Properties of Bacteriophage T4 Thymidylate Synthase following Mutagenic Changes in the Active Site and Folate Binding Region†

Laura LaPat-Polasko, Gladys F. Maley, and Frank Maley*

Wadsworth Center for Laboratories and Research, New York State Department of Health, Empire State Plaza, Albany, New York 12201-0509

Received February 23, 1990; Revised Manuscript Received June 19, 1990

ABSTRACT: Amino acid replacements have been introduced in specific sites of bacteriophage T4 thymidylate synthase (T4-TS) to assess the role that these changes have on enzyme activity. Each of the conserved amino acids in the active-site region of T4-TS was modified, and the effects that these changes had on the kinetic and physical properties of this enzyme were measured. The mutations introduced were Pro-155-Ala (P155A), Cys-156-Ser (C156S), and His-157-Val (H157V) with the resulting synthases possessing k_{cat}'s of 10.3, 0.008, and 2.70 s^{-1}, respectively, relative to that of the wild-type enzyme of 11.8 s^{-1}. Equilibrium dialysis was performed on the wild-type and mutant enzymes to determine the binding constants for 2′-deoxyuridylate and 5-fluoro-2′-deoxyuridylate, and while in most cases the extent of binding of these nucleotides to the mutant proteins was reduced when compared with wild-type TS, the number of binding sites involved remained about 1 or less for the binary complex and almost 2 for the ternary complex. Heat and urea stability studies revealed that the mutant with the highest enzyme activity, P155A, was the most unstable, while spectrofluorometric analyses revealed that the structures of P155A and H157V were perturbed relative to the C156S and wild-type TSs. These studies are in agreement with others implicating the phylogenetically conserved active-site cysteine as playing an essential mechanistic role in the catalytic process promoted by TS. The proximal amino acids on either side of this cysteine, although also highly conserved, do not appear to affect the catalytic mechanism directly, but may do so indirectly through their influence on the conformation at the active site as well as other regions of the enzyme. Amino acids replacements were introduced also into the folate and deoxynucleotide 5′-phosphate binding sites of the T4-phage TS to ascertain the potential role that these amino acids play in the catalytic process. These positions were selected on the basis of previous chemical modification and X-ray crystallographic studies on *Lactobacillus casei* TS. Amino acid residues 48 and 49, which are in the putative folate binding site, were converted from lysines to arginines; in the former case, the mutated enzyme had less than 7% of the wild-type activity while in the latter, the mutated enzyme still retained about 60% of its activity. Spectrofluorometric studies revealed the K49R T4-TS mutation to affect a conformational change in the enzyme's structure, but little or no change was observed in the spectra of the T4-TS from K48R. The latter enzyme was impaired in its interaction with $CH_2H_4PteGlu$, as evidenced by a greater than 3-fold increase in its K_m. On the basis of these and our previous folylpolyglutamate fixation studies (Maley et al., 1982), it would appear that Lys-48 of T4-TS (Lys-50 in *L. casei* TS) contributes to the binding of folate substrates and their analogues to a greater degree than Lys-49 of T4-TS (Lys-51 in *L. casei* TS). Replacement of Arg-137 and Arg-176 in the phosphate binding sites of T4-TS with lysine residues diminishes enzyme activity by 70% in the former case, and almost completely in the latter. The TS from R137K does not show a spectrofluorometric shift, while the synthase from R176K does. However, the mutant enzyme from R137G shows a blue shift in its fluorescence spectrum, which is associated with a complete loss in activity. From these studies, it would appear that while both Arg-137 and Arg-176 promote nucleotide binding, the latter contributes more to this phenomenon than the former.

Thymidylate synthase (TS)[1] (EC 2.1.1.45) catalyzes one of the most unusual reactions in nature wherein $CH_2H_4PteGlu$ serves as both a one-carbon donor and a reductant in the conversion of dUMP to dTMP (Friedkin et al., 1957). The most plausible mechanism for this reaction involves an initial attack of a nucleophile from the enzyme on the 6-position of dUMP, followed by the directed addition of the methylene group from $CH_2H_4PteGlu$ to the 5-position of dUMP (Pogolloti & Santi, 1977). Kinetic studies with TSs from both eukaryotic (Lorenson et al., 1967) and prokaryotic (Daron & Aull, 1978) sources, as well as substrate binding studies (Galivan et al., 1976a,b), are in agreement with this sequential order of substrate addition, at least with $CH_2H_4PteGlu_1$ as the second substrate. However, when folylpolyglutamates are used as substrates, the reversed order of substrate addition appears to be in effect (Lu et al., 1984).

Studies involving the formation of a ternary complex with *Lactobacillus casei* TS plus FdUMP and $CH_2H_4PteGlu$ revealed unequivocally that the sulfur of the active-site cysteine is the nucleophile most likely involved in promoting the catalysis (Bellisario et al., 1976; Pogolotti et al., 1976). These results are consistent with chemical modification studies using sulfhydryl reagents that strongly implicated this amino acid

† This work was supported in part by National Cancer Institute Grant CA44355 from the U.S. Public Health Service, Department of Health and Human Services, and by a grant from the National Science Foundation (DMB86-16273).

* Address correspondence to this author.

[1] Abbreviations: TS, thymidylate synthase; PDDF, N^{10}-propargyl-5,8-dideazafolate; CH_2H_4F, 5,10-$CH_2H_4PteGlu$ or 5,10-methylenetetrahydrofolate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; IPTG, isopropyl β-thiogalactoside; TCA, trichloroacetic acid; BSA, bovine serum albumin; WT, wild type.

0006-2960/90/0429-9561$02.50/0

Table I: Amino Acid Sequence around the Active-Site Cysteine of Various Thymidylate Synthases[a]

Human	M-A-L-P-P C H-T-K-Y-Q	(Takeishi et al. 1985)
Mouse	M-A-L-P-P C H-T-L-Y-Q	(Perryman et al. 1989)
H. saimiri	M-V-L-P-P C H-V-L-S-Q	(Honess et al. 1986)
H. ateles	M-A-L-P-P C H-V-I-S-Q	(Richter et al. 1988)
Lei. major[b]	M-A-L-P-P C H-L-L-A-Q	(Beverley et al. 1986)
Lei. tropica[b]	M-A-V-P-P C H-L-L-A-Q	(Grumont et al. 1986)
E. coli	M-A-L-A-P C H-A-F-F-Q	(Belfort et al. 1983)
P. carinii	M-A-L-P-P C H-M-F-C-Q	(Edman et al. 1989)
V. zoster	M-V-L-P-P C H-T-L-C-Q	(Thompson et al., 1987)
C. fasiculata	M-A-V-R-P C H-L-L-G-Q	(Hughes et al. 1989)
T4-phage	M-A-L-P-P C H-M-F-Y-Q	(Chu et al. 1984)
S. cerevisiae	M-A-L-P-P C H-I-F-S-Q	(Taylor et al. 1987)
L. casei	M-A-L-P-P C H-T-L-Y-Q	(Maley et al. 1979)
Phage 3T	M-A-L-T-P C V-Y-E-T-Q	(Kenny et al. 1985)
Pl. falciparum	M-A-L-P-P C H-I-L-C-Q	(Bzik et al. 1987)
Ca. albicans	M-A-L-P-P C H-V-F-C-Q	(Singer et al. 1989)
B. subtilis	M-A-L-A-P C H-C-L-F-Q	(Iwakura et al. 1988)

[a] Abbreviations: *Lei*, *Leishmania*; *E.*, *Escherichia*; *P.*, *Pneumocystis*; *V.*, *Varicellavirus*; *L.*, *Lactobacillus*; Pl., *Plasmodia*; *Ca.*, *Candida*; *B.*, *Bacillus*; *H.*, *Herpesvirus*; *C*, *crithidia*. [b] There are the same organisms.

in the catalytic reaction (Dunlap et al., 1971; Galivan et al., 1977a). In agreement with these findings, evidence has been obtained by Moore et al. (1986) that the natural substrate dUMP forms a binary complex with TS at this cysteine. It is still unclear, however, whether the active-site cysteine at residue 198 in each of the two identical subunits of the *L. casei* TS is involved in promoting the conversion of dUMP to dTMP, and what role the highly conserved amino acids surrounding this cysteine play in the catalytic process.

The TS amino acid sequences from at least 16 diverse species encompassing man to bacteriophage reveal that this enzyme is among the most highly conserved of all proteins (Hardy et al., 1987; Perry et al., 1990) and suggest that the various TSs have similar conformations, particularly at their catalytic centers. The active-site cysteine and preceding proline are completely conserved in all of the synthase species studied to date, whereas the amino acid following the cysteine is histidine in all but one case (Table I), that induced by a *Bacillus subtilis* phage (Kenny et al., 1985).

A high degree of conservation was also found in the folate binding region, once it was established through the covalent fixation of folylpolyglutamate to the *L. casei* TS that Lys-50 and Lys-51 and possibly Lys-58 were involved in promoting the binding of the folyl substrate ligand (Maley et al., 1982). This proposal was supported in part by the invariant nature of the sequence in this region from various TSs (Table II). However, despite the conservation of basic amino acids in this region, when the inhibition of *Escherichia coli* and T4-TSs by $PteGlu_6$ was compared (Maley et al., 1979), the latter enzyme was much more susceptible to inhibition than the former. Since a distinctive difference in the two enzymes was a lysine at residue 49 in T4-TS and an arginine at this position in the *E. coli* synthase, it was of interest to determine whether converting the lysine to an arginine would make the phage synthase less responsive to inhibition by the folylpolyglutamates.

Similar studies to define the nature of the functional groups involved in dUMP binding of the nucleotide substrates revealed previously that phenylglyoxal, an arginine-modifying reagent, inactivated *L. casei* TS (Belfort et al., 1980). While our results indicated that a single arginine per enzyme subunit was affected, others have reported that about four arginines had to be modified before enzyme activity was lost (Cipollo et al., 1979). Subsequent X-ray crystallographic analyses have revealed that the invariant guanidinium groups of arginines-23, and -178, -179, and -218 of the *L. casei* TS (Hardy et al., 1987) form seven hydrogen bonds with the oxygen atoms of the phosphate group. Similar results have been obtained with the enzyme from *E. coli* (Montfort et al., 1990).

The present paper presents a more complete view of our earlier studies (Frasca et al., 1988) on the effect that amino acid alterations in these sites have on T4-TS activity in an effort to define the role that they play in contributing to catalysis by this enzyme.

Experimental Procedures

Materials

Strains and Vectors. *E. coli* JM103, *E. coli* JM105, and PUC18 were obtained from Bethesda Research Laboratories. JM103 was maintained on M9 minimal media (Maniatis et al., 1982) and DH5αF′ on TBYE (1% Bacto-tryptone, 0.5% NaCl, and 0.5% yeast extract). The *thy*A$^-$ mutant, χ2913, a gift from Dr. D. V. Santi (Pinter et al., 1988), was maintained on M9 plus thymine and trimethoprim (50 μg/mL each). M13tdΔI was constructed by West et al. (1986) and was employed for all of the mutagenesis experiments. pKK223.3 (Ahmann et al., 1983) was purchased from Pharmacia.

Radioisotopes and Restriction Enzymes. [γ-^{32}P]ATP (>5000 Ci/mmol) and [α-^{32}P]dCTP (400 Ci/mmol) were purchased from Amersham Corp. The former isotope was used with a highly purified thymidine kinase from *E. coli* to synthesize [^{32}P]FdUMP of a comparable specific activity (F. Maley, unpublished experiments) and for the 5′-end labeling of oligodeoxynucleotide probes with cloned T4-phage polynucleotide kinase (U.S. Biochemical Corp.). The latter was used for DNA sequencing. [6-^{3}H]FdUMP and also [5-^{3}H]- and [6-^{3}H]dUMP were purchased from Moravek Biochemicals, Inc. These compounds were diluted to 75–200 cpm/pmol for use in the equilibrium dialysis and binding studies. Restriction enzymes were obtained from Bethesda Research Laboratories, U.S. Biochemical Corp., and American Allied Laboratories.

Oligonucleotides. The following oligonucleotides, prepared with an Applied Biosystems Model 381A DNA synthesizer, were used for mutagenesis; the underlined regions represent those where mutagenic changes were introduced:

(P155A) 5′-CATATGACAAGCCGGTAATGC

(H157V) 5′-GAACATAACACAAGGCGG

(C156S) 5′-GAACATATGGCTAGGCGGTAA

(C156R) 5′-GAACATATGGCGAGGCGGTAA

(K48R) 5′-GGGGAGCTTCCGAGTTGTTAC

(K48M) 5′-GGCGAGCTTCATAGTTGTTAC

(K49R) 5′-GGCGAGCCGCTTAGTTGTTAC

(R137G) 5′-AATTTGGCCCCTATCATTTGG

(R137K) 5′-AACAATTTGTTTCCTATCATTTGG

(R176K) 5′-ATCTACTGATTTTTGATACCACT

Methods

Growth Conditions. *E. coli* JM103 and 105 were grown in TBYE (plus 5 μg/mL ampicillin if transformed with the plasmids pUC18 or pKK223.3) at 37 °C in a New Brunswick G25 shaker until log phase was reached (A_{650} = 0.2). The cells were then infected with the appropriate M13*td*ΔI mutant (at a multiplicity of 10) and 20 min later were induced with 1 mM IPTG. After 3.5 h of incubation, the cells were harvested, washed with TM buffer (10 mM Tris-HCl, pH 7.5, and 1 mM $MgCl_2$), and stored at −80 °C as a centrifuged pellet.

Table II: Amino Acid Sequence at the Folate Binding Region of Various Thymidylate Synthases[a]

Human	P - L - L - T - T K - R V - F - W - K	(Takeishi et al. 1985)
Mouse	P - L - L - T - T K - R V - F - W - K	(Perryman et al. 1989)
H. saimiri	P - L - L - T - T K - R V - F - W - K	(Honess et al. 1986)
H. ateles	P - L - L - T - T K - R V - F - W - R	(Richter et al. 1988)
Lei. major[b]	P - L - L - T - T K - R V - F - W - R	(Beverley et al. 1986)
Lei. tropica[b]	P - L - L - T - T K - R V - F - W - R	(Grumont et al. 1986)
E. coli	P - L - V - T - T K - R C - H - L - R	(Belfort et al. 1983)
P. carinii	P - A - V - T - T K - R V - F - I - R	(Edman et al. 1989)
C. fasiculata	P - L - L - T - T K - R V - F - W - R	(Thompson et al., 1987)
V. zoster	P - L - L - T - T K - R V - F - W - R	(Hughes et al. 1989)
T4-phage	P - A - V - T - T K - K L - A - W - K	(Chu et al. 1984)
S. cerevisiae	P - L - L - T - T K - K V - F - T - R	(Taylor et al. 1987)
L. casei	P - L - L - T - T K - K V - P - F - G	(Maley et al. 1979)
Phage-3T	P - I - L - T - T K - K V - A - W - K	(Kenny et al. 1985)
Pl. falciparum	P - L - L - T - T K - K L - F - L - R	(Bzik et al. 1987)
Ca. albicans	P - L - L - T - T K - K V - F - S - K	(Singer et al 1989)
B. subtilis	P - M - L - T - T K - K L - H - F - K	(Iwakura et al. 1988)

[a] Same abbreviations as in Table I. [b] Same as in Table I.

Mutagenesis Procedure. The procedure used for the preparation of P155A and H157V was basically that described by Zoller and Smith (1984). C156S, K48R, K49R, R137K, and R176K were isolated by using the Taylor et al. (1985) procedure as described by Amersham, but with 3 times the DNA recommended for the ligation step. M13*td*ΔI RF DNA was prepared by the cesium chloride gradient procedure of Maniatis et al. (1982).

Single-stranded M13tdΔI DNA (2 μg) was mixed with 2.5 ng of 5′-phosphorylated mutant oligonucleotide and 1.2 μL of 10× mutagenesis buffer (100 mM Tris-HCl, pH 7.5, and 100 mM $MgCl_2$) in a final volume of 12 μL. The mixture was placed in an 80 °C water bath, which was cooled slowly to room temperature (usually 1.5–2 h). Then, the following reagents were added: 0.8 μL of 10× mutagenesis buffer, 4 μL of 2 mM dNTPs, 1 μL of 10 mM ATP, 1 μL of 100 mM DTT, 1 μL of Klenow DNA polymerase (5 units/μL), and 0.5 μL of T4-phage DNA ligase (2.0 units/μL). The mixture was incubated at 15 °C in a circulating water bath for 16 h, and heat-inactivated for 10 min at 65 °C, after which aliquots were diluted 1 to 10 and 1 to 100. log-phase JM103-competent cells (100 μL), prepared as described by Maniatis et al. (1982), were mixed with 1 μL of the diluted mutagenized DNA, placed on ice for 30 min, and heat-shocked for 1.5 min at 42 °C, after which 200 μL more of the log-phase cells was added. The resulting suspension was transferred to a sterile glass tube to which 3 mL of top agar was added. The mixture was poured onto agar plates, which were incubated overnight at 37 °C to promote plaque formation.

Selection of Mutants. Mutants were selected by a modification of the nitrocellulose plaque lift oligonucleotide screening procedure described by Zoller and Smith (1984). Those plaques that still hybridized with the ^{32}P-labeled mutant oligonucleotides (prepared as described below) under stringent conditions (65 °C in 6 × SSC) were plaque-purified at least once more. Mutant and wild-type M13*td*ΔI were dot-blotted onto nitrocellulose or Hybond filters and washed at 37 °C in 20 × SSC for 30 min. This was followed by a wash at 65 °C for 30 min in 0.1 × SSC plus 0.5% SDS. Under these conditions, the mutant oligonucleotides were usually removed from the wild-type plaques but not from the mutants.

Mutant Cloning. The mutated forms of M13*td*ΔI RF DNA (100 μg) were treated with 5 units of *Eco*RI/μg of DNA at 37 °C for 4 h, and the resulting digests were subjected to electrophoresis on 1.5% agarose gels. The DNA fragments were isolated from the gels and purified by using the Gene Clean procedure according to the manufacturer's instructions (Bio101 Corp., La Jolla, CA) or by the Spin-X centrifuge method (Costar, Cambridge, MA). The corresponding H157V and P155A (50 ng), 1.7-kb *td*ΔI fragments were cloned into *Eco*RI, calf alkaline phosphatase treated pKK223.3 (10 ng) using 2.5 μL of optimal ligation buffer (Bethesda Research Laboratories) which contained 250 mM Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 25% PEG 8000, 5 mM ATP, 10 mM spermidine, 5 μL of fresh 0.1 M dithiothreitol, and 0.25 μL of T4 DNA ligase (0.4 unit/μL). The reaction was incubated overnight at 15 °C followed by heating at 65 °C for 10 min to inactivate the ligase. Five microliters of the ligated DNA was added to 100 μL of the appropriate competent cells. The mixture was incubated on ice for 10 min and then placed at 37 °C for 10 min, followed by a 10-min incubation at room temperature. One milliliter of TBYE was added to the transformation mixture, and the contents were transferred to a sterile glass tube. The transformation reaction was then placed in a 37 °C shaking water bath for at least 1 h, and various amounts of the mixture were spread onto agar plates containing ampicillin (100 μg/mL). Isolated colonies were selected by a modified colony hybridization procedure (Grunstein & Hogness, 1975). The plasmid DNA was purified (Holmes & Quigley, 1981) and cut with various restriction enzymes to determine the orientation of the DNA.

Oligonucleotide Labeling. About 500 ng of each specific mutant oligonucleotide was mixed with 10 μL of [γ-^{32}P]ATP (100 μCi, 5000 Ci/mmol), 2.5 μL of 10× kinase buffer (500 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, and 1 mM spermidine), and 1 μL of (10 units/μL) T4-phage polynucleotide kinase in a final reaction volume of 25 μL. The reaction mixture was incubated at 37 °C for 30 min, then treated with 4 μL of 10 mM ATP, 1.5 μL of 10× kinase buffer, 1 μL of the polynucleotide kinase, and 8.5 μL of water, and incubated for another 30 min at 37 °C. After heat inactivation at 65 °C for 10 min, the reaction mixture was passed through a Nensorb column (New England Nuclear Corp.), and the labeled oligonucleotide was isolated according to the manufacturer's instructions. The methanol–water solution was concentrated to dryness in a Speed Vac apparatus (Savant) and dissolved in water to give a final oligonucleotide concentration of 2.0 ng/μL (Zoller & Smith, 1984).

DNA and Amino Acid Sequencing. The mutants were all verified by using Sanger (1981) dideoxy sequencing. Additional verification was obtained by amino acid analysis of a heptapeptide encompassing amino acids 152–158 of the active-site region (Chu et al., 1984), that was isolated by reverse-phase HPLC of CNBr-treated mutant and wild-type TSs. The peptides were also sequenced by using an Applied Biosystems 477 protein/peptide sequencer.

Purification of Mutant and Wild-Type TSs. The various mutant synthases were induced with 1 mM IPTG for at least 3 h from JM103 infected with the appropriate M13*td*ΔI mutants (K48R, K49R, R137K, R176K) or the following plasmid/cell transformants: P155A and pKK223.3/JM105; C156S from pUC18/$_{\chi}$2913; H157V from pKK223.3/JM103. R137G was cloned into pUC18 and transformed into the *thy*A$^-$ host $_{\chi}$2913, and the transformed cells were induced with 1 mM IPTG for 12–18 h at which point the mutant enzyme was induced to about 5% of the cellular protein. The M13-transfected cells, after IPTG induction, yielded the mutant enzymes to about 1% of the cellular protein. The wild-type synthase was induced as described previously (Belfort et al., 1983b). All of the procedures in the purification were performed at 0–4 °C and yielded enzyme protein after the final step that was at least 95% pure based on SDS–PAGE.

Step 1: Cell-Free Extract. Harvested cells (55 g) were thawed and resuspended in 3 volumes of a solution containing 50 mM Tris-HCl, pH 7.1, 0.1 mM EDTA, 20 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and DNase (5 μg/mL of suspension). The mixture was stirred and incubated at room

temperature for 30 min. The slurry was placed in an ice–ethanol bath, and the cells were disrupted by sonication (Bronwill Biosonic IV large probe, setting of 80) for three 2-min cycles. Unbroken cells and cell debris were removed by centrifugation at 20000*g* for 30 min.

Step 2: *Streptomycin Treatment*. A 5% solution of streptomycin sulfate was added to the cell-free extract (15 mL/100 mL). The suspension was stirred for 10 min and then centrifuged at 20000*g* for 30 min.

Step 3: *Ammonium Sulfate*. The supernatant fraction from the previous step was brought to 0.30 saturation ammonium sulfate with solid ammonium sulfate and centrifuged at 20000*g* for 30 min, and the pellet was discarded. The resulting solution was then brought to 0.80 saturation and centrifuged as before. The precipitate was dissolved in a solution of 50 mM Tris-HCl, pH 7.1, 0.1 mM EDTA, and 20 mM 2-mercaptoethanol and dialyzed overnight against two 1-L changes of the same buffer.

Step 4: *Phosphocellulose Chromatography*. The dialyzed enzyme solution was applied to a 2 × 15 cm phosphocellulose (Schleicher & Schuell) column equilibrated with 20 mM potassium phosphate buffer, pH 7.1, 0.1 mM EDTA, and 20 mM 2-mercaptoethanol. The column was eluted with a 50 mM aliquot of the same buffer until the A_{280} was minimal (about 100 mL). Then 100 mM buffer was applied until the enzyme began to elute, at which point the buffer was changed to 200 mM to remove all of the enzyme (50–100 mL). The active fractions were pooled, and the enzyme was precipitated with 0.80 saturation ammonium sulfate. After centrifugation, the pellet was stored at −80 °C. The C156S, R137G, and R176K synthases were rechromatographed on a second phosphocellulose column (2 × 7 cm), since they did not absorb to the affinity column.

Step 5: *Affinity Column Chromatography*. This procedure was a modification of the method of Rode et al. (1979), which utilizes an affinity column made with the quinazoline derivative N^{10}-formyl-5,8-dideazafolate. The ammonium sulfate pellet from the previous step was dissolved in a small volume of 100 mM potassium phosphate, pH 7.1, and 20 mM 2-mercaptoethanol, and dialyzed overnight against two 1-L changes of the same buffer. The enzyme solution was made 40 μM in dUMP and applied to a 2 × 5 cm affinity column, previously equilibrated with a solution containing 20 mM potassium phosphate, pH 7.1, 20 mM 2-mercaptoethanol, and 40 μM dUMP. The column was washed with 100 mL of the equilibration buffer, followed by 100 mL of a solution containing 100 mM potassium phosphate, pH 7.1, 20 mM 2-mercaptoethanol, and 40 μM dUMP. Fractions of 5 mL were collected throughout the washing procedure. The column was then washed with the latter buffer containing 200 mM KCl, until TS activity began to elute. At this point, the remaining enzyme was eluted with the same buffer minus the dUMP. Fractions containing activity were pooled and concentrated in an Amicon ultrafiltration concentrator fitted with a PM-30 membrane to a volume of about 10 mL (A_{280} of at least 0.4). The enzyme was precipitated by adding solid ammonium sulfate to 0.8 saturation, and after centrifugation, the pellet was stored at −80 °C.

Protein and Enzyme Analysis. TS activity was determined spectrophotometrically (Wahba & Friedkin, 1961). One unit of activity is defined as the amount of enzyme that converted 1 μmol of dUMP to dTMP in 1 min at 30 °C. Protein concentrations were determined by a modification of the dye binding assay of Bearden (1978). Proteins were separated by SDS–PAGE (Laemmli, 1970) with the separating gel at 12.5% and the stacking gel at 4.5% acrylamide. The gels were visualized by staining with Coomassie Blue. The best-fit curves for the various kinetic constants, as well as ligand binding analyses, were performed by using the Enzfitter program of Robin Leatherbarrow (Elsevier Science Publishers, Amsterdam, The Netherlands). All of the enzymes used in these studies were purified to homogeneity.

Equilibrium Dialysis. Equilibrium dialysis was performed as described by Galivan et al. (1976b). The protein concentration was 3–5 μM, and radioactive ligands were present at 1–50 μM. 2-Mercaptoethanol was included in all experiments at a concentration of 20 mM. TS activity was determined before and after dialysis to be certain that the enzymes had retained at least 90% of their activity. All dialyses were performed at 4 °C for approximately 18 h. Duplicate 20-μL samples were counted and routinely agreed to within ±5%.

Results

Comparison of Kinetic Parameters for the Active-Site Mutants. The mutagenic substitutions introduced by either the Zoller and Smith (1984) or the Taylor et al. (1985) procedures were verified by both dideoxy sequencing and amino acid sequence analysis of an HPLC-isolated seven-residue CNBr peptide encompassing residues Ala-152 to Met-158 (Chu et al., 1984). The k_{cat} values for wild-type and mutant TSs presented in Table II reveal that the TS from C156S is the most dramatically affected, which is consistent with the proposed role for a nucleophile in the substrate binding site (Santi et al., 1974; Danenberg et al., 1974). This result is in basic agreement with that reported by Dev et al. (1988), who mutated the active-site cysteine in *E. coli* TS to a serine residue, and our findings (Frasca et al., 1988), where replacement of the cysteine with an arginine resulted in a complete loss in activity. In view of the 2–3-fold lower specific activity of pure *E. coli* TS activity relative to T4-TS, it was perhaps not unexpected to find that the T4-phage C156S enzyme was 5–10-fold higher in activity than reported for the corresponding *E. coli* TS, even when corrected for temperature differences and the assays employed. The possibility of contamination of C156S by *E. coli* TS was minimized by the use of an *E. coli* strain containing a deletion in the thyA gene (Pinter et al., 1988) and by the fact that the *E. coli* and phage TSs separate from one another at the phosphocellulose step of the purification procedure (Maley et al., 1983). However, since it would take only a very minor contamination by the wild-type TS, which could be introduced by reversion of the mutant, it was necessary to eliminate this possibility. Vector-transformed *E. coli* containing the deletion in the synthase were grown in the presence of thymine, since growth in the absence of this base resulted in reversion. At the end of the growth period, the cells were tested for reversion by growth on culture plates devoid of thymine. In no case was growth detected, indicating that reversion to wild-type cells was not a problem and that the activity in the isolated C156S TS was inherent in the protein.

Of interest is the finding that the k_{cat} of P155A is almost identical with that of the wild-type TS despite the considerably higher K_ms for its substrates. Since the k_{cat}/K_m represents the rate of association of dUMP with the enzyme (Santi et al., 1990) at saturation levels of $CH_2H_4PteGlu$, there appears to be a 5-fold decrease in the mutant relative to the wild-type k_{cat}/K_m (Table III) which is probably related to the difference in conformational states of the two proteins. This difference is also reflected in the altered binding constants for dUMP (Table IV) and for FdUMP in the ternary complex for this mutant (Table V). In contrast to P155A, H157V possessed K_m's for its substrates closer to that of the wild-type enzyme,

Table III: Kinetic Parameters for Wild-Type and Mutant T4-Phage Thymidylate Synthases[a]

enzyme	K_m(dUMP) (μM)	$K_m(CH_2H_4F)$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m(dUMP) (μM^{-1} s^{-1})	$k_{cat}/K_m(CH_2H_4F)$ (μM^{-1} s^{-1})
WT	2.73 ± 0.5 (4)[b]	49.9 ± 0.3	11.8 ± 0.4	4.32	0.237
P155A	11.8 ± 1.2 (3)	99.1 ± 4.9	10.3 ± 0.3	0.873	0.104
H157V	4.12 ± 0.7 (3)	75.2 ± 1.2	2.70 ± 0.5	0.655	0.036
C156S	4.92 ± 0.5 (2)	81.9 ± 0.8	0.008 ± 0.002	0.002	0.0001
K48R	5.90 ± 0.7	187.0 ± 7.4	0.76 ± 0.02	0.128	0.004
K49R	6.49 ± 1.7	77.9 ± 2.3	6.65 ± 0.8	1.04	0.085
R137K	7.02 ± 1.2	108.0 ± 5.4	3.80 ± 0.2	0.541	0.035
R137G			<0.01		
R176K			<0.01		

[a] To obtain the desired parameters, the method of Dalziel (1957) was used. Initial velocity determinations were obtained by measuring the change in A_{340} with a Gilford 240 recording spectrophotometer on adding 1 milliunit of enzyme to 1 mL of a reaction solution at 30 °C containing 50 mM Tris-HCl, pH 7.0, 50 mM β-mercaptoethanol, 20 mM $MgCl_2$, 5 mM ascorbate, and 3.1 mM formaldehyde; dUMP concentrations in the reaction solutions ranged from 1 μM to 1 mM while CH_2H_4PteGlu concentrations varied from 6 to 150 μM. Five to six fixed concentrations of the latter were used to obtain its K_m, with the intercepts on the ordinate replotted to obtain the K_m for dUMP. For further details, see Methods. [b] The numbers in parentheses in this column represent the number of individual determinations.

Table IV: Binding of dUMP to Mutant and Wild-Type T4-Phage Thymidylate Synthase in the Presence and Absence of PDDF As Determined by Equilibrium Dialysis

enzyme	K_d(dUMP)[a] (μM)	η[b]	K_d(dUMP)[c] (μM)	η[b]
WT	2.3 ± 1.2	0.9 ± 0.4	1.1 ± 0.1	1.5 ± 0.2
P155A	6.9 ± 1.8	0.4 ± 0.2	5.8 ± 1.5	1.5 ± 0.1
C156S	4.9 ± 0.3	0.9 ± 0.0	2.2 ± 0.7	1.4 ± 0.1
H157V	10.8 ± 1.5	0.9 ± 0.1	5.0 ± 0.5	1.5 ± 0.2

[a] Determined in the absence of PDDF. [b] Number of binding sites per mole of enzyme (based on two determinations). [c] Determined in the presence of 30 μM PDDF.

Table V: Binding of FdUMP to Mutant and Wild-Type Thymidylate Synthase in the Presence and Absence of CH_2H_4PteGlu As Determined by Equilibrium Dialysis

	binary complex		ternary complex	
enzyme	K_d-(FdUMP)[a] (μM)	η[b]	K_d-(FdUMP)[c] (μM)	η[b]
WT	6.9 ± 0.6	0.3 ± 0.1	<0.01	1.9 ± 0.1
P155A	5.8 ± 1.3	0.3 ± 0.2	2.7 ± 1.2	1.6 ± 0.2
C156S	14.0 ± 2.4	0.3 ± 0.1	7.8 ± 1.3	1.7 ± 0.2
H157V	23.1 ± 0.8	0.4 ± 0.1	13.1 ± 1.9	1.4 ± 0.3

[a] Determined in the absence of CH_2H_4PteGlu. [b] Number of binding sites per mole of enzyme (based on two determinations). [c] Determined in the presence of 30 μM CH_2H_4PteGlu.

although its k_{cat} was only 23% that of the wild type. This was still about 4 times that found on comparing the comparable valine mutant in *E. coli* (Dev et al., 1989). Because of histidine's role in promoting acid–base catalysis reactions in many enzymes, a similar role could be envisaged for His-156, particularly since a hydrogen abstraction step at C5 is required in the catalytic mechanism (Pogolotti et al., 1979). The fact that replacement of this residue with a valine attenuated TS activity but did not completely eliminate it suggests that another explanation is required for histidine's function in this highly conserved active-site region. This would appear not to be, as suggested by Dev et al. (1989), to maintain the protonated state of a catalytically important lysine or arginine, since the ϕ3T enzyme (Kenney et al., 1985), which contains a valine in place of histidine (Table I), is about 4–5 times more active than the *E. coli* TS (Maley & Maley, 1989). However, it is possible that amino acids in other regions of the ϕ3T-TS compensate for the replacement of histidine in this enzyme.

Comparison of Kinetic Parameters for the Folate Binding Mutants. It is clearly seen in Table III that substituting an arginine for Lys-48 reduced the activity of the wild-type enzyme by 94%. This change appeared to have no major effect on the conformation of the enzyme (see below). However, when methionine was substituted for Lys-48, the mutated enzyme had less than 1% the activity of the wild-type TS (data not shown). Replacement of Lys-49 with an arginine had less of an impact on synthase activity, as the enzyme still retained about 56% of its activity. The major purpose of this substitution was to determine if the enzyme was now more susceptible to inhibition by $PteGlu_6$ (Maley et al., 1979b). Since it was not (data not shown), it appears that another structural element in these enzymes is responsible for the differential inhibition by the folylpolyglutamates. The reduction of activity in both instances was accompanied by increases in the K_m's for each of the substrates, with the K_m for CH_2H_4PteGlu being most notably affected in the case of K48R (Table II). As a consequence, the resulting effect on the catalytic efficiencies (k_{cat}/K_m) of dUMP and CH_2H_4PteGlu was even greater. It is seen that the rate of association of dUMP with the enzyme is reduced about 30-fold, which could be due to the impaired binding of CH_2H_4PteGlu as a result of the synthase being conformationally altered at this site in K48R.

Nucleotide Binding As Determined by Equilibrium Dialysis. In contrast to the case for the K_m values presented in Table II, where the presence of both substrates is required, the influence of amino acid replacements on the direct binding of dUMP and FdUMP, was determined by equilibrium dialysis. The K_d values reported in Table IV reveal that the binding of dUMP to the H157V synthase is weaker than to the other synthases. Somewhat similar results were obtained for the binding of both FdUMP and CH_2H_4 folate to the mutant enzyme (Table V). However, in both cases, nucleotide binding approached its expected value of about 2.0 in the presence of a folate derivative (PDDF in the case of dUMP and CH_2H_4PteGlu in the case of FdUMP). The fact that the number of binding sites per mole for the FdUMP binary complex is so low (Table V) was surprising, since this nucleotide gave higher values than dUMP with the *E. coli* TS (Maley & Maley, 1988). However, despite the low n value of the FdUMP binary complex, the FdUMP ternary complex approached the expected value of 2. The potentiation by folate and its derivatives, particularly the folylpolyglutamates, on the binding of dUMP and its analogues, as well as the reverse, was shown earlier with the *L. casei* synthase (Galivan et al., 1976a,b, 1977b). The only instance where the K_d was not reduced was with dUMP and the P155A synthase (Table IV). It is of interest to note that while the K_d for dUMP in its ternary complex with PDDF was about 5 times higher for the P155A synthase relative to the wild type (Table IV) it was several orders of magnitude higher when the corresponding

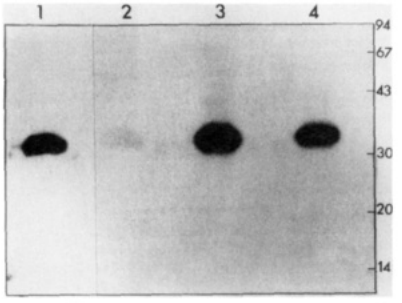

FIGURE 1: Ternary complex formation by wild-type and active-site mutant T4-TSs. Ternary complexes were formed as previously described (Belfort et al., 1983) but with 1.05×10^6 cpm of [^{32}P]FdUMP (5000 Ci/mmol) and 1.7 nmol of C156S, 0.3 nmol of wild-type synthase, and 0.78 nmol of P155A in a total volume of 50 μL; after incubation of the solutions at room temperature for 30 min, 30 μL of each sample was mixed with 10 μL of Laemmli buffer (Laemmli, 1970) and heated at 100 °C for 3 min. The entire sample of each was electrophoresed in a 12.5% SDS–PAGE gel, after which the gel was dried on Whatman 3MM paper and subjected to autoradiography. Lane 1 is C156S exposed for 24 h; lanes 2, 3, and 4 are C156S, wild-type synthase, and P155A, respectively, exposed for 4 h.

FdUMP, CH_2H_4PteGlu ternary complexes were compared (Table V). In these cases where binary complex formation was measured, only one of the two potential binding sites was occupied, but all were increased to about two in the presence of the indicated folate derivatives (Tables IV and V). Similar results were obtained by Dev et al. (1988) with the C146S mutant of *E. coli* TS. Despite the fact that FdUMP binding was greatly reduced in C156S, the existence of a ternary complex could be detected by using highly labeled [^{32}P]-FdUMP and extended autoradiography (Figure 1, lanes 1 and 2) of the SDS–PAGE gels containing the mutant ternary complexes. It is obvious that the stability of the ternary complexes of the wild-type enzyme (lane 3) and of the P155A synthase (lane 4) is much greater than that from the C156S synthase, where only a slight hint of ternary complex was seen after 4 h of exposure relative to lanes 3 and 4 but was clearly evident after 24 h of autoradiography (lane 1). These results are in contrast to those obtained with the *E. coli* C146S mutant [^{32}P]FdUMP ternary complex, which could not be detected (Dev et al., 1988), perhaps because long enough exposures were not employed. Binding constants for the other mutants are not reported, since the yields of pure enzyme were insufficient to undertake these studies.

Properties of the Active-Site Mutant TSs. One possible explanation for the differences in k_{cat} and binding constants between the mutant and wild-type enzymes is that the structure of the mutant protein has been so perturbed that although substrate binding can still occur at the active site and amino acids involved are now sufficiently removed from one another so as to impair formation of the transition-state complex. That the structures of some of the mutants are probably altered was observed on comparing the heat and urea stabilities of the mutants relative to the wild-type enzyme, as well as their fluorescence spectra. Thus, it is seen in Figure 2 that the P155A TS is markedly less stable to heat than the other mutants, in spite of the fact that the k_{cat} of the P155A synthase is comparable to the wild-type enzyme. By contrast, H157V with only about 20% and C156S with less than 0.1% the k_{cat} of the wild type are as stable as the wild-type TS. Somewhat similar results were obtained on incubation of these enzymes in 1 M urea at 25 °C (data not shown). That the structures of the enzymes from P155A and H157V have been altered is reflected in the blue shift of their fluorescent spectra (Figure 3A,C), indicating that some of these proteins' tryptophans are

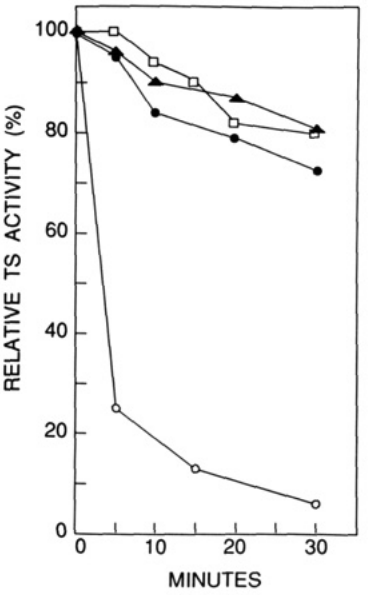

FIGURE 2: Stability of wild-type and active-site mutant TSs to incubation at 40 °C. Each of the enzymes (20 milliunits except for C156S where 10 milliunits was used) was incubated in 100 μL of a solution containing 50 mM Tris-HCl, pH 7.1, 20 mM 2-mercaptoethanol, and 20 mM $MgCl_2$. At the indicated times, 10 μL was removed and assayed spectrophotometrically (Wahba & Friedkin, 1961). (□) WT-TS; (○) P155A; (▲) H157V; (●) C156S.

in a more hydrophobic environment than those in the wild-type or C156S synthases (Figure 3B). From this information, it would appear that the perturbation of an enzyme's structure may or may not be reflected in its activity or in its stability. This is particularly true of the TS mutant from P155A.

Properties of the Nucleotide or Phosphate Binding Mutants of TS. The binding studies of Dev et al. (1988), and those presented in Tables IV and V, suggest that the interaction between dUMP or FdUMP and the active-site cysteine contributes only marginally toward the binding energy required for binding of these nucleotides. This is evident on examining the K_d and K_m values for the synthase from C156S and the wild-type enzyme (Tables III–V) where only small differences between the two enzymes were found. In fact, the nucleoside portion of the molecule is not necessary as phosphate alone is an effective competitor of dUMP and FdUMP (Galivan et al., 1976b; Lewis et al., 1978). The X-ray crystallographic studies with *L. casei* TS strongly implicate Arg-179′ and Arg-218 in facilitating nucleotide binding by a charge–charge interaction (Hardy et al., 1987). To verify the involvement of these arginines, the corresponding amino acids in T4-TS (Arg-137 and Arg-176) were replaced with lysine and glycine in the former case and lysine in the latter. As shown in Table III, these changes had a dramatic effect on enzyme activity, particularly with the TSs from R137G and R176K, since their activities were virtually eliminated. However, in the case of R137K, about 32% of the enzyme activity was retained, which is reflected in the impaired capacity of this enzyme to form a dUMP–PPDF–enzyme complex (Figure 4) relative to the wild-type synthase (0.96 vs 0.22 s^{-1}). The K_m values for both dUMP and CH_2H_4PteGlu were increased by about 3-fold, and the rate of dUMP association with R137K was reduced about 4-fold. The reduced activity in the R137K synthase does not appear to be associated with a perturbation in this protein's structure as indicated by the lack of a shift in its fluorescence spectrum relative to the wild-type enzyme. However, this was not the case for the inactive synthase from R176K where a definite blue shift was seen (data not shown). A blue shift was obtained also for the inactive synthase from R137G.

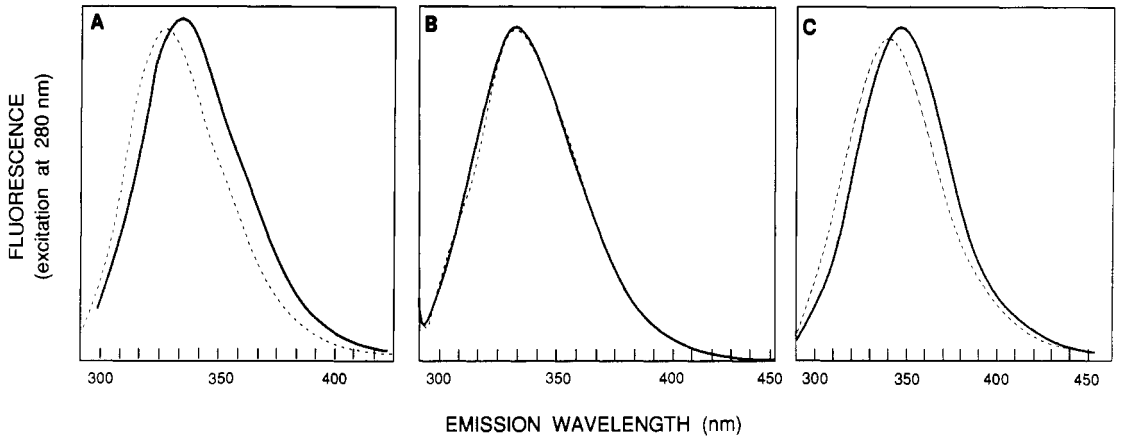

FIGURE 3: Fluorescence spectra of active-site mutant and wild-type TSs. The spectra were obtained from 3-mL solutions containing an A_{280} of 0.05 for each enzyme in 50 mM potassium phosphate, pH 7.1, and 20 mM 2-mercaptoethanol. A Perkin-Elmer MP544A variable-wavelength fluorescence spectrophotometer was used to obtain the spectra. The excitation wavelength was 280 nm. (—) in panels A–C represents WT-TS, while (– – –) in panel A is P155A, (– – –) in panel B is C156S, and (– – –) in panel C is H157V.

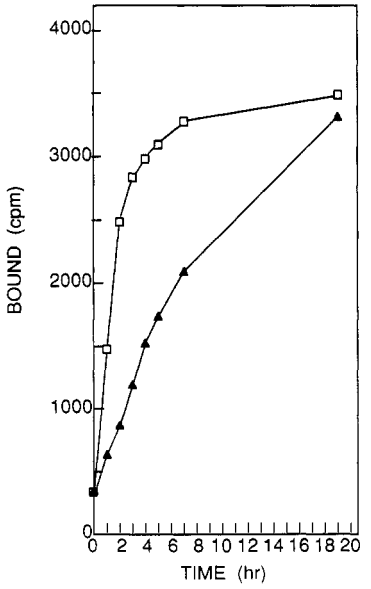

FIGURE 4: Kinetics of ternary complex formation with a phosphate binding site mutant and wild-type TSs in the presence of [6-^{3}H]dUMP and PDDF. The assay employed is a modification of that described by Pogolotti et al. (1986). The mutant and wild-type enzymes (about 1 μM) were incubated at 25 °C in 200 μL of a solution containing 5 mM ascorbate, 50 mM 2-mercaptoethanol, 25 mM $MgCl_2$, 100 μg of BSA, 100 μM PDDF, 50 mM Tris-HCl, pH 7.0, and 10 μM [6-^{3}H]dUMP (75–200 cpm/pmol). Aliquots of 20 μL were removed at the times indicated after addition of the [6-^{3}H]dUMP and added to 50 μL of 50 mg/mL BSA followed by 1 mL of ice-cold 5% TCA. The precipitate was allowed to settle overnight in the cold and then collected on a GFA filter (Whatman) with suction. After the precipitate was washed with two 5-mL volumes of 5% TCA, the filters were added to scintillation vials containing 6 mL of Aquasol (Du Pont) and counted in a Beckman Model LS3801 scintillation counter, (□) WT-TS; (▲) R137K.

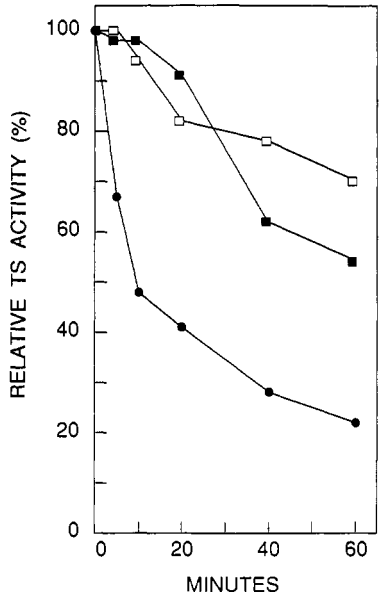

FIGURE 5: Stability of folate binding mutants and wild-type TSs to incubation at 40 °C. Conditions were similar to those described in Figure 2. (□) WT-TS; (●) K49R; (■) K48R.

Properties of the Folate Binding Mutants of TS. A reasonable assumption at this time for the much greater effect on the k_{cat} of the T4-TS from K48R relative to that from K49R, in view of our previous results on the fixation of $PteGlu_7$ to either Lys-50 or -51 of *L. casei* TS (Maley et al., 1982), is that Lys-50 (Lys-48 in T4-TS) is most likely involved. This would be consistent with the results from the K48R mutation in T4-TS. To determine if structural perturbations were associated with the observed effects on catalytic activity, stability and spectrofluorometric analyses were undertaken. It is readily apparent from the data in Figures 5 and 6 that the decreased stability of K49R to incubation at 40 °C relative to the

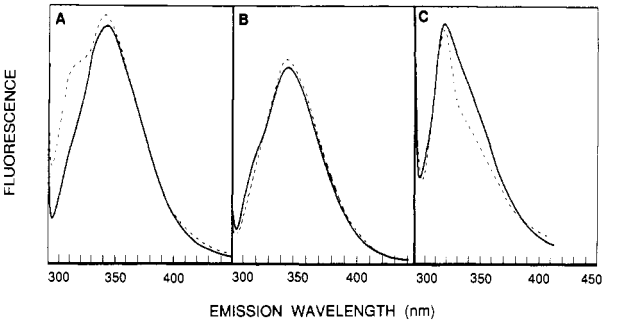

FIGURE 6: Comparison of the fluorescence spectra of folate binding mutants of thymidylate synthase with wild-type thymidylate synthase. Conditions were similar to those described in the legend of Figure 3 except for panel C where the excitation wavelength was 300 nm. The excitation wavelength for panels A and B was 280 nm. (—) WT-TS in (A); (– – –) K49R in (A) and (C); (– – –) K48R in (B).

wild-type and K48R synthases is associated with an altered fluorescent spectrum. Thus, it is seen on excitation at 280 nm a shoulder appears at about 310 nm (Figure 6A), suggesting that a tyrosine-containing region in the mutant has been perturbed (Weber, 1961; Cuatrecasas et al., 1967). On ex-

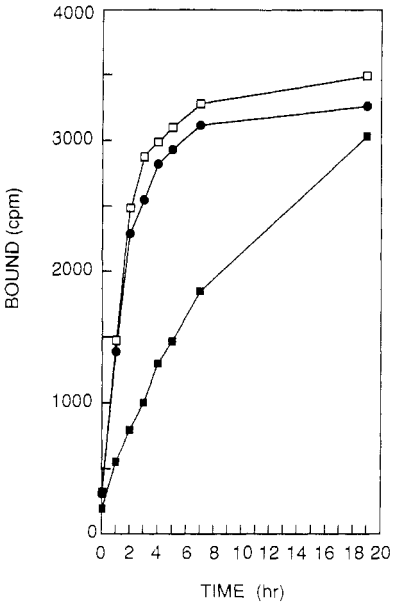

FIGURE 7: Kinetics of ternary complex formation of folate binding mutants and wild-type TSs in the presence of [6-^{3}H]dUMP and PDDF. See legend of Figure 4 for details. (□) WT-TS; (●) K49R; (■) K48R.

citation at 300 nm (Figure 6C), the spectral shift in the 330–340-nm region indicates that a change in the environment of some of the mutant's tryptophans has occurred (Gally, 1976).

While an altered conformation in the active-site regions (nucleotide and/or folate) of the synthase from K49R (Figure 6A,C) could in effect explain its reduced activity and higher K_m values (Table II), this does not appear to be the case for that from K48R where a structural perturbation could not be detected (Figure 6B). However, on comparing the rates of irreversible ternary complex formation by both mutant enzymes relative to the wild-type synthase in the presence of PDDF and dUMP, it is evident that the synthase from K48R alone is greatly impaired in its ability to bind [5-^{3}H]dUMP (Figure 7). The extent of this impairment is about 5-fold greater than the rate of complex formation for the synthases from K49R and the wild-type (0.96 vs 0.18 s^{-1}) and is consistent with the greatly impaired k_{cat} for this enzyme. A possible explanation for the apparent lack of a strucural perturbation in the K48R synthase is that a conformational change not affecting tyrosines or tryptophans is involved.

Discussion

The rather high degree of conservation in specific amino acids or specific regions of TSs from evolutionary diverse origins implies that they were conserved because of their functional importance, whether it be substrate binding, catalysis, stability, or some combination of these properties. To explore the role that amino acids might play in contributing to these processes, three highly conserved regions known to be directly or indirectly involved in the catalytic process were selected for amino acid replacements in T4-TS, which encompassed the proposed active-site cysteine, the nucleotide binding residues, and the folate binding region.

Amino Acid Replacements in the Active-Site Region. The most dramatic effect on TS activity was obtained by replacing the active-site Cys-156 with an arginine, which resulted in a complete loss in enzyme activity (Frasca et al., 1988). Replacement of the adjacent Pro-155 with an alanine and His-157 with a valine was much less detrimental to T4-TS activity. However, conversion of Cys-156 to a serine provided an enzyme with only 0.06% the activity of the wild-type TS (Table III). The trace of activity that remained is most likely associated with the modified enzyme and not due to contamination by wild-type TS. This belief is based on the fact that the enzyme was prepared in a *thy*A$^-$ deletion mutant of *E. coli*, as well as the rather clear-cut separation of the two enzymes on phosphocellulose chromatography (Maley et al., 1983). In addition, the normal sensitivity of the wild-type TS to sulfhydryl reagents was not observed with C156S. These findings are consistent with those reported by Dev et al. (1988) using a comparable mutant C146S from *E. coli*, where the residual activity was about 0.02% of the wild-type enzyme. Whether the 3-fold difference in residual activities of the phage and *E. coli* synthases is related to the fact that T4-TS is 2–3 times greater in activity than the *E. coli* TS or the difference in assay procedures employed is not known. However, it is of interest to note that the H157V mutation in the *E. coli* TS (Dev et al., 1989) is one-third as active as the comparable T4-TS mutation (H157V), where their respective residual activities were 7 and 23%, relative to their corresponding wild-type enzymes. Recently, it was shown with His-199 mutants of *L. casei* TS that amino acid replacements at this position can be tolerated by the enzyme, providing the amino acids have small hydrophobic side chains (Climie & Santi, 1990). Somewhat similar results were obtained by Michaels et al. (1990) with *E. coli* TS. It is of interest to note that despite the fact that even in those T4-TS mutants where enzyme activity was drastically decreased or even eliminated, substrate and analogue binding constants were only minimally affected (Tables IV and V), which is consistent with comparable studies of Dev et al. (1988, 1989) with *E. coli* TS. It would appear therefore that binding energy alone is not sufficient to reduce the energy of activation of minimally active mutants of TS to the point where significant activity can be restored. Productive binding is most likely accompanied by conformational changes induced by the substrates to enable their most effective interaction for reductive methylation to occur at the C5 position of dUMP.

An example of nonproductive binding was seen earlier in the case of carboxypeptidase A inactivated *L. casei* TS, where little impairment in the binding of dUMP and $CH_2H_4PteGlu_4$ when present together was observed (Galivan et al., 1977b). However, activity could not be detected even with [5-^{3}H]-dUMP. As indicated recently by Matthews et al. (1989), the binding of folate-like molecules to the *E. coli* dUMP–TS binary complex results in a repositioning of Arg-21 so that it is in proximity to the carboxy terminus of the enyzme. This would position the C6 hydrogen of $CH_2H_4PteGlu$ above the C5 of dUMP in such a manner as to enable hydride transfer to the C5 methylene intermediate. Removal of the C terminus of TS would prevent the conformationally active TS structure from forming, although not preventing ligand binding, which would explain why we could not detect tritium release from [5-^{3}H]dUMP (Galivan et al., 1977b). This ligand-promoted shift has been shown recently to involve six residues at the carboxy terminus, which moves 4.0 Å to cover the active-site partially (Montfort et al., 1990).

Ample evidence for the perturbation of the synthase structure on substrate binding has been documented from hydrodynamic (Lockshin & Danenberg, 1980), circular dichroism (Galivan et al., 1975; Donato et al., 1976), fluorescent quenching (Donato et al., 1976), NMR (Lewis, et al., 1980), differential absorbance at 320–340 nm (Santi et al., 1974), and Raman spectra (Fitzhugh et al., 1986). Consistent with these findings is the marked enhancement in binding of several orders of magnitude that the substrates and analogues exert

on each other to form ternary complexes. In addition, the assymetric nature of this binding (Galivan et al., 1976b; Danenberg & Danenberg, 1979; Santi et al., 1987) suggested that binding at the first substrate site altered binding at the second site, which would be consistent with substrate-induced conformational changes. More recently, support for the conformational change thesis has been demonstrated by comparing the X-ray crystallographic structures of liganded and nonliganded *E. coli* TS (Montfort et al., 1990), where it has been shown that ligand binding is accompanied by movement of segments of β-sheet, α-helix, and connecting loops toward the active center. This phenomenon, termed *segmental accommodation*, appears to involve many highly conserved regions in the enzyme.

Since substrates can bind to the C156S mutant, as also described for carboxypeptidase-inactivated *L. casei* TS (Galivan et al., 1977), conversion of the noncovalent ternary complex to the active covalent structure must be impaired. This was apparent in being able to measure the C156S ternary complex under nondenaturing conditions (Table III) but only barely under denaturing conditions (Figure 1), relative to the P155A and H157V mutants. While the thermodynamics of these interconversions have been clearly delineated (Santi et al., 1987), the first indication that a perturbation in enzyme structure most probably involving an isomerization of a reversible to an irreversible enzyme–ligand complex came from FdUMP inhibition studies, which revealed the inhibition to change from competitive to noncompetitive on preincubation of the enzyme with FdUMP (Mathews & Cohen, 1963; Lorenson et al., 1967). A somewhat similar temporal change from a dissociable complex of dUMP and PDDF to a much less dissociable form accompanied by structural alterations of the protein has also been reported (Pogolotti et al., 1986).

The mechanistic role of Cys-156 as a nucleophile in the catalytic reaction is simpler to rationalize than the contribution of P155A and H157V to catalysis. The fluorescence spectra in Figure 3 suggest that the mutant forms of these proteins differ in conformation from the wild-type and C156S synthases. While binding was affected to some degree in each case, it was insufficient to explain why the P155A possessed almost wild-type activity while H157V was reduced in activity by about 70%. That the structure of the former was indeed altered is indicated by its greatly decreased stability, which apparently did not affect this protein's ability to form a transition-state complex. Unlike the synthase from P155A, that from H157V was as stable as the wild-type enzyme, but its altered conformation must have affected its capacity to form a transition-state intermediate, and as a consequence affected its k_{cat} adversely. There would appear, therefore, to be distinct differences among these mutants in their thermodynamics of transition-state complex formation, which may or may not be affected by conformational changes induced by amino acid replacements at positions other than the active site.

It has been proposed that His-157 might act as an acid–base catalyst in the reaction (Hardy et al., 1987), which could be compensated for by another functional group on replacement of histidine. This scenario appears unlikely, however, since it has been shown that the $\phi 3T$ enzyme is the most active of the synthases described to date (Maley & Maley, 1989) despite the fact that it contains a valine (Table I) in place of histidine normally encountered following the active-site cysteine.

Amino Acid Replacements in the Phosphate Binding Region. The apparent involvement of one (Belfort et al., 1980) or more (Cipollo et al., 1979) argininyl residues in the binding of dUMP or its analogues is based on the finding that the Arg-modifying reagents phenylglyoxal and 2,4-butanedione inactivate *L. casei* TS, an effect that can be prevented by dUMP, but not by CH_2H_4PteGlu. Earlier NMR studies (Beckage et al., 1979) revealed dUMP to be associated with the synthase as a dianion, the binding of which could be promoted by the basic ϵ-amino group of lysine or the even more basic guanidinium of arginine through salt bridges. The arginine-modifying reagents suggested the latter amino acid to be involved as did molecular modeling studies with *L. casei* TS, which revealed that the phosphate of dUMP is in close contact with the guanidinium groups of Arg-218 on one subunit and Arg-179′ on the other, thus providing a bidentate locus for binding (Hardy et al., 1987). More recently it has been shown that a phosphate group, whether it be inorganic or a nucleotide phosphate, is bound between the guanidinium groups of Arg-23, -218, -178, and -179, and also to Ser-219. Although arginine-23 does not hydrogen bond to the phosphate, seven hydrogen bonds are made with the oxygen of the phosphate (Perry et al., 1990; Montfort et al., 1990). Of those residues in T4-TS corresponding to Arg-218 and Arg-179 of the *L. casei* synthase, Arg-176 appears to be more critical than Arg-137, reflecting this arginine's significant role in the binding of dUMP (Hardy et al., 1987). This is shown in Table III where it is indicated that changing Arg-176 to a lysine almost completely inactivates the enzyme, while replacing Arg-137 with a lysine yields an enzyme that still retains about 30% its original activity. The latter result is consistent with that found for the corresponding mutation (R179K) in the *L. casei* synthase (Santi et al., 1990), where about 26% of the wild-type activity was retained. Similar results were obtained by these workers whether the amino acid replacement was an alanine, threonine, or glutamate. The essential nature of Arg-166 in *E. coli* TS, comparable to *L. casei* TS Arg-218 and T4-TS Arg-176, was also observed recently by Michaels et al. (1990).

It is of interest to note that while the R137K T4-TS retains considerable activity, it differs from the wild-type synthase in the rate at which it forms a ternary complex with PDDF and dUMP (Figure 4) and its greater apparent heat stability (data not shown). These effects are not associated with an appreciable conformation change as reflected by its unaltered fluorescent spectra relative to the wild-type synthase. Since R137K is active and that form R137G is basically inactive (Table III), it appears that the basic lysine residue contributes to substrate binding while the neutral glycine does not. Alternatively, the conformational change associated with R137G, as indicated by a blue shift in its fluorescence spectra and similarly with R176K (data not shown), could be a major determinant in influencing enzyme activity. In contrast, the fact that changing Arg-179 in the *L. casei* synthase to a neutral residue still yields considerable activity suggests that compensatory changes in the structure might enable other basic residues to contribute to the binding of the nucleotide phosphate as a result of the inherent "plastic" nature of TS (Santi et al., 1990; Perry et al., 1990).

Amino Acid Replacements in the Folate Binding Region. It was shown previously that the folate and nucleotide binding sites were distinct from one another in that impairing the binding of one substrate (or analogue) did not prevent the binding of the other (Galivan et al., 1977). However, their impact on each other's binding was clearly evident by the extent to which they enhanced each other's K_D's (Galivan et al., 1976a,b). This was particularly true in the case of the folates, where the naturally occurring folylpolyglutamates could bind to TS in the absence of dUMP (or an analogue), in contrast to the folylmonoglutamate. Although the former

compound's binding could be prevented by the treatment of *L. casei* TS with carboxypeptidase A, its binding could be restored on the addition of dUMP (Galivan et al., 1977b). These studies reveal the dependency of the two substrates of TS on each other for their degree as well as affinity of binding, effects which are undoubtedly related to substrate-induced conformational changes in the enzyme. In earlier studies (Maley et al., 1982), it was shown that three amino acids were implicated in the binding of $PteGlu_7$ to *L. casei* TS. These involved Lys-50, Lys-51, and Lys-58 of *L. casei*. Once the sequences of the T4-phage and *E. coli* synthases were known, it became evident that amino acids 50 and 51 of the *L. casei* synthase are comparable to those at positions 48 and 49 of the former enzymes (Table II) and that replacing these amino acids by mutagenesis, particularly Lys-48 in T4-TS, since it is the only invariant residue in this region, might support our thesis for the importance of this amino acid in folate binding. A role for Lys-58 of *L. casei* TS in folate binding appears unlikely since it is the only species that possesses a lysine at this site.

The rationale for converting Lys-49 of the T4-phage synthase to an arginine was based on our finding that $PteGlu_6$ could, in the absence of Mg^{2+}, effectively inhibit T4-TS, but not the *E. coli* synthase (Maley et al., 1979b). Since the *E. coli* synthase contains an arginine at residue 49, it was reasoned that if this arginine was responsible for the enzyme's resistance to inhibition by the folylpolyglutamates, then replacement of Lys-49 in the T4-TS with an arginine might attenuate this enzyme's susceptibility to $PteGlu_6$. That this effect was not observed (data not shown) indicates that another explanation must be sought for the difference in susceptibility of these two enzymes to inhibition by the folylpolyglutamates.

While the influence of Lys-49 on mediating the inhibitory effects of the folylpolyglutamates was minimized by the above studies, replacing the amino acids at Lys-48 and Lys-49 in T4-TS with arginines revealed the resulting mutant enzymes to differ considerably in activity, with the synthase from K48R retaining only about 6% of the wild-type activity, but that from K49R retaining more than 50% (Table III). The importance of a positive charge at residue 48 is emphasized by replacing Lys-48 with neutral amino acids such as methionine or glutamine, both of which yielded TS activity with less than 1% the normal enzyme activity (data not shown). If ion-pair interactions are required for the binding of folate or analogues such as PDDF and their corresponding polyglutamates, the more basic arginine residue in place of lysine at residue 48 of the synthase from K48R should conceivably have a lower K_m than the wild-type enzyme, that is, unless the protonated arginine is sharing its proton with another group in the enzyme, which would limit access of the folate carboxyl to the positively charged guanidinium group, relative to the less basic lysyl residue. This proposal is supported by the fact that the K_m for folate binding by the K48R mutant is about 4 times that of the wild-type enzyme (Table III). As pointed out by Wigley et al. (1987), the ion-pair binding between a carboxyl and a guanidinium group is much tighter than between a carboxyl and the ϵ-amino group of lysine, which could increase an enzyme's energy of activation and accordingly decrease its rate of catalysis. The structure of the enzyme from K48R does not appear to be perturbed, as revealed by the spectral fluorescence analyses in Figure 3B, but its rate of ternary complex formation is impaired relative to the enzyme from K49R or the wild-type synthase (Figure 7). If the latter data are any indication of the rate of transition-state formation by these enzymes, an explanation could in part be provided for the dramatic decrease in activity for the enzyme from K48R. This does not appear to be the case for the synthase from K49R, where the observed 44% decrease in activity appears to be associated with a structural change, as evidenced by the diminished stability of this enzyme on incubation at 40 °C (data not shown), in addition to the blue shift in its fluorescence spectrum. Other changes in this region can also have dramatic effects on enzyme activity as we have found with a mutant (C50Y) in the folate binding region of *E. coli* TS. From a computer graphic analysis, it appears that access of the folate carboxyl groups to lysine-48 of *E. coli* TS is impaired by the tyrosyl hydroxyl group, yielding an enzyme with only 0.2% the activity of the wild-type synthase (Maley et al., 1988). Replacing this tyrosine with a phenylalanine, to determine the extent to which the benzene ring affects TS activity, resulted in a 25-fold increase in activity, implicating the importance of charge–charge interactions in the binding of folate (unpublished results).

From these studies, it would appear that Lys-48 of T4-TS and the other conserved lysines at this position in the synthases described to date (Table II) play a major role in the binding of the folylmono- and polyglutamates. The fact that enzyme activity is greatly reduced in Lys-48, although not eliminated, may be a consequence of an attendant alteration in enzyme structure (plasticity) on substrate binding which enables other residues to compensate for the impaired binding at this site (Bone et al., 1989; Santi et al., 1990; Perry et al., 1990).

An interesting speculation on the nature of substrate binding was presented recently by Matthews et al. (1989), who suggested that the binding of a folylpolyglutamate to TS does not restrict the channel through which dUMP or its analogues must pass to enter the active site. After dUMP or FdUMP enters the active site, it is proposed that a conformational shift occurs to seal off the channel.

An observation consistent with this interpretation comes from our earlier CD studies (Galivan et al., 1975) which revealed that a potential conformational change is associated with the binding of $CH_2H_4PteGlu_4$ to *L. casei* TS in the absence of FdUMP. A similar result was obtained with *N*-ethylmaleimide-inactivated TS which cannot bind FdUMP. When FdUMP was added to active enzyme to which $CH_2H_4PteGlu_4$ was bound, an additional spectral shift occurred possibly due to a reorientation of the enzyme's structure.

Conclusions. It is often difficult to target those amino acids in an enzyme that contribute directly to the catalytic process, whether one employs amino acid specific modifying agents or site-specific mutagenesis. This is due, in part, to the fact that the loss in enzyme activity could result from the introduction of peripheral changes that can alter a protein's conformation to the extent that enzyme activity is affected. Thus, any attempt to attribute a functional role to the amino acids modified in this study must be tempered by this consideration. Another factor that can confuse the issue still further is that while an amino acid may be essential for a specific reaction, replacing it with another may not eliminate enzyme activity due to the plastic or flexible nature of various proteins. This is particularly true in the presence of substrates that can alter a protein's conformation during the course of binding to the enzyme. Of those sites that were modified in T4-TS, major losses were encountered in C156S, K48R, R137G, and R176K. While it is known from other studies that these amino acids are involved in substrate binding, verificiation of the precise manner in which this was accomplished has been provided by X-ray crystallographic analysis of ligand bound *E. coli* TS (Montfort et al., 1990; Perry et al., 1990). Future X-ray

studies will no doubt delineate how TS orchestrates the synthesis of dTMP from dUMP and 5,10-CH_2H_4PteGlu and the amino acids involved in this process.

Added in Proof

TS mutants K48Q and K49Q, recently prepared by Verna Frasca in our laboratory, were found in the former case to have little or no activity, but in the latter the activity was comparable to that of the wild-type enzyme. This information lends further support to the importance of Lys-48 in T4-TS in the binding of CH_2H_4PteGlu and its analogues.

Acknowledgments

We express our appreciation to Joan Pedersen-Lane for her aid in preparing P155A and to Dr. Li-Ming Changchien for the amino acid and peptide sequence analyses used to confirm the mutants. We also thank Judith Valentino for her excellent assistance in typing the manuscript.

References

Amann, E., Brosius, J., & Ptashne, M. (1983) *Gene 25*, 167–178.

Bearden, J. C., Jr. (1978) *Biochim. Biophys. Acta 533*, 525–529.

Beckage, M. J., Blumenstein, M., & Kisliuk, R. L. (1979) *Biochim. Biophys. Acta 571*, 157–161.

Belfort, M., Maley, G. F., & Maley, F. (1980) *Arch. Biochem. Biophys. 204*, 340–349.

Belfort, M., Maley, G. F., Pedersen-Lane, J., & Maley, F. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4914–4918.

Belfort, M., Moelleken, A., Maley, G. F., & Maley, F. (1983b) *J. Biol. Chem. 258*, 2045–2051.

Bellisario, R. L., Maley, G. F., Galivan, J. H., & Maley, F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1848–1852.

Bellisario, R. L., Maley, G., Guarino, D., & Maley, F. (1979) *J. Biol. Chem. 254*, 1296–1300.

Beverley, S. M., Ellenberger, T. E., & Cordingley, J. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2584–2588.

Bone, R., Silen, J. L., & Agard, D. A. (1989) *Nature 339*, 191–195.

Bzik, D. J., Li, W. B., Horii, T., & Inselberg, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8360–8364.

Chu, F. K., Maley, G. F., Maley, F., & Belfort, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3049–3053.

Cipollo, K. L., & Dunlap, R. B. (1979) *Biochemistry 18*, 5537–5541.

Climie, S., & Santi, D. V. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 633–637.

Cuatrecasas, P., Edelhoch, H., & Anfinsen, C. B. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 2043–2050.

Danenberg, K. D., & Danenberg, P. V. (1979) *J. Biol. Chem. 254*, 4345–4348.

Danenberg, P. V. (1977) *Biochim. Biophys. Acta 473*, 73–92.

Danenberg, P. V., Langenbach, R. J., & Heidelberg, C. (1974) *Biochemistry 13*, 1073–1080.

Daron, H. H., & Aull, J. L. (1978) *J. Biol. Chem. 253*, 940–945.

Dev, I. K., Yates, B. B., Leong, J., & Dallas, W. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8360–8364.

Dev, I. K., Yates, B. B., Atashi, J., & Dallas, W. S. (1989) *J. Biol. Chem. 264*, 19132–19137.

Donato, H., Jr., Aull, J. L., Lyon, J. A., Reinseh, J. W., & Dunlap, R. B. (1976) *J. Biol. Chem. 251*, 1301–1310.

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

Edman, U., Edman, J. C., Lundgren, B., & Santi, D. V. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6503–6507.

Fitzhugh, A., Fodor, S., Kaufman, S., & Spiro, T. G. (1986) *J. Am. Chem. Soc. 108*, 7422–7424.

Frasca, V., LaPat-Polasko, L., Maley, G. F., & Maley, F. (1988) in *Proceedings of the 1988 Miami Bio/Technology Winter Symposium* (Brew, K., et al., Eds.) p 149, IRL Press, Washington, DC.

Friedkin, M., & Kornberg, A. (1957) *The Chemical Basis of Heredity* (McElroy, W. D., & Glass, B., Eds.) pp 609–614, The Johns Hopkins Press, Baltimore, MD.

Galivan, J. H., Maley, G. F., & Maley, F. (1975) *Biochemistry 14*, 3338–3344.

Galivan, J. H., Maley, F., & Baugh, C. M. (1976a) *Biochem. Biophys. Res. Commun. 71*, 527–534.

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

Galivan, J., Noonan, J., & Maley, F. (1977a) *Arch. Biochem. Biophys. 184*, 336–345.

Galivan, J., Maley, F., & Baugh, C. M. (1977b) *Arch. Biochem. Biophys. 184*, 346–454.

Gally, F. (1976) in *Biochemical Fluorescence* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 2, pp 409–439, Marcel Dekker, New York.

Grumont, R., Washtein, W. L., Caput, D., & Santi, D. V. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5387–5391.

Grunstein, M., & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3961–3965.

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

Holmes, D. S., & Quigley, M. (1981) *Anal. Biochem. 114*, 193–197.

Honess, R. W., Bodemer, W., Cameron, K. R., Niller, H.-H., Fleckenstein, B., & Rall, R. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3604–3608.

Hughes, D. E., Shonekan, O. A., & Simpson, L. (1989) *Mol. Biochem. Parasitol. 34*, 155–166.

Iwakura, M., Kawata, M., Tsuda, K., & Tanaka, T. (1988) *Gene 64*, 9–20.

Kenney, E., Atkinson, T., & Hartley, B. S. (1985) *Gene 34*, 335–342.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lewis, C. A., Jr., Munroe, W. A., & Dunlap, R. B. (1978) *Biochemistry 17*, 5382–5387.

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

Lockshin, A., & Danenberg, P. V. (1980) *Biochemistry 19*, 4244–4251.

Lorenson, M. Y., Maley, G. F., & Maley, F. (1967) *J. Biol. Chem. 242*, 3332–3344.

Lu, Y.-Z., Aiello, P., & Matthews, R. (1984) *Biochemistry 23*, 6870–6876.

Maley, G. F., & Maley, F. (1988) *J. Biol. Chem. 263*, 7620–7627.

Maley, G. F., & Maley, F. (1989) *Adv. Enzyme Regul. 29*, 181–187.

Maley, G. F., Bellisario, R. L., Guarino, D. U., & Maley, F. (1979a) *J. Biol. Chem. 254*, 1301–1304.

Maley, G. F., Maley, F., & Baugh, C. M. (1979b) *J. Biol. Chem. 254*, 7485–7487.

Maley, G. F., Maley, F., & Baugh, C. M. (1982) *Arch. Biochem. Biophys. 216*, 551–558.

Maley, G. F., Belfort, M., & Maley, F. (1983) in *Chemistry and Biology of the Pteridines* (Blair, J. A., Ed.) pp 645–650, Walter deGruyter & Co., Berlin.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mathews, C. K., & Cohen, S. S. (1963) *J. Biol. Chem. 238*, 367-370.
Matthews, D. A., Appelt, K., & Oatley, S. J. (1989) *Adv. Enzyme Regul. 29*, 47-60.
Michaels, M. L., Kim, C. W., Matthews, D. A., & Miller, J. H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3957-3961.
Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G. F., Hardy, L., Maley, F., & Stroud, R. M. (1990) *Biochemistry 29*, 6964-6977.
Moore, M. A., Ahmed, F., & Dunlap, R. B. (1986) *J. Biol. Chem. 261*, 12745-12749.
Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Montfort, W. R., Maley, G. F., Maley, F., & Stroud, R. M. (1990) *Proteins: Struct., Funct., Genet.* (in press).
Perryman, S. M., Rossana, C., Deng, T., Vanin, E. F., & Johnson, L. F. (1980) *Mol. Biol. Evol. 3*, 313-321.
Pinter, K., Davison, V. J., & Santi, D. V., (1988) *DNA 7*, 235-241.
Pogolotti, A. L., Jr., & Santi, D. V. (1977) in *Bioorganic Chemistry* (Van Tamelin, E., Ed.) Vol. I, pp 277-311, Academic Press, Orlando, FL.
Pogolotti, A. L., Jr., Ivanetich, K. M., Sommer, H., & Santi, D. V. (1976) *Biochem. Biophys. Res. Commun. 70*, 772-778.
Pogolotti, A. L., Jr., Weil, C., & Santi, D. V. (1979) *Biochemistry 18*, 2794-2804.
Pogolotti, A. L., Jr., Danenberg, P. V., & Santi, D. V. (1986) *J. Med. Chem. 29*, 478-482.
Richter, J., Puchtler, I., & Fleckenstein, B. (1988) *J. Virol. 62*, 3530-3535.
Rode, W., Scanlon, K. J., Hynes, J., & Bertino, J. R. (1979) *J. Biol. Chem. 254*, 11538-11543.
Sanger, F. (1981) *Science 214*, 1205-1210.
Santi, D. V., McHenry, C. S., & Sommer, H. (1974) *Biochemistry 13*, 471-481.
Santi, D. V., McHenry, C. S., Raines, R. T., & Ivanetich, K. M. (1987) *Biochemistry 26*, 8606-8613.
Santi, D. V., Pinter, K., Kealey, K., & Davisson, V. J. (1990) *J. Biol. Chem. 265*, 6770-6775.
Singer, S. C., Richards, C. A., Ferone, R., Benedict, D., & Ray, P. (1989) *J. Bacteriol. 171*, 1372-1378.
Takeishi, K., Kaneda, S., Ayusawa, D., Shimizu, K., Gotoh, O., & Seno, T. (1985) *Nucleic Acids Res. 13*, 2035-2043.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8765-8785.
Taylor, G. R., Lagosky, P. A., Storms, R. K., & Haynes, R. H. (1987) *J. Biol. Chem. 262*, 5298-5307.
Thompson, R., Honess, R. W., Taylor, L., Moran, J., & Davison, A. J. (1987) *J. Gen. Virol. 68*, 1449-1455.
Wahba, A. J., & Friedkin, M. (1961) *J. Biol. Chem. 236*, PC11-12.
Weber, G. (1961) *Nature 190*, 27-29.
West, D. K., Belfort, M., Maley, G. F., & Maley, F. (1986) *J. Biol. Chem. 261*, 13446-13450.
Zoller, M. J., & Smith, M. (1984) *DNA 3*, 479-488.

Activator-Dependent Preinduction Binding of σ-70 RNA Polymerase at the Metal-Regulated *mer* Promoter†

Andreas Heltzel,‡ Ike Whan Lee, Paul A. Totis, and Anne O. Summers*
Department of Microbiology, University of Georgia, Athens, Georgia 30602
Received March 27, 1990; Revised Manuscript Received July 2, 1990

ABSTRACT: Expression of the Tn*21* mercury-resistance (*mer*) locus is controlled by the *merR* gene product, which represses *mer* structural gene (*merTPCAD*) transcription in the absence of mercuric ion [Hg(II)] and activates it in the presence of Hg(II). In vivo DNA methylation of the *mer* regulatory region (*merOP*) shows that, with or without the inducer Hg(II), MerR strongly protects four guanine residues in a dyadic region located between the −10 and −35 hexamers of the structural gene promoter (P_{TPCAD}). Prior to induction by Hg(II), RNA polymerase is also bound at P_{TPCAD}; occupancy of the uninduced promoter by RNA polymerase is dependent on MerR. Methylation and permanganate footprinting demonstrate that induction by Hg(II) results in MerR/Hg(II)-dependent promoter DNA melting in the −10 region of P_{TPCAD} and in additional DNA structural distortions within the region of dyad symmetry. Thus, MerR fosters the binding of RNA polymerase to an inactive promoter, and upon induction, MerR/Hg(II) facilitates DNA distortions suitable for efficient formation of the active transcription complex.

The bacterial mercury-resistance operon (*mer*) confers resistance to inorganic mercury [Hg(II)]. The most extensively studied examples of *mer* are encoded by the Gram-negative transposons Tn*21* and Tn*501* [reviewed in Summers (1986), Foster (1987), and Walsh et al. (1988)]. In Tn*21*, *mer* (Figure 1) consists of five structural genes encoding a Hg(II)-uptake system (*merT* and *merP*), the mercuric reductase enzyme (*merA*), and a small, low-abundance protein (*merD*) whose precise function is unknown (Lee et al., 1989). The role of an additional structural gene (*merC*), encoding an inner membrane protein present only in Tn*21*, has also yet to be established (Summers, 1986).

The expression of *merTPCAD* is under negative and positive control of the product of the *merR* gene (Figure 1; Foster et al., 1979; Ni'Bhriain et al., 1983; Foster & Brown, 1985; Lund et al., 1986; Heltzel et al., 1987). MerR also negatively

† This work was supported in part by a grant from the National Institutes of Health (GM28211).
* Correspondence should be addressed to this author.
‡ Present address: Zentrum für Molekulare Biologie, Universität Heidelberg, INF.282, D-6900 Heidelberg, Federal Republic of Germany.

0006-2960/90/0429-9572$02.50/0