM E60A or E60L TS, 6.7 μM [6-^{3}H]dUMP (15 Ci/mmol) and 400 μM CH_2H_4folate, or (b) 4.5 μM E60A or E60L TS, 400 μM dUMP and 560 μM [6-^{3}H]CH_2H_4folate (26.6 mCi/mmol) in standard TES assay buffer at 25° C. Aliquots (15 μL) were denatured at various time and analyzed on 12% SDS-PAGE as described (20).

For quantitation and assessment of kinetic parameters of the E60A or E60L TS-[6-^{3}H]dUMP-CH_2H_4folate covalent complex, the radioactivity associated with the protein band at its optimum formation was compared to that of the wild-type TS-[6-^{3}H]FdUMP-CH_2H_4folate covalent complex which contains one FdUMP molecule per TS monomer. Coomassie-stained protein bands were excised, solubilized using Solvable (NEN), and counted in 10 mL of Aquasol-2 (NEN). The reaction mixtures contained 4.5 μM E60A or E60L TS, 200 μM [6-^{3}H]dUMP (0.5 Ci/mmol) and 400 μM CH_2H_4folate, in standard TES assay buffer at 25° C. The rate constant for disappearance of the covalent complex was obtained by adding 100-fold excess of non-radioactive dUMP after the maximum formation of the complex, and then monitoring the first order rate decrease of the radioactivity associated with the complex. The apparent first order rate constant for formation of the covalent complex was calculated by dividing the initial rate of formation by the concentration of TS monomer.

RESULTS AND DISCUSSION

The TS reaction can be monitored by following either dTMP formation, or the release of tritium from [5-^{3}H]dUMP which accompanies dTMP formation. With wild-type TS, the observed rates of these reactions are essentially identical (**Table 3.1**), indicating that tritium release occurs concomitantly with methylation. With E60L and E60A TS , there was a retardation of both the CH_2H_4folate-dependent tritium release from [5-^{3}H]dUMP (600- to 1,400-fold) and dTMP formation (25,000-fold). Importantly, with the E60A and E60L mutants, the CH_2H_4folate-dependent tritium release from [5-^{3}H]dUMP was 20- and 40-fold faster, respectively, than dTMP formation. In the absence of cofactor, tritium release was negligible (<0.02 min^{-1}). The uncoupling of 5-tritium release from 5-methylation indicates a change in the partitioning of an intermediate in the reaction pathway.

The rapid release of tritium from [5-^{3}H]dUMP compared to dTMP formation by the E60 mutants can only be explained by an exchange reaction, where the tritium of the substrate is replaced by solvent protons faster than by one carbon units of the cofactor. Indeed, exchange of tritium from [5-^{3}H]dUMP was directly demonstrated by analysis of the HPLC-isolated substrate during the course of the reaction. From the reaction mechanism (**Figure 3.1**) it can be seen that the exchange likely occurs from (a) proton abstraction from **III** to give **IV**, followed by (b) re-protonation with water, and (c) reversal of intermediate **III** to yield dUMP. The tritium released from **III** must equilibrate with solvent protons in order to observe the exchange; if this were slow, the observed rate of tritium exchange would be a low estimate of the net rate of formation of **III**. In itself, the cofactor-dependent exchange reaction provides evidence for the putative intermediate **III**.

Table 3.1: Kinetic parameters for E60A and E60L TS-catalyzed tritium release from [5-^{3}H]dUMP and dTMP formation. dTMP formation catalyzed by wild-type TS was measured spectrophotometrically; dTMP formation catalyzed by E60A and E60L TS was measured by HPLC. Tritium release from [5-^{3}H]dUMP was monitored by decrease in $^{3}H/^{14}C$ ratio of [2-^{14}C,5-^{3}H]dUMP during the course of the reaction. K_m values for wild-type TS were obtained from dTMP formation and, for E60A and E60L TS, from the tritium release reaction. The standard errors from nonlinear least squares fit of the experimental data are less than 20% for all values.

	k_{cat} (min^{-1})			K_m (μM)	
TS	Tritium release	dTMP formation	$\frac{\text{Tritium release}}{\text{dTMP formation}}$	K_m, dUMP	K_m, CH_2H_4folate
Wild-type	590	540	1.1	6.8	18.8
E60L	0.98	0.024	40.8	47.6	20.7
E60A	0.42	0.021	20.0	31.4	220.3

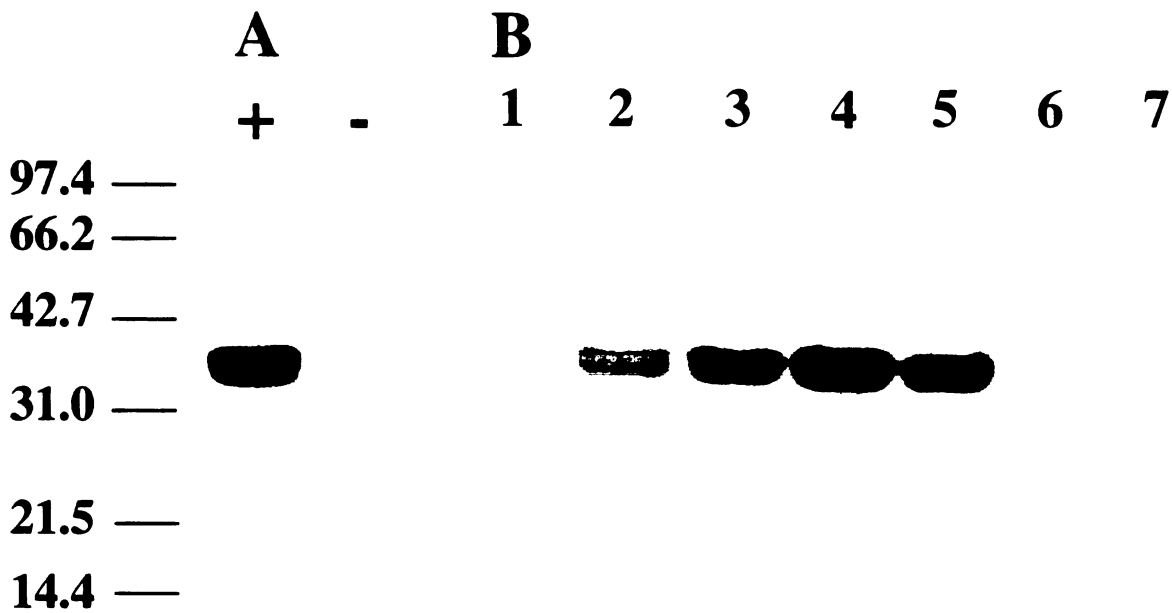

Figure 3.3: Autoradiogram of SDS-PAGE showing the covalent complexes containing E60L TS, dUMP and CH_2H_4folate. (**A**) E60L TS was incubated with [6-^{3}H]dUMP, in the presence (+) or absence (-) of CH_2H_4folate, and denatured after 10 min and subjected to SDS-PAGE. (**B**) Formation of the covalent complex over time. E60L TS was incubated with [6-^{3}H]dUMP and CH_2H_4folate, and denatured at 20 s (lane 1), 40 s (lane 2), 1.5 min (lane 3), 10 min (lane 4) 30 min (lane 5), 2 h (lane 6), and 5 h (lane 7).

The observation that the E60A and E60L TS-catalyzed tritium exchange is faster than methylation indicates that the rate-determining step of dTMP formation is subsequent to formation of putative intermediate **III**. This suggested to us that **III** may accumulate. Mixture containing E60L TS, [6-^{3}H]dUMP and CH_2H_4folate was incubated, denatured at various times, and subjected to SDS-PAGE. As shown in Figure 3.3A, radioactive dUMP became associated with the protein. When CH_2H_4folate was omitted, no radioactive protein band was observed. The protein-bound radioactivity reached a maximum of 2% of TS monomers at about 10 min, and then slowly disappeared (**Figure 3.3B**). As estimated by SDS-PAGE, the apparent first order rate constant for formation of the ternary complex was 1.4 min^{-1}, and the rate constant for its disappearance was 0.07 min^{-1}. After all radioactivity was lost from the covalent complex, HPLC analysis of the reaction mixture demonstrated that the radioactive dUMP was completely converted to dTMP. When [6-^{3}H]CH_2H_4folate was used with unlabeled dUMP, a similar radioactive protein band was formed (data not shown). The results for E60A mutant were essentially the same as that of the E60L mutant except that the rate constants for the covalent complex of E60A mutant were not measured.

Thus, E60A and E60L TS form stable covalent ternary complexes which contain both dUMP and CH_2H_4folate. The observation that the complex was converted to dTMP shows that it is chemically competent as an intermediate in the TS reaction. The rate constant for the disappearance of **III** in the E60L mutant (0.07 min^{-1}) is in accord with k_{cat} for dTMP synthesis (0.03 min^{-1}) under steady-state conditions. Thus, the covalent complex is kinetically competent as an intermediate in dTMP formation. In the accepted mechanism of TS (**Figure 3.1**), **III** is the only candidate for a stable, covalent ternary complex which could fulfill these criteria for a covalent intermediate.

The present results can be correlated with the structure of TS to provide insight into the specific role of Glu 60 in catalysis. Mutation of Glu 60 would modify or disrupt the hydrogen bond network which stabilizes the incipient negative charge and facilitates

proton transfers at O-4 of dUMP (**Fig. 1**). Functional consequences of mutation might be a retardation of the formation of intermediate **III**, the tritium release and exchange reactions, and dTMP formation, all of which were observed (**Table 3.1**). In addition, it has been proposed that opening of the imazolidine ring of CH_2H_4folate to the reactive 5-iminium ion would be facilitated by general acid assisted protonation of N-10 in the transition state (13, 22). In structures of TS ternary complexes (4,12), Glu 60 interacts with N-10 of the cofactor either directly or through a hydrogen-bonded water molecule, and mutation of Glu 60 might retard the formation of III by not allowing this interaction. According to the accepted mechanism of TS, conversion of **IV** to **V** also requires proton transfers at O-4 which would likewise be effected by mutation of Glu 60, and this might lead to further retardation of dTMP formation and the observed accumulation of **III**. Further, since Glu 60 is coordinated through water molecules to both O-4 of dUMP and N-10 of CH_2H_4folate, it is possible that it plays a role in maintaining proper orientations of the activated pyrimidine ring and cofactor (**V**) for hydride transfer.

In summary, taken together, the experiments described here provide proof of the formation of a covalent intermediate in the TS reaction, localize the rate-determining step of the reaction, and provide insight for the molecular role of Glu 60. First, the CH_2H_4folate-dependent TS Glu 60 mutant-catalyzed exchange of tritium from [5-^{3}H]dUMP for solvent protons provides evidence for an intermediate with an exchangeable proton; the expected properties of **III** make it the likely candidate for this intermediate. Second, isolation of a covalent complex containing both dUMP and CH_2H_4folate which is chemically and kinetically competent to form dTMP is only consistent with intermediate **III**. Third, the 5-H exchange reaction and accumulation of the covalent intermediate show that the rate determining step occurs during conversion of **III** to dTMP. The results of our experiments meet the basic criteria for demonstration of a covalent intermediate in enzymatic catalysis (23, 24). Finally, structure-function considerations lead us to suggest that Glu 60 serves a role in coordinating a hydrogen

bond network that promotes proton transfer reactions at O-4 of dUMP and N-10 of the cofactor, and orienting the pyrimidine ring of substrate and the pterin ring of the cofactor for hydride transfer. The ability to directly observe intermediate **III** with TS Glu 60 mutants provides a unique opportunity to study the catalytic details of the formation and reaction of a steady-state intermediate in the TS reaction.

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CHAPTER IV

REPLACEMENT SET MUTAGENESIS OF GLUTAMATE 60 OF THYMIDYLATE SYNTHASE: CHARACTERIZATION OF THE REACTION PATHWAY AND ISOLATION OF A STEADY-STATE INTERMEDIATE¶

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ABSTRACT

The codon for Glu-60 of *Lactobacillus casei* thymidylate synthase (TS) was replaced with those for the nineteen other natural amino acids. Normal and partial TS reactions were used to dissect the reaction pathway and define the functional defects caused by the mutations. In contrast to many conserved residues of TS which are permissive to amino acid substitutions, mutation of Glu-60 resulted in pronounced decreases in TS activity. Although most mutant enzymes showed dissociation constants for dUMP binding similar to the wild-type enzyme, k_{cat} values for 2'-deoxythymidine-5'-monophosphate (dTMP) formation decreased by more than 5,000-fold, except for the E60D mutant. Surprisingly, most mutant enzymes catalyzed dehalogenation of 5-bromo-2'-deoxyuridine-5'-monophosphate faster (up to 70-fold) than wild-type TS. The CH_2H_4folate-dependent tritium release from [5-^{3}H] 2'-deoxyuridine-5'-monophosphate (dUMP) was 5- to 40-fold faster than dTMP formation (when measurable) for all Glu-60 mutant enzymes except the aspartate mutant E60D, indicating changes in partitioning of an intermediate in the reaction. Covalent ternary complexes of mutant TS, dUMP and 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) that were isolated on SDS-PAGE were chemically and kinetically competent to form dTMP. The addition of carboxylic acids, such as acetic acid and formic acid did not affect the k_{cat} values for wild-type TS and the E60D mutant, but increased the k_{cat} values for dTMP formation for the remaining Glu-60 mutants up to 400-fold. For E60A, the carboxylic acids produced small (<3-fold) changes in the K_m values for dUMP and CH_2H_4folate, but decreased the ratio of tritium exchange/dTMP formation and eliminated the accumulation of the covalent steady-state intermediate. The present work demonstrates that the major defect in the reaction pathway for TS Glu-60 mutants is the conversion of the covalent intermediate to the products.

Thymidylate synthase (TS[5]) catalyzes the reductive methylation of dUMP by CH_2H_4folate to give dTMP and H_2folate. TS has been the subject of extensive structure-function studies and is a well characterized enzyme (for reviews, see Santi & Danenberg, 1984; Carreras & Santi, 1995). Sequences from some 29 species of TS have been determined and deposited in the DNA databases (Perry *et al.*, 1990; Carreras & Santi, 1995), and the three-dimensional structures of free and ligand-bound enzyme forms have been solved (Hardy *et al.*, 1987; Matthews *et al.*, 1990a; Montfort *et al.*, 1990; Finer-Moore *et al.*, 1990). The catalytic mechanism of TS is well understood, and a large number of mutants have been prepared and characterized (for review see Carreras & Santi, 1995).

Although TS is one of the most conserved enzymes known (Perry *et al.*, 1990; Perryman *et al.*, 1986), it is permissive to amino acid substitutions at many conserved positions (Santi *et al.*, 1990; Kim *et al.*, 1992; Climie *et al.*, 1990; Climie *et al.*, 1992; Liu & Santi, 1993). Glu-60[6] is a completely conserved residue among all known TS sequences (Carreras & Santi, 1995) and a component of an extensive hydrogen-bond network believed to be involved in proton transfer reactions of the pyrimidine of dUMP (**Scheme 4.1**) (Montfort *et al.*, 1990; Finer-Moore *et al.*, 1990). Although other residues of the hydrogen-bond network (e.g. Asn-229, His-199, see Scheme 4.1) are tolerant to amino acid substitutions (Climie *et al.*, 1990; Liu & Santi, 1993), Glu-60 is very sensitive to mutation to Ala or Leu in *L. casei* TS (Huang & Santi, 1994b) and to Gln or in *E. coli* TS (Zapf *et al.*, 1993; Hardy *et al.*, 1995). The lack of tolerance of Glu-60 toward mutagenesis, together with its involvement in the

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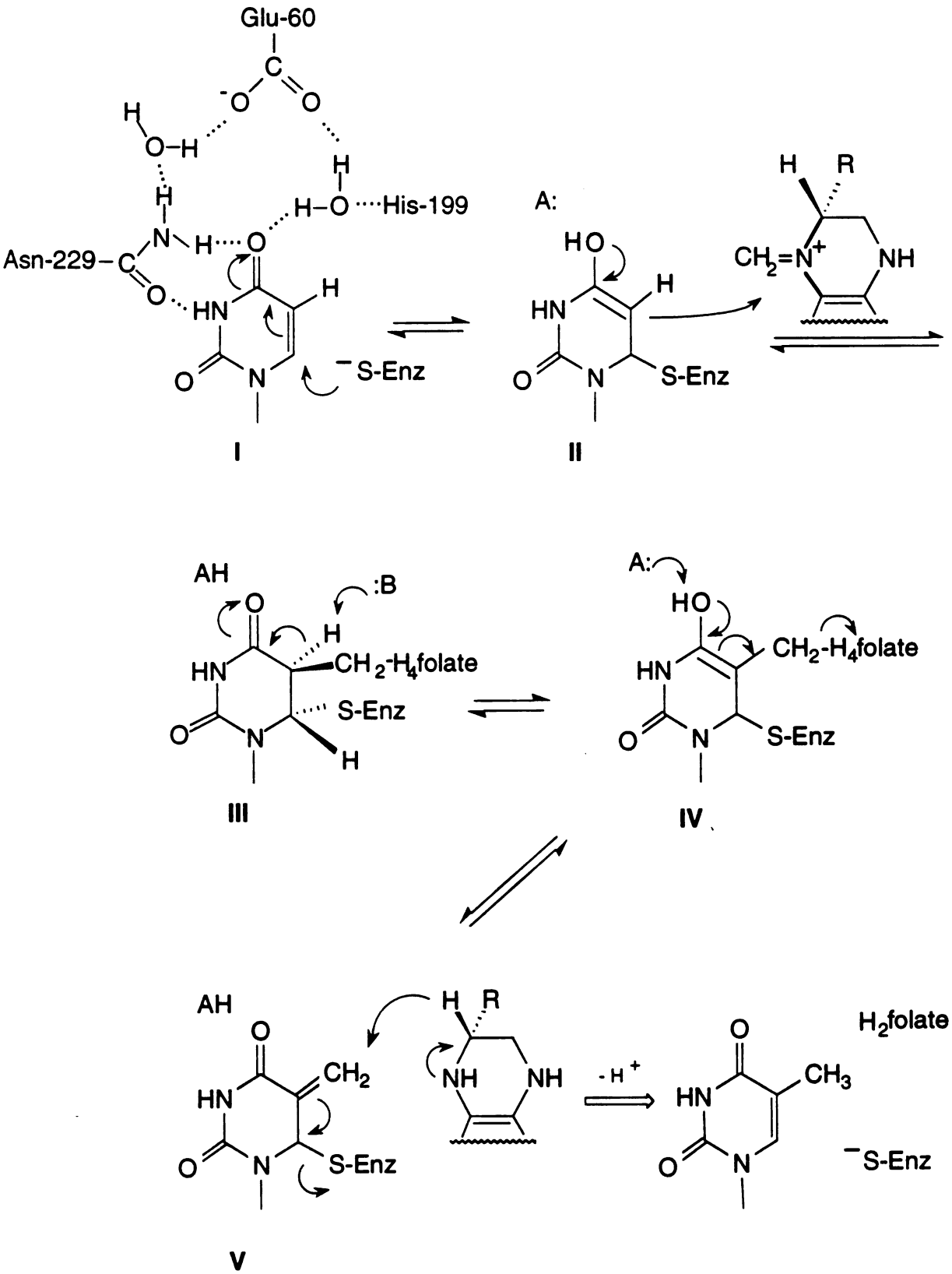
[5] Abbreviations used : TS, thymidylate synthase; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dUMP, 2'-deoxyuridine-5'-monophosphate; BrdUMP, 5-bromo-2'-deoxyuridine-5'-monophosphate; IdUMP, 5-iodo-2'-deoxyuridine-5'-monophosphate; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; HmdUMP, 5-hydroxymethyl-2'-deoxyuridine-5'-monophosphate; dTMP, 2'-deoxythymidine-5'-monophosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TES, N-tris[hydroxymethyl]-methyl-2-amino ethane sulfonic acid; PLP, pyridoxal 5'-phosphate; DTT, dithiothreitol; HPLC, high pressure liquid chromatography.

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

Scheme 4.1


Glu-60
Asn-229
His-199
S-Enz
I
A:
II
AH
:B
CH2-H4folate
III
IV
V
H2folate
- H+

hydrogen-bond network in the TS active site suggested an important role for this residue. Several possible roles for Glu-60 have been suggested based on crystallographic studies of wild-type TS and kinetic studies of Glu-60 mutant enzymes. These include (i) protonation of N-10 of the cofactor to promote iminium ion formation (Matthews *et al.*, 1990a; Matthews *et al.*, 1990b); (ii) stabilization of the incipient enolate intermediate of dUMP via a water molecule hydrogen bonded to O-4 of dUMP (Finer-Moore *et al.*, 1990); (iii) assisting water-mediated abstraction of the C-5 proton from dUMP (Matthews *et al.*, 1990b); (iv) playing a role in CH_2H_4folate binding (Zapf *et al.*, 1993; and (v) facilitating bond cleavage between N-5 and the methylene group of CH_2H_4folate via electrostatic destabilization by the side-chain carboxylate of Glu-60 (Hardy *et al.*, 1995).

Several mutagenesis studies aimed at understanding the catalytic role of Glu-60 have been reported. Preliminary studies showed that most TS mutants at Glu-60 exhibited very low or insignificant activity of TS (Huang & Santi, 1994a). In addition, a pronounced decrease in catalytic activity and CH_2H_4folate binding was reported for the E60Q mutant of *E. coli* TS (Zapf *et al.*, 1993). In a preliminary communication (Huang & Santi, 1994b), we described the E60A and E60L mutants of *L. casei* TS, demonstrated the uncoupling of 5-tritium release of dUMP from its 5-methylation for these mutants, and reported the isolation of covalent steady-state ternary complexes with these mutant TSs. Recently, a similar uncoupling of 5-deprotonation from 5-alkylation was reported for E60D and E60Q mutants of *E. coli* TS (Hardy *et al.*, 1995). It appears that Glu-60 is critical to TS catalysis, but its catalytic role remains to be defined.

In the present work, we prepared a complete replacement set of Glu-60 mutants of *L. casei* TS and analyzed the mutational effects on several steps along the TS reaction pathway. We have also investigated the restoration of catalytic activity of the mutant enzymes by exogenous carboxylic acids. In the context of the previous structural and mechanistic studies of TS, we discuss the catalytic role of Glu-60 in the TS reaction.

MATERIALS AND METHODS

Materials. E. coli strain χ2913recA (*ΔthyA572, recA56*) (Climie *et al.*, 1992) and plasmid pSCTS13 (Climie & Santi, 1990) have been described. Automated oligonucleotide synthesis and purification [Ivanetich, 1991 #43] and automated DNA fluorescent dye terminator cycle sequencing were performed at the UCSF Biomolecular Resource Center. (6*R*)-CH_2H_4folate was a generous gift from SAPEC S.A. (Lugano, Switzerland) and EPROVA A.G. (Schaffhansen, Switzerland). HmdUMP was prepared and purified as described (Kunitani & Santi, 1980). [5-^{3}H]dUMP (13.2 Ci/mmol), [6-^{3}H]FdUMP (20 Ci/mmol), and [2-^{14}C] dUMP (56 mCi/mmol) were from Moravek Biochemicals. (6*R*)-[6-^{3}H]CH_2H_4folate (56 mCi/mmol) was prepared as described (Bruice & Santi, 1982).

Mutagenesis. The Glu-60 mutants were constructed by cassette mutagenesis of the *L. casei* TS synthetic gene in plasmid pSCTS13 (Climie *et al.*, 1990). The plasmid DNA of pSCTS13 was digested with *Bcl* I and *Pst* I and the *Bcl* I/ *Pst* I fragment (56 bp) of pSCTS13 was replaced by a "stuffer" oligonucleotide to give pSCTS13-stuffer as described (Huang & Santi, 1994a). A unique *Stu* I site was introduced into pSCTS13-stuffer to reduce the size of the synthetic oligonucleotide cassette needed for mutagenesis from 56 bp (*Bcl* I/*Pst* I) in the original synthetic TS gene to 32 bp (*Bcl* I/*Stu* I). The *Stu* I/*Pst* I component of the "stuffer" retains the wild-type TS sequence; the *Bcl* I/ *Stu* I component does not encode TS and has a unique *Not* I site for restriction purification (Wells *et al.*, 1986). Two oligonucleotide mixtures were designed to replace Glu-60 with all natural amino acids (Huang & Santi, 1994a). The first mutagenic oligonucleotide duplex cassette contains 5'-GATCAAAAGC (ACT)(ACTG) (GC) CTGCTGTGGTT-3' and the reverse complement. The underlined degenerate codon at position 60 encodes all amino acids except Ala, Asp, Gly, Val and Glu. The second oligonucleotide cassette was as above but contained (G)(ACGT)(C) at position 60 and

encodes Ala, Asp, Gly and Val. The two oligonucleotide cassettes were individually ligated into the *Bcl* I/ *Stu* I large fragment of pSCTS13-stuffer. DNA from the ligation reaction was purified using Magic Clean-up (Promega) and restricted by *Not* I to selectively eliminate the parent plasmid pSCTS13-stuffer. The plasmid DNA from the restricted ligation mixtures was used to transform Thy$^-$ *E. coli* χ2913 recA cells. TS activity of the Glu-60 mutants was first screened for the ability of transformed Thy- χ2913 recA cells to grow on minimal agar lacking thymine. DNA was prepared from individual transformants and all mutations were identified by DNA sequencing. Fifteen mutants were isolated from the two oligonucleotide mixtures described above. The remaining four mutants, TS E60C, F, H and W, were constructed with two oligonucleotide cassettes as described above but containing (T)(G)(GC) and (CT)(AT)(C) at position 60 for TS E60C and W and for TS E60 F, H, L, Y and P, respectively. The four Glu-60 mutants were isolated and identified as described above.

Protein purification. The wild-type and mutant enzymes were purified by automated sequential chromatography on phosphocellulose and hydroxyapatite as described (Kealey & Santi, 1992). The purification was monitored by 12% SDS-PAGE and, in the case of wild-type TS and TS E60D, also by specific activity determinations. Enzyme preparations were concentrated using Centriprep-30 concentrators (Amicon) and stored at -80 °C in 10 mM KH_2PO_4, pH 7.0, 1 mM EDTA until use. The concentrations of the purified enzymes was determined spectrophotometrically using ε_{278} = 125,600 M^{-1} cm^{-1} (Carreras *et al.*, 1994), it was assumed that the mutations at position 60 had no significant affect on the extinction coefficient.

Enzyme assays. TS activity was monitored spectrophotometrically at 340 nm as described (Pogolotti *et al.*, 1986). The standard TES assay buffer contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA and 75 mM β-mercaptoethanol.

The K_d value for dUMP was determined by UV/visible difference spectroscopy as described (Santi *et al.*, 1993). Binary complexes of PLP with wild-type or Glu-60 mutant enzymes were formed using 20-50 μM PLP and 2-3 μM protein in 50 mM TES, pH 7.4 and 50 μM DTT. Dissociation constants for dUMP were obtained by competitive replacement of PLP by dUMP.

TS-catalyzed dehalogenation of BrdUMP and IdUMP was monitored by the decrease in absorbance that accompanies dehalogenation ($\Delta\varepsilon_{285}$ = 5,320 M^{-1} cm^{-1} for BrdUMP, and $\Delta\varepsilon_{290}$ = 6,520 M^{-1} cm^{-1} for IdUMP, Garrett *et al.*, 1979). Reaction mixtures contained TES/DTT assay buffer (50 mM TES, pH 7.4, 25 mM $MgCl_2$, 1 mM EDTA and 10 mM DTT), 3 to 200 μM BrdUMP or IdUMP, and 0.12 to 3 μM wild-type or mutant enzymes.

TS-catalyzed tritium release from [5-^{3}H]dUMP was monitored at 25 °C by following the decrease in $^3H/^{14}C$ ratio of [2-^{14}C, 5-^{3}H]dUMP with time as described (Pogolotti *et al.*, 1979). Reaction mixtures (200 μl) contained 0.5 to 3 μM wild-type or mutant enzyme, 200 μM [2-^{14}C, 5-^{3}H]dUMP (20 mCi ^{3}H/mmol, 4 mCi ^{14}C/mmol), and 400 μM of either (6*R*)-CH_2H_4folate or folic acid in standard TES assay buffer. Aliquots (20 μL) were assayed as described (Carreras *et al.*, 1992). To measure the exchange of 5-tritium of dUMP for solvent protons, aliquots (30 μl) of the reaction mixtures described above were monitored by following the decrease in the $^3H/^{14}C$ ratio of HPLC-isolated [2-^{14}C, 5-^{3}H]dUMP.

Steady-state kinetic parameters were obtained by a non-linear least squares fit of the data to Michaelis-Menten equation using the program Kaleidagraph (Abelbeck Software). For kinetic measurements in which enzyme concentrations exceeded 1 μM, data were fit to an equation which corrects for ligand depletion by protein (Segel, 1975).

Activation of mutant TSs by carboxylic acids. Carboxylic acids were adjusted to pH 7.4 and added to the standard reaction mixtures for TS and partial TS reactions. For

the pH dependent activation experiments, acetic acid was adjusted to pH 6.2 to 7.8, and the total ionic strength was maintained at 2.0 by addition of NaCl.

HPLC analysis of nucleotide products. HPLC was performed using a Rainin Rabbit HPLC equipped with a Hewlett Packard 1040A diode array detector (Carreras *et al.*, 1992). dUMP, HmdUMP and dTMP were separated isocratically on an Alex Ultrasphere IP column (4.6 mm × 25 cm) using 5 mM KH_2PO_4, pH 7.0, 5 mM tetra-n-butylammonium sulfate and 2.5% (v/v) acetonitrile as eluant at a flow rate of 1 mL/min. Retention volumes for dUMP, HmdUMP and dTMP were 15, 19 and 26 mL, respectively. For analysis of radioactive reactants and products, dUMP, dTMP and HmdUMP carriers (25 nmol each) were added to samples prior to chromatography.

SDS-PAGE of the covalent ternary complex. Covalent ternary complexes were formed by incubating (i) 3 μM wild-type or Glu-60 mutant enzymes, 200 μM [6-^{3}H]dUMP (8 Ci/mmol) and 400 μM CH_2H_4folate, or (ii) 4.5 μM TS E60A, TS E60L or TS E60Q, 200 μM dUMP and 400 μM [6-^{3}H]CH_2H_4folate (26.6 mCi/mmol) in standard TES assay buffer (200 μl) at 25°C. Aliquots (15 μL) were denatured at various times and analyzed on 12% SDS-PAGE as previously described (Huang & Santi, 1994b). Coomassie-stained protein bands were excised, solubilized using Solvable (New England Nuclear), and counted in 10 mL of Aquasol-2 (New England Nuclear). For assessment of kinetic parameters of the mutant TS-[6-^{3}H]dUMP-CH_2H_4folate covalent complex, the radioactivity associated with the protein band of TS E60A, TS E60L or TS E60Q at optimum complex formation was compared to that of the wild-type TS-[6-^{3}H]FdUMP-CH_2H_4folate covalent complex which contains one FdUMP molecule per TS monomer. It was assumed that the ratio of nucleotide to TS monomer was the same for wild-type and Glu-60 mutant enzymes.

RESULTS

Sensitivity of TS to mutation at Glu-60. A pool of Thy⁻ χ2913 recA cells containing all Glu-60 mutants but lacking wild-type TS was prepared as described (Huang & Santi, 1994a). Over 2,000 colonies were selected for growth on minimal agar lacking thymine, and plasmids from three positive colonies were sequenced. All three plasmids contained the GAC codon for Asp-60, indicating that TS E60D was the only mutant with sufficient activity to support growth of Thy⁻ *E. coli*. This was subsequently verified by isolation and analysis of each individual Glu-60 mutant as described below.

Mutagenesis and protein purification. Nineteen Glu-60 mutants were constructed by cassette mutagenesis using the synthetic *L. casei* TS gene and degenerate oligonucleotides. Initially, two degenerate oligonucleotide cassettes were used that contained codons for all natural amino acids except the wild-type Glu at position 60 (Huang & Santi, 1994a). 15 mutants were obtained after sequencing the DNA of 35 randomly chosen isolates. To avoid sequencing a large number of isolates to identify the remaining C, F, H and W mutants, we constructed two additional mutagenic DNA cassettes consisting of mixtures of two (C and W) and four (H, L, Y and F) codons at position 60. These cassettes were used in separate experiments to generate two Glu-60 mutant pools. The four remaining mutants (TS E60C, F, H and W) were identified after sequencing the DNA of 12 isolates. All Glu-60 mutants except TS E60P were expressed at 8 to 30% of the total soluble protein in crude extracts as estimated by Coomassie-stained protein bands on SDS-PAGE. Expression of TS E60P was less than 0.5% of the total protein, and the enzyme was insoluble; therefore, this mutant was not studied further. Each of the eighteen other Glu-60 mutants was purified to apparent homogeneity by automated chromatography on phosphocellulose and hydroxyapatite (Kealey & Santi, 1992).

TS activity of Glu-60 mutants. The Glu-60 mutants were assayed for dTMP formation by monitoring the change at 340 nm which accompanies the conversion of CH_2H_4folate to H_2folate with a sensitivity of ~10^{-3} units/mg (Pogolotti *et al.*, 1986). TS E60D, the only mutant that had measurable TS activity by this assay, showed a 140-fold decrease

in k_{cat} for dTMP formation but no significant change in K_m for dUMP and a 2-fold increase in the K_m for CH_2H_4folate (Tables 4.1and 4.2). The rate of formation of dTMP by other Glu-60 mutants was obtained by monitoring the TS-catalyzed conversion of [2-^{14}C, 5-^{3}H]dUMP to [2-^{14}C]dTMP by a sensitive HPLC assay which can detect TS activity as low as 4×10^{-8} units/mg (Carreras *et al.*, 1992). The k_{cat} values (from dTMP formation) for all Glu-60 mutants except TS E60D were more than 5,000-fold lower than that for wild-type TS (Table 4.1). HPLC analysis also confirmed that dTMP was the only major product derived from dUMP; this ruled out formation of 5-HmdUMP which could have arisen from hydrolysis of the putative intermediate **V** (Scheme 4.1) but gone undetected by the spectrophotometric assay.

Dissociation constants for dUMP. Dissociation constants for dUMP were determined by a competitive binding assay in which PLP is displaced from the TS-PLP complex by dUMP (Santi *et al.*, 1993). The K_d values for binding of PLP to TS E60A, C, D, G, L, M, N, S, T and V were 6 to 30-fold higher than for the wild-type enzyme, whereas the K_d value for the E60Q mutant was similar to that for wild-type TS (Table 4.3). TS E60F, H, I, K, R, W and Y did not form detectable complexes with PLP, and K_d values for these mutants were not determined. The K_d values for binding of dUMP to the Glu-60 mutants studied were 2 to 33-fold higher than for wild-type TS, with the highest values for TS E60A, L and V (Table 4.3).

Table 4.1: k_{cat} values for dTMP formation and tritium release from [5-^{3}H]dUMP catalyzed by wild-type and Glu-60 mutant TSs.

TS	k_{cat}, s^{-1}		Ratio of k_{cat}	Covalent Ternary
	^{3}H release[a]	dTMP formation[b]	^{3}H release/dTMP	Complex Isolation[c]
Wild-type	9.8	9.2	1.1	-
E60A[d]	7×10^{-3}	3.5×10^{-4}	20	+
E60C	4×10^{-3}	7.7×10^{-4}	5.2	+
E60D	7.7×10^{-2}	6.5×10^{-2}	1.2	-
E60F	1×10^{-4}	ND[e]		-
E60G	3.5×10^{-3}	5.7×10^{-4}	6.2	+
E60H	6×10^{-4}	ND		-
E60I	2.2×10^{-3}	ND		-
E60K	4×10^{-3}	7×10^{-4}	5.9	+
E60L[d]	1.6×10^{-2}	4×10^{-4}	40	+
E60M	4.3×10^{-3}	3.8×10^{-4}	11	+
E60N	5.6×10^{-3}	1.1×10^{-3}	5.1	+
E60Q	6×10^{-2}	1.5×10^{-3}	40	+
E60R	1.5×10^{-5}	ND		-
E60S	9.8×10^{-3}	2×10^{-3}	4.9	+
E60T	5.4×10^{-3}	1×10^{-3}	5.4	+
E60V	4.1×10^{-3}	6.8×10^{-4}	6	+
E60W	1×10^{-4}	ND		-
E60Y	2.8×10^{-4}	ND		-

[a]Tritium release from [5-^{3}H]dUMP was monitored by a decrease in the ^{3}H/^{14}C ratio of [2-^{14}C,5-^{3}H]dUMP during the course of the reaction. [b]dTMP formation catalyzed by wild-type TS or TS E60D was measured spectrophotometrically at 340 nm, and dTMP formation catalyzed by Glu-60 mutant enzymes except TS E60D was measured by HPLC.
[c]Covalent complex containing TS, [6-^{3}H]dUMP and CH_2H_4folate was detected by SDS-PAGE.
[d]Data are from Table I of Huang and Santi (1994b). [e]ND, not determined.
The standard errors from nonlinear squares fit of the experimental data are less than 20%.

Table 4.2: Steady-state kinetic parameters for tritium release from [5-^{3}H]dUMP catalyzed by wild-type and selected Glu-60 mutant TSs[a].

		K_m, μM		k_{cat}/K_m, s^{-1} μM^{-1}	
TS	k_{cat}, s^{-1}	dUMP	CH_2H_4folate	dUMP	CH_2H_4folate
Wild-type	9.8	6.8	19	1.4	0.5
E60D	7.7×10^{-2}	6.9	37	1.1×10^{-2}	2.1×10^{-3}
E60Q	6×10^{-2}	11	76	5.4×10^{-3}	7.9×10^{-4}
E60L	1.6×10^{-2}	48	21	3.3×10^{-4}	7.6×10^{-4}
E60A	7×10^{-3}	34	220	2.1×10^{-4}	3.2×10^{-5}

[a]The standard errors from nonlinear squares fit of the experimental data are less than 20% for all values.

Table 4.3: Dissociation constants for PLP and dUMP for wild-type and Glu-60 Mutant TSs[a].

	K_d[c], μM	
TS[b]	PLP	dUMP
Wild-type	1.6	0.7
E60Q	1.1	1.2
E60G	11	2.8
E60N	9.6	3.7
E60M	21	4.5
E60T	15	4.6
E60C	13	5.1
E60D	26	8.1
E60S	22	8.8
E60A	22	14
E60L	23	14
E60V	48	23

[a]Assays are described in Materials and Methods. [b]The other seven Glu-60 mutant enzymes that are not included in this table did not form complexes with PLP. [c]The standard errors from nonlinear squares fit of the experimental data are less than 20% for all values.

Dehalogenation of BrdUMP and IdUMP. In the presence of thiols, TS catalyzes the CH_2H_4folate-independent dehalogenation of BrdUMP and IdUMP to produce dUMP (Garrett *et al.*, 1979). All 18 of the Glu-60 mutants catalyzed CH_2H_4folate-independent debromination of BrdUMP (Table 4.4). Most Glu-60 mutants had k_{cat} values for debromination of BrdUMP higher than that for wild-type TS, with the E60L and E60Q mutants showing about 70-fold increase. The K_m values of most Glu-60 mutants for BrdUMP were either similar to or somewhat higher (<10-fold) than that of wild-type TS. The kinetic parameters for dehalogenation of IdUMP by eight selected Glu-60 mutants were similar to those for debromination of BrdUMP by the corresponding mutants, except for the K_m for E60A and the k_{cat} values for E60D and E60R (Table 4.4).

CH_2H_4folate-dependent tritium release from [5-^{3}H]dUMP. As shown in Table 4.1, Glu-60 mutants catalyzed the CH_2H_4folate dependent release of tritium from [5-^{3}H]dUMP with k_{cat} values 10^2- to 10^5-fold lower than that of wild-type TS. For TS E60D and wild-type TS, the ratio of k_{cat} for tritium release to k_{cat} for dTMP formation was ~1. For other Glu-60 mutants, the k_{cat} values for the CH_2H_4folate-dependent tritium release were 5- to 40-fold greater than those for dTMP formation. For the selected mutants examined (TS E60A, L and Q) and wild-type TS, tritium release from [5-^{3}H]dUMP was insignificant ($< 1 \times 10^{-4}$ unit/mg) in the absence of CH_2H_4folate or in the presence of 400 μM folic acid.

Steady-state kinetic parameters for the CH_2H_4folate-dependent tritium release catalyzed by TS E60A, D, L and Q are given in Table 4.2. The k_{cat} values for these mutants except TS E60D were >6,000-fold lower than that for wild-type TS. The K_m values for dUMP increased by less than 7-fold compared to wild-type TS; the K_m for CH_2H_4folate for TS E60L was similar to that for wild-type TS, whereas the K_m values of CH_2H_4folate for TS E60A, D and Q increased by less than 12-fold compared to

Table 4.4: Steady-state kinetic parameters for dehalogenation of BrdUMP and IdUMP by wild-type and Glu-60 mutant TSs.

TS	BrdUMP			IdUMP		
	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m, min^{-1} μM^{-1}	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m, min^{-1} μM^{-1}
WT	1.2	5.4	0.2	4.1	9	0.5
E60L	84	22	3.8	90	6.9	13
E60Q	74	14	5.3	82	7.3	11
E60S	45	16	2.7	nd	nd	
E60C	28	26	1.1	nd	nd	
E60M	24	28	0.8	nd	nd	
E60I	22	53	0.4	nd	nd	
E60V	22	16	1.3	nd	nd	
E60N	18	44	0.4	nd	nd	
E60T	14	9.9	1.4	5.6	7.6	0.7
E60G	6.3	41	0.2	nd	nd	
E60A	3.6	35	0.1	8.2	7.1	1.2
E60F	3.2	31	0.1	8.9	19	0.5
E60Y	3.0	11	0.3	5	9.8	0.5
E60H	2.1	23	0.1	nd	nd	
E60K	2.0	18	0.1	nd	nd	
E60W	1.5	13	0.1	nd	nd	
E60R	0.6	20	0.03	8.5	17	0.5
E60D	0.4	20	0.02	3.7	13	0.3

Assays are described in Materials and Methods. The standard errors from nonlinear squares fit of the experimental data are less than 15% for all values. nd: not determined.

wild-type TS. Tritium release catalyzed by TS E60A, L and Q was shown to be due to exchange of solvent protons for the tritium of [5-^{3}H, 2-^{14}C]dUMP, by HPLC analysis, which showed a decrease in the ^{3}H/^{14}C ratio of the isolated dUMP. The k_{cat} values for tritium exchange (Data not shown) were similar to those for tritium release (Table 4.1) for these mutant enzymes.

Isolation of covalent intermediates. Wild-type and the mutant enzymes were incubated with [6-^{3}H]dUMP and CH_2H_4folate, and the complexes were denatured at various times between 20 s and 5 h, and subjected to SDS-PAGE. In eleven of the Glu-60 mutants, radioactivity was associated with the 35 kDa protein band (Figure 4.1)(Table 4.1), indicating formation of covalent complexes. Interestingly, the mutants which formed the covalent complexes were those that showed a high ratio (5- to 40) of tritium release from [5-^{3}H]dUMP to dTMP formation (Table 4.1). The covalent complex was not observed with wild-type TS or TS E60D, each of which had a ratio of tritium release to dTMP formation of approximately one, or in Glu-60 mutants that had very low rates of tritium release ($k_{cat} < 2 \times 10^{-3}$ s^{-1}). TS E60A, L and Q were chosen for analysis of the stability and quantity of the complexes. When CH_2H_4folate was omitted, no radioactive protein band was observed with TS E60A, L or Q (Figure 4.1), but when [6-^{3}H]CH_2H_4folate was used with unlabelled dUMP, the radioactive protein band was formed (Data not shown). Thus, the covalent complex contained both dUMP and CH_2H_4folate. The covalent complexes of these mutants were stable in SDS-gel loading buffer kept overnight on ice or immersed in boiling water for 5 min. The protein-bound radioactivity reached a maximum of 1% of the total amount of TS monomer for TS E60A and Q, and 2% for TS E60L at about 10 min, and then slowly disappeared (Figure 4.1).

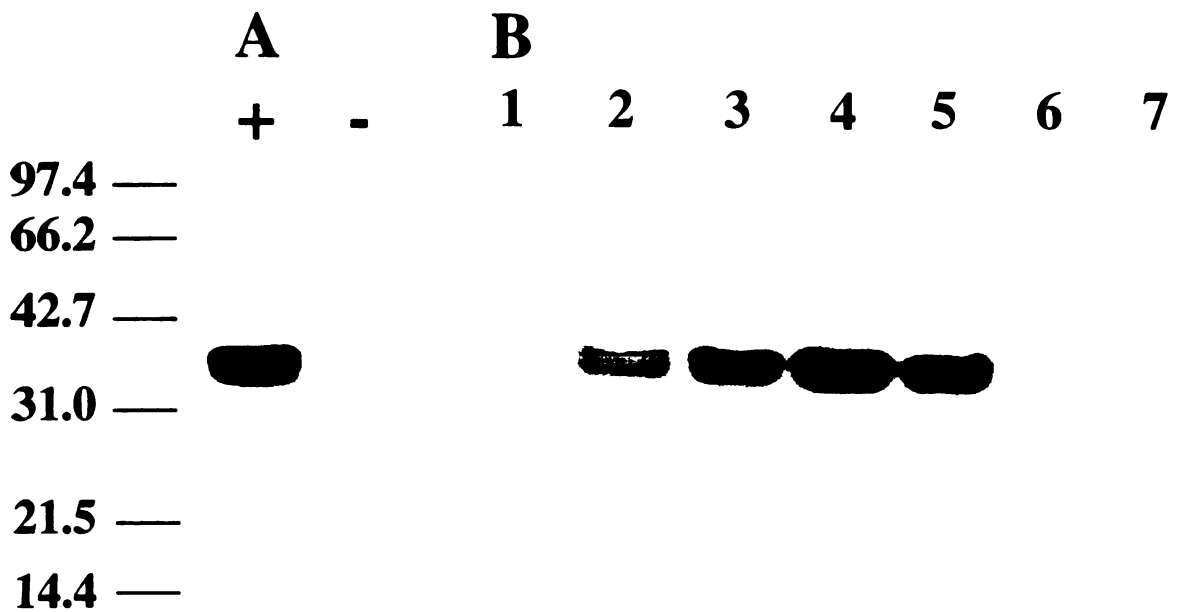


Figure 4.1: Autoradiogram of SDS-PAGE showing the covalent complexes containing TS E60L, dUMP and CH_2H_4folate. (**A**) TS E60L was incubated with [6-^{3}H]dUMP, in the presence (+) or absence (-) of CH_2H_4folate, denatured after 10 min and subjected to SDS-PAGE. (**B**) Formation of the covalent complex over time. TS E60L was incubated with [6-^{3}H]dUMP and CH_2H_4folate, and denatured at 20 s (lane 1), 40 s (lane 2), 1.5 min (lane 3), 10 min (lane 4) 30 min (lane 5), 2 h (lane 6), and 5 h (lane 7). This figure is taken from our previous communication (Huang, 1994b).

The rates of formation and dissociation of the covalent complex with TS E60L were monitored by quantitation of the radioactivity of [6-^{3}H]dUMP associated with the 35 kDa protein band in SDS-PAGE as a function of time. With saturating [6-^{3}H]dUMP and CH_2H_4folate, the apparent first order rate constant for formation of the covalent complex ($k_{app.}$) was calculated (by dividing the initial rate of complex formation by enzyme concentration) to be 1.4 min^{-1}. The first order rate constant for dissociation ($k_{dissoc.}$) of the covalent complex was obtained by adding a 100-fold excess of non-radioactive dUMP after maximal formation of the complex, and monitoring the first order loss of radioactivity associated with the complex; under these conditions, the dissociation of [6-^{3}H]dUMP from the complex is essentially irreversible since once dissociated it is replaced by unlabelled dUMP. The $k_{dissoc.}$ value for TS E60L was 0.07 min^{-1}. After all radioactivity was lost from the covalent complex, HPLC analysis of the reaction mixture demonstrated that the radioactive dUMP was quantitatively converted to dTMP.

Effects of carboxylic acids on Glu-60 mutants. The addition of acetic acid or formic acid to a reaction mixture containing TS E60G, dUMP and CH_2H_4folate greatly increased the rate of dTMP formation (Figure 4.2). That the enhancement by exogenous carboxylic acids was a specific effect on the rate of dTMP formation was shown by the following observations: (i) dTMP formation was blocked by the TS inhibitor FdUMP; (ii) no rate enhancement was seen with TS E60G and 0.5 to 2 M NaCl or sodium phosphate; and (iii) the activity of wild-type TS was not affected by up to 2 M acetic acid or formic acid.

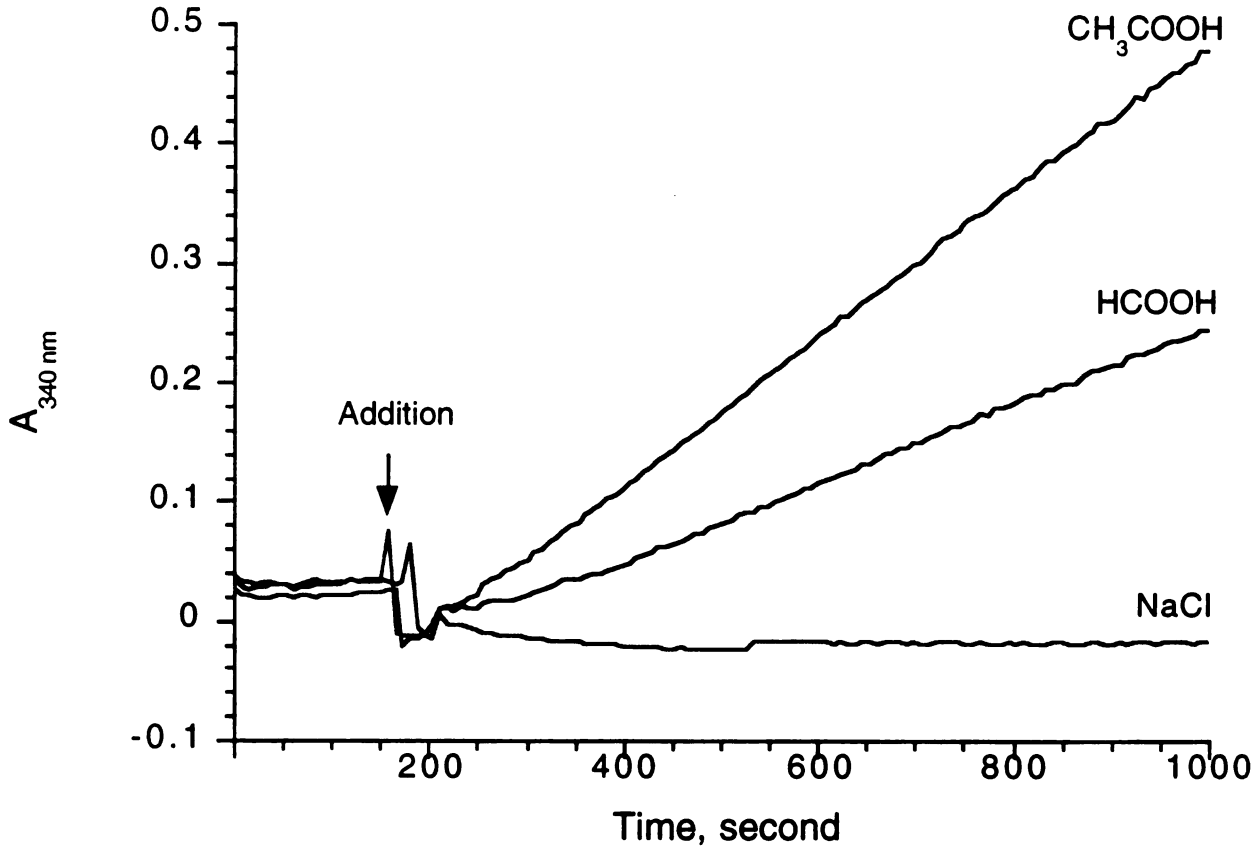


Figure 4.2: Activation of TS E60G by acetic acid and formic acid. The reaction mixture contained 200 μM dUMP, 200 μM CH_2H_4folate, 2 μM TS E60G in standard TES buffer at pH7.4. Sodium acetate, sodium formate or sodium chloride (2 M, final concentration) adjusted to pH7.4 was added to the reaction mixture as indicated.

Table 4.5 shows the effects of 2 M acetic acid and formic acid on the k_{cat} values for dTMP formation by wild-type TS and ten Glu-60 mutants. Wild-type TS and the E60D mutant were not affected by the acids, but the remaining nine Glu-60 mutant enzymes showed 3 to 388-fold rate enhancements for dTMP formation. Four Glu-60 mutants were more efficiently activated by formic acid, one (E60G) was activated more effectively by acetic acid, with equivalent effects of formic acid and acetic acid on the five remaining mutants (Table 4.5). Mutants with smallest side chain volumes, TS E60G and TS E60A, were most efficiently activated by the carboxylic acids. Eight Glu-60 mutants, TS E60F, H, I, K, M, R, W and Y, showed no spectrophotometrically detectable dTMP formation either in the presence or the absence of the carboxylic acids (Table 4.5).

Propionic, glycolic and lactic acids (concentrations up to 2 M) also activated TS E60G (Figure 4.3). Acetic acid analogs containing α-halogen atoms (α-F_2, F_3, Cl_2, Cl_3) activated TS E60G at concentrations <0.1 M (pH 7.4), then inactivated the mutant enzyme at higher concentrations (data not shown). Since these acids also inactivated wild-type TS at concentrations >0.1 M, the latter effects were probably due to denaturation of the protein.

Effects of carboxylic acids on partial TS reactions of TS E60A. The steady-state kinetic parameters for debromination of BrdUMP by TS E60A in the present of 2 M acetic acid or formic acid are given in Table 4.6. The k_{cat} and K_m values for the debromination reaction remained essentially unchanged, but the k_{cat} values for tritium release and dTMP formation by TS E60A were enhanced (Table 4.6). The magnitude of the rate enhancement was greater for dTMP formation than for tritium release.

Table 4.5: k_{cat} values for dTMP formation by wild-type and Glu-60 mutant TSs in the presence of carboxylic

	k_{cat}, min^{-1}, dTMP formation			Ratio of k_{cat}[d]	
TS[a]	No addition[b]	Formic Acid[c]	Acetic Acid[c]	+/- Formic Acid	+/- Acetic Acid
WT	532	486	471	0.9	0.9
E60D	4.0	3.2	2.8	0.8	0.7
E60G	0.034	7.2	13	212	388
E60A	0.021	3.5	1.2	167	57
E60T	0.061	1.2	0.8	20	12
E60L	0.024	0.4	0.4	18	18
E60V	0.041	0.6	0.3	15	6.3
E60Q	0.090	0.8	0.7	8.3	7.2
E60C	0.046	0.3	0.4	6.7	8.3
E60N	0.066	0.4	0.2	5.8	3.2
E60S	0.118	0.4	0.4	3.6	3.4

[a]The eight Glu-60 mutants that are not included in this table did not show spectrophometrically detectable dTMP formation either in the presence or the absence of formic acid and acetic acid. [b]dTMP formation in the absence of carboxylic acids. Data are from Table I with conversion of the units of k_{cat} from s^{-1} to min^{-1}. [c]Formic acid or acetic acid was added to a fininal concentration of 2 M, pH 7.4. [d]Ratio of k_{cat} values in the presence (+) and absence (-) of formic acid or acetic acid.

Table 4.6: Kinetic parameters for TS E60A-catalyzed tritium release from [5-^{3}H]dUMP, dTMP formation, and BrdUMP debromination in the presence of acetic acid and formic acid.[a]

	Tritium release from [5-^{3}H]dUMP and dTMP formation					BrdUMP dbromination	
	k_{cat} (min^{-1})			K_m (μM)			
TS	Tritium release	dTMP formation	Tritium release / dTMP formation	dUMP	CH_2H_4folate	k_{cat} (min^{-1})	K_m (μM)
WT[b]	590	540	1.1	6.8	19	1.2	5.4
E60A[b]	0.42	0.021	20	31	220	3.6	35
E60A + Acetic Acid[c]	1.6	1.2	1.3	6.9	53	3.3	49
E60A + Formic Acid[c]	3.8	3.5	1.1	22	43	5.9	53

[a]Kinetic parameters for tritium release reaction, dTMP formation, and debromination reaction were obtained as described in Table I, Table IV and Materials and Methods. [b]Data are from Table I and Table IV. [c]Acetic acid or formic acid was added to a final concentration of 2 M at pH 7.4.

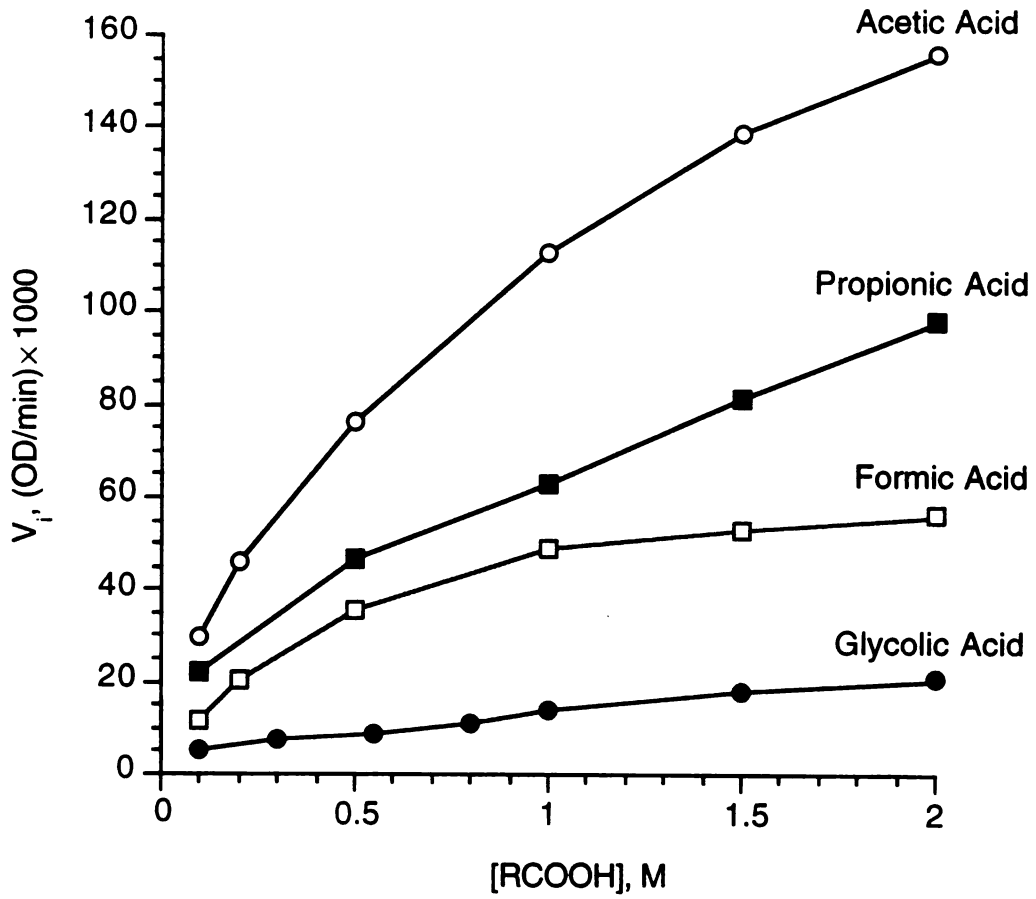


Figure 4.3: Concentration-dependent activation of TS E60G by carboxylic acids. The reaction mixture contained 200 μM dUMP, 200 μM CH_2H_4folate, 2 μM TS E60G in standard TES buffer at pH 7.4. The carboxylic acids were adjusted to pH 7.4 before adding to the reaction mixture.

The ratio of k_{cat} for tritium release to that for dTMP formation, which was 20 for E60A in the absence of added acids, in the presence of 2 M acetic or formic acid decreased to unity, which was equal to the value for the wild-type enzyme. In the presence of 2 M acetic or formic acid, the K_m values for E60A for dUMP for CH_2H_4folate decreased moderately (< 6-fold). In the presence of 2 M acetic or formic acid, no covalent complex of TS E60A was detected by SDS-PAGE (Data not shown).

pH dependent activation of TS E60G by acetic acid. Activation of TS E60G by acetic acid was measured from pH 6.2 to 7.8 (Figure 4.4). dTMP formation increased linearly with concentration of acetic acid (up to 0.8 M) at each pH tested. The rates for dTMP formation in the presence of acetic acid were greater at lower pHs, consistent with a role for the anionic species of the acid in catalysis; however, the rate constants were not linear functions of the concentrations of hydronium ion, and were not further analyzed.

DISCUSSION

Extensive mutagenesis of TS has revealed that many conserved residues are tolerant to amino acid substitutions (Carreras & Santi, 1995). In contrast, mutagenesis of Glu-60 results in a large loss of activity (Tables I and II) (Hardy *et al.*, 1995, Huang & Santi, 1994a, Huang & Santi, 1994b, Zapf *et al.*, 1993), indicating that this residue may represent a truly "essential" residue of TS. Glu-60 has been proposed to be involved in various steps of the TS reaction, including binding and activation of the cofactor CH_2H_4folate; stabilization of the incipient enolate intermediate of dUMP; abstraction of C-5 proton from dUMP, and conversion of the intermediate **IV** (Scheme 4.1) to the products (Finer-Moore *et al.*, 1990, Hardy *et al.*, 1995, Matthews *et al.*, 1990a, Matthews *et al.*, 1990b, Zapf *et al.*, 1993). However,

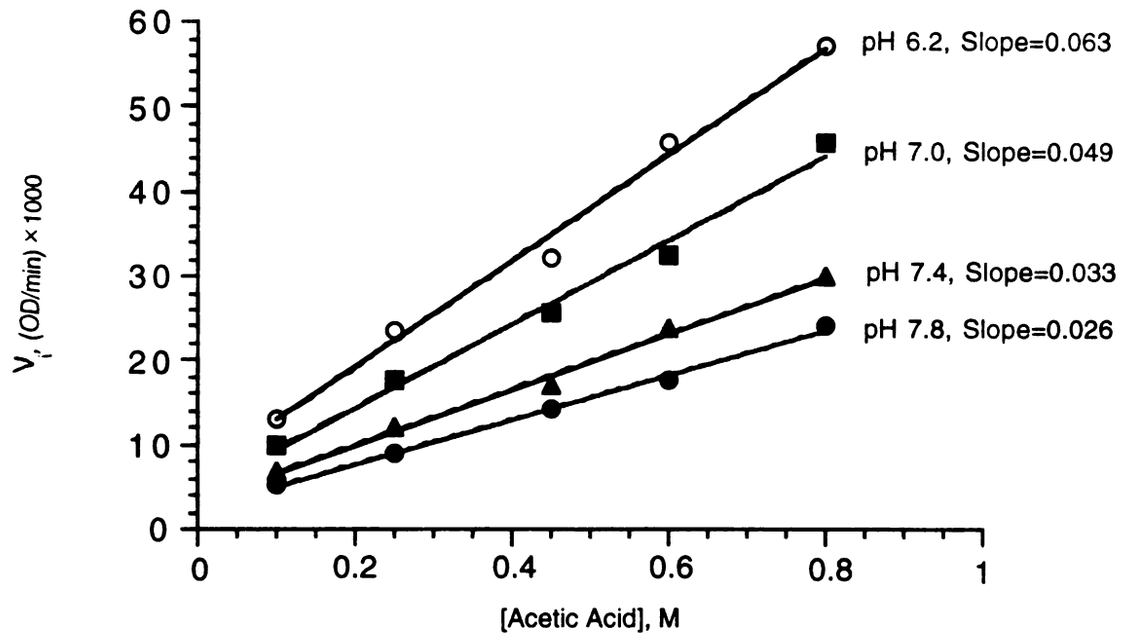


Figure 4.4: The pH dependent activation of TS E60G by acetic acid. The reaction mixture contained 400 μM dUMP, 400 μM CH_2H_4folate, 2 μM TS E60G in standard TES buffer at pH 6.2, 7.0, 7.4, or 7.8. Acetic acid adjusted to pH 6.2, 7.0, 7.4, or 7.8 was added to the reaction mixture with a final concentration ranging from 100 to 800 mM. The ionic strength was maintained at 2.0 by addition of NaCl. dTMP formation was monitored spectrophotometrically at 340 nm.

the catalytic role of Glu-60 has not been clearly identified. The objective of this study was to identify the steps and intermediates in the TS reaction which are affected by mutation of Glu-60.

We report here the preparation, purification and characterization of a replacement set of 18 of the 19 possible Glu-60 mutants. E60P was not studied because it was expressed in an insoluble form. All of the active mutants studied showed large decreases in their ability to catalyze dTMP formation, with reductions of 140-fold for E60D and greater than 5,000-fold for all other mutants (Table 4.1). Selected mutants were subjected to binding studies and steady-state kinetic analysis. Although substrate binding was generally reduced compared to wild-type TS (Tables 4.2 and 4.3), the effects on binding varied depending on the side chain of the mutant.

The TS-catalyzed dehalogenation of BrdUMP or IdUMP is a partial TS reaction, which includes early events of substrate binding and initial covalent complex formation, but does not require CH_2H_4folate as cofactor (Garrett *et al.*, 1979; Carreras *et al.*, 1992). The reaction involves nucleophilic attack at C-6 of Br(I)dUMP by Cys-198 to form a 5-halo-5,6-dihydropyrimidine intermediate analogous to intermediate II in the normal TS reaction (Scheme 4.1), followed by a thiol-mediated dehalogenation, and β-elimination of the enzyme. Despite the large decreases in activity for dTMP formation (Table 4.1), most Glu-60 mutants catalyzed the dehalogenation of BrdUMP and IdUMP as well or more efficiently than did wild-type TS (Table 4.4). For most Glu-60 mutants, the k_{cat} values for the dehalogenation reaction were greater (up to 70-fold) than that for wild-type TS, and the K_m values were within a factor of ten of that for the wild-type enzyme, suggesting that the productive binding of nucleotides was not impaired by the mutations. We conclude that the binding and catalytic events in the dehalogenation of Br(I)dUMP, and probably their counterparts in the normal TS reaction, are not detrimentally affected by mutation of Glu-60.

With wild-type TS, the rate of tritium release from [5-^{3}H]dUMP into solvent correlated with the replacement of the 5-H of dUMP by a CH_3- group; once the 5-H of dUMP is released, the reaction is committed to go on to products. With all Glu-60 mutants except E60D, the k_{cat} for CH_2H_4folate-dependent tritium release from [5-^{3}H]dUMP was significantly greater than that for dTMP formation (Table 4.1), indicating that tritium release occurs primarily from an exchange reaction with solvent protons, rather than by the methylation.

The 5-^{3}H exchange of dUMP by Glu-60 mutants requires the CH_2H_4folate-dependent formation of an intermediate with an acidic proton at C-5, which undergoes ionization (i. e. tritium loss to solvent), proton recapture from solvent and reversal to dUMP faster than methylation. Intermediate **III** (Scheme 4.1), which is believed to be a steady-state intermediate in the TS reaction, fulfills these criteria. Thus, the 5-tritium exchange of [5-^{3}H]dUMP with Glu-60 mutants occurs by a change in partitioning of intermediate **IV** whereby it captures a solvent proton and reverts to intermediate **III** faster than it is methylated to intermediate **V**.

The rapid formation and reversal of putative intermediate **III** compared to its methylation in Glu-60 mutants suggested that the intermediate might accumulate in pre-equilibrium forms of the bound enzyme complex, most likely intermediate **III**, and be isolable by common techniques. The Glu-60 mutants which catalyzed 5-H exchange of [5-^{3}H]dUMP (but not those that did not) also catalyzed the formation and disappearance of a stable covalent complex which was isolable on SDS-PAGE. The complex, which contained TS, dUMP and CH_2H_4folate and slowly converted to dTMP. These results and kinetic considerations suggest that the most likely assignment for the covalent complex is the steady-state intermediate **III**.

To assess whether the isolable covalent complex was kinetically competent as an intermediate in the formation of dTMP, we examined its rates of formation and disappearance. To be a viable intermediate, the rate constant for formation of the

putative intermediate must be equal to or greater than that for tritium exchange from [5-^{3}H]dUMP, and the rate constant for disappearance of the complex must be equal to or greater than dTMP formation. For TS E60L, the apparent rate constant for formation of the ternary complex at saturating concentrations of both substrates was 1.4 min^{-1}, which is equal to or greater than the k_{cat} value (0.98 min^{-1}) for tritium release, and the rate constant for disappearance of the ternary complex of 0.07 min^{-1}, was greater than the k_{cat} (0.03 min^{-1}) for dTMP synthesis (Table 4.1). Thus, the covalent intermediate appears to be kinetically competent as an intermediate in the TS reaction.

The activity of an enzyme mutated at the side chain of a key functional group may sometimes be restored by addition of the functional group as an exogenous, small molecule (Toney & Kirsch, 1989; Phillips *et al.*, 1992;Perona *et al.*, 1994; Carlow *et al.*, 1995). The addition of certain carboxylic acids to TS E60G caused a striking, concentration-dependent increase in the k_{cat} for dTMP formation. The most effective acids were formic, acetic and propionic. In the presence of 2 M formic or acetic acid, nine of the ten Glu-60 mutants examined showed rate enhancements in dTMP formation of up to 400-fold, with the greatest rate enhancements seen for mutants with the smallest side chains, i.e. glycine and alanine, at position 60 (Table 4.5). Thus, it appeared that exogenous carboxylatic acids served as surrogates for the Glu-60 side chain in these mutants.

Wild-type TS and TS E60D were not activated by formic or acetic acids. The mutants with positively charged or aromatic side chains at position 60 did not show measurable TS activity in the absence and the presence of the acids.

If the carboxylic acids were true surrogates for Glu-60, the same reaction steps affected by the mutations should be partially restored by the acids. We therefore studied the effects of added formic and acetic acids on the steady-state kinetic parameters for dTMP formation, 5-H^3 release and BrdUMP dehalogenation of TS E60A (Table 4.6). As mentioned earlier, in the presence of either acid, the k_{cat} values for

dTMP formation and tritium release were increased for the E60A mutant, with a relatively greater effect on dTMP formation. The E60A mutant seems to have undergone a change in rate determining step from before formation of intermediate **IV** to after. Mutantion at Glu-60 did not affect the dehalogenation of BrdUMP (Table 4.4), and for E60A, dehalogenation of BrdUMP was essentially unchanged upon treatment with formic or acetic acids. Thus, exogenous carboxylic acids partially restore or do not affect the same steps as does the Glu-60 mutation, and we conclude that they act as true surrogates of Glu-60.

In summary, Glu-60 is critical to the TS reaction. The residue does not contribute greatly to substrate or cofactor binding, but plays a role in both the formation and breakdown of the covalent intermediate **IV**. TS Glu-60 mutants show differential effects on these partial reactions, with the greater effect occurring during the conversion of **IV** to products. The previously proposed roles for Glu-60 that remain consistent with our data include (i) stabilizing the incipient enolate intermediate of dUMP via a water molecule hydrogen bonded to O-4 of dUMP (Finer-Moore *et al.*, 1990), and (ii) facilitating bond cleavage between N-5 and the methylene group of CH_2H_4folate via electrostatic destabilization by the side-chain carboxylate of Glu-60 (Hardy *et al.*, 1995). Because of the change in partitioning of **IV**, there is a buildup of the steady-state intermediate **III** which can be isolated under denaturing conditions. The ability to separate the TS reaction pathway into components may allow experimental approaches to a further assignment of the role of Glu-60 in the TS reaction.

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CHAPTER V

Active Site General Catalysts are not Necessary for Some Proton Transfer Reactions of Thymidylate Synthase¶

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ABSTRACT

Several steps of the reaction catalyzed by thymidylate synthase (TS) require proton transfers to and from O-4 and C-5 of the pyrimidine moiety of substrate dUMP. It has been proposed that one or more of three active site residues — Glu60, His199 and Asn229 — together with ordered water molecules serve as general catalysts in facilitating such proton transfers. These three residues, individually and together, were mutated to residues incapable of proton transfer, and the mutant enzymes were purified and tested for activity in the formation of dTMP and the dehalogenation of 5-bromo- and 5-iodo-dUMP. The dehalogenation reaction pathway shares at least two direct chemical counterparts with the TS reaction pathway which are believed to involve general acid/base catalysis — namely, the addition and elimination of the catalytic Cys of TS at C-6 of the pyrimidine substrate. Generally, the mutations had detrimental effects on dTMP synthesis with the triple mutant being completely inactive. In contrast, single mutants TS E60L and H199A and, interestingly, the triple mutant stripped of all three active site catalysts catalyzed the dehalogenation reaction as well as or better than the wild-type enzyme. It was concluded that addition and elimination reactions involving the 5,6-bond of pyrimidine substrates do not require general acid/base catalysis or, alternatively, the water molecules in the TS active site serve this role. The function(s) of the triad of general catalysts resides elsewhere in the reaction pathway leading to dTMP synthesis.

Thymidylate synthase (TS[7]; EC 2.1.1.45) catalyzes the reductive methylation of dUMP by 5,10-CH_2H_4folate to give dTMP and H_2folate. The structure, catalytic mechanism and mutagenesis of TS has been of continuing interest to this laboratory (for a recent review see Carreras & Santi, 1995).

An initiating event in the catalytic mechanism of TS involves nucleophilic attack of a conserved Cys residue of the enzyme at C-6 of dUMP to give a 5,6-dihydropyrimidine intermediate (**II** in Scheme 5.1) (Santi & Danenberg, 1984; Carreras & Santi, 1995). All subsequent covalent bond modifications involved in the reductive methylation at C-5 of dUMP occur on the covalently bound intermediate, and upon completion there is a β-elimination of the catalytic Cys from C-6 of the pyrimidine to give the product dTMP and regenerated enzyme.

The chemistry of the TS reaction requires several proton transfers to and from O-4 and C-5 of the pyrimidine of dUMP (Scheme 5.1). It has been proposed that three active site residues — Glu60[8], His199 and Asn229 — in a hydrogen bond network together with ordered water molecules serve as general catalysts in facilitating these proton transfers (Liu & Santi, 1993; Huang & Santi, 1994; Carreras & Santi, 1995). Because of the multiple steps in the TS pathway, it has been difficult to identify the contributions of given residues to a particular step in the pathway. For this reason, "partial reactions", which undergo one or a few of the steps in the normal catalytic pathway, have been developed and used to study isolated steps in the TS pathway (Garrett *et al.*, 1979; Carreras & Santi, 1995). One such reaction is the CH_2H_4folate-independent dehalogenation of 5-

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7 Abbreviations used : TS, thymidylate synthase; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; BrdUMP, 5-bromo-2'-deoxyuridine-5'-monophosphate; IdUMP, 5-iodo-2'-deoxyuridine-5'-monophosphate; TES, N-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

8 The numbering system of *L. casei* TS is used.

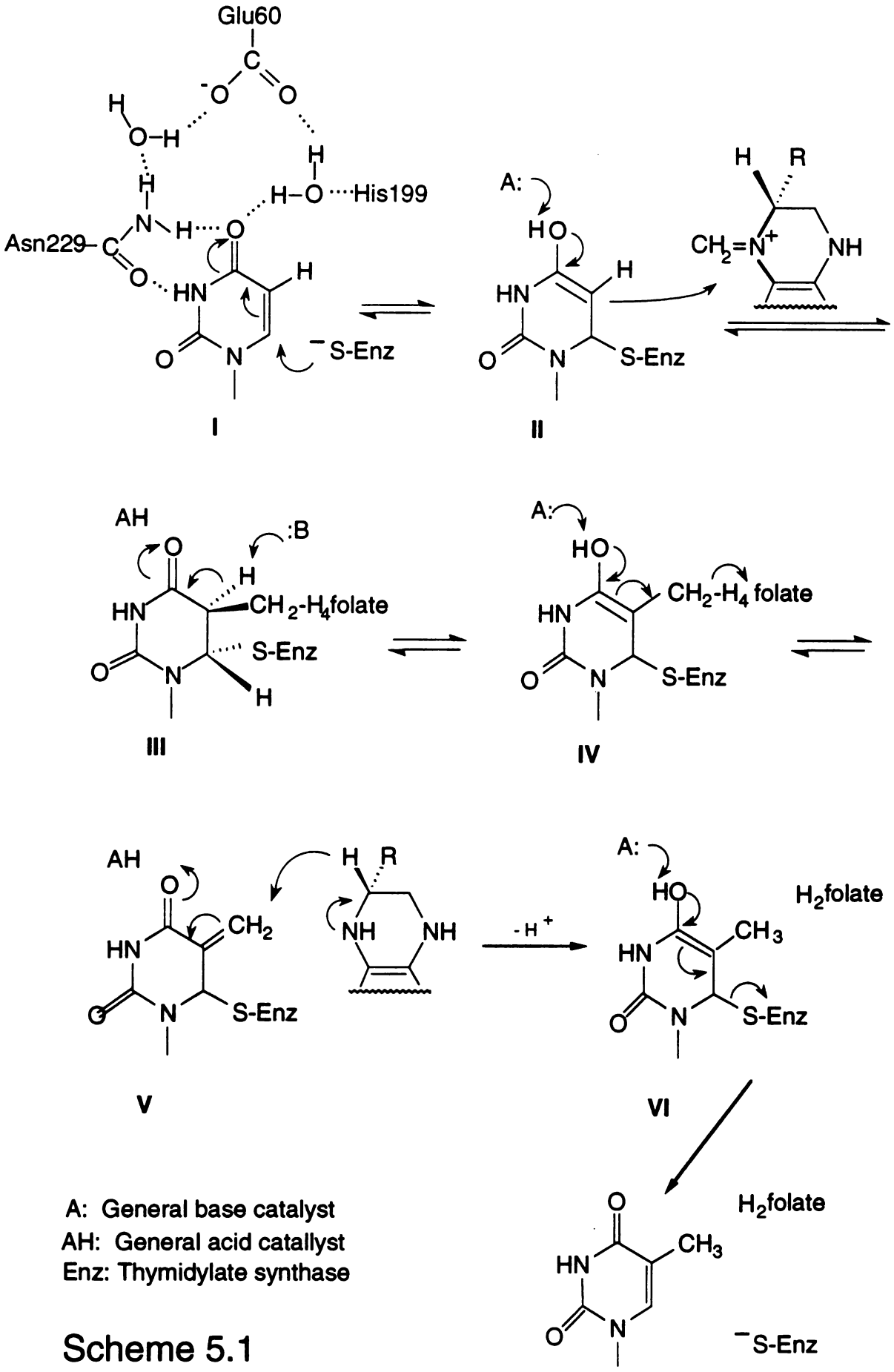
Br- and 5-IdUMP (Scheme 5.2) (Garrett, *et al.*, 1979), which shares at least two direct chemical counterparts with the TS pathway: (i) nucleophilic addition of the catalytic Cys to C-6 of the pyrimidine to form a 5-halo-5,6-dihydropyrimidine intermediate (**III** in Scheme 5.2), and (ii) elimination of the Cys from C-6 in the final step of the dehalogenation reaction.

The objective of the present work was to assess and compare the importance of residues of the hydrogen bond network in dTMP synthesis and in dehalogenation of 5-Br(I)dUMP. We first mutated Glu60, His199 and Asn229 individually to residues incapable of proton transfer reactions; then we mutated all the residues together to produce an enzyme stripped of active site general catalysts. Our results indicate that the hydrogen bond network is essential for dTMP formation, but not for the catalytic events that occur in the dehalogenation reaction.

MATERIALS AND METHODS

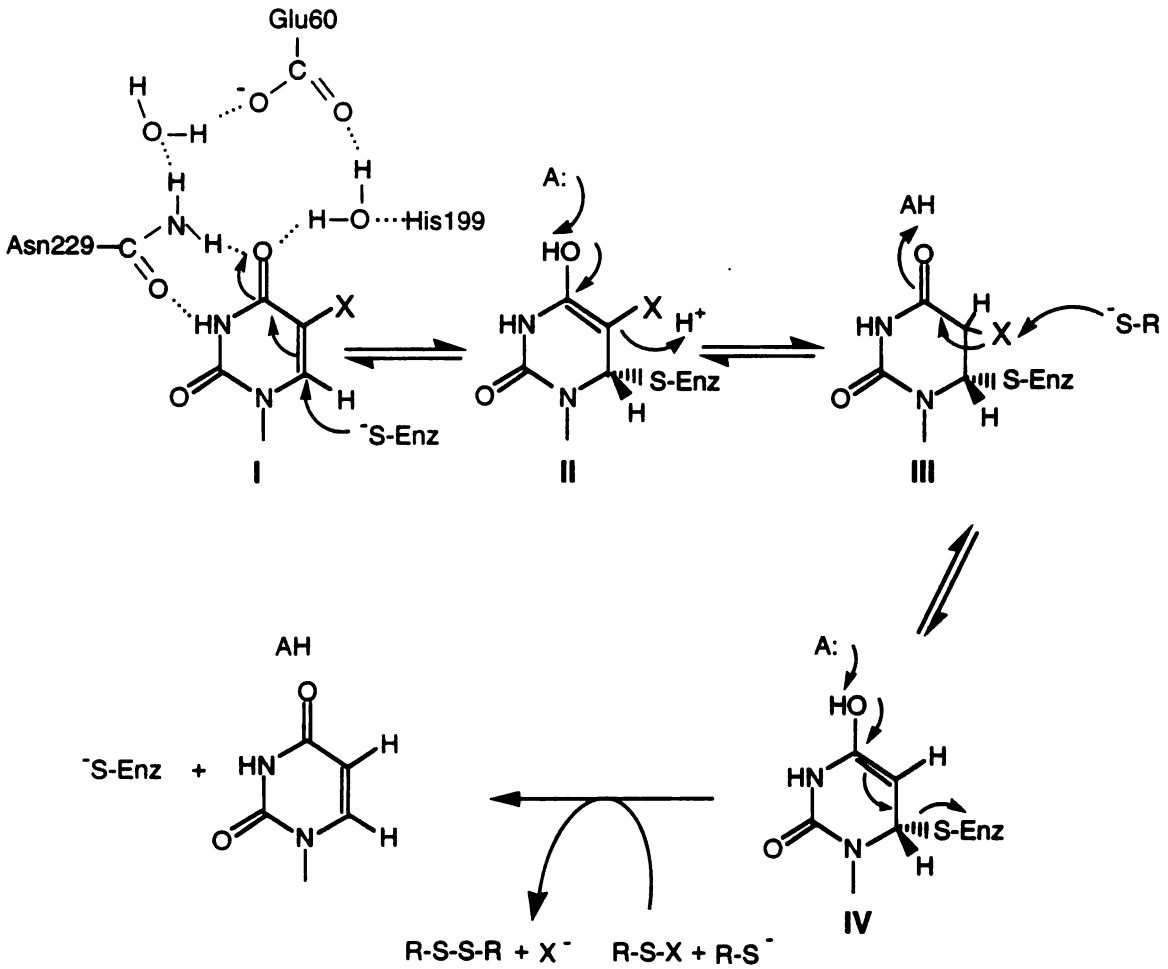
Materials. E. coli χ2913recA (*Δthy*A572, *recA*56) (Climie *et al.*, 1992) and plasmid pSCTS13 and pSCTS9 have been described (Climie & Santi, 1990). Oligonucleotide synthesis and DNA sequencing were performed at the UCSF Biomolecular Resource Center. (6*R*)-CH_2H_4folate was a generous gift from SAPEC S.A. (Lugano, Switzerland) and EPROVA AG (Schaffhausen, Switzerland).

Mutagenesis and Protein Purification. The E60L, H199A and N229V single mutants were constructed by cassette mutagenesis of the *L. casei* TS synthetic gene in plasmid pSCTS13 or pSCTS9 as described (Huang & Santi, 1994; Climie *et al.*, 1990; Liu & Santi, 1993). The E60L/H199A/N229V triple mutant was constructed from the E60L single mutant and the H199A/N229V double mutant as follows. First, the H199A/N229V double mutant was constructed as follows: plasmid DNAs of TS



A: General base catalyst
AH: General acid catalyst
Enz: Thymidylate synthase

Scheme 5.1



X: Br or I
A: General base catalyst
AH: General acid catalyst
Enz: Thymidylate synthase

Scheme 5.2

H199A and N229V were each digested with *EcoR* I and *Bgl* II; the small DNA fragment containing the H199A mutation and the large DNA fragment containing the N229V mutation were purified by 1% agarose gel electrophoresis and ligated to give the H199A/N229 double mutant. The E60L/H199A/N229V triple mutant was then constructed as follows: plasmid DNAs of TS E60L and TS H199A/N229V were each digested with *Nco* I and *EcoR* I; the small DNA fragment containing the E60L mutation and the large DNA fragment containing the H199A/N229V mutations were purified and ligated to give the E60L/H199A/N229V triple mutant. The plasmid DNAs of the single and triple mutants were individually isolated and sequenced. Mutant enzymes were purified by chromatography on phosphocellulose and hydroxyapatite as described (Kealey & Santi, 1992). Enzyme preparations were concentrated using Centriprep-30 concentrators (Amicon) and stored at -80 °C in 10 mM KH_2PO_4, pH 7.0, 1 mM EDTA, until use. The concentrations of the purified enzymes were determined spectrophotometrically using ε_{278} = 125,600 M^{-1} cm^{-1} (Carreras *et al.*, 1994), with the assumption that the mutations did not affect the extinction coefficient.

Enzyme assays. dTMP formation was monitored spectrophotometrically at 340 nm as described (Pogolotti et al., 1986). The standard TES buffer contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA and 75 mM β-mercaptoethanol. For the E60L mutant and the E60L/H199A/N229V triple mutant, dTMP formation was monitored by HPLC analysis (Huang & Santi, 1994). The steady-state kinetics for TS E60L were monitored by the TS-catalyzed release of tritium from [2-^{14}C, 5-^{3}H]dUMP as described (Huang & Santi, 1994).

TS-catalyzed dehalogenation of BrdUMP or IdUMP was monitored by the decrease in absorbance at 285 nm ($\Delta\varepsilon_{285}$ = 5,320 M^{-1} cm^{-1}) or 290 nm ($\Delta\varepsilon_{290}$ = 6,520 $M^{-1}cm^{-1}$) that accompanies dehalogenation of BrdUMP or IdUMP, respectively (Garrett, *et al.*, 1979). Reaction mixtures contained TES/DTT assay buffer (50 mM TES, pH 7.4, 6.5 mM formaldehyde, 25 mM $MgCl_2$, 1 mM EDTA and 10 mM DTT), 3 to 200 μM BrdUMP

or IdUMP and 0.12 to 3 μM of enzyme. Upon completion of the reaction, 0.5 μM wild-type TS and 200 μM CH_2H_4folate were added to the reaction mixture. The product of the dehalogenation reaction, dUMP, was then confirmed by conversion of dUMP to dTMP which was measured spectrophotometrically at 340 nm (Garrett, *et al.*, 1979). For TS N229V, enzyme concentrations of up to 10 μM and incubations of up to 4 h were used in attempts to detect dehalogenation. The limit of detection for k_{cat} was 0.006 min^{-1} in this assay.

Steady-state kinetic parameters were obtained by a non-linear least squares fit of the data to the Michaelis-Menten equation using the program Kaleidagraph (Abelbeck Software, Reading, PA, 1989). For kinetic measurements in which the enzyme concentration exceeded 1 μM, data were fit to an equation that corrects for ligand depletion by protein (Segel, 1975).

RESULTS

TS mutants were constructed by cassette mutagenesis of the *L. casei* TS synthetic gene, and the mutations were confirmed by DNA sequencing. The TS mutants were expressed at levels of 10 to 30% of the total cellular protein in soluble crude extracts, as determined by 12% SDS-PAGE and comparison of specific activity of the purified proteins to that of the crude extract. The mutant enzymes were purified by phosphocellulose and hydroxyapatite chromatography (Kealey & Santi, 1992) to apparent homogeneity as assessed by SDS-PAGE.

dTMP formation catalyzed by wild-type and mutant TSs were determined (Table 5.1). Decreases in rates of dTMP formation were observed for all mutant enzymes. There was an approximately 10-fold decrease in k_{cat} of dTMP formation for TS

Table 5.1: dTMP formation by wild-type and mutant TSs[a]

TS	k_{cat}, min^{-1}	K_m, μM		k_{cat}/K_m, min^{-1} μM^{-1}	
		dUMP	CH_2H_4folate	dUMP	CH_2H_4folate
Wild-type	540	6.8	19	79	28
E60L	2×10^{-2}	220	21	9×10^{-5}	10×10^{-3}
H199A	41	5.2	19	7.9	2.2
N229V	57	128	40	0.4	1.4
E60L/H199A/N229V	$< 1 \times 10^{-4}$	ND[b]	ND		

[a]dTMP formation by wild-type TS, TS H199A and TS N229V was monitored spectrophotometrically at 340 nm. k_{cat} values for TS E60L and TS E60L/H199A/N229V were obtained from HPLC analysis. K_m values for dUMP and CH_2H_4folate for TS E60L were obtained by the TS-catalyzed tritium release from [5-^{3}H]dUMP as previously described (Huang & Santi, 1994). The standard errors from nonlinear least squares fit of the experimental data are less than 20% for all values. [b]ND: Not determined.

H199A and TS N229V. Similar data for TS N229V has been reported (Liu & Santi, 1993). There was a pronounced (~3×10^4-fold) decrease in k_{cat} value for TS E60L, and no detectable dTMP formation (k_{cat} value < 1×10^{-4} min^{-1}) for the E60L/H199A/N229V triple mutant. For the TS mutants studied, the K_m for dUMP was either unchanged or moderately increased (<32-fold), and the K_m for CH_2H_4folate increased less than 3-fold, compared to wild-type TS (Table 5.1).

As described for wild-type TS (Garrett, *et al.*, 1979), TS E60L, TS H199A and TS E60L/H199A/N229V catalyzed the thiol dependent CH_2H_4folate-independent dehalogenation of BrdUMP and IdUMP to produce dUMP. When BrdUMP or IdUMP was reacted with TS E60L in the presence of DTT, the spectra were converted to that of dUMP (Figure 5.1). After completion of the reaction, wild-type TS and CH_2H_4folate were added, and the product was verified to be >90% of dUMP by its conversion to dTMP. Thus, the major product of dehalogenation of either BrdUMP or IdUMP was dUMP. The conversion of BrdUMP to dUMP by TS E60L was also confirmed by HPLC analysis (Data not shown). The rate of debromination of BrdUMP by TS E60L increased with increasing amount of DTT, and reached half-maximum rate when the concentration of DTT was 0.7 mM. The concentration of DTT used in the standard reaction mixture was 10 mM, which is more than sufficient to saturate the enzyme for the dehalogenation reaction.

Table 5.2 shows the steady-state kinetic parameters for dehalogenation of BrdUMP and IdUMP by the wild-type and mutant enzymes. The dehalogenation reaction was monitored spectrophotometrically at 285 nm for BrdUMP and at 295 nm for IdUMP. For TS E60L, there were 70-fold and 22-fold increases in k_{cat} values for dehalogenation of BrdUMP and IdUMP, respectively, compared with wild-type TS. For TS H199A, k_{cat} values for dehalogenation of BrdUMP and IdUMP were similar to and 6-fold higher than those for wild-type TS, respectively. TS N229V was inactive in dehalogenation of BrdUMP or IdUMP (k_{cat} < 0.006 min^{-1}). The k_{cat} values for

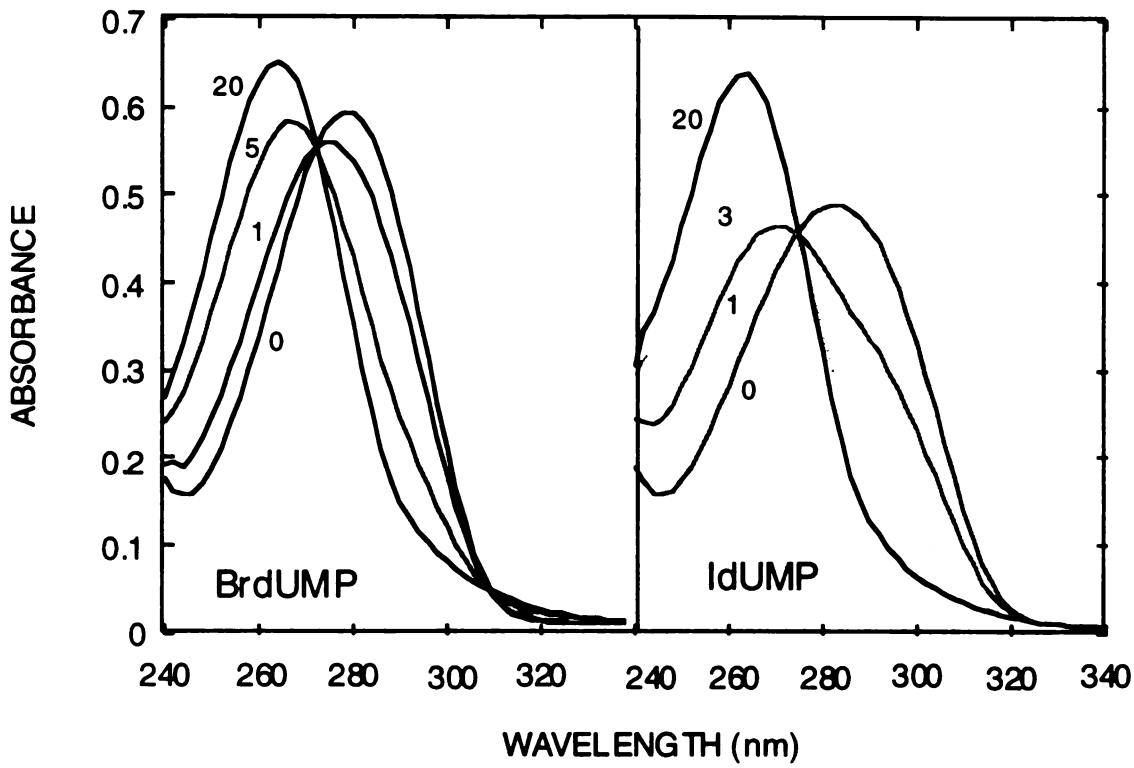


Figure 5.1: Ultraviolet spectral changes during TS E60L catalyzed dehalogenation of BrdUMP and IdUMP. Reaction mixtures contained standard TES buffer, 0.18 μM TS E60L, 10 mM DTT and 50 μM BrdUMP or IdUMP. The numbers shown indicate the reaction time in minutes.

Table 5.2: Dehalogenation of BrdUMP and IdUMP by wild-type and mutant TSs[a]

TS	BrdUMP			IdUMP		
	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m, $min^{-1}.\mu M^{-1}$	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m, $min^{-1}.\mu M^{-1}$
Wild-type	1.2	5.4	0.2	4.1	9	0.5
E60L	84	22	3.8	90	6.9	13
H199A	0.9	8.5	0.1	24	19	1.3
N229V	<0.006	ND[b]		<0.006	ND	
E60L/H199A/N229V	0.8	39	0.02	3.4	40	0.08

[a]Dehalogenation of BrdUMP and IdUMP was monitored spectrophotometrically at 285 nm and 290 nm, respectively For TS N229V, the dehalogenation reaction was also monitored by HPLC. The standard errors from nonlinear least squares fit of the experimental data are less than 15% for all values. [b]ND: not determined.

dehalogenation of BrdUMP and IdUMP for the E60L/H199A/N229V triple mutant were similar to those for wild-type TS. K_m values for BrdUMP and IdUMP for all mutant enzymes were either unchanged or moderately increased (< 8-fold) compared to those for wild-type TS.

DISCUSSION

Several steps of the TS reaction require proton transfer to and from O-4 and C-5 of the pyrimidine of substrate dUMP (Scheme 5.1). These include (1) formation of the 5,6-dihydropyrimidine covalent intermediate (**II**) by nucleophilic attack of the catalytic Cys198 at C-6 of dUMP, (2) condensation of C-5 of the 5,6-dihydropyrimidine intermediate with cofactor CH_2H_4folate, (3) β-elimination of H_4folate from the intermediate (**IV**) to give a reactive exocyclic methylene intermediate (**V**), (4) reduction of that intermediate, and finally (5) elimination of the enzyme from C-6 of intermediate **VI** to give dTMP and free enzyme. Chemical considerations of proton transfer reactions at such poorly basic or acidic sites[9] led to the proposal that general acid/base catalysts of the enzyme might assist in these reactions (Santi & Danenberg, 1984; Carreras & Santi, 1995). Structural evidence suggests that the side chains of three conserved residues— Glu60, His199 and Asn229 — of the protein are contenders for the putative general catalysts. These three residues, together with at least two conserved water molecules, form a hydrogen bond network which positions labile protons within hydrogen bonding distance of O-4 of dUMP, and which is in close proximity to C-5 of dUMP (Finer-Moore *et al.*, 1990; Matthews *et al.*, 1990;

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[9] The pK_a of protonated a O-4 of a uracil is estimated to be ca. -4 (Sobell & Tomita, 1964; Shapiro & Danzig, 1972). The pK_a of the 5-H of the 5,6-dihydropyrimidine is estimated to be at least 20, and tof he pK_a of its corresponding enol is estimated to be at least 12 (Liu & Santi, 1993).

Finer-Moore *et al.*, 1993). As such, these residues, together with active site water molecules, are the only contenders for the general catalyst(s) in the TS reaction.

In multi-step pathways as complex as the TS reaction, it is difficult to assign the contributions of specific amino acids to individual steps. For this reason, several "partial reactions" of TS, which undergo one or a few steps in the normal catalytic pathway, have been developed (Garrett, *et al.*, 1979). Studies of such reactions greatly simplify the chemistry, and permit analysis of the effects of mutations on isolated steps of the TS pathway. One such reaction is the CH_2H_4folate-independent dehalogenation of Br(I)dUMP which shares two direct chemical counterparts with the TS pathway: (i) nucleophilic addition of the catalytic Cys to C-6 of the pyrimidine substrate to form a 5-halo-5,6-dihydropyrimidine intermediate, and (ii) elimination of the enzyme from C-6 of the dihydropyrimidine intermediate in the final step of the reaction.

We have mutated each of the possible three general catalysts of TS to a residue incapable of proton transfer, and then mutated all three residues together to strip the active site of all possible general catalysts. The mutants were examined for their effects on dTMP synthesis and the reactions involved in dehalogenation of Br- and IdUMP. The uncoupling of effects of the mutations on dTMP synthesis and the dehalogenation reactions allowed us to speculate on the contributions of these residues in the TS pathway.

TS E60L, H199A and N229V were chosen for study because individually they provided catalytically active enzymes and side chains incapable of proton transfer. TS H199A showed the smallest alteration in the kinetics of dTMP formation compared to wild-type TS, with similar substrate K_m values, but about a 10-fold decrease in k_{cat}. TS N229V showed a similar K_m for the cofactor, a 20-fold increase in K_m of dUMP and a 10-fold decrease in k_{cat} of dTMP formation compared to wild-type TS. The most affected single mutant was TS E60L which showed a large increase (30-fold) in the K_m for dUMP, and a very large decrease (3×10^4-fold) in k_{cat}. The k_{cat} value for dTMP formation with

the E60L/H199A/N229V triple mutant was decreased by at least 5×10^6-fold; the mutant was essentially inactive in catalyzing dTMP formation. It is not surprising that the triple mutant was inactive, since the active site has been stripped of all residues containing groups which could serve as general catalysts.

The detrimental effects of these mutations on dTMP formation contrasted with their effects on the partial reactions involving the dehalogenation of BrdUMP and IdUMP (Tables 5.1 and 5.2). Whereas TS E60L was an extremely poor catalyst for dTMP formation, it catalyzed the dehalogenation reaction *more* effectively than wild-type TS. Mutation of His199 to Ala resulted in a 10-fold decrease in k_{cat} for dTMP formation, but no significant effects on k_{cat} for the dehalogenation reactions. Inexplicably, TS N229V, which showed only a 10-fold decrease in k_{cat} for dTMP formation, was inactive in the dehalogenation reactions (Liu & Santi, 1993). Most importantly, the E60L/H199A/N229V triple mutant, which was inactive in dTMP formation, had k_{cat} values for dehalogenation of BrdUMP and IdUMP that were about the same as wild-type TS. Thus, completely stripping the active site of the putative general catalysts had little effect on the dehalogenation reaction.

Our results clearly show that the triad of active site residues — Glu60, His199 and Asn229 — are required for dTMP formation, but do not contribute to the dehalogenation of BrdUMP or IdUMP. Since the dehalogenation reaction mimics certain catalytic events in dTMP synthesis, it is reasonable to conclude that these three residues of the active site may not be essential for the counterpart events in the normal TS pathway. Thus, nucleophilic attack of Cys198 at C-6 of the pyrimidine of the substrate and formation of a 5,6-dihydropyrimidine may only require juxtapositioning of the reactive moieties within the protected confines of the active site of TS, and may not require other catalysts. The caveat to this proposal is that the 5-halo pyrimidines may be sufficiently reactive towards nucleophiles that they might not require general acid assistance, whereas dUMP does. Alternatively, the structural water molecules of the active site may provide assistance

with the 5-halo pyrimidines, but may be insufficient in doing so in dTMP synthesis. The other reaction common to pathways of both dTMP formation and Br(I)dUMP dehalogenation is the elimination of the enzyme from the 5,6-dihydropyrimidine adduct. Here, the elimination occurs from covalent intermediates which differ only by the 5-methyl group for dTMP formation, versus a 5-H for the dehalogenation reaction. Since β-elimination of the enzyme occurs in the absence of any active site general catalysts, we conclude that the elimination occurs independently of such catalysts, but possibly with the assistance of water molecules in the active site.

In summary, we have shown that the TS-catalyzed dehalogenation of Br(I)dUMP, which has two direct counterparts to catalytic steps in dTMP synthesis, does not require the assistance of putative active site general catalysts. We propose that either these steps do not require general catalysis or, more likely, that water molecules of the active site of the enzyme serve such roles. Thus, at least some steps of the TS reaction — i. e., nucleophilic attack of Cys and β-elimination of Cys to give products — may occur simply by the appropriate positioning of the substrate in an active site possessing strategically positioned water molecules and favorable thermodynamic properties of the reactions catalyzed. It will be of interest to determine whether the active site water molecules of native TS believed to directly donate to and accept protons from O-4 of dUMP (Finer-Moore *et al.*, 1990) are also present in the triple mutant which is completely stripped of putative active site general catalysts.

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CHAPTER VI

Functional Interactions among the Glu60, His199 and Asn229 Catalysts in the Active Site of Thymidylate Synthase¶

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Key Words: thymidylate synthase; nonadditivity; energetic interaction; multiple mutant cycle.

Running Title: Functional interactions of active site residues in TS.

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ABSTRACT

The side chains of E60, H199 and N229 hydrogen bond to ordered water molecules and the pyrimidine of substrate dUMP, forming an extensive hydrogen-bond network in the active site of thymidylate synthase (TS). Quantitative evaluation of the interactions among the network residues is important to understanding how the hydrogen-bond network functions in the TS reaction. Here we report an energetic analysis of the mutational effects of the single E60D, H199A, N229V, and the corresponding double and triple mutant enzymes. Increases in free energy barriers to dissociation constant (K_d) for dUMP, k_{cat} of dTMP formation, and rate of association (k_1) for dUMP to free enzyme were observed in all mutant enzymes, indicating contributions of the network to various steps of the TS reaction pathway. Nonadditive mutational effects were observed for all multiple mutants and the free energy changes measured in the multiple mutants were generally less than the sum of those derived from the corresponding single mutations. Thus, the network residues interact and synergism is likely a common underlying mechanism by which these residues function. Interaction free energies between E60, H199, and N229 and their dependency on the third residue were quantified by using a triple mutant cube (First & Fersht, 1995). The results provide a detailed picture of the energetic interactions among the network residues and quantitative contributions of these interactions to various steps of the TS reaction pathway.

INTRODUCTION

Thymidylate synthase (TS[10], EC 2.1.1.45) catalyzes conversion of CH_2H_4folate and dUMP to dTMP and H_2folate. X-ray crystallographic studies have revealed a hydrogen bond network in the active site of TS which includes the side chains of conserved residues E60[11], H199 and N229, ordered water molecules, and the pyrimidine ring of dUMP (**Figure 6.1**) (Finer-Moore *et al.*, 1990; Matthews *et al.*, 1990). The hydrogen-bond network has been proposed to facilitate proton transfer reactions to and from the 4-oxygen of dUMP, which are believed to be required for several steps in the TS reaction (Finer-Moore *et al.*, 1990; Huang & Santi, 1994b; Carreras & Santi, 1995). Since the side chains of E60, H199 and N229 provide the only potential general catalysts in the TS active site, understanding the roles of these residues and the functional interactions among them is important toward understanding how the hydrogen-bond network functions in the TS reaction.

Numerous conserved residues of TS have been individually mutated in attempts to determine their contributions to catalysis (Climie *et al.*, 1990; Carreras & Santi, 1995). The surprising general finding has been that only a few residues in TS seem to be truly essential. One hypothesis to explain this finding is that many residues of TS may interact, and when one residue is disabled, another can support its function – an effect termed functional "plasticity" (Santi *et al.*, 1990; Perry *et al.*, 1990). Individual mutations of residues E60, H199 and N229 have indicated that none of these are completely essential for the TS reaction, although mutants of E60 have very low TS activity (Huang & Santi, 1996b; Climie *et al.*, 1990; Liu & Santi, 1993). Given that the

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[10] Abbreviations used : TS, thymidylate synthase; dUMP, 2'-deoxyuridine-5'-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dTMP, 2'-deoxythymidine-5'-monophosphate; H_2folate, 7,8-dihydrofolate; TES, N-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid; PLP, pyridoxal 5'-phosphate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

[11] We use the numbering system of *L. casei* TS.

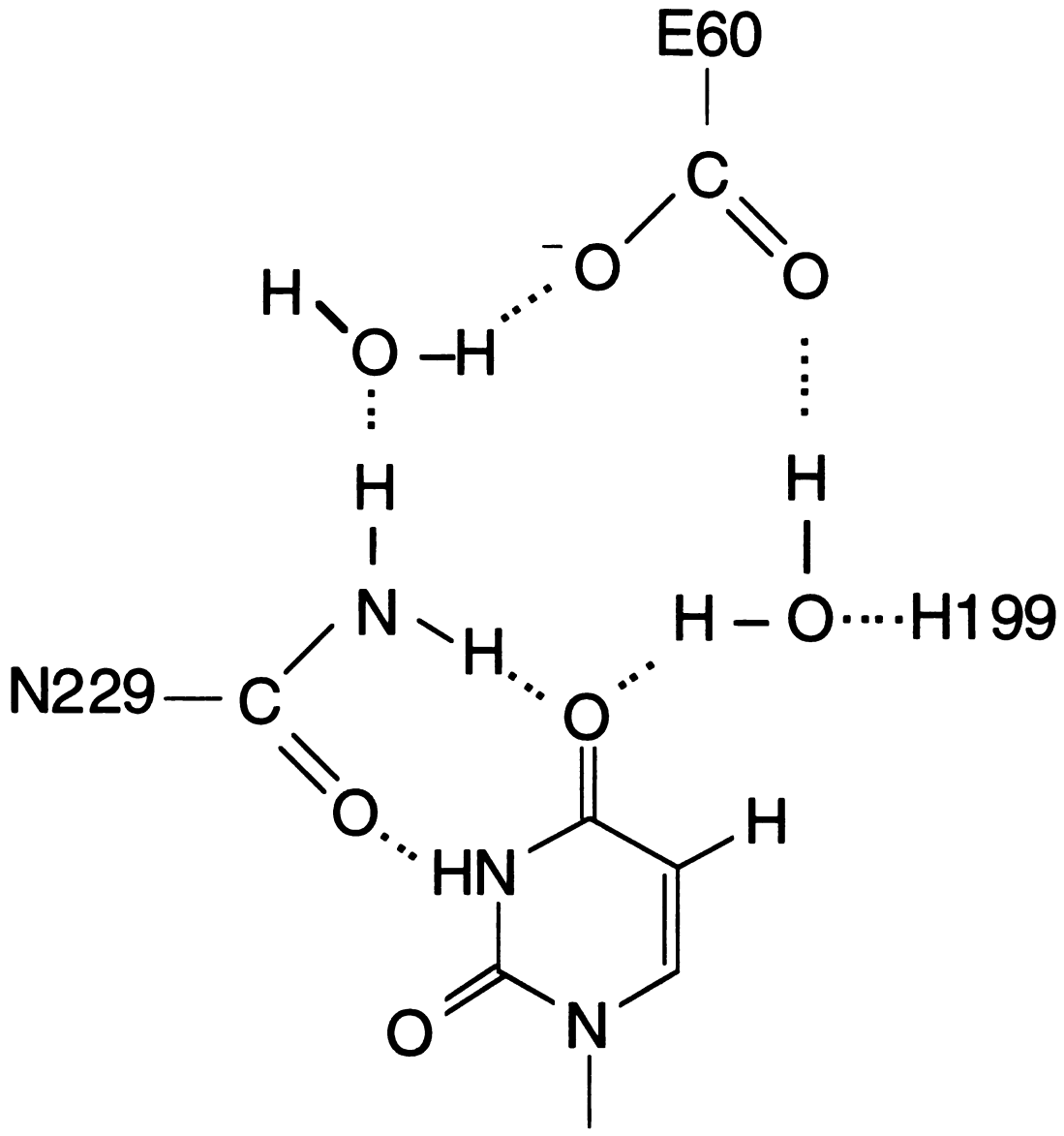


Figure 6.1: Schematic diagram of the water-mediated hydrogen bond network in the active site of thymidylate synthase (Finer-Moore *et al.*, 1990).

side chains of these residues are involved in a common hydrogen-bond network, it is reasonable to speculate that they may interact with each other; if so, the individual mutations may not reveal the true importance of the H-bond network in catalysis.

In the present work, we sought to ascertain whether there was a functional interaction among the three residues of the hydrogen bond network, and if so, to quantify effects of the interaction on the TS reaction. TS E60D, H199A, N229V, and the corresponding double and triple mutants were constructed, expressed, and purified. Effects of the mutations on binding constants, catalytic constants, and rates of association of dUMP with free enzyme were determined. Energetic interactions between E60, H199, and N229 on these parameters were quantified and analyzed using a triple mutant cube (Horovitz & Fersht, 1990; Steyaert & Wyns, 1992; First & Fersht, 1995).

MATERIALS AND METHODS

Materials. E. coli χ2913recA (*ΔthyA*572, *recA*56) (Climie *et al.*, 1992) and plasmid pSCTS13 and pSCTS9 (Climie & Santi, 1990) have been described. Oligonucleotide synthesis and DNA sequencing were performed at the UCSF Biomolecular Resource Center. (6*R*)-CH_2H_4folate was a generous gift from SAPEC S.A. (Lugano, Switzerland) and EPROVA AG (Schaffhausen, Switzerland).

Mutagenesis and Protein Purification. The single E60D, H199A, and N229V mutants were constructed by cassette mutagenesis of the *L. casei* TS synthetic gene in plasmid pSCTS13 or pSCTS9 as previously described (Huang & Santi, 1994b; Climie *et al.*, 1990; Liu & Santi,, 1993). Plasmids of double and triple mutants were constructed by interchanging unique restriction fragments between plasmids of the corresponding single and double mutants. Construction of the H199A/N229V double mutant has been

described (Huang & Santi, 1996a). The E60D/H199A and E60D/N229V double mutants were constructed as following: (1) plasmid DNA of TS E60D, H199A, and N229V was each digested with *Hind* III and *Pst* I; (2) the small DNA fragment containing the E60D mutation and the large DNA fragment containing the H199A mutation or the N229V mutation were each purified by 1% agarose gel electrophoresis; (3) the small fragment with the E60D mutation was ligated to the larger fragment with the H199A mutation or the N229V mutation to give the E60D/H199A or E60D/N229V double mutant, respectively. The E60D/H199A/N229V triple mutant was constructed as follows: plasmid DNAs of TS E60D and TS H199A/N229V were each digested with *Nco* I and *EcoR* I; the small DNA fragment containing the E60D mutation and the large DNA fragment containing the H199/N229V mutations were each purified by agarose gel electrophoresis and ligated to give the E60D/H199A/N229V triple mutant. All mutant plasmids were individually purified and sequenced. Wild-type and mutant enzymes were purified by automated sequential chromatography on phosphocellulose and hydroxyapatite as described (Kealey & Santi, 1992). Enzyme preparations were concentrated and stored as described (Huang & Santi, 1994b).

Determination of K_d of dUMP. Binary complexes of PLP with wild-type TS or the mutant enzymes were formed using concentration of PLP of 20 to 50 μM and 3 μM protein in buffer containing 50 mM TES, pH 7.4 and 50 μM DTT. Dissociation constants (K_d) for dUMP were obtained by monitoring the competitive replacement of PLP from the TS-PLP complex by dUMP as described (Santi *et al.*, 1993).

Steady-state Kinetics. dTMP formation catalyzed by wild-type TS, TS E60D, TS H199A, TS N229V or TS H199A/N229V was monitored spectrophotometrically at 340 nm as described (Pogolotti *et al.*, 1986). The standard assay buffer contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 75 mM β-mercaptoethanol. For the less active mutants (TS E60D/H199A, TS E60D/N229V, and TS E60D/H199A/N229V), the kinetic parameters were obtained by monitoring the TS-

catalyzed tritium release from [2-^{14}C, 5-^{3}H]dUMP as previously described (Huang & Santi, 1994b). dTMP formation catalyzed by TS E60D/H199A, TS E60D/N229V, or TS E60D/H199A/N229V at saturating substrates was also monitored by HPLC analysis (Huang & Santi, 1994b).

Energetic Analysis of the Mutational effect. The magnitude of the mutational effect can be conveniently expressed as change in free energy ($\Delta\Delta G$) for substrate binding or catalysis. In the present work, $\Delta\Delta G$ values for K_d of dUMP, k_{cat} value for dTMP formation, and $k_{cat}/K_{m\text{-}dUMP}$ are defined as in Equation 1, 2, and 3, respectively,

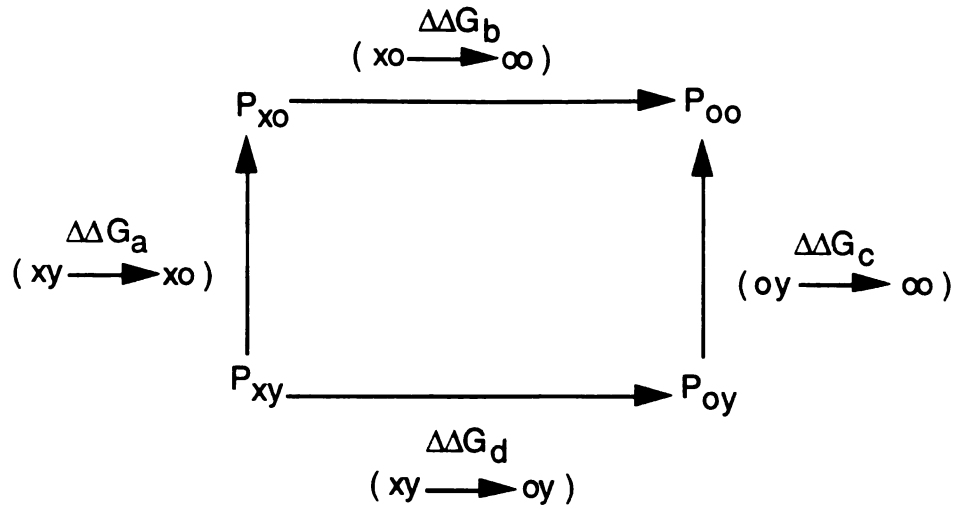
$$\Delta\Delta G_{Kd} = RT \ln[(K_d)_{mutant}/(K_d)_{wild\text{-}type}] \quad (1)$$

$$\Delta\Delta G_{kcat} = - RT \ln[(k_{cat})_{mutant}/(k_{cat})_{wild\text{-}type}] \quad (2)$$

$$\Delta\Delta G_{kcat/Km\text{-}dUMP} = - RT \ln[(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wild\text{-}type}] \quad (3)$$

where the gas constant (R) is 1.99 cal/K/mol, the absolute temperature (T) is 298K, and the unit for $\Delta\Delta G$ is kcal/mol. Note that $\Delta\Delta G$ is so defined that a positive $\Delta\Delta G$ indicates an increase of free energy barrier, whereas, a negative $\Delta\Delta G$ indicates a decrease of free energy barrier for the functional property studied.

The functional interaction between two residues can be analyzed using the double mutant free energy cycle (Carter *et al.*, 1984; Horovitz, 1987). Scheme I shows two wild-type amino acid residues (x and y) and the corresponding mutant residues in a double mutant cycle. The mutation of a wild-type amino acid residue is denoted by "o" as shown in Scheme I,



Scheme I

where , P_{xy} stands for the wild-type protein, a single mutant is denoted as P_{xo} or P_{oy}, and the double mutant as P_{oo}. $\Delta\Delta G_a$ and $\Delta\Delta G_c$ are the free energy changes upon mutations of residue y with an unchanged or an already mutated residue x, respectively. $\Delta\Delta G_b$ and $\Delta\Delta G_d$ are similarly defined for the mutation of residue x. If the change of free energy due to mutation of residue y is independent of residue x, then $\Delta\Delta G_a=\Delta\Delta G_c$; and by symmetry of the cycle, it follows that $\Delta\Delta G_b=\Delta\Delta G_d$. The interaction term, $\Delta\Delta G_{int(xy)}$, measures the interaction free energy between residue x and y in wild-type enzyme, and is expressed as following:

$$\Delta\Delta G_{int(xy)} = \Delta\Delta G_d - \Delta\Delta G_b \text{ , and, } = \Delta\Delta G_c - \Delta\Delta G_a \qquad (4)$$

Note that the interaction free energy between two residues is defined thermodynamically, and no assumptions about the underlying structural mechanisms are made.

The double mutant free energy cycle can be extended to a triple mutant cube to analyze the dependence of the interaction between two residues on a third residue in proteins (Horovitz & Fersht, 1990). In this work, wild-type and the mutant TSs were constructed into a triple mutant cube (Fig. 3) similar to those used in studies of functional interactions among catalytic elements of other enzymes (Steyaert & Wyns, 1992; First & Fersht, 1995). Each face of the triple mutant cube contains a double mutant cycle,

which measures interaction free energy between two residues. The pair of cycles located on opposing faces of the triple mutant cube measures the same pair interactions between two residues except that the third residue is unchanged in one cycle and mutated in another. For example, the front and the back faces of the triple mutant cube measure the interaction between E60 and H199 while the third residue is N229 or V229, respectively. The ternary interaction free energy, which is the difference between interaction free energies of the two cycles of opposing faces, measures the effect of a third residue on the interaction between two other residues. For example, the ternary interaction free energy, which measures the effect of N229 on the interaction between E60 and H199, is the difference of interaction free energies of the front and back cycles (i.e. $\Delta\Delta G_{int(ternary)} = \Delta\Delta G_{int(front\ cycle)} - \Delta\Delta G_{int(back\ cycle)}$.

RESULTS

Mutagenesis and Protein Purification. Mutant TSs were constructed by cassette mutagenesis of the *L. casei* TS synthetic gene. All mutations were confirmed by DNA sequencing. The mutant enzymes were expressed at levels of 10 to 30% of the total cellular protein in soluble crude extracts as determined by 12% SDS-PAGE. The mutant enzymes were purified by phosphocellulose and hydroxyapatite chromatography to apparent homogeneity as described (Kealey *et al.*, 1992).

Dissociation constants of dUMP. Dissociation constants (K_d) for dUMP binding to wild-type and mutant enzymes were determined using a competitive binding assay in which PLP is displaced from the TS-PLP complex by dUMP (Santi *et al.*, 1993). K_d values for the mutant TSs increased 5 to 95-fold compared to that of wild-type TS (corresponding to 1 to 2.7 kcal/mol increased free energy barrier for dUMP binding), with the highest value for the TS N229V single mutant (Table 6.1). The free energy

changes measured in the double and triple mutants were 0.4-2.3 kcal/mol smaller than those expected from the sums of the individual mutations.

Steady-state Kinetics for dTMP formation. dTMP formation catalyzed by TS E60D, H199A, N229V or H199A/N229V was determined spectrophotometrically by monitoring the change at 340 nm, which accompanies the conversion of CH_2H_4folate to H_2folate (Pogolotti *et al.*, 1986). dTMP formation catalyzed by TS E60D/N229V, E60D/H199A, or E60D/H199A/N229V was monitored using the more sensitive tritium release assay (Huang & Santi, 1994b), which measures the TS-catalyzed release of tritium from [2-^{14}C, 5-^{3}H]dUMP. Since the k_{cat} values obtained by tritium release and spectrophotometric assays are different for some E60 mutant TSs (Huang & Santi, 1994b), the rates of dTMP formation catalyzed by TS E60D/N229V, E60D/H199A, and E60D/H199A/N229V at saturating substrate concentrations were also monitored by HPLC. Like wild-type TS, the rates of tritium release catalyzed by these mutant TSs were essentially identical to the corresponding rates of dTMP formation as determined by HPLC analysis (data not shown). Therefore, tritium release is a valid measurement of dTMP formation for these mutant TSs.

The kinetics of dTMP formation were analyzed according to Scheme II (Santi *et al.*, 1990):

$$\text{TS} \underset{k_2}{\overset{k_1\,[\text{dUMP}]}{\rightleftharpoons}} \text{TS-dUMP} \underset{k_4}{\overset{k_3\,[\text{CH}_2\text{H}_4\text{folate}]}{\rightleftharpoons}}$$

$$\text{TS-dUMP-CH}_2\text{H}_4\text{folate} \xrightarrow{k_5} \text{dTMP} + \text{H}_2\text{folate}$$

Scheme II

The interaction of the substrates with *L. casei* TS is an ordered Bi Bi mechanism with dUMP binding first, followed by CH_2H_4folate binding (Danenberg & Danenberg, 1978). We assume the same mechanism for binding of dUMP and CH_2H_4folate for all mutant

Table 6.1: Dissociation Constants of the Wild-type and the Mutant Enzyme Complexes with PLP and dUMP[a].

TS	K_d, μM		ΔΔG, kcal/mol[b]	
	PLP	dUMP	K_d,dUMP	Sum[c]
Wild-type	1.7	0.5	0	
E60D	27	3.9	1.2	
H199A	3.0	2.6	1.0	
N229V	10	48	2.7	
E60D/H199A	32	9.9	1.8	2.2
E60D/N229V	40	17	2.1	3.9
H199A/N229V	6.0	43	2.6	3.7
E60D/H199A/N229V	26	43	2.6	4.9

[a]Assays are as described in Materials and Methods. The standard errors from nonlinear squares fit of the experimental data are less than 15% for all values. [b]ΔΔG is calculated according to eq. 1. [c]This value is the sum of ΔΔGs of the corresponding single mutants.

TSs in this study. The kinetic expressions relevant to this study are given in Equation 5 and 6 (adapted form Santi *et al.*, 1990) .

$$k_{cat} = k_5 \qquad (5)$$

$$\frac{k_{cat}}{K_m^{dUMP}} = k_1 \qquad \text{At saturating } CH_2H_4\text{folate} \qquad (6)$$

The catalytic constant, k_{cat}, measures conversion of the ternary complex (both substrates bound to the enzyme) to the products. At saturating CH_2H_4folate, k_{cat}/K_m for dUMP is a measure of rate of association (k_1) for dUMP with free enzyme (Equation 6).

Table 6.2 shows the kinetic parameters of dTMP formation for wild-type and mutant enzymes. k_{cat} values for the mutant TSs decreased 5 to 2,200-fold compared with that of wild-type TS (corresponding to 1 to 4.6 kcal/mol increase in free energy of catalysis) with the highest value for TS E60D/H199A/N229V. The free energy changes for the multiple mutants were 0.6-1.4 kcal/mol smaller than those expected from sum of the free energy changes for the individual mutations. The values of $k_{cat}/K_{m\text{-}dUMP}$ for the mutant TSs decreased 18 to 307,000-fold compared to that of wild-type TS (corresponding to 1.7 to 7.5 kcal/mol increase in free energy). The free energy changes measured in TS H199A/N229V and TS H199A/N229V were smaller than those expected from the sums of the individual mutations. The free energy changes measured in TS E60D/N229V and TS E60D/H199A were similar to and larger than those expected from the sums of the individual mutations, respectively.

Cycles of interactions. Each face of the triple mutant cube (**Figure 6.2**) contains a double mutant cycle, which measures the interaction free energy between two residues. The pair of cycles located on opposing faces of the triple mutant cube measures the interactions between the same pair of residues except that a third

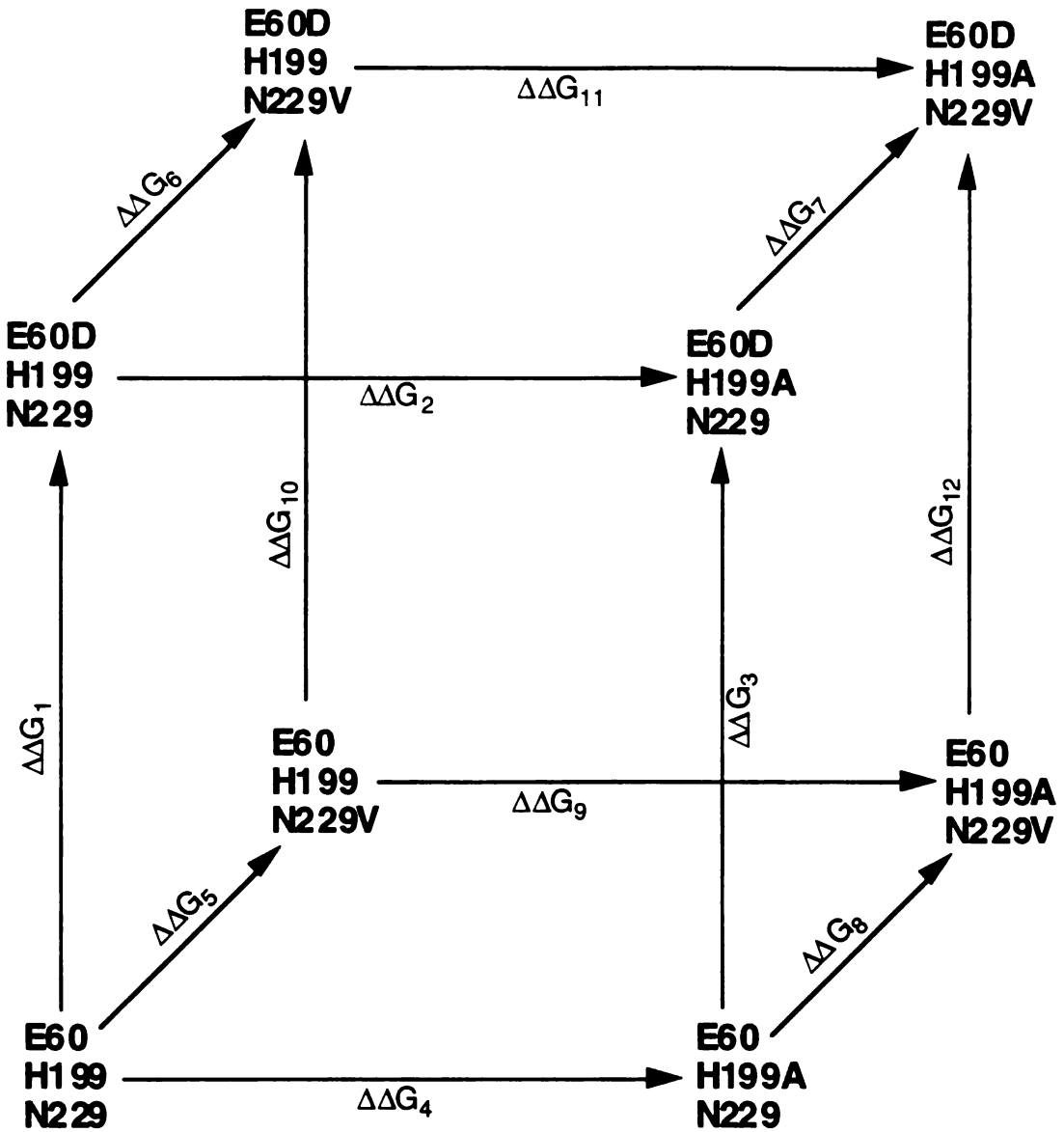


Figure 6.2: The triple mutant cube constructed to illustrate the interrelationships between the wild-type enzyme and TS mutants of E60, H199, and N229. The starting point of the cube is the wild-type enzyme. Arrows point from less mutated enzymes to more highly mutated enzymes. Each face of the cube represents a double mutant cycle. $\Delta\Delta G_{(1\text{-}12)}$ is free energy change imposed by a mutation and is calculated by subtraction of $\Delta\Delta G$ of the less mutated enzyme from that of the more highly mutated enzyme, e.g., $\Delta\Delta G_2 = \Delta\Delta G_{(E60D/H199A)} - \Delta\Delta G_{(E60D)}$ in the front cycle.

Table 6.2: Steady-state Kinetic Parameters for dTMP Formation Catalyzed by Wild-type and Mutant Enzymes[a].

TS	k_{cat}			K_m (μM)		$k_{cat}/K_{m\text{-dUMP}}$		
	(min^{-1})	ΔΔG, kcal/mol[d]	Sum[e]	dUMP	CH_2H_4folate	(μM^{-1}, min^{-1})	ΔΔG, kcal/mol[c]	Sum[d]
Wild-type	534	0		5.8	19	92	0	
E60D[b]	2.4	3.2		6.9	27	0.35	3.3	
H199A	27	1.8		5.4	19	5	1.7	
N229V[b]	102	1.0		135	87	0.76	2.8	
E60D/H199A[c]	0.4	4.3	5.0	46	ND[f]	0.008	5.5	5.0
E60D/N229V[c]	1.6	3.4	4.2	620	ND	0.003	6.2	6.2
H199A/N229V	13	2.2	2.8	92	40	0.14	3.8	4.6
E60D/H199A/N229V[c]	0.2	4.6	6.0	790	ND	0.0003	7.5	7.9

[a]Assays are as described in Materials and Methods. The standard errors from nonlinear squares fit of the experimental data are less than 20% for all values. [b]Simillar results have been reported (Huang & Santi, 1994b). [c]The values for k_{cat} and K_m for these enzymes were ontained from the tritium release assay. [d]ΔΔG is calculated according to Equation 2 and Equation 3 for k_{cat} and k_{cat}/K_m, respectively. [e]The expected ΔΔG for mutiple mutant is the sum of ΔΔGs for mutiple mutant is the sum of ΔΔGs for the corresponding single mutants. [f]ND, not determined.

residue is unchanged in one cycle and mutated in another. For example, the front and back cycles measure the interaction between E60 and H199 while the third residue is N229 or V229, respectively. Similarly, the left and right cycles measure the interaction between E60 and N229 while the third residue is H199 or A199, respectively; and the top and bottom cycles measure the interaction between H199 and N229 while the third residue is D60 or E60, respectively. Each mutation is defined by a $\Delta\Delta G$ indicated as $\Delta\Delta G_1$ through $\Delta\Delta G_{12}$ in Figure 2. The $\Delta\Delta G$ value is calculated by subtraction of free energy change of the less mutated enzyme (the beginning of an arrow) from that of the more highly mutated enzyme (the end of the arrow), e.g., $\Delta\Delta G_2 = \Delta\Delta G_{(E60D/H199A)} - \Delta\Delta G_{(E60D)}$ in the front cycle.

Table 6.3 shows the free energy changes for K_d, k_{cat} and k_1 imposed by each mutation. $\Delta\Delta G_1$ through $\Delta\Delta G_{12}$ are the free energy changes for the mutations studied in the context of the triple mutant cube(Figure 6.2). A positive $\Delta\Delta G$ indicates an increase in the free energy barrier to the reaction process studied, whereas, a negative $\Delta\Delta G$ indicates a decrease. The magnitude for free energy change is considered negligible in this study if it is less than $\pm$ 0.2 kcal/mol because of the experimental variations on measurements of the parameters. The parameters (e.g. k_{cat}, K_d) for all mutant TSs were measured on the same day and with the same buffer and reagents, in an attempt to minimize the experimental errors. The errors from non-linear square fit for all data are less than 15%. Change of 0.2 kcal/mol corresponds to 1.4-fold (140%) change of a parameter (e.g. k_{cat}) due to mutation. Thus, the errors of all data in this study are less than this values ($\pm$ 0.2 kcal/mol). Most $\Delta\Delta G$ values for K_d of dUMP were positive except that the $\Delta\Delta G_{10}$ value was negative, and the $\Delta\Delta G_9$ and $\Delta\Delta G_{12}$ values were nearly zero. All $\Delta\Delta G$ values for k_{cat} and k_1 were positive and generally larger than the corresponding $\Delta\Delta G$ values for K_d, indicating that the mutations studied had greater effects on k_{cat} and k_1 than on K_d. The data in Table 6.3 were used to calculated the interaction free energies between

Table 6.3: Changes in Free Energies for K_d, k_{cat}, and k_1 imposed by TS mutations

Mutation	ΔΔG for Functional Properties, kcal/mol[a]		
	K_d, dUMP	k_{cat}	k_1[b]
WT to E60D (ΔΔG1)	1.2	3.2	3.3
H199A to E60D/H199A (ΔΔG3)	0.8	2.5	3.8
N229V to E60D/N229V (ΔΔG10)	-0.6	2.4	3.3
H199A/N229V to E60D/H199A/N229V (ΔΔG12)	0	2.4	3.7
WT to N229V (ΔΔG5)	2.7	1.0	2.8
E60D to E60D/N229V (ΔΔG6)	0.9	0.2	2.8
H199A to H199A/N229V (ΔΔG8)	1.6	0.4	2.1
E60D/H199A to E60D/H199A/N229V (ΔΔG7)	0.8	0.3	2.0
WT to H199A (ΔΔG4)	1.0	1.8	1.7
E60D to E60D/H199A (ΔΔG2)	0.6	1.1	2.2
N229V to H199A/N229V (ΔΔG9)	-0.1	1.2	1.0
E60D/N229V to E60D/H199A/N229V (ΔΔG11)	0.5	1.2	1.4

[a]Changes in free energies are derived from data on the mutational effects in Table 1 and Table 2, and calculated by subtraction of free energy change of the less mutated enzyme from that of the more highly mutated enzyme, e.g., $\Delta\Delta G_2 = \Delta\Delta G_{(E60D/H199A)} - \Delta\Delta G_{(E60D)}$ in the front cycle of Figure 2. [b]k_1, the rate of association of dUMP with free enzyme, is defined as $k_{cat}/K_{m\text{-}dUMP}$ (Equation 6).

the network residues as described below.

Interaction free energy. The interaction free energies ($\Delta\Delta G_{int}$) for K_d, k_{cat} and k_1 between E60, H199, and N229 in wild-type enzyme are summarized in Table 6.4. $\Delta\Delta G_{int}$ measures the energetic interaction between two residues, e.g., the interaction between E60 and H199 is determined by using the front cycle of the triple mutant cube (Figure 6.2). $\Delta\Delta G_{int}$ is the difference between the free energy changes for opposing sides of a cycle, e.g., $\Delta\Delta G_{int} = \Delta\Delta G_4 - \Delta\Delta G_2$ for the front cycle. The interaction between the two residues in wild-type enzyme can either enhance or reduce a functional property studied depending on whether the $\Delta\Delta G_{int}$ is positive or negative, respectively. When the value of $\Delta\Delta G_{int}$ is zero, the two residues function independently in wild-type enzyme. As shown in Table 6.4, values of $\Delta\Delta G_{int}$ ranged from -0.6 to 1.8 kcal/mol, indicating diverse interactions depending on the residues involved and the parameters studied. The interactions between E60, H199, and N229 generally enhanced binding of dUMP to TS (K_d) except (1) the interaction between E60 and H199 reduced binding of dUMP when N229 was mutated, and (2) H199 and N229 function independently when E60 was mutated. The interactions between E60, H199, and N229 enhanced catalysis (k_{cat}) when the third residue was not altered, but had no effect when the third residue is mutated. The interactions between E60, H199, and N229 resulted in diverse effects on rate of association (k_1) for dUMP with free enzyme (Table 6.4C).

Ternary interaction free energy, $\Delta\Delta G_{int(E60,\ H199,\ N229)}$, measures the effect of the third residue on the interaction of the other two residues. $\Delta\Delta G_{int(E60,\ H199,\ N229)}$ is the difference in interaction free energies of the two cycles on the opposite faces of the triple mutant cube (i.e. front-back, left-right, top-bottom cycles in Figure 6.2) For example, $\Delta\Delta G_{int(E60,\ H199,\ N229),\ Kd} = \Delta\Delta G_{int(Front\ cycle),\ Kd} - \Delta\Delta G_{int(Back\ cycle),\ Kd} = 0.4 - (-0.6) = 1.0$ kcal/mol (Table 6.4A), for binding of dUMP to TS. For a given parameter, the value of ternary interaction free energy calculated from each of the

Table 6.4: Free Energies of Pairwise Interactions Between E60, H199, and N229 in Wild-type TS.

A. Free Energy of interaction in Binding of dUMP to TS (K_d of dUMP)

Cycle[a]	Interacting Residues[a]	Unchanged Residue	Mutant Enzyme	$\Delta\Delta G_{int}$[b] kcal/mol	Effect of Interaction[c] on Binding of dUMP
Front	E60-H199	N229	E60D/H199A	0.4	Enhancing
Back	E60-H199	N229V	E60D/H199A/N229V	-0.6	Reducing
Left	E60-N229	H199	E60D/N229V	1.8	Enhancing
Right	E60-N229	H199A	E60D/H199A/N229V	0.8	Enhancing
Bottom	H199-N229	E60	H199A/N229V	1.1	Enhancing
top	H199-N229	E60D	E60D/H199A/N229V	0.1	Negligible

B. Free Energy of Interaction in Catalysis (k_{cat} for dTMP formation)

Cycle	Interacting Residues	Unchanged Residue	Mutant Enzyme	$\Delta\Delta G_{int}$ kcal/mol	Effect of Interaction on Catalysis
Front	E60-H199	N229	E60D/H199A	0.7	Enhancing
Back	E60-H199	N229V	E60D/H199A/N229V	0	Negligible
Left	E60-N229	H199	E60D/N229V	0.8	Enhancing
Right	E60-N229	H199A	E60D/H199A/N229V	0.1	Negligible
Bottom	H199-N229	E60	H199A/N229V	0.6	Enhancing
top	H199-N229	E60D	E60D/H199A/N229V	-0.1	Negligible

C. Free Energy of Interaction in Rate of Association of dUMP with Free Enzyme (k_1)

Cycle	Interacting Residues	Unchanged Residue	Mutant Enzyme	$\Delta\Delta G_{int}$ kcal/mol	Effect of Interaction on k_1
Front	E60-H199	N229	E60D/H199A	-0.5	Reducing
Back	E60-H199	N229V	E60D/H199A/N229V	-0.4	Reducing
Left	E60-N229	H199	E60D/N229V	0	Negligible
Right	E60-N229	H199A	E60D/H199A/N229V	0.1	Negligible
Bottom	H199-N229	E60	H199A/N229V	0.7	Enhancing
top	H199-N229	E60D	E60D/H199A/N229V	0.8	Enhancing

[a]Cycles and interacting residues are defined in Figure 2. [b]ΔΔGint is the difference between the free energy changes for opposing sides of a cycle, e.g., ΔΔGint = ΔΔG4 - ΔΔG2 for the front cycle (Fig. 2). [c]The interation between two residues can either enhance or reduce a functional property, dependeding on whether the ΔΔGint is positive or negative, respectively (see text for detail).

two cycles on the opposite faces of the cube (Figure 6.2) is equivalent, due to energetic additivity of the triple mutant cube. As calculated form Table 6.4, $\Delta\Delta G_{int(E60, H199, N229)}$ was 1.0, 0.7, and -0.1 kcal/mol for K_d, k_{cat}, and k_1, respectively.

DISCUSSION

The change in function of a protein that accompanies mutation of a single residue is often used to measure the contribution of the altered residue to function. However, single mutation experiments may be prone to misinterpretations because the residue mutated may interact with other residues in a co-operative manner, and such interactions can not be observed by analysis of single mutations. Multiple mutations have been used to measure the functional interactions between residues (for review see Wells, 1990; Mildvan *et al.*, 1992) . When non-interacting single mutants are combined, the sum of the free energy changes derived from the single mutations should be equal to the free energy changes in the multiple mutant (Wells, 1990). Deviations from simple additivity can occur when the mutated residues interact with each other and/or the mutations causes a change in mechanism (Weber *et al.*, 1990; Mildvan *et al.*, 1992; Steyaert & Wyns, 1992). Analysis of multi-mutant free energy cycles (or triple mutant cube) have been introduced to quantify interactions between three or more catalytic elements in enzymes (Carter *et al.*, 1984; Horovitz, 1987; Steyaert & Wyns, 1992)

In the present work, we used multi-mutant free energy cycles – a triple mutant cube – to investigate functional interactions among the side chains of E60, H199 and N229 on various kinetic and thermodynamic properties of the TS reaction. The residues mutated are involved in a water-mediated hydrogen bond network believed to be important in binding of dUMP and in proton transfer reactions of the TS reaction. The amino acid replacements used for the three residues (E60 to D60, H199 to A199, and N229 to

V229) were chosen because the kinetic properties of the single mutants suggested that the double and triple mutants would have sufficient activity for measurement (Climie *et al.*, 1990; Huang & Santi, 1994a; Liu & Santi, 1993), and because the altered side chains are incapable of forming hydrogen bonds which might complicate interpretations. The parameters measured – K_d of dUMP, k_{cat} of dTMP formation and rate of association (k_1) of dUMP with the free enzyme – reflect steps of the reaction believed to involve changes in the H-bond network which might manifest cooperativity of network interactions.

Mutations of the network residues, individually or in combinations, resulted in increases in free energy of one or more thermodynamic or kinetic parameters of the TS reaction, pointing to contributions of the residues to specific steps in the reaction pathway. Further, mutational effects on K_d, k_{cat}, and k_1 were nonadditive in each of the multiple mutants, except for the effect of TS E60D/N229 on k_1. The $\Delta\Delta$Gs measured from the multiple mutants were generally smaller than sum of those derived from the corresponding single mutations, indicating that the three network residues function synergistically to reduce the free energy barriers to various steps of the TS reaction.

Effects on Kd of dUMP. The interaction between E60 and H199 enhanced binding of dUMP (K_d) to wild type TS, but reduced binding when N229 was mutated to Val (Table 6.1) N229 directly hydrogen bonds to the pyrimidine ring of dUMP, and it is speculated that the intact network residues contribute to and extend the binding site by positioning the N229 appropriately; mutation of N229 disrupts the network, or extended binding site, which in turn is manifested as an decrease in interaction energy of E60 and H199. E60 and N229 contributed cooperatively to binding of dUMP independently of H199, as indicated by the positive $\Delta\Delta$Gint values with either H199 or A199 (Table 6.4A). This observation, together with the relatively small mutational effects of H199 on all parameters studied suggest that mutation of H199 causes limited change to the overall structure of the hydrogen bond network. H199 and N229 contributed cooperatively to

binding of dUMP when E60 is unchanged. However, H199 and N229 function independently when E60 was mutated. Since E60 seems to coordinate the entire network, it is not surprising that mutation of E60 disrupts the interaction between H199 and N229.

Effects on k_{cat}. Interactions between any two of the network residues- E60, H199 and N229-enhanced catalysis (k_{cat}) (Table 6.4B), indicating synergistic interactions between the network residues in the wild-type enzyme. However, the synergistic effect on kcat was abolished when any of the three residue was mutated. Thus, alteration of any of the three residues disrupts the synergism between the other two residues on catalysis. It is apparent that the integrity of the hydrogen bond network affects the interactions and is required for the synergistic interaction on catalysis.

Effects on k1 of dUMP. The rate of association (k_1) for dUMP to free enzyme is measured by $k_{cat}/K_{m\text{-}dUMP}$ at saturating CH_2H_4folate (Equation 6). k_1 is proposed to reflect a conformational change of TS to accommodate binding of dUMP before cofactor binding (Santi *et al.*, 1990). Effects of interactions on k_1 between E60, H199 and N229 free enzyme were diverse: interactions between E60 and H199 reduced k_1; interactions between H199 and N229 enhanced k_1; and interactions between E60 and N229 on k_1 was negligible. Effects of these interactions on k_1 were independent of the third residue, which is clearly different from those on k_{cat}. Effects of all interactions on k_{cat} between E60, H199 and N229 were dependent of the third residue. Since k_1 may reflect a conformational change of TS before cofactor binding and k_{cat} measures dTMP synthesis in which cofactor binding and the subsequent conformational changes are involved, differences in effects on the interactions on k_1 and k_{cat} may reflect different structures and mechanisms of the hydrogen bond network before and after the binding of CH_2H_4folate. In fact, differences in the hydrogen bond network are observed between crystal structures of TS-dUMP binary complex and TS-dUMP-folate analog ternary complex (Finer-Moore *et al.*, 1993).

Mutational effects are typically considered nonadditive when the interaction free energy is large (5 to 10 kcal/mol); and the nonadditivity is frequently interpreted to indicate direct contact between two residues or short-range steric interaction (Carter *et al.*, 1984; Wells, 1990; Mildvan *et al.*, 1992). Interaction free energies below about 1-1.5 kcal/mol are usually considered additive and are correlated with compounding errors when summing the single mutants, or/and week interactions between residues (Carter *et al.*, 1984; Wells, 1990). However, small magnitude nonadditivity of mutational effects have been found in residues that distantly separated in several protein systems (LiCata *et al.*, 1990; Howell *et al.*, 1990; Robinson & Sligar, 1993; Green & Shortle, 1993). The long-rang, small magnitude nonadditivity of mutational effects between sites that have no direct molecular contact is now appreciated as a general feature of proteins (see LiCata & Ackers, 1995 for recent review). In this work, interaction free energies between E60, H199, and N229 were less than 5 kcal/mol, but similar to or greater than the small magnitude nonadditivity (generally < 1 kcal/mol) found between sites in proteins that can not interact directly via steric contact, salt bridge or hydrogen-bonding (LiCata & Ackers, 1995). The magnitude of the nonadditive mutational effects in the TS mutants is consistent with structural interrelationships between E60, H199, and N229 in that the side chains of these residues interact indirectly through mediating waters and the substrate molecule.

In summary, mutations of the hydrogen bond network residues -E60, H199, and N229 - increased free energy barriers to dissociation constant of dUMP, k_{cat} for dTMP formation, and rate of association of dUMP to free enzyme, indicating an important role of the network in the TS mechanism. Nonadditive mutational effects and the smaller-than-expected changes of free energies in the multiple mutants suggest that synergism is a common underlying mechanism by which the network residues functions. Interactions between these residues as well as their dependency on the third residues were quantified using a triple mutant cube. The results provide a detailed picture for

energetic basis of the cooperative interactions between the network residues, which could not have been elucidated from analysis of single mutations alone.

ACKNOWLEDGMENTS

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CHAPTER VI REFERENCES

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CHAPTER VII

The separate effects of E60Q in *L. casei* Thymidylate Synthase delineate between mechanisms for intermediates in catalysis

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SHORT TITLE : Crystal Structures of *L. casei* E60 mutant TS Complexes

ABSTRACT

X-ray crystal structures of *L. casei* thymidylate Synthase mutant complexes E60D with dUMP and E60Q with dUMP, FdUMP, and these nucleotides with folate analog inhibitor CB3717 are described. The extreme decrease in Kcat from greater than 9 s^{-1} to less than $2x10^{-3}$ s^{-1} for the E60Q mutant (and down to even less than 4x10-4 s^{-1} for other E60 mutants), related primarily to covalent complex formation and separately in breakdown of the covalent complex, is associated with slight changes within the active site cavity indicated by side chain orientations of some hydrophobic residues and a greater freedom of rotation for the catalytically important residue Asn-229. These changes help to disorder water molecules which normally mediate interactions between residue side chains and substrate ligands. Initial orientations of substrate ligands favorable for catalysis may thus be affected in the mutant complexes, leading to a slower rate of formation of the necessary ternary covalent intermediate. This may allow the reaction to reverse itself before product formation is achieved.

RESULTS

Thymidylate Synthase (TS) is a widely studied enzyme responsible for the *de novo* synthesis of dTMP in most organisms[1]. It has been extensively characterized chemically, kinetically[2] and structurally as the free enzyme and in binary and ternary complexes with its substrates dUMP and CH_2THF[3], folate analog inhibitor CB3717[4,5] and products dTMP and DHF[6]. TS is a highly conserved enzyme across species[7] with several catalytic site mutations retaining activity[8,9]. In this aspect it is remarkable that the mutation E60Q (*L. casei* numbering) decreases the activity of the enzyme 760 to 10,000-fold *in vitro* and renders bacterial recombinants thy-[10,11]. The side chain of residue 60 is too far away from

the substrate nucleotide for any direct interactions. In contrast, the only residue which normally hydrogen bonds to the pyrimidine base, Asn-229, has been found not to be necessary for catalysis[12] and serves mainly for substrate nucleotide specificity[13].

The E60Q mutation (in the LCTS enzyme) does not greatly hinder substrate binding to the active site[11]. However, E60 A, L, and Q mutations allow for the accumulation of a ternary covalent intermediate to such an extent that it is readily isolatable with SDS PAGE[14,11]. The wild type reaction occurs too quickly for this isolation; therefore, slow resolution of the covalent intermediate into products of the reaction (dTMP and DHF) suggests that E60 mutations uncover or highlight a rate determining step in the mutant TS reaction that occurs between the formation of the covalent intermediate and the reduction of the transferred methylene group[14,11]. The E60Q mutant is also able to enhance (by 40-fold over the wild type enzyme) the folate independent dehalogenation of dUMP brominated or iodinated at the C-5 position of the pyrimidine[11], suggesting that *in vitro* an initial binary complex of TS with nucleotide orients the substrate dUMP to form a covalent adduct of nucleotide to the reactive thiol of active site residue Cys-198, although fluorine resonance evidence for FdUMP in covalent complex with the *E. coli* mutant was lacking[10].

Since the E60 residue must exert its influence on catalysis indirectly, it has been postulated that its side chain promotes and maintains a hydrogen bonding network mediated by catalytically important conserved water molecules which can link active site residues Asn-229, His-199, Tyr-146, and Glu-60 to the pyrimidine substrate[1,3]. This would allow proton transfers necessary for the formation of pyrimidine base enolate species which must be established before and during methylene transfer to C-5[3]. However, it is not intuitively obvious how in particular an E60Q mutation would disrupt such a hydrogen bonding network, nor how the mutation would indirectly cause such a drastic reduction in catalytic activity. The only other mutation of residue 60 which allows successful

complementation of thy- bacteria is the most conservative one (Glu -> Asp)[9]. K_d values for binding of dUMP to E60 TS mutants are highest for the hydrophobic mutants E60A, E60L, and E60V[11].

To study the structural consequences of the E60Q mutation in *L. casei* TS x-ray crystallographic studies of cocrystals of mutant enzyme binary complexes with dUMP and FdUMP (a nucleotide inhibitor mimicking the product dTMP) and cocrystals soaked with folate analog CB3717 were made and are presented here. The cocrystal binary complex between E60D and dUMP was also studied for comparison. Analogous ECTS ternary complexes, with both ligands cocrystalized to wild type enzyme, have revealed that these ligands can orient themselves within the active site in manners that would be favorable for the initiation of catalysis[4,5,15]. Direct comparisons of the LCTS E60Q mutant complex structures to wild type LCTS ternary complexes[16] help to reveal significant structural changes at the active site induced by the E60Q mutation.

E60D-dUMP

The structure of the most conservative mutant binary complex, E60D-dUMP, allows the nucleotide to bind to the active site and make hydrogen bond interactions with the protein similar to those seen for the wild type enzyme (Figures. 7.2a, 7.4a). The dUMP phosphate is coordinated by the guanidinium groups of arginines 23 and

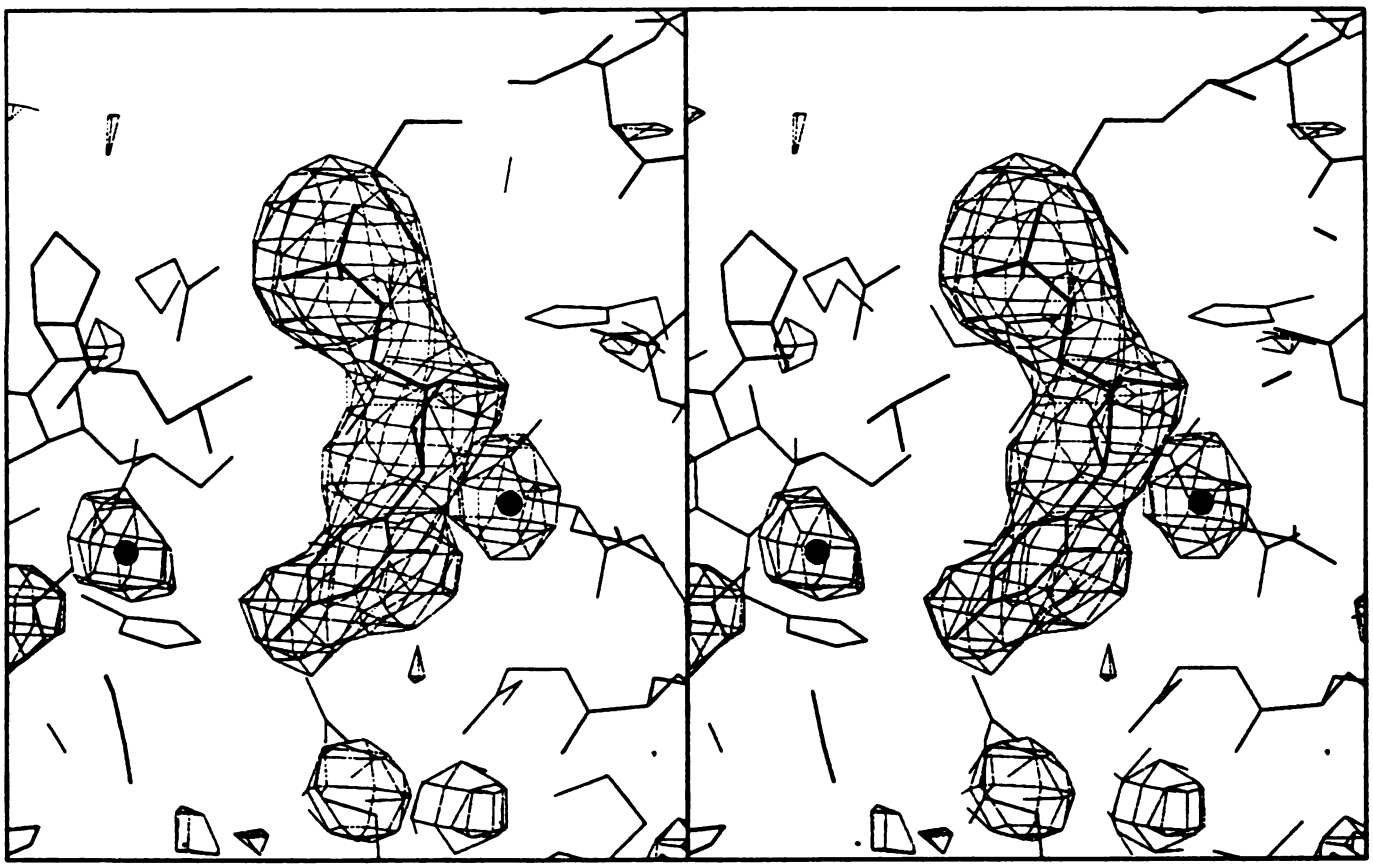


Figure 7.2a: E60D-dUMP 3.0 Å map at 2.5 σ. Fo-Fc omit maps for ligands of the residue 60 mutants binary and ternary complexes. complex. Ligands and water molecules were removed and the protein subjected to positional refinement before calculation of difference density maps. These were made using the graphics program MOLSCRIPT[35]

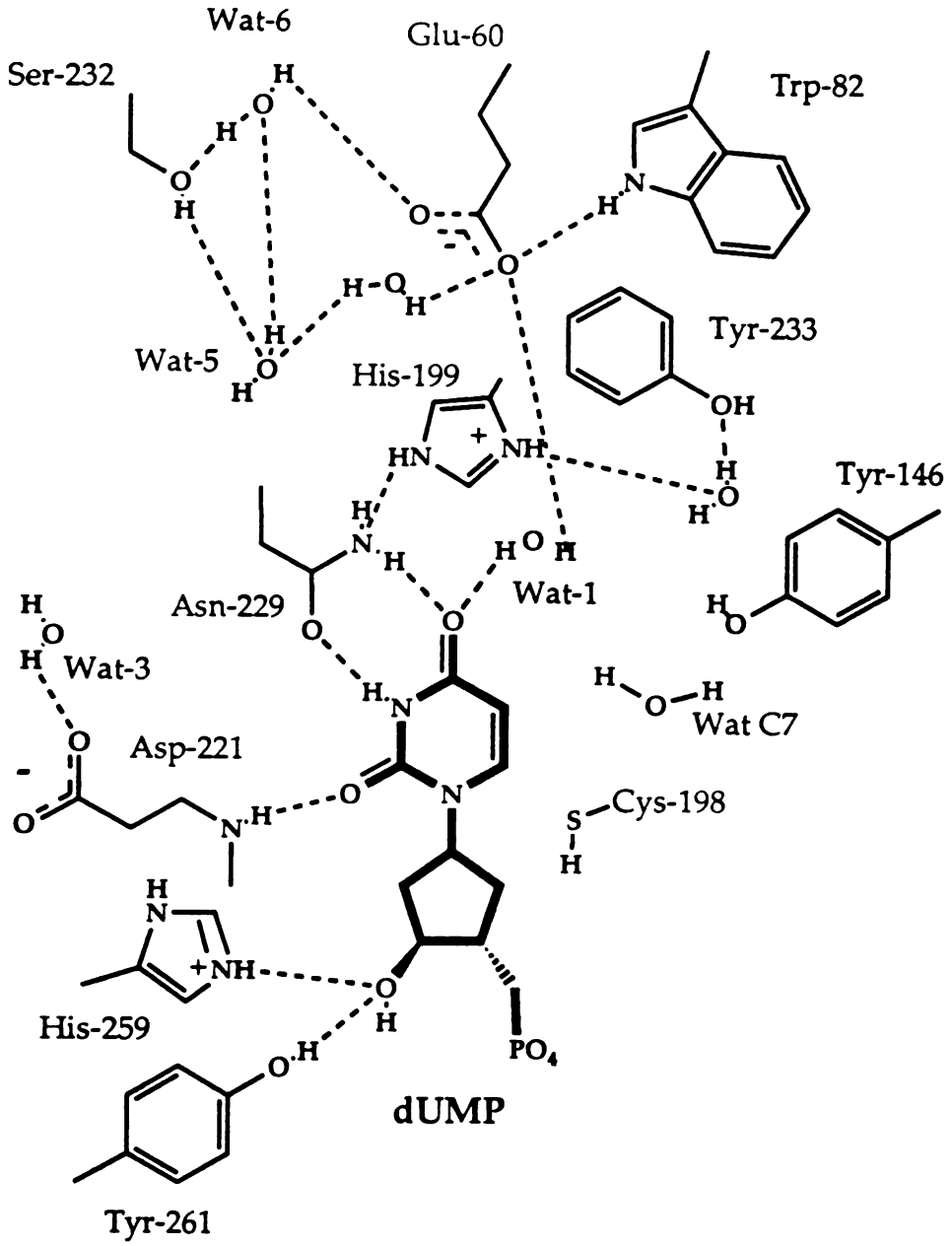


Figure 7.4a: Schematics LCTS active site of WT LCTS-dUMP.

218, as well as 178' and 179' from the active site of the dimer's monomer partner. The phosphate coordination is aided by interaction with serine 219. The pyrimidine base orientation is promoted by hydrogen bonding of Asp-221 N-H to dUMP O-2, as seen in wild type complexes, and by His-259 Nε2-Hε2 to dUMP O-3' and Tyr-261 O-H to dUMP O-3'. Asparagine 229 is able to make the normally observed hydrogen bonds of its side chain to the pyrimidine, namely Nδ2-Hδ2 to dUMP O-4 and Oδ1 to dUMP N3-H3. A water molecule is within hydrogen bonding distance of O-1' of the pyrimidine.

Asp-60 is able to retain hydrogen bonding for one of its side chain oxygens to the indole of Trp-82. This allows both Trp-82 and Asp-60 to adopt side chain orientations similar to that seen in wild type complexes, although for the shortened side chain at residue 60 there can be found no evidence of water molecules between it and Ser-232. There is no covalent contact between the pyrimidine base (at C-6) and the reactive thiol of Cys-198; however, the Sγ of Cys-198 can probably be polarized via hydrogen bonding with the guanidinium of Arg-218, which has been suggested as favoring enzymatic activity[17]. The imidizole of His-199, in a doubly protonated state, is able to hydrogen bond to both Tyr-146 and Asn-229 in the active site. His-199 in this complex is also able to hydrogen bond to dUMP O-4, further enhancing the importance of this non-essential residue to activity. The importance of hydrophobic residues along the J α-helix is suggested by backbone and side chain interactions between Asp-221 and residues Phe-223, Leu-224, Gly-225, and Val-226 and between side chain atoms of Asn-229 and residues Gln-217 and Gly-225. Although the mutant binary complex is very similar to wild type, difference electron densities for usually conserved water molecules considered important for activity, between residue 60 and dUMP O-4 (Water I) and between residue 60 and Asn-229, can not be discerned. The C terminus is in an open conformation, as usual for dUMP-bound TS.

E60Q-dUMP

In the E60Q-dUMP cocrystallization binary complex the nucleotide does not bind atypically to the TS active site (Figures 7.2b, 7.3b, 7.4b), although there is no covalent attachment of the dUMP base to Cys-198 (which is not unusual for LCTS crystal binary complexes). The Michaels addition necessary for the linkage is known to be promoted by folate or folate analog binding[3]. Energy minimization refinement of this complex with doubly protonated histidine 199 results in a rotation of the Asn-229 carboxamide group to allow hydrogen bonding between Asn-229 Oδ1 and His-199 Nε2 at the expense of hydrogen bonding between Oδ1 and H-3 of the pyrimidine base, even though the rotation introduces hydrogen atom clashing between H-3 of dUMP and Nδ2-H of Asn-229. When the mono-protonated δH tautomer of His-199 is used in refinement, the Asn-229 side chain remains in the wild type orientation making the usual hydrogen bonds to the pyrimidine base : Asn-229 Nδ2-H to O-4 of dUMP and Asn-229 Oδ1 to H-3 of dUMP. The tendency of the Asn-229 side chain to adopt a less favorable orientation for hydrogen bonding to the pyrimidine is characteristic of each of the E60Q complexes studied, and not of E60D or wild type TS complexes. Even when the Asn-229 carboxamide group refines to its usual orientation (i.e., when the δH tautomer of His-199 is used), the pyrimidine base of dUMP in the E60Q ternary complexes and in E60Q-FdUMP is not positioned to form the conserved double-hydrogen bond network with Asn-229. When the mono-protonated εH tautomer of His-199 is refined, a hydrogen bond between it and Asn-229 Oδ1 is maintained. When, instead, the δH tautomer is refined, Nδ1 forms a hydrogen bond

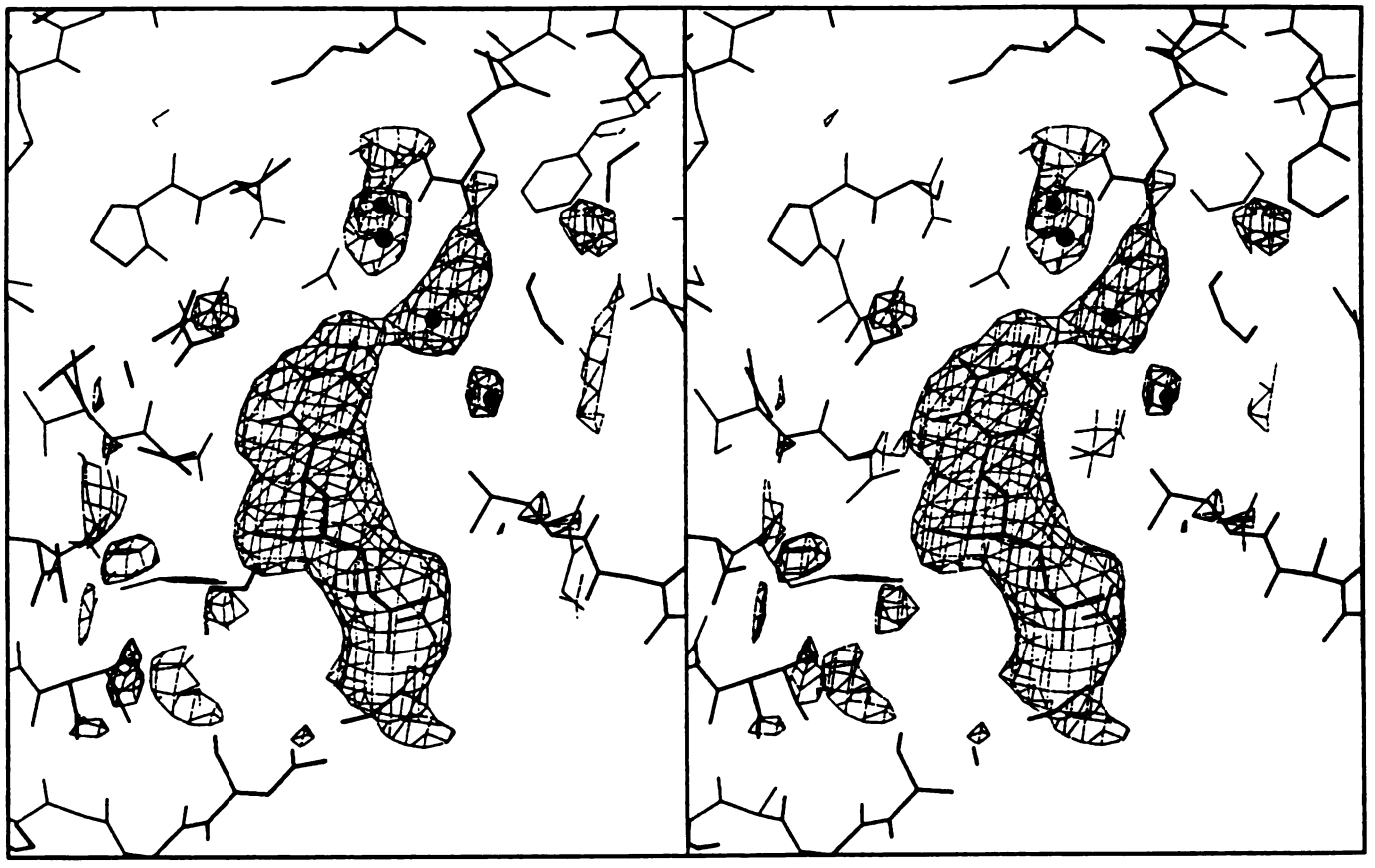


Figure 7.2b: E60Q-dUMP 3.0 Å map at 2.5 σ. Fo-Fc omit maps for ligands of the residue 60 mutants binary and ternary complexes. complex. Ligands and water molecules were removed and the protein subjected to positional refinement before calculation of difference density maps. These were made using the graphics program MOLSCRIPT[35].

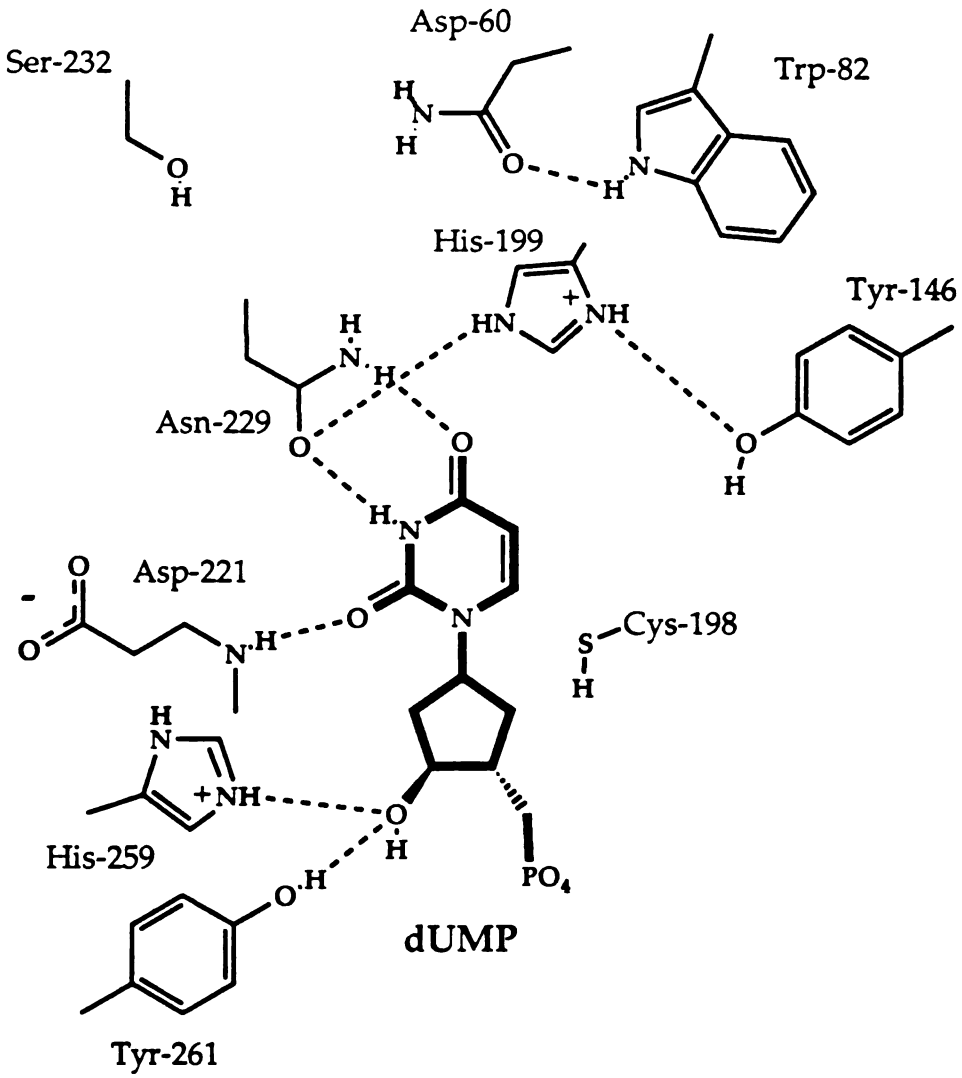


Figure 7.4b: Schematics LCTS active site of E60D-dUMP.

to the hydroxyl of Tyr-146; however, the orientation of the Tyr-146 side chain does not change if His-199 Nδ1 is left unprotonated. At 1σ contour level, 2Fo-Fc density for the His-199 side chain in the E60Q-dUMP binary complex is spherially voluminous enough that the imidazole ring remains in density as it is rotated 360° about the end of the Cβ-Cγ bond, so it is not possible to determine which nitrogen of the imdazole is protonated (if indeed His-199 is only mono-protonated at the active site).

The enzymatic dimmer remains in an 'open' conformation as the C terminus faces away from the active site. The Arg-23 side chain, however, is able to lie close to the C terminus while also helping to coordinate the nucleotide phosphate. The overall structure of the active site remains similar to wild type LCTS binary complexes. Electron densities for water molecules between the Gln-60 side chain and the side chains of residues Asn-229 and Ser-232 , and O-4 of dUMP (Water I) are present. However, the usually conserved water C-7 between dUMP C-5 and Tyr-146[6] appears to be disordered. The side chain of Ile-57 points away from that of Ile-81, while the Leu-195 side chain is able to point directly towards the pyrimidine base. Doubly protonated His-199 is able to hydrogen bond to both Asn-229 and Tyr-146, as for the E60D-dUMP complex. This would provide support (scaffolding) for the 'back' of the active site cavity. The Gln-60 side of the cavity, which includes Tryptophans 82 and 85, is similar to that found for wild type complexes with Gln-Oε1 able to hydrogen bond to the Trp-82 indole. Overall, the E60Q-dUMP complex is structurally similar to the corresponding wild type complex[18], but hints at changes seen in the other E60Q complexes.

E60Q-FdUMP

The E60Q-FdUMP cocrystal binary complex structure (Figures 7.2c, 7.3c, 7.4c) shares major features with the ternary E60Q complexes, including movement of the pyrimidine

away from Asn-229 and repositioning of the Asn-229 side chain. Strong 2Fo-Fc density near and around O-2 of the pyrimidine suggests a possible water molecule may be partially occupied in the area to facilitate interactions with the Asn-229 side chain in an unusual orientation. The Gln-60 residue remains oriented like Glu-60 of the wild type enzyme.

The C terminus has not moved towards the active site region and one of the terminal carboxylic acid oxygens can hydrogen bond to a water molecule located near the backbone atoms of Thr-24 and His-25, far away from the nucleotide. The side chain of Arg-23, which is often disordered in free enzyme structures[17,18], helps to coordinate the nucleotide's phosphate while being distal to the C terminus but is not located in well defined difference density and is probably somewhat disordered. It may be equally well refined with the guanidinium group closer to the C terminus. In this case the Val-316 residue rotates to bring the carboxylic acid end closer to the active site center, although the C terminus itself does not move further into the active site. While a monomer serves as the asymmetric unit of the $P6_122$ crystal space group, the enzyme itself exists as an obligate dimmer. It is possible, therefore, that both orientations of the Arg-23 side chain and the Val-316 residue are present. There is no covalent linkage between the FdUMP base and Cys-198. Interestingly, the side chain of Leu-195 bends away from the pyrimidine as if to make space F-5 of the pyrimidine This further suggests that Leu-195 may help influence nucleotide positioning[16].

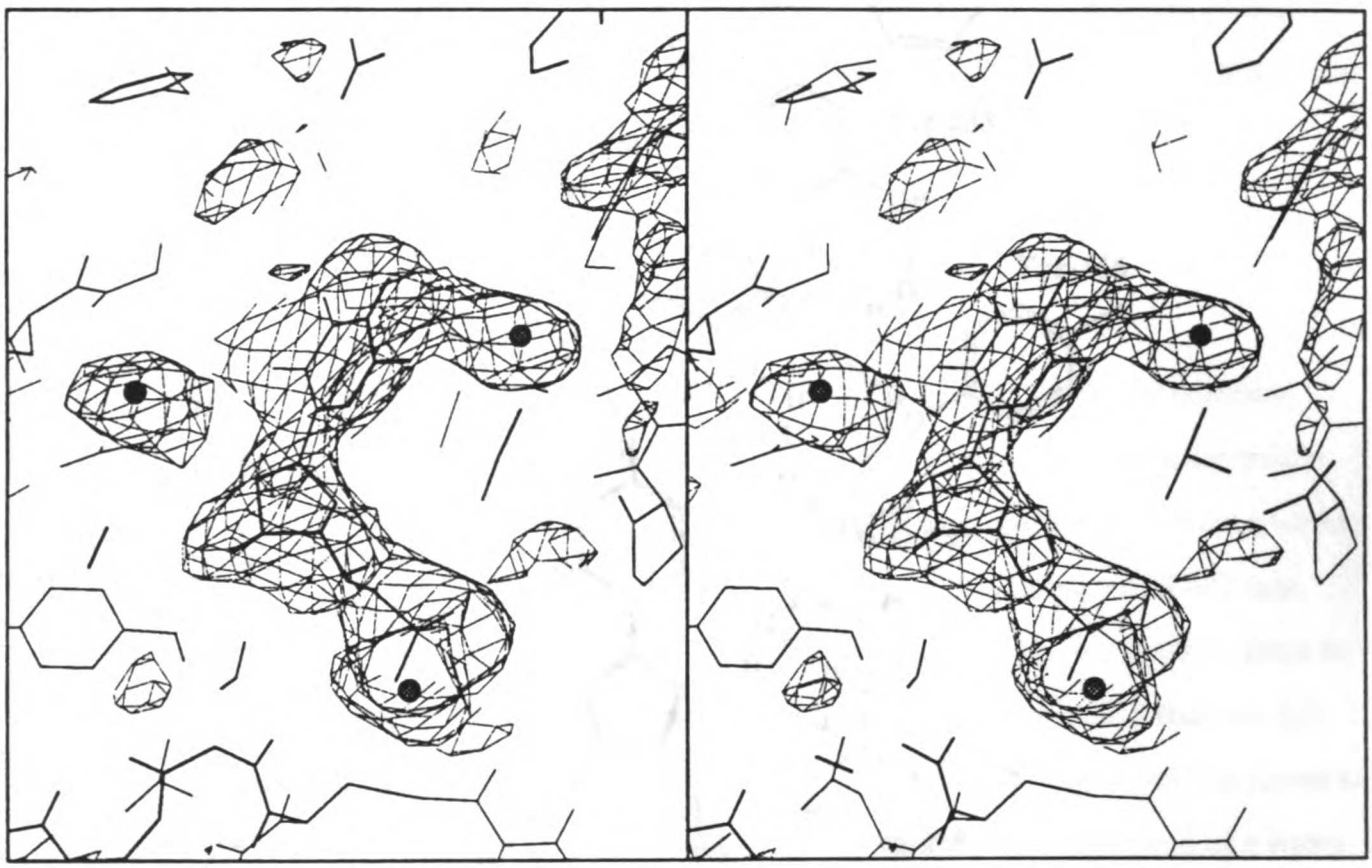


Figure 7.2c: E60Q-FdUMP 3.0 Å map at 2.0 σ. Fo-Fc omit maps for ligands of the residue 60 mutants binary and ternary complexes. complex. Ligands and water molecules were removed and the protein subjected to positional refinement before calculation of difference density maps. These were made using the graphics program MOLSCRIPT[35].

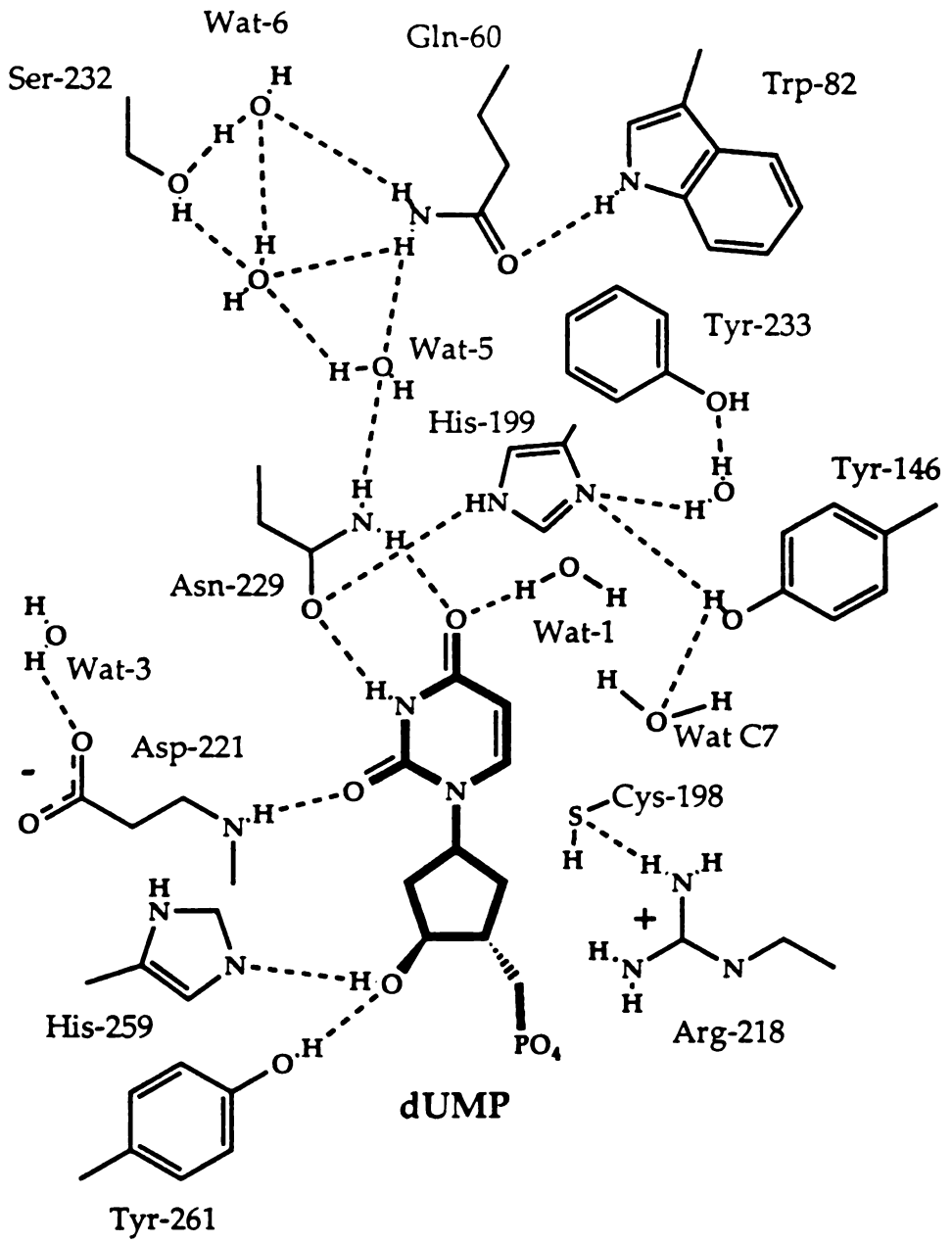


Figure 7.4c: Schematics LCTS active site of E60Q-dUMP.

Electron densities for most of the active site water molecules typical for LCTS binary complexes are found. However, as for the E60Q ternary complexes, Water I is not convincingly located and may not be well ordered within the active site. A shift in the orientation of the pyrimidine may be responsible for (or due to) the disordering of this water. Therefore, it is difficult to determine if the movement of the pyrimidine is due to the presence of the F5 atom or indirectly caused by the E60Q mutation.

E60Q-dUMP-CB3717

While the overall protein structure of the E60Q-dUMP-CB3717 ternary complex (Figures 7.2d, 7.3d, 7.4d) is similar to other LCTS ternary complexes[19,16], there are major and significant changes at the active site concerning ligand (esp. nucleotide) binding when compared to the equivalent wild type complex[16]. The nucleoside portion of dUMP has shifted orientation to position it farther from Asn-229 and Gln-217 and somewhat closer to Cys-198. This prohibits hydrogen bonding between the pyrimidine base and the Asn-229 side chain. The Asn-229 side chain is now free to rotate such that its amine (Nδ2) is closer to O-2 rather than O-4 of the pyrimidine. This is further stabilized by the inclusion of a water molecule that is able to hydrogen bond to both (Asn-229 Nδ2 amine and dUMP O-2).

Table 2a Common non-hydrogen distances within 3.3 Å for two or more binary complexes

contact	wild type dUMP	e60d dUMP	e60q dUMP	e60q FdUMP
R23 NH2 to dUMP PO_4	2.6	---	2.5,2.9, 3.2	---
res. 60 Oε,δ to W82 Nε1	2.9	3.0	2.7	2.8
res. 60 N/Oε,δ to water(s)	2.8, 2.9	—	2.9, 3.0, 3.0	—
H199 Nδ1 to Y146 OH	3.1	2.7	2.9	3.0
H199 Nε2 to N229 Oδ1	3.1	2.8	3.3	---
H199 Nε2 to dUMP O4	3.1	---	3.3	---
R218 $NH_{1,2}$ to dUMP PO_4	3.0,3.0, 3.3	2.7,3.1	2.9,3.1, 3.2	3.2
S219 Oγ to dUMP PO4	2.9	2.8	3.2	2.6
D221 N to dUMP O2	2.6	3.0	2.6	---
N229 Nδ2 to dUMP O4	3.1	---	2.5	---
N229 Oδ1 to dUMP N3	---	3.2	2.6	---
S232 Oγ to water(s)	2.9	---	2.5,2.5	---
H259 Nε2 to dUMP O3'	2.9	2.9	2.9	---
Y261 OH to dUMP O3'	2.7	3.1	2.7	---

Table 2b Common distances within 3.3 Å for two or more ternary complexes

contact	wild type dUMP CB3717	e60q dUMP CB3717	e60q FdUMP CB3717
R23 $NH_{1,2}$ to dUMP PO_4	3.1	---	2.7
res.60 Oε to W82 Nε1	3.2	2.9	3.2
res.60 Oε to CB3717 CP3-HP	---	2.8	2.8
C198 Sγ to dUMP C6	3.1	2.8	---
H199 Nδ1 to Y146 OH	2.9	3.0	3.1
R218 $NH_{1,2}$ to dUMP PO_4	2.9, 3.3	2.9, 3.3	3.2, 3.2, 3.3
S219 Oγ to dUMP PO4	2.8	3.1	---
N229 Nδ2 to G225 O	---	2.6	2.7
N229 Nδ2 to water	---	3.0	3.3
D221 N-H to water	---	2.7	2.9
S232 Oγ to F228 O	2.6	3.3	2.8
H259 Nε2 dUMP O3'	3.0	---	2.8
Y261 OH to CB3717 NA2-H1	3.1	2.1	---
CB3717 CP3 to water	2.3	2.6	2.8

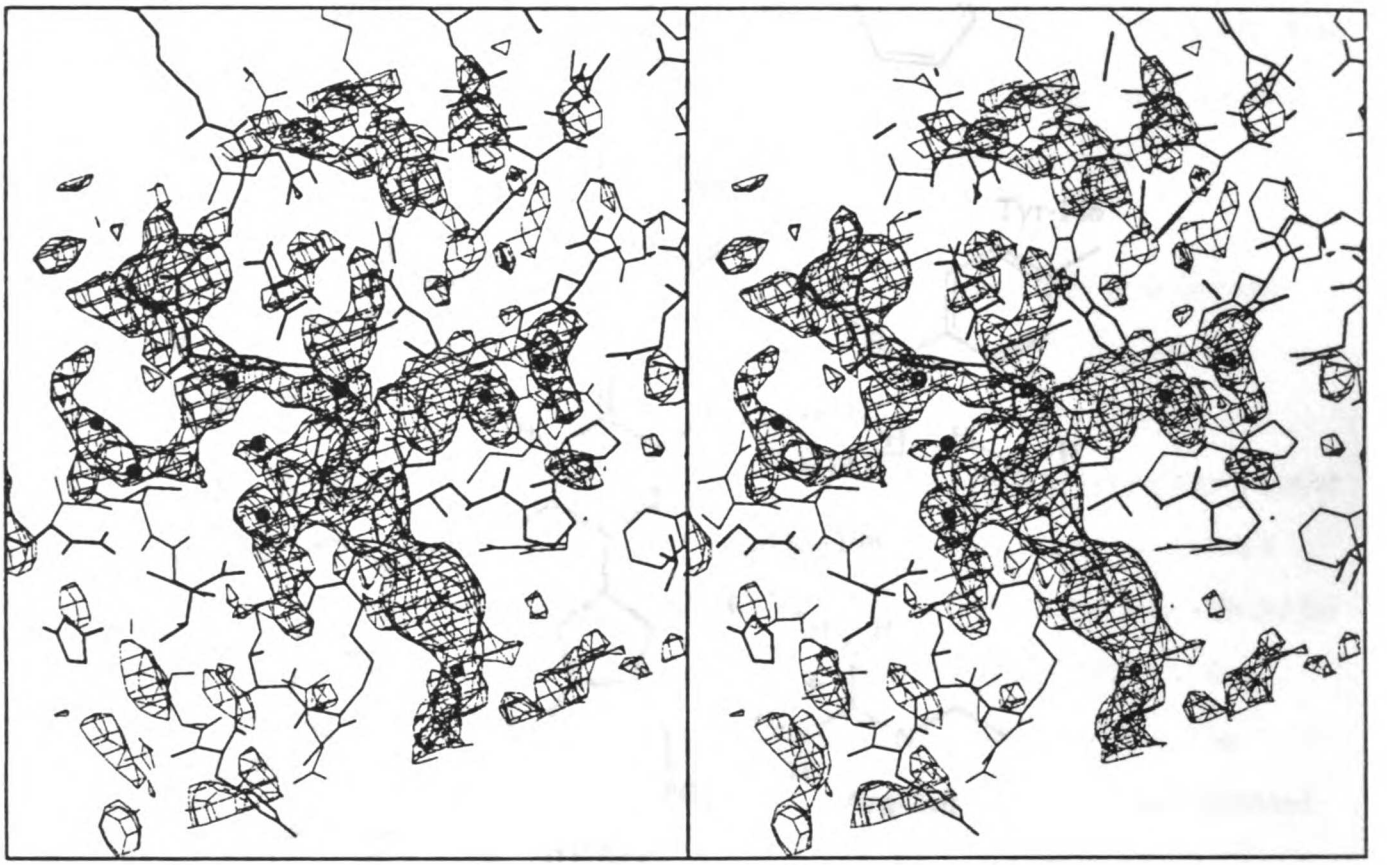


Figure 7.2d: E60Q-dUMP-CB3717 3.0 Å map at 1.5 σ. Fo-Fc omit maps for ligands of the residue 60 mutants binary and ternary complexes. complex. Ligands and water molecules were removed and the protein subjected to positional refinement before calculation of difference density maps. These were made using the graphics program MOLSCRIPT[35].

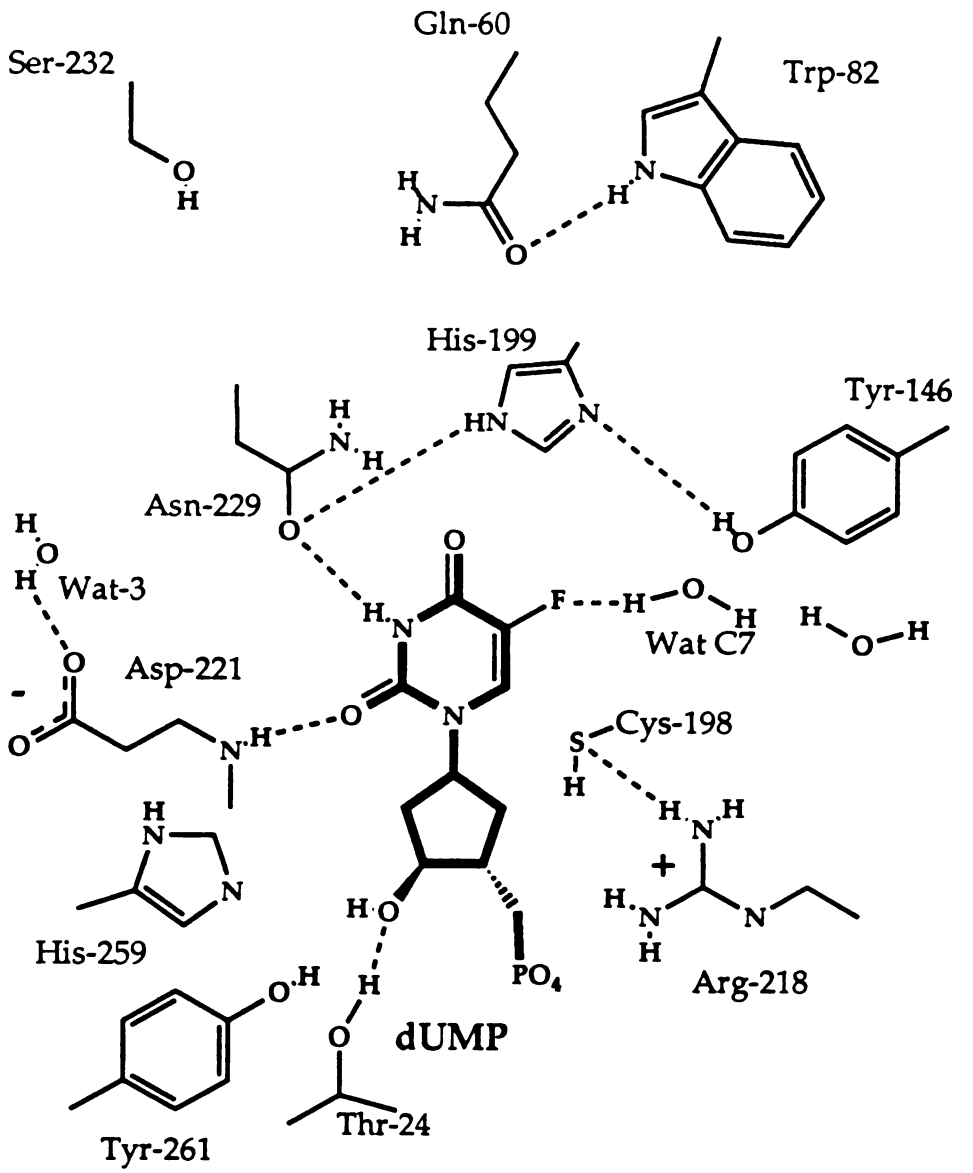


Figure 7.4d: Schematics LCTS active site of E60Q-FdUMP.

As for most TS ternary complexes[3] Water I maintains a location between residue 60 and O-4 of dUMP but is only transiently seen in electron density maps, suggesting partial occupancy. The transientness of Water I may be related to the protonation state of the His-199 imidazole since electron density for the water appears when Nε2 is protonated and Nδ1 is not. Oε1 of Gln-60 is still able to retain a hydrogen bond to the indole of Trp-82. This interaction probably helps to keep the mutant residue in the same orientation as for the wild type enzyme. The side chain of residue Ser-232 remains near residue 60.

A noticeable difference between the ternary E60Q mutant and wild type ternary complexes is the location of the propargyl group of the CB3717 folate analog. For the mutant complex it points directly towards Gln-60 while for the wild type complexes it points towards Phe-228 or towards the pyrimidine[5,16]. The propargyl moiety plays a major role in CB3717 binding since an equivalent folate analog utilizing a propyl group has a much lower binding affinity for TS[20,21]. CB3717 has a 4-fold increase in binding affinity for the mutant over the wild type enzyme (Table 3). The glutamate end of CB3717 in the mutant complex lies farther from Lys-50 than observed in wild type complexes. The quinazoline moiety of CB3717, while parallel in plane to the nucleotide base and oriented similarly to wild type complexes, shows little overlap with the pyrimidine ring.

The C terminus is pulled towards the active site cavity, which is known to be induced by folate or folate analog binding[19,3]. The guanidinium of Arg-23 is able to help coordinate the nucleotide phosphate while also being situated between the phosphate and the C terminus. This allows Arg-23 to directly

Table 3 kinetic parameters for LCTS wild type and E60Q complexes

parameter	wild type	E60Q
Thy^- Medium[a]	+	-
k_{cat}, s^{-1} for ^3H release[b]	9.8	6×10^{-2}
k_{cat}, s^{-1} for dTMP formation[c]	9.2	1.5×10^{-3}
Ratio of k_{cat} : ^3H rel./dTMP	1.1	40
Covalent Complex[d]	-	+
K_m[e] (mM) for dUMP	6.8	11
K_m[e] (mM) for CH_2H_4folate	19	76
k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$) dUMP	1.4	1.4×10^{-4}
k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$) CH_2H_4folate	0.5	2×10^{-5}
K_d, PLP (mM)[f]	1.6	1.1
K_d, dUMP (mM)[f]	0.7	1.2
Dehalogenation Reactions[g] (BrdUMP, IdUMP)		
k_{cat} (min^{-1})	1.2, 4.1	74, 82
K_m (mM)	5.4, 9.0	14, 7.3
k_{cat}/K_m ($min^{-1}\cdot mM^{-1}$)	0.2, 0.5	5.3, 11

[a] Thy^- medium data are complementation of c2913 *recA E. coli* in Thy^- medium.
[b] Tritium release from [5-^{3}H]dUMP was monitored by a decrease in the $^3H/^{14}C$ ratio of [2-^{14}C,5-^{3}H]dUMP during the course of the reaction.
[c] dTMP formation catalyzed by wild type TS was measured spectrophotometrically at 340 nm. dTMP formation for the E60Q mutant was measured by HPLC.
[d] Covalent complex containing TS, [5-^{3}H]dUMP and CH_2H_4folate was detected by SDS-PAGE.
[e] K_m value for wild type TS was obtained by spectrophotometric assay and that for E60Q TS was measured by tritium release reaction.
[f] The K_d value of dUMP was determined by UV/visable difference spectroscopy as described[32]. Binary complexes with wild type or E60Q TS enzymes were formed using 20 or 50 mM PLP and 2-3 mM protein in buffer containing 50 mM TES, pH 7.4 and 50 mM DTT. Dissociation constants for dUMP were obtained by competitive replacement of PLP by dUMP.
[g] TS-catalyzed dehalogenation of BrdUMP and IdUMP was monitored by the decrease in absorbency that accompanies dehalogenation ($\Delta\varepsilon_{285}$ = 5,320 $M^{-1}\cdot cm^{-1}$ for BrdUMP or $\Delta\varepsilon_{290}$ = 6,520 $M^{-1}\cdot cm^{-1}$ for IdUMP[26]. Reaction mixtures contained TES/DTT assay buffer (50 mM TES, pH 7.4, 25 mM $MgCl_2$, 1 mM EDTA and 10 mM DTT), 3 to 200 mM BrdUMP or IdUMP, and 0.12 to 3 mM of wild type or E60Q TS. The standard errors from nonlinear squares fit of the experimental data are less than 20% to 15% for all values.

hydrogen bond to the protein's terminal carboxylic acid, as can the side chain of Thr-24. These interactions help to stabilize the 'closure' of the active site[16].

There is a slight reordering of the clustering of hydrophobic side chains above the CB3717 molecule. Compared to the equivalent wild type complex[16] the side chain of Ile-57 has rotated away from that of Ile-81. For the wild type complex the terminal (δ) carbon of the Ile-57 side chain points towards Glu-60 and one of the hydrogenous from this methyl group may be able to form a weak polar bond with one of the Glu-60 side chain (ε) oxygens (to further support a hydrophobic clustering). In the mutant complex, with the CB3717 propargyl group pointing towards Gln-60 and away from Phe-228 and lying closer to the indole of Trp-82, the hydrophobic cluster formed by Trp-82, Ile-81, CB3717 propargyl, Phe-228, and Ile-57 (and secondarily supported by Trp-85 and CB3717 PABA) may be somewhat altered. This is apparently enough to subtly alter long-range ternary structure interactions between the side of the active site cavity wall involving Ser-232 and Asn-229 and the side of the cavity wall holding the catalytically reactive thiol of Cys-198 to favor movement of the pyrimidine closer to Cys-198 (and farther from Asn-229). Electron density maps suggest a possible covalent attachment of the pyrimidine from C-6 to Sγ of Cys-198, whereas for the wild type complex there is no evidence of covalence[16].

Partial occupancy of the cofactor "docking site" by the CB3717 quinazoline in E60Q ternary complex

Omit maps for the ligands (Figure 7.2d) show somewhat patchy densities for the CB3717 molecule which may suggest that it has multiple occupancies in the crystal. Difference electron density suggests partial occupancy of the quinazoline of CB3717 at an alternate site similar to that observed for the pterin of CH_2H_4folate in a wild type enzyme substrate complex in which CH_2H_4folate was bound with a closed imidazolidine ring; that

is, before the conversion of the cofactor to the reactive iminium ion[16]. We have proposed this alternate site to be the initial docking site for cofactor[16]. When the E60Q-dUMP-CB3717 complex is refined with the quinazoline ring in the alternate "docking" site the Q60 side chain rotates farther away from Ser-232 but is able to maintain a hydrogen bond to the indole of Trp-82. The OA4 atom of the quinazoline may also form long hydrogen bonds to the Q60 side chain (3.7 Å) and to the imidazole of His-199 (3.6 Å), while the NA2 amine of the quinazoline can hydrogen bond to the catalytic thiol of Cys-198 (2.2 Å). The epsilon amine of the Q60 side chain can no longer hydrogen bond to a water molecule conserved between Ser-232 and Glu-60, thereby reducing any structural influence residue 60 might have on the J α-helix (through water mediated interaction with Ser-232).

With the quinazoline positioned at this alternate site the dUMP nucleoside moiety is pushed farther away from Cys-198 to prevent covalent contact, as was seen for the wild type complex. It can not be directly determined whether the alternate site occupancy for the quinazoline is restricted to one or both monomers of the obligate dimmer, but partial density (from omit maps) for the nucleotide when it is (probably) covalently attached to the active site suggests that one or both monomers have a mixture of CB3717 orientations in the crystal.

Under X-plor[30] it is possible to refine both complexes at once, each with half occupancy, although this results in a doubling of the parameters to observations ratio. Results of this refinement presented non-overlapping ligands (from the two complexes), suggesting that both orientations of the CB3717 quinazoline might occur in either monomer's active site. The propargyl for CB3717 with alternate site quinazoline points towards Q60, while that of CB3717 with normal site quinazoline runs astride it's complex's dUMP pyrimidine. Noticeably, the side chain of Asn-229 is 'flipped' (as seen with the other E60Q complexes) for the complex having the CB3717 molecule bound in the normal

fashion, while for the complex with the alternate site quinazoline the Asn-229 side chain retains a more normal orientation with the delta oxygen hydrogen bonded to the imidazole of His-199 and the delta amine possibly making a long hydrogen bond (> 3 Å) to the quinazoline induced displaced pyrimidine base of dUMP. The C terminus for the complex with the alternate site quinazoline is moved farther into the active site cavity, in conjunction with movement of the guanidinium of Arg-23. Refined occupancies yielded 0.52 and 0.48 for the two complexes. A propensity for the E60Q mutant to allow alternate site binding for the pterin of a cofactor (greater than that allowed in wild type complexes with folate analog or cofactor) would support a diminished activity of the enzyme. Since quinazoline binding is most greatly mediated by hydrophobic interactions, its observed mixture of orientations in the crystal structure supports the hypothesis that the E60Q mutation affects the hydrophobicity of the active site pocket surrounding residue 60.

E60Q-FdUMP-CB3717

The structure of the E60Q-FdUMP-CB3717 ternary complex (Figures 7.2e, 7.3e, 7.4e) is similar to that of the dUMP ternary complex in that the side chain of Asn-229 is refined to position the Oδ1 atom distal to H-3 of the pyrimidine, but in this case the nucleotide has shifted even farther from the Asn-229 side of the active site cavity so that even with the Asn-229 side chain placed in a 'normal' orientation no direct

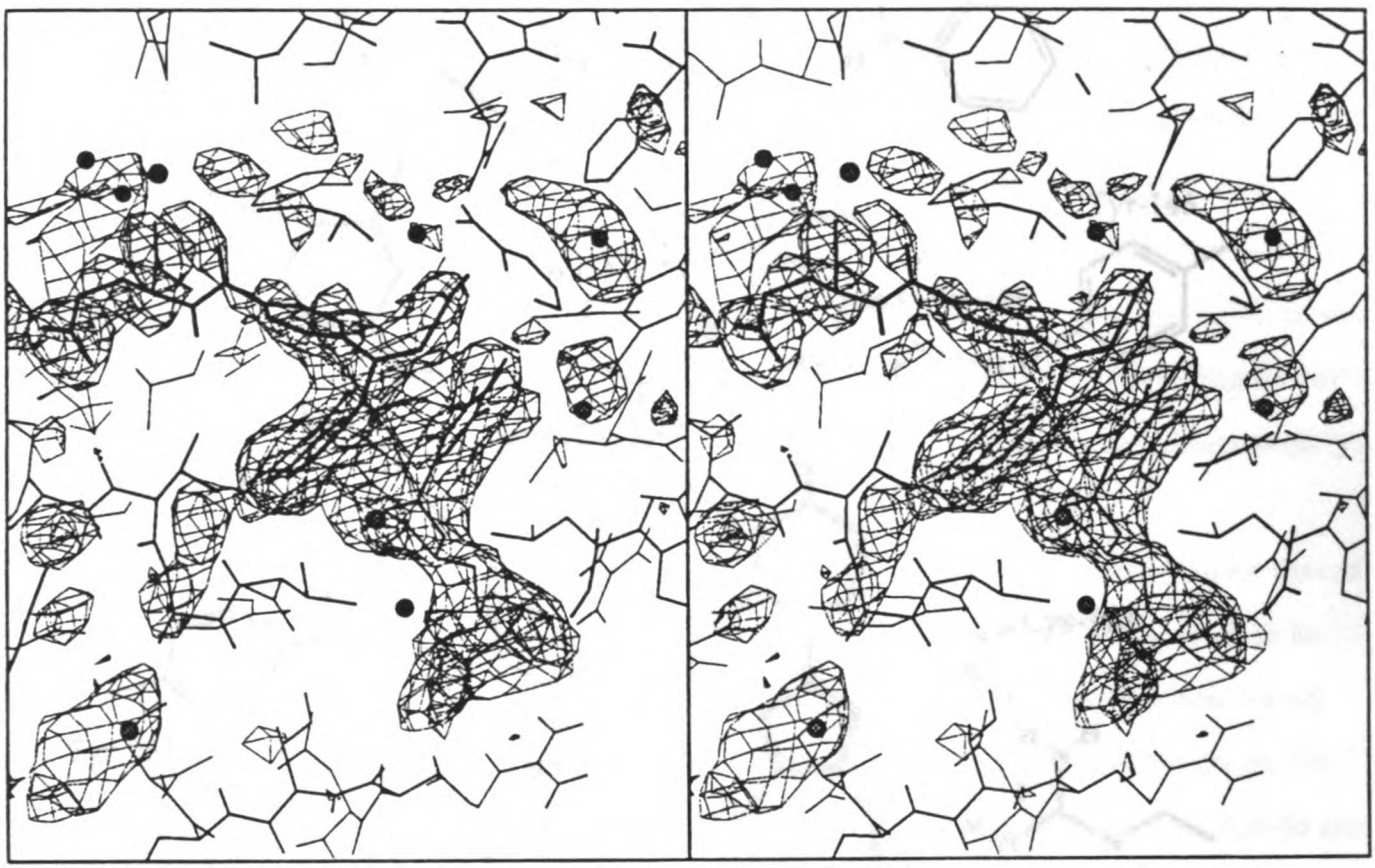
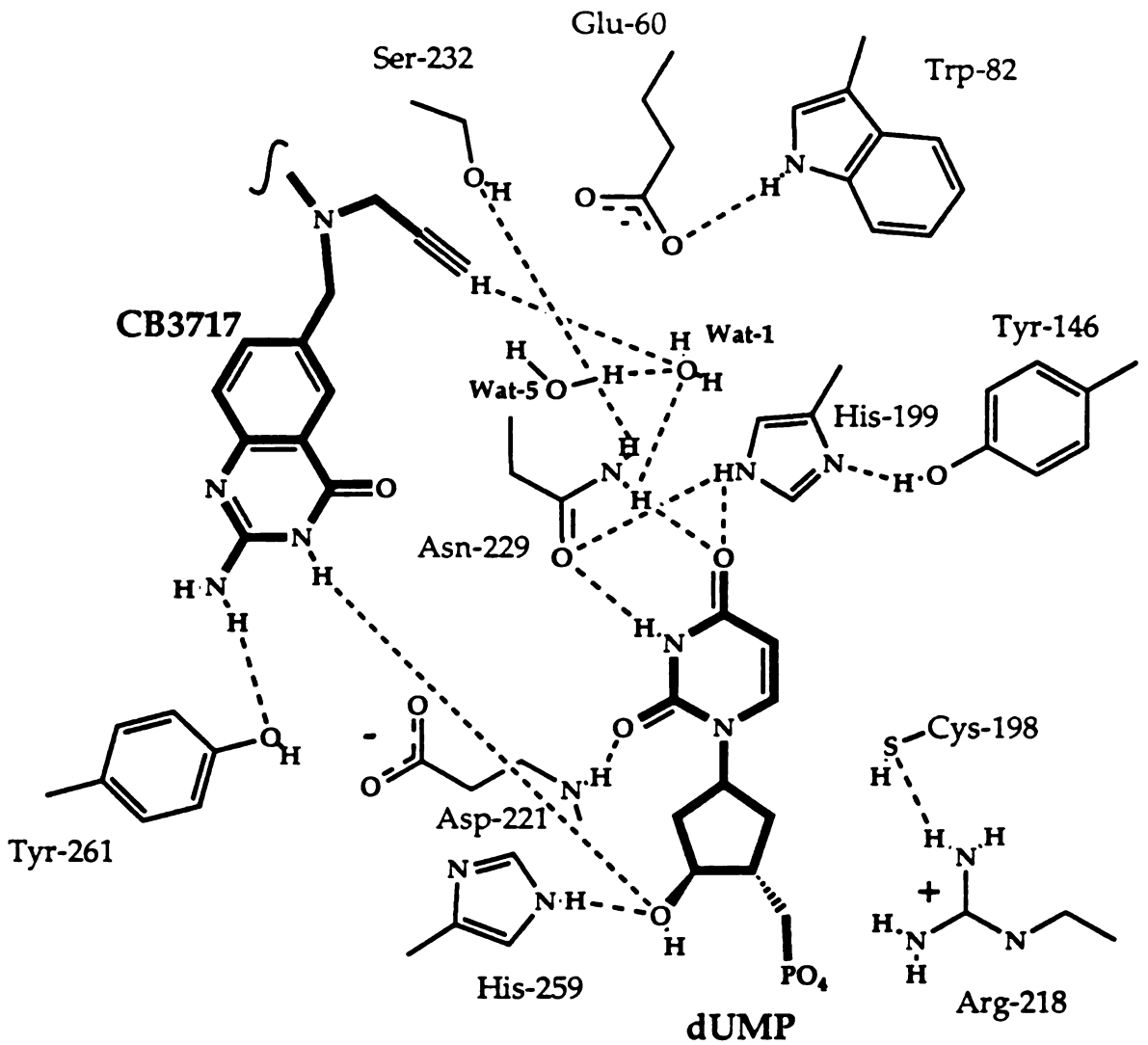


Figure 7.2e: E60Q-FdUMP-CB3717 3.0 Å map at 1.5 σ. Fo-Fc omit maps for ligands of the residue 60 mutants binary and ternary complexes. complex. Ligands and water molecules were removed and the protein subjected to positional refinement before calculation of difference density maps. These were made using the graphics program MOLSCRIPT[35].



TS wild type - dUMP - CB3717

Figure 7.4e: Schematics LCTS active site of WT LCTS-dUMP-CB3717.

interactions to the pyrimidine are possible. Yet, the FdUMP base is oriented in such a way as to also prevent its covalent attachment to Cys-198. The positioning of the CB3717 molecule does not appear to be unusual, but as for the mutant dUMP ternary complex there is no overlap of the quinazoline and pyrimidine ring systems and the propargyl group points directly towards Gln-60. Here a long (> 2.5 Å) hydrogen bond is possible between the propargyl's terminal hydrogen and Oε1 of Gln-60. The shifting of the nucleotide allows the quinazoline to move deeper within the active site cavity, and noticeably the C terminus of this complex has moved farther into the active site than for the E60Q-dUMP-CB3717 complex. The guanidinium of Arg-23, however, lies distal to the C terminus relative to the nucleotide phosphate. Compared to the corresponding dUMP complex there is less evidence of alternate site binding of the CB3717 quinazoline moiety.

As for the dUMP ternary complex the side chain of Ile-57 points away from that of Ile-81. However, the indole of Trp-85 has changed orientation. Water C-7, known to be involved in promoting the release of product dTMP from the active site[6], is positioned farther away from the pyrimidine. This may be due to the presence of the F-5 atom. No electron density can be discerned for Water I in its usual position (between residue 60 and pyrimidine O-4) regardless of the protonation state of the His-199 imidazole. It may have shifted to a position between Q60 and the propargyl group of CB3717, placing it closer to Oε1 of Q60 and farther from the pyrimidine base. As for the other mutant complexes a water molecule between Asn-229 and O-2 of the pyrimidine base is found well ordered.

Effects of the E60Q mutation

It is striking that a single atomic mutation relatively far from substrate ligands can result in an almost complete loss of catalytic activity for an enzyme. Residues Asn-229 and Cys-198 are the only amino acids which directly interact with the nucleotide base. Cys-198 has a fundamental role in catalysis, namely activation of C-5 of dUMP prior to

methylation. Asn-229 helps determine substrate specificity since it is used to exclude most pyrimidine nucleotides in favor of the appropriate substrate[13,22, 23]. However, Asn-229 is not as important in terms of effects on k_{cat}/K_m as is Glu-60, any mutation of which cuts down the catalytic rate by at least 10^{-4} , with the single exception of E60D which preserves the charge on residue 60, for which it is 10^{-2} [12,9,11].

The TS reaction involves several steps[3] and the mutation of Glu-60 affects several of these (Table 3). Therefore, correlation of structural and functional effects of the mutations is complex. The structures we report here are analogs of intermediates in the first half of the reaction (Figure 7.5); binary substrate complexes, and noncovalent ternary complexes. They provide insight into the role of Glu-60 in substrate and cofactor binding, alignment of substrates in a productive orientation for catalysis, and activation of C-5 of dUMP by covalent addition of Cys-198 Sγ to C-6. We assign effects of Gln-60 mutation primarily within these reaction steps. Our structures are less useful for understanding defects in the breakdown of the covalent ternary complex. One or more of the steps subsequent to covalent bond formation in ternary complex formation are affected by mutation of Glu-60 to such an extent that the later steps become rate determining[14,24]. We have analyzed effects of Glu-60 in these latter reaction steps to be presented independently[25].

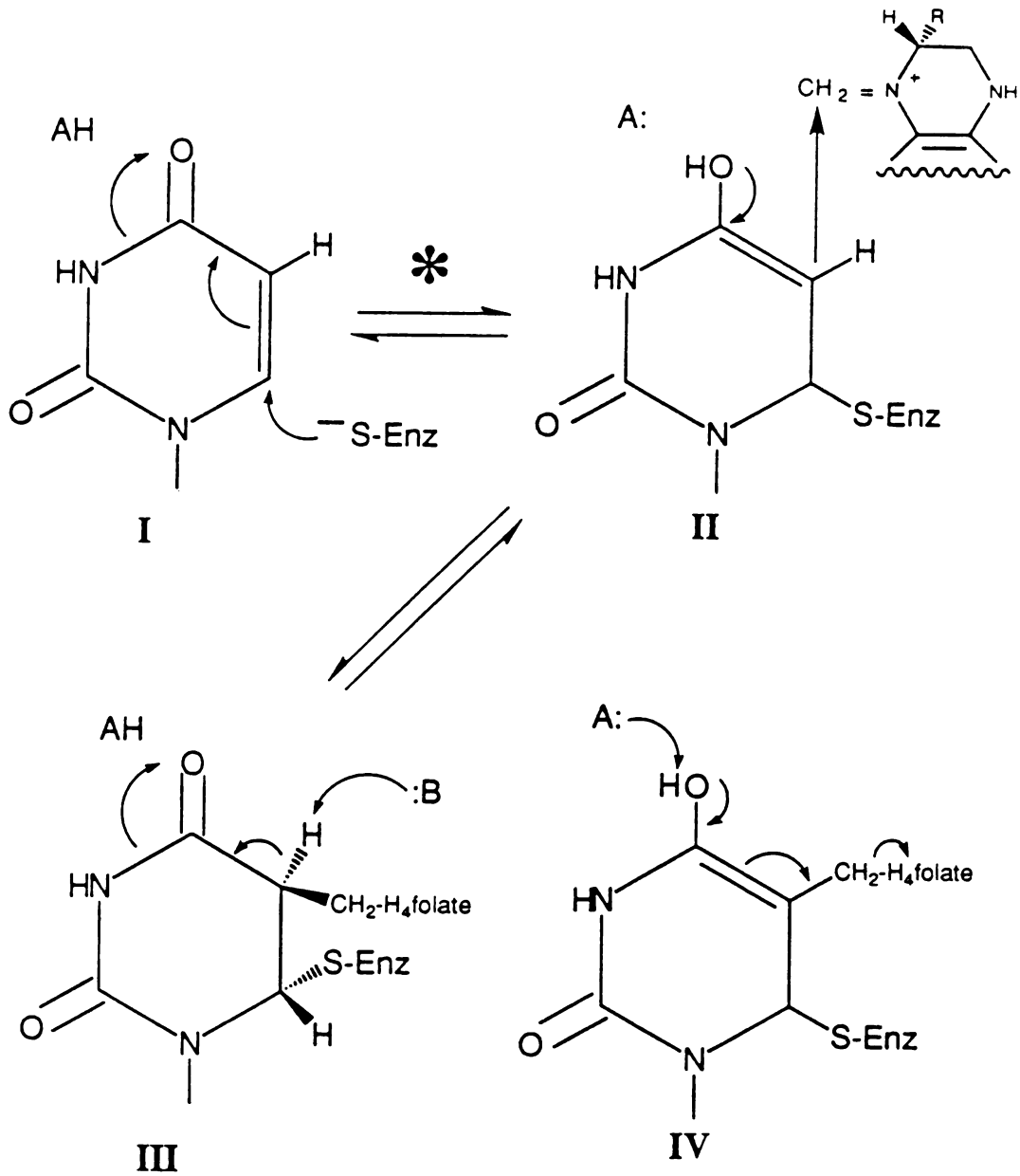


Figure 7.5: TS reaction scheme with intermediates labeled by roman numerals. An asterisk marks the initial states of associations of enzyme with ligands which would be addressed by the structures presented in this paper.

A. Effects on the mechanism of initial docking and alignment of substrate and cofactor for catalysis

The rate of covalent ternary complex formation is greatly depressed in Glu-60 variants as indicated by covalent complex formation with FdUMP and CH_2H_4 folate[10,24,11]. All of the structures of the E60 mutants with the exception of E60Q complex with dUMP alone show that direct interactions between Asn-229 and the nucleotide are broken, and so might hinder catalysis due to misorientation. The mutant structures show that deviation of the Asn-229 side chain from the optimal dUMP-binding orientation occurs not only in ternary complexes, where it completely eliminates interactions with the pyrimidine, but also in binary complexes where some interactions between side chain and pyrimidine are possible. In contrast, the cocrystal structure of a binary complex of the most conservative LCTS mutant, E60D with dUMP (Figures 7.3a and 7.4a) reveals more conventional interactions of the nucleotide with the active site residues.

In wild type TS, Asn-229 Oδ1 and Nδ2-H are hydrogen bond acceptor and donor, respectively, for N3-H and O-4 of dUMP. The two Asn-229 hydrogen bonds optimally align the pyrimidine of dUMP with the cofactor pterin ring and the catalytic cysteine in covalent ternary complexes[4,15]. In a structure proposed to represent the initial noncovalent ternary complex, prior to activation of the cofactor for addition to dUMP, the cofactor pterin ring is bound in the space that is occupied by the pyrimidine base in the covalent ternary complex (as well as in binary substrate complexes of TS) and dUMP does not form hydrogen bonds to Asn-229[16]. Thus, the hydrogen bonds formed by Asn-229 to dUMP may be key factors in the energetically costly transition between the initial loose ternary complex and the covalent ternary complex. Structural evidence that the Gln-60 mutation affects the transition from noncovalent to covalent ternary complex is that in both the *L. casei* TS E60Q-dUMP-CB3717 and in the crystal structure of the *E. coli* TS E58Q (*L. casei*

residue 60) complex with dUMP and CB3717[25], the substrate and cofactor analog are disordered, and appear to be bound in both the productive orientation seen in covalent complexes, and in the orientation seen in our proposed initial noncovalent ternary complex[16].

The Asn-229 residue position does not change greatly with respect to wild type TS in the active sites of the mutant complexes we report here but the entire region seems less highly constrained. The absence of interactions between Asn-229 and dUMP in the presence of folate or anti-folate can result from greater mobility of the Asn-229 side chain, allowing unconstrained rotation of the carboxamide group. This is related to rotational freedom of the imidizole of His-199 with feasible hydrogen bonding to Oδ1 of Asn-229 and/or to the hydroxyl of the Tyr-146 side chain. This may depend on whether the His-199 imidazole is doubly or mono-protonated at the active site. Electron density maps show a spherical volume for the His-199 side chain able to accommodate a 360° rotation of the imidazole about the Cβ-Cγ bond. Thus, in the E60Q mutant, His-199 can act as a 'gate' for allowing the Asn-229 side chain to orient itself to hydrogen bond productively to the pyrimidine. In the E60Q-dUMP binary complex where the N229 side chain is allowed to remain flipped, the NH_2 nevertheless retains hydrogen bonding to O-4. The structural changes and altered conformation of Asn-229 seen for the E60Q mutant are due to the indirect, relayed effects of mutation at residue 60 which also affect activity.

The electrostatic and structural effects of Glu-60 are relayed to Asn-229 by two mechanisms as detailed in 1,2 below. First, Glu-60 maintains a hydrogen bonding network of water molecules which mediate interactions between the pyrimidine base and the catalytically important residues Glu-60, Asn-229, and His-199[3]. The water network extends from Glu-60 to N-10 of the folate cofactor, the protonation of which would induce the opening of the imidazolidine ring, a necessary step in the TS reaction[3]. Second,

hydrophobic interactions between Phe-228 and the PABA moiety of the cofactor could indirectly affect the mobility and orientation of Asn-229, which is also on the hydrophobic helix.

1. *Evidence that water mediates productive orientation*

Gln-60 adopts a position in the active site almost identical to that of Glu-60 in wild type TS, maintaining a highly conserved hydrogen bond from the indole ring of Trp-82. However, there are two significant differences in the water structure around the side-chain. First, the electron density for the conserved water that normally hydrogen bonds to O-4 of dUMP, Water I, is diminished for the mutant structures so that it may be less ordered, or at a partially occupied site compared to wild type complexes. There is also a lack of electron density for a water molecule (**water #5**) connecting residue 60 with the side chains of Asn-229 and His-199 in most of these complexes. Therefore, the hydrogen bonding network is disrupted due to the E60Q mutation versus wild type complexes[16]. The same changes in water structure are also seen in E60Q of another species, the *E. coli* TS[25]. Hydrogen bonding to Asn-229 via a water (**#5**) in wild type TS is one mechanism by which Glu-60 indirectly constrains the orientation of the Asn-229 carboxamide so that it can make two hydrogen bonds to the pyrimidine substrate. In TS E60Q, this constraint is removed, and the side chain of Asn-229 is more free to adopt new orientations in which not only are the hydrogen bonds to dUMP not made, but also the carboxamide of Asn-229 interferes with productive orientation of substrates. In the *L. casei* TS mutant complexes, the Nδ2-Hδ2 of the Asn-229 side chain is hydrogen bonded to O-2 of the pyrimidine via a new water molecule. This interaction maintains both the non-productive orientations of Asn-229 and the nucleoside.

2. *How the polar E60Q substitution changes hydrophobicity at the cofactor binding site*

Glu-60 is located in the center of a cluster of hydrophobic residues involved in the initial binding and proper orientation of the folate cofactor or cofactor analog. The positioning of the pterin/quinazoline moiety is promoted by the hydrophobicity of the pyrimidine base; however, the orientation of the PABA ring is more influenced by hydrophobic residues Phe-228 and Ile-81. N-10, in the imidazolidine ring of the cofactor, is positioned directly under Oε1 of Glu-60 in "productive" wild type ternary complexes[4,5,16]. Thus, the hydrophilicity at the cofactor binding site is modulated for optimal binding of CH_2H_4folate. Both ternary E60Q mutant complexes presented here show a subtle change in the hydrophobicity of the cofactor binding site most consistently indicated by movement of the CB3717 propargyl moiety away from both the side chain of Phe-228 and the pyrimidine base, and towards the Gln-60 side chain itself (Figures 7.3d and 7.3e). There is also less stacking of the CB3717 PABA ring with that of the Phe-228 side chain, and a reordering of the side chains of Ile-57 and Ile-81. These small changes in hydrophobicity on mutation from Glu to Gln have little impact on K_m for CH_2H_4folate (Table 3).

Residue 60 is intimately associated with the hydrophobic core of the protein by virtue of a direct hydrogen bond from the indole of Trp-82. The hydrogen bonds on the donor N-H_2 of Q60 are more restricted than the acceptor O of E60. The decrease in degrees of freedom for the E60Q side chain is accompanied by a corresponding increase in mobility for the side chain of Asn-229, which is normally constrained by its direct interactions with the dUMP pyrimidine and with His-199. The cumulative effects of changes in the hydrophobic core, and in the water structure, make the ternary covalent intermediate form much more slowly.

B. Effects of Glu-60 mutation on activation of C-5 of dUMP for catalysis

The E60Q mutation does not appear to inhibit covalent attachment of nucleotide to protein. On the contrary, dehalogenation of 5-Br-dUMP (dUMP brominated at the C-5 position), a reaction used to assay binary complex formation and C-5 activation independent of cofactor, is actually enhanced by mutation of Glu-60 to Gln (Table 3). The loss of a negative charge at residue 60 might (via long range electrostatic effects) promote the formation of the negative Br^- ion during the dehalogenation. Folate or anti-folate binding to the binary complex should also promote Michael addition of the reactive Sγ of Cys-198 to C-6 of dUMP during the chemical reaction. Alternatively, the looser binding site might allow increased access to C-5 (of dUMP) of the required DTT for the dehalogenation reaction[26]. Indeed, TS activity in Glu-60 mutants can be rescued by the inclusion of simple organic acids, to high concentrations, which may take advantage of an altered active site volume or shape (Huang, W., personal communication).

C. Electrostatic effects of Glu-60 mutation on breakdown of the ternary complex

Kinetic data on *E. coli* TS mutants E58D and E58Q and of Bacteriophage T4 deoxycytidine hydroxymethylase (CH) mutants E60D and E60Q (*E. coli* numbering) suggest that cleavage of N-5 of the substrate folate from the methylene nascently transferred to C-5 of the substrate pyrimidine base is inhibited or slowed down[24]. They propose that the essential role of E60 (*L. casei* numbering) is to couple this cleavage with the abstraction of the pyrimidine C-5 hydrogen mediated by the side chain's electrostatic repulsion of anionic intermediates formed during the reaction. This, it is argued, could aid in movement of the conversion of the ternary covalent intermediate III to intermediate IV (Figure 7.5) along the reaction pathway so that for wild type enzymes (as opposed to the E60 mutants) the reaction is too fast to allow for isolation of intermediate III *in vitro*[14]. The maintenance of the E60 electrostatic repulsion is argued to be due to the residue's isolation

from bulk solvent, despite the presence of active site waters, since it is buried within a cluster of hydrophobic side chains (Figure 7.1).

The structural studies presented here for *L.casei* E60Q mutant complexes show that the Q60 residue is situated with an almost identical orientation as for the wild type E60 residue and that the Oε1 atom, nearest Water I and O-4 of the pyrimidine base, makes a commonly observed hydrogen bond to the indole of Trp-82. The Oε1 atom is located a similar distance from the pyrimidine O-4 (5.4 Å), despite movement of the nucleotide, as in the equivalent wild type complex. Therefore, it should have a similar influence on active site (nucleotide enolate) anionic species for both the wild type and mutant complexes (compared to Oε2 of E60). Support for a negative charge at Oε1 of Q60 is provided by the propensity of the CB3717 propargyl group in the mutant ternary complexes to point towards the Oε1 atom in an effort to form a hydrogen bond with the propargyl's terminal CH. The Q60 amine (Nε2) appears to lie too far away from N-10 of CB3717 and O-4 of dUMP (> 6.5 Å for both) in the LCTS E60Q-dUMP-CB3717 ternary complex to have much of a direct electrostatic effect and the water structure around it and nearby residue Ser-232 is not dissimilar to that found for the wild type complex. Our structures, therefore, lie in sharp contrast with the electrostatic proposals of Dunlop and Hardy[24] which do not provide for the structural changes seen in the mutant complexes reported here. The primary role of E60 cannot be protonation of N-5.

METHODS

Cocrystals of E60D/E60Q mutant *L. casei* TS-dUMP and TS-FdUMP binary complexes were grown in hanging drops at a pH range of 6.5 to 7.4 and concentrations of ammonium sulfate from 1% to 2% as described[17]. The crystals adopted a bipyramidal habit typically seen for liganded *L. casei* TS, with space group $P6_122$. Water dilutions of stock

solutions of CB3717 (K_i = 1.1 nM[21] were made and 1µl aliquots were tested on 4µl hanging drops until folate analog concentrations were determined which did not dissolve or degrade crystals (due to a conformational change in the protein) to the point of disallowing x-ray data collection. The final concentration of CB3717 was around 20 µM. Crystals were allowed to sit for no more than one week after CB3717 additions before mounting and x-ray diffraction. Treated and untreated crystals were selected for data collection at room temperature with an RAXIS II image plate detector system mounted on a Rigaku rotating anode generator using CuKα x-rays. Reduction of data was done using the RAXIS software[27,28].

The complex structures were solved by difference Fourier techniques[29] using protein phases (α_{calc}) from a highly refined wild type *L. casei* structure of TS-dUMP[18]. Evidence for ligand positioning was found by densities in initial 2Fo-Fc maps. Inclusions of first dUMP and later CB3717 molecules were followed by successive rounds of positional and molecular dynamics refinements using X-PLOR[30], combined with manual rebuilding under the Frodo graphics program[31] and calculation of new 2Fo-Fc, Fo-Fc, and simulated annealing 2Fo-Fc omit maps to confirm ligand positioning and side chain orientations. Inclusion of water molecules and refinement of individual thermal factors were done as last steps. Table 1 gives a summary of the data collection and refinement statistics.

Table 1a Data Collection and Refinement Statistics : ternary complexes

	e60q-dUMP-CB3717	e60q-FdUMP-CB3717
Data Collection		
Unit Cell lengths ($P6_122$) Å		
a,b	78.9	78.5
c	230.7	230.6
#reflections[1]	12343	9386
R-factor[2]	0.186	0.190
R-merge[3]	0.0752(54.8)[4]	0.1154(60.7)
Average B-factors after refinement ($Å^2$)		
backbone	16.0	10.5
side-chain	16.3	10.9
nucleotide	19.6	17.6
folate	27.9	22.5
waters(#)	18.8(238)	10.2(125)
rms deviations from ideality		
bonds > .06 A	0.020	0.021
number found	22	26
angles > 10°	4.087	4.565
number found	103	155
dihedrals > 90°	26.30	27.21
violations	none	none
impropers > 20°	1.758	1.885
violations	none	none
rms differences from initial protein structure		
backbone atoms	0.646	0.680
side-chain atoms	1.408	1.268

Table 1a (continued)

[1] resolution range 15 Å to 2.3 Å for e60q-dUMP-CB3717

resolution range 15 Å to 2.5 Å for e60q-FdUMP-CB3717

[2] R-factor = $\Sigma|(|F_o|-|F_c|)|_{hkl} / \Sigma|F_o|_{hkl}$

[3] R-merge = $(\Sigma\Sigma|F^2(i)-\langle F^2(\mathbf{h})\rangle|)/\Sigma\Sigma F^2(i)$ where $F^2(i)$ = ith reflection intensity, $\langle F^2(\mathbf{h})\rangle$ = mean intensity of h, k, and l

[4] (): percent of total reflections collected from 15 Å to highest refinement resolution

Table 1b Data Collection and Refinement Statistics : binary complexes

	e60q-dUMP	e60q-FdUMP	e60d-dUMP
Data Collection			
Unit Cell lengths ($P6_122$) Å			
a,b	78.3	78.5	79.1
c	242.3	229.1	230.3
#reflections[1]	11429	9385	11162
R-factor[2]	0.214	0.219	0.210
R-merge[3]	0.1078(71.0)[4]	0.0980(61.2)	0.0799(71.9)
Average B-factors after refinement ($Å^2$)			
backbone	23.9	12.5	22.7
side-chain	24.3	13.3	23.4
nucleotide	28.7	23.3	27.3
waters(#)	31.6(43)	13.0(40)	30.4(29)
rms deviations from ideality			
bonds > .06 A	0.023	0.023	0.023
number found	44	51	36
angles > 10°	4.260	4.585	4.245
number found	118	156	115
dihedrals > 90°	26.23	27.23	26.94
violations	none	none	none
impropers > 20°	1.914	1.993	1.870
violations	none	none	none
rms differences from initial protein structure			
backbone atoms	0.573	0.890	0.558
side-chain atoms	1.350	1.547	1.197

Table 1b (continued)

[1] resolution range 15 Å to 2.5 Å

[2] R-factor = $\Sigma|(|F_o|-|F_c|)|_{hkl}$ / $\Sigma|F_o|_{hkl}$

[3] R-merge = $(\Sigma\Sigma|F^2(i)-\langle F^2(\mathbf{h})\rangle|)/\Sigma\Sigma F^2(i)$ where $F^2(i)$ = ith reflection intensity, $\langle F^2(\mathbf{h})\rangle$ = mean intensity of h, k, and l

[4] (): percent of total reflections collected from 15 Å to 2.5 Å

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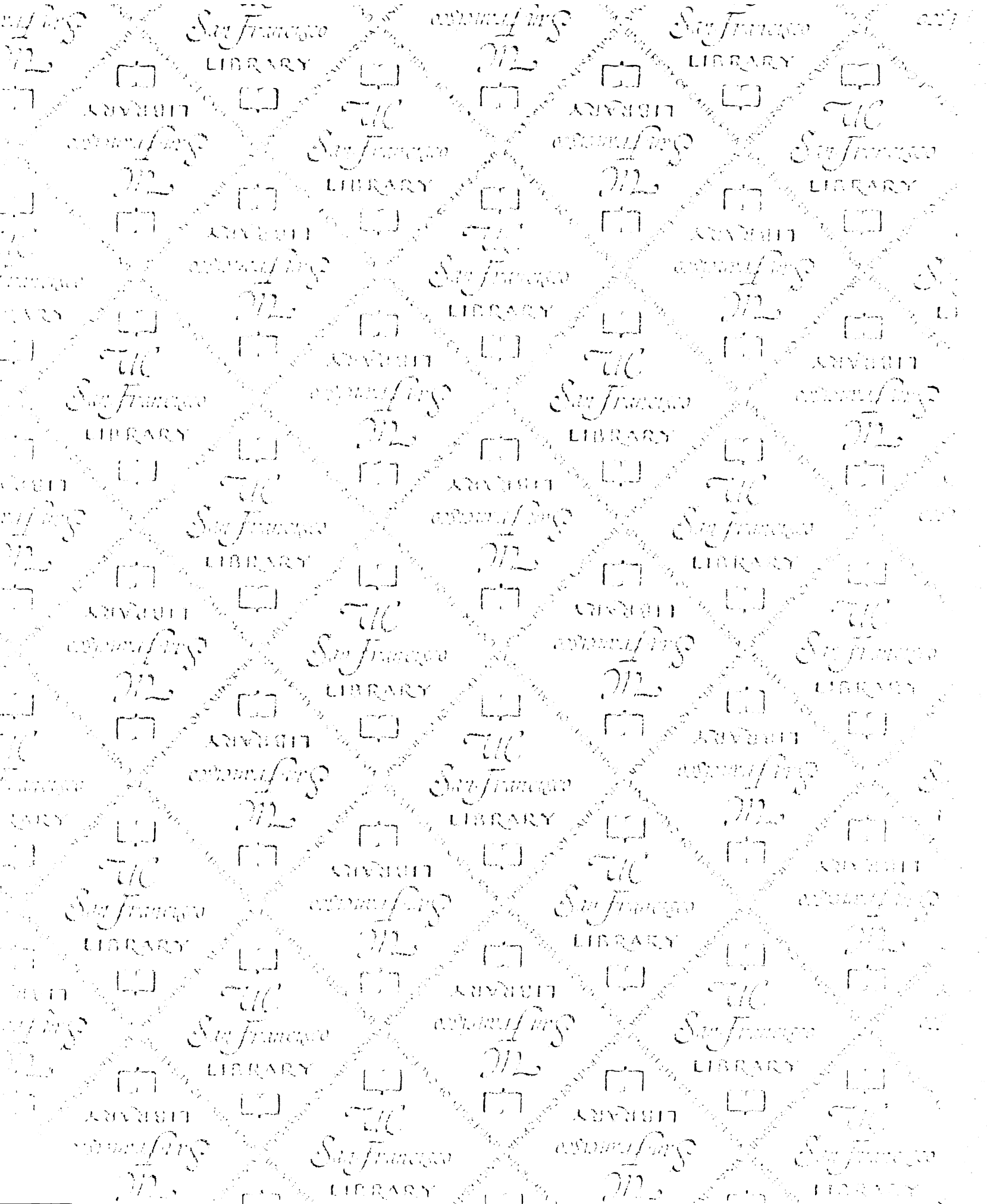
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