0026-895X/93/020316-08$03.00/0

MOLECULAR PHARMACOLOGY, 44:316-323

Functional Effects of a Naturally Occurring Amino Acid Substitution in Human Thymidylate Synthase

C. TODD HUGHEY,[1] KAREN W. BARBOUR, FRANKLIN G. BERGER, and SONDRA H. BERGER

Departments of Basic Pharmaceutical Sciences (C.T.H., S.H.B.) and Biological Sciences (K.W.B., F.G.B.), University of South Carolina, Columbia, South Carolina 29208

Received March 22, 1993; Accepted May 19, 1993

SUMMARY

A major mechanism underlying the cytotoxicity of fluoropyrimidine analogs such as 5-fluorouracil and 5-fluoro-2′-deoxyuridine (FdUrd) occurs via the formation of 5-fluoro-2′-deoxyuridylate (FdUMP), a tight-binding inhibitor of thymidylate synthase (TS). Genetic variation in the structure of the TS molecule is an important determinant of response to fluoropyrimidines, because such variation may affect the binding of FdUMP to the enzyme. Previous studies have shown that the colonic tumor cell line HCT116 expresses two structurally distinct TS polypeptides that differ by the presence of tyrosine or histidine at residue 33. Compared with the Tyr-33 form, the His-33 form confers a 3–4-fold level of FdUrd resistance to cells; this was postulated to be derived from the reduced affinity of the enzyme for FdUMP and N^5,N^{10}-methylenetetrahydrofolate, ligands required for the formation of a stable inhibitory complex. In the present study, the Tyr-33 and His-33 forms have been purified to homogeneity, and their properties have been compared in detail. The K_m values for dUMP and N^5,N^{10}-methylenetetrahydrofolate in the TS reaction were not significantly different between the two enzymes. In contrast, the catalytic efficiency (k_{cat}) was 8-fold lower for the His-33 form. Kinetic and equilibrium binding measurements demonstrated that the dissociation constant for FdUMP binding into the ternary complex was 3–4-fold higher for the His-33 form; this was shown to be due to both a decrease in the rate of FdUMP association with the enzyme and an increase in the rate of FdUMP dissociation from the ternary complex. A TS form containing phenylalanine at residue 33 was created by site-directed mutagenesis and was shown to be very similar to the Tyr-33 enzyme with regard to k_{cat}, pH/activity profile, and effect on FdUrd response. Thus, it is the presence of histidine at residue 33, rather than the absence of tyrosine, that is responsible for the alterations in catalytic and ligand-binding functions exhibited by the His-33 form. Possible mechanisms by which the histidine residue perturbs the structure of the TS active site are discussed.

Fluoropyrimidine analogs, such as 5-fluorouracil and FdUrd, have been used in the therapy of carcinomas of the breast, ovary, and gastrointestinal tract (reviewed in Refs. 1 and 2). The cytotoxic effects of these agents may occur through several mechanisms. One involves the formation of fluorinated derivatives of dUTP and UTP, which can be incorporated into DNA and RNA, respectively (1, 2). A second mechanism derives from conversion of the analogs to FdUMP, a potent inhibitor of the enzyme TS (EC 2.1.1.45) (1, 2). TS, which is a dimer containing identical subunits of 36 kDa, catalyzes the synthesis of TMP via the reductive methylation of dUMP by CH_2H_4PteGlu (3, 4). FdUMP, being an analog of dUMP, inhibits TS activity through formation of a stable covalent ternary complex with the enzyme and CH_2H_4PteGlu (3, 4). The ternary complex is catalytically inactive, resulting in TMP depletion, cessation of DNA biosynthesis, and cell death.

Drug resistance is a major obstacle to the successful use of chemotherapeutic agents in the treatment of neoplastic disease. Investigations of naturally occurring resistance in model cell lines provide useful insights into the mechanisms that may underlie innate clinical resistance in patients not previously exposed to drugs. Numerous studies have been directed at assessing the role of TS in fluoropyrimidine resistance, and several mechanisms have been identified. These include higher concentrations of TS, which in some cases derive from amplification of the TS structural gene (5, 6); decreases in FdUMP levels, resulting from either reduced conversion of FdUrd to FdUMP, increased catabolism of FdUMP, or increased efflux of FdUrd (7–9); changes in intracellular CH_2H_4PteGlu levels, impacting upon the formation and stability of ternary complexes (10, 11); and structural changes in the TS polypeptide, generating altered affinities between FdUMP or CH_2H_4PteGlu and the enzyme (12, 13).

Recent studies of FdUrd cytotoxicity in human colonic tumor

This work was supported by a grant (CA44013) from the National Institutes of Health.

[1] Research reported in this article was performed in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Immunology, Duke University Medical Center, Box 3010, Durham, NC 27710.

ABBREVIATIONS: FdUrd, 5-fluoro-2′-deoxyuridine; TS, thymidylate synthase; CH_2H_4PteGlu, N^5,N^{10}-methylenetetrahydrofolate; FdUMP, 5-fluoro-2′-deoxyuridylate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid.

cell lines have revealed the importance of TS structure in fluoropyrimidine response. Analysis of seven cell lines established from primary cultures of human colonic tumors revealed one, HCT116, that had a unique phenotype. HCT116 cells were found to be relatively resistant to FdUrd; this resistance was not associated with particularly high levels of TS, nor was it a consequence of an inability to form FdUMP (12, 14). Structural studies showed that HCT116 expresses two electrophoretically distinguishable forms of TS; one form is common to all cell lines examined, whereas a variant form, having a higher pI, is present only in HCT116 (12, 14). Of particular interest was the finding that derivatives of HCT116 overexpressing the variant form were more resistant to FdUrd than were derivatives overexpressing the ubiquitous form (12). Studies of ligand binding in crude extracts indicated that the variant TS has a lower affinity for FdUMP and CH_2H_4PteGlu, a property that may account for the relative FdUrd resistance of HCT116 cells (12).

Sequence analysis of TS-specific cDNAs led to the identification of a single amino acid substitution that distinguishes the two TS forms (15). The mRNA encoding the variant TS contains a thymidine to cytidine transition mutation at codon 33; this mutation, which causes the loss of a *Rsa*I restriction site within the TS structural gene, results in the replacement of a tyrosine residue by histidine at residue 33 of the polypeptide chain (15, 16). Tyr-33 is invariant among the TS polypeptides of 18 species for which sequence information is available (17), implying a critical role for the amino acid in TS function. Direct demonstration that the Tyr-33 to His-33 substitution has an impact upon FdUrd response was provided by DNA transfer experiments, which showed that mammalian or bacterial cells expressing the His-33 form were 3–4-fold more resistant to FdUrd than were cells expressing the Tyr-33 form (16).

To acquire more detailed information on the effects of the Tyr-33 to His-33 replacement on TS function, we have purified the two forms to homogeniety and have shown that they differ with regard to catalytic properties and the kinetics of interactions with ligands. In addition, we demonstrate that the altered properties of the variant TS form are caused by the presence of histidine, rather than by the absence of tyrosine, at residue 33.

Experimental Procedures

Materials. [6-^{3}H]FdUMP (20 Ci/mmol) and [5-^{3}H]dUMP (25 Ci/mmol) were purchased form Moravek Biochemicals (Brea, CA). Folic acid, CF, FdUMP, dUMP, and MES were purchased from Sigma Chemical Co. (St. Louis, MO). CH_2H_4PteGlu was prepared from tetrahydrofolate as described previously (10); folate concentrations are reported in terms of the active isomer. Bradford protein dye-binding reagent and Gel Silver Rapid Stain were purchased from Bio-Rad Laboratories (Richmond, CA). RPMI 1640 medium and fetal bovine serum were purchased from GIBCO Inc. (Grand Island, NY). Fetal bovine serum supplemented with iron was obtained from Hyclone Laboratories (Logan, UT).

Cell culture. Cell lines HCT116/200–10 and HCT116/200–11, which overproduce the Tyr-33 and His-33 forms of TS, respectively (see Results), were maintained as monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 nM FdUrd, and 10 μM folinic acid (12). The cells were routinely monitored for *Mycoplasma* contamination by using the Mycoplasma T.C. Rapid Detection System (Gene-Probe, San Diego, CA).

Purification of TS. HCT116/200–10 and HCT116/200–11 cells were grown for 12–14 days in drug-free medium containing 8% fetal bovine serum, to allow for the *in situ* dissociation of intracellular ternary complexes. Enzymes were purified by previously described procedures (18), with minor modifications. Cell-free extracts were prepared in buffers containing 20 μg/ml leupeptin, 50 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (19). Purity of the TS preparations was assessed by SDS-PAGE, using silver staining to detect enzyme, as described previously (18).

TS assays. TS polypeptide concentrations were quantitated by FdUMP binding in the presence of CH_2H_4PteGlu (14). Enzyme activity was assayed by measuring formation of [^{3}H]H_2O from [5-^{3}H]dUMP in reactions containing 30 μM [5-^{3}H]dUMP and 150 μM CH_2H_4PteGlu (18). One unit of activity is defined as the amount of enzyme required to release 1 nmol of [^{3}H]H_2O/min. For determination of initial velocities, reactions containing 0.013 units of purified TS, 1–60 μM [5-^{3}H]dUMP, and 10–200 μM CH_2H_4PteGlu were analyzed as described previously (18). Michaelis constants were calculated by computer-assisted linear regression analysis of double-reciprocal plots. The effects of pH on enzyme reaction rates were determined by incubating 0.012 units of purified TS with 30 μM [5-^{3}H]dUMP and 150 μM CH_2H_4PteGlu (18) in the pH range of 4.5–8.5, using a buffer composed of 60 mM MES, 60 mM acetic acid, and 120 mM Tris (20).

Kinetic analysis of FdUMP binding. Characterization of FdUMP binding to TS was based upon previously described kinetic models (21, 22), using specific methods that have been outlined in detail (18). Rate constants for FdUMP association into the ternary complex (k_{on}) were measured in reactions containing enzyme (1.5 nM ligand binding sites, assuming 1.7 mol of FdUMP/mol of protein dimer), 0.5–1.5 nM [6-^{3}H]FdUMP, and 150 μM CH_2H_4PteGlu; constants were calculated from linear plots based upon a second-order rate equation described previously (22).

Rate constants for dissociation of FdUMP from ternary complexes (k_{off}) were determined from the half-lives ($t_{½}$) of preformed complexes (21, 22). Complexes were isolated from reactions containing enzyme (1.7 nM ligand binding sites), 30 nM [6-^{3}H]FdUMP, and 150 μM CH_2H_4PteGlu. The stabilities of complexes were quantitated by charcoal adsorption assays (18) at various times during incubation with 100 μM unlabeled FdUMP and 30–200 μM CH_2H_4PteGlu. The $t_{½}$ values were determined and k_{off} was calculated from the relationship $k_{off} = \ln 2/t_{½}$.

Equilibrium studies of FdUMP binding were carried out in reactions containing 1.7 nM ligand binding sites, 0.5–32 nM [6-^{3}H]FdUMP, and 150 μM CH_2H_4PteGlu. Apparent dissociation constants (K_d) were determined by computer-assisted linear regression analysis of data graphed according to the Scatchard equation (23).

Analysis of a Phe-33 mutant of TS. A mutant TS cDNA with a phenylalanine codon at residue 33 was produced by site-directed mutagenesis, using methods described earlier (16). Plasmid pDHTS-S1, containing a full length cDNA corresponding to the Tyr-33 form of human TS in a vector designed for expression in *Escherichia coli* (19), was used as template. The mutagenic oligonucleotide was the antisense sequence 5′-TGCCCCAGGAACTGCAGCTC-3′ (the phenylalanine codon is underlined), complementary to the codon 33 region of the TS mRNA. The mutant plasmid was transformed into a *thyA*$^-$ strain of *E. coli* (χ2913) (19); cells expressing functional TS were selected in thymine-free medium. FdUrd sensitivities were measured as described previously (16).

Results

Purification of the Tyr-33 and His-33 forms of TS. Cell lines HCT116/200–10 and HCT116/200–11 were used for the purification of the Tyr-33 and His-33 forms of TS, respectively. These clonal derivatives of HCT116 express one form of TS in approximately 20-fold excess, relative to the other (12). The purification procedure consisted of ammonium sulfate fractionation, chromatography on AffiGel Blue-Sepharose, affinity chromatography on 10-formylfolate-Sepharose, and ion

exchange chromatography using Mono Q Sepharose (18). Table 1 shows the specific activities and yield of enzymes during the procedure. For each enzyme, approximately 250-fold purification was achieved, with a yield of about 14–22%. Each preparation contained a single protein species of molecular mass 36 kDa, as assessed by SDS-PAGE (Fig. 1). Isoelectric focusing gel electrophoresis of [^{32}P]FdUMP-containing ternary complexes, which provides a very sensitive method for distinguishing the two TS forms (12, 14), indicated that the purified His-33 form was, as expected, more basic than the Tyr-33 form (data not shown); this is consistent with the known electrophoretic properties of the two TS forms (12). No contamination of either purified enzyme with the alternative form was detectable on these gels (data not shown). Based upon the levels of expression of the two TS forms in the tumor cell lines used for enzyme purifications, we expected a 2–5% contamination of each preparation, which would be detectable by the electrophoretic methods used. Because no such contamination was observed, it is likely that the two TS forms were resolved to some extent at one or more steps in the purification procedure (18).

TABLE 1

Purification of the Tyr-33 and His-33 forms of TS

Step	Total activity	Total protein	Specific activity	Fold purification	Yield
	units	*ms*	*units/mg*		%
Tyr-33 form					
Crude	1009	320	3.2	1	100
$(NH_4)_2SO_4$	868	138	6.3	2	86
AffiGel Blue	565	6.0	94	29	56
Affinity	262	0.4	655	205	26
Mono Q	223	0.3	743	232	22
His-33 form					
Crude	63	180	0.35	1	100
$(NH_4)_2SO_4$	42	64	0.65	2	66
AffiGel Blue	26	4.0	6.6	19	41
Affinity	13	0.2	65	186	21
Mono Q	9	0.1	90	257	14

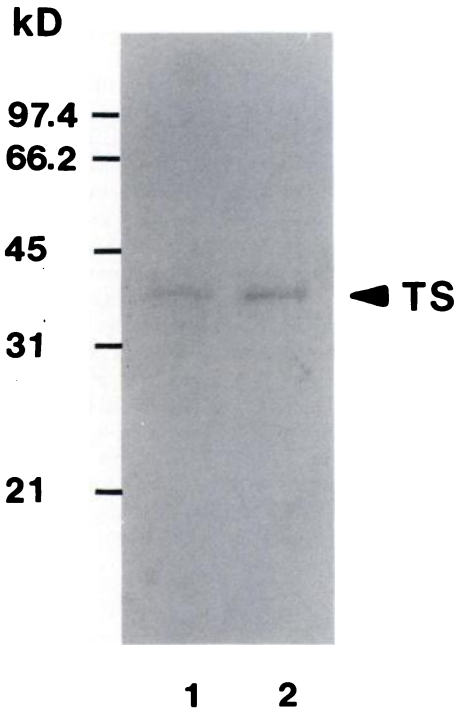

Fig. 1. Analysis of purified TS forms by SDS-PAGE. Purified preparations of the Tyr-33 (*lane 1*) and His-33 (*lane 2*) enzymes were fractionated by SDS-PAGE and detected by silver staining. Molecular mass standards, shown on the *left*, were rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

The specific activity of the Tyr-33 form was 743 units/mg, which is similar to what was reported for human TS purified from *E. coli* (19). In contrast, the specific activity of the His-33 enzyme was only 90 units/mg. This difference between the two TS forms is not an artifact of the purification, because in crude cell-free extracts of appropriate cell lines the catalytic activity of the His-33 form is about 8–10-fold lower than that for the Tyr-33 form (data not shown). The large difference in specific activities between the two TS forms must be due to an intrinsic difference in catalytic efficiency, i.e., k_{cat}.

Kinetics of ligand binding to the Tyr-33 and His-33 forms of TS. In earlier studies with crude enzyme preparations, the His-33 form of TS was observed to have reduced affinities for FdUMP and $CH_2H_4PteGlu$, relative to the Tyr-33 form (12). To verify this finding and to obtain more detailed information on the impact of the Tyr-33 to His-33 substitution on TS function, kinetic parameters describing the catalytic and ligand-binding properties of purified preparations of the two enzymes were compared.

K_m values for dUMP and $CH_2H_4PteGlu$ were not significantly different between the two TS forms (Table 2). However, the catalytic efficiency (k_{cat}) differed markedly. The k_{cat} for the Tyr-33 enzyme was 1.6 sec^{-1}, whereas that for the His-33 enzyme was 0.2 sec^{-1} (Table 2). This is consistent with the difference in specific activities described above and indicates that the Tyr-33 to His-33 substitution affects substrate turnover.

The binding of FdUMP and $CH_2H_4PteGlu$ to TS in the formation of the ternary complex can be considered to occur via the following reactions (21, 22):

$$\text{TS} + \text{FdUMP} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{TS-FdUMP} \underset{k_4}{\overset{k_3[\text{CH}_2\text{H}_4\text{PteGlu}]}{\rightleftharpoons}} \text{TS-FdUMP-CH}_2\text{H}_4\text{PteGlu} \quad \text{Scheme 1}$$

Rates of FdUMP binding into a ternary complex with TS and $CH_2H_4PteGlu$, described by the kinetic constant k_{on}, are a function of the rate constants in scheme 1, according to the following equation (21, 22):

$$k_{on} = \frac{k_1 k_3[\text{CH}_2\text{H}_4\text{PteGlu}]}{k_2 + k_3[\text{CH}_2\text{H}_4\text{PteGlu}]} \quad (1)$$

At high concentrations of $CH_2H_4PteGlu$, $k_3[CH_2H_4PteGlu] \gg k_2$, so that k_{on} approximates k_1. Thus, by measuring the initial rate of [^{3}H]FdUMP binding into ternary complexes in the presence of excess $CH_2H_4PteGlu$, an estimate of k_{on} can be obtained (21, 22). Under these conditions, k_{on} can be determined from the following second-order equation (22):

$$\frac{1}{[\text{E}_0] - [\text{FdUMP}_0]} \ln \frac{[\text{FdUMP}_0]([\text{E}_0] - [\text{X}])}{[\text{E}_0]([\text{FdUMP}_0] - [\text{X}])} = k_{on} t \quad (2)$$

where $[E_0]$ is the initial concentration of binding sites, $[FdUMP_0]$ is the initial concentration of FdUMP, and [X] is the concentration of ternary complex measured at time t (22); eq. 2 can be plotted as a straight line, with slope k_{on}. Rates of

TABLE 2

Kinetic constants for the Tyr-33 and His-33 forms of TS

All values represent the average ± standard deviation of two or three independent determinations.

Parameter	Tyr-33 enzyme	His-33 enzyme
K_m for dUMP	1.4 ± 0.3 μM	2.3 ± 0.5 μM
K_m for $CH_2H_4PteGlu$	36 ± 5 μM	22 ± 4 μM
k_{cat}	1.6 ± 0.3 sec^{-1}	0.2 ± 0.08 sec^{-1}
k_{on} for FdUMP	1.2 ± 0.1 × 10^8 M^{-1} min^{-1}	0.48 ± 0.06 × 10^8 M^{-1} min^{-1}
k_{off} for FdUMP[a]	0.0094 ± 0.0008 min^{-1}	0.021 ± 0.002 min^{-1}

[a] At 150 μM $CH_2H_4PteGlu$.

FdUMP association into a ternary complex with the Tyr-33 and His-33 forms of TS were observed to be linear with respect to time, dependent upon the FdUMP concentration, and independent of the $CH_2H_4PteGlu$ concentration above 10 μM (data not shown). Rates were measured with 150 μM $CH_2H_4PteGlu$, and the apparent k_{on} values were calculated from the slopes of the straight lines plotted according to eq. 2 (Fig. 2). For the Tyr-33 form, k_{on} was found to be 1.2 × 10^8 M^{-1} min^{-1} (Table 2), which is very similar to the value of 1.7 × 10^8 M^{-1} min^{-1} determined for the enzyme from human leukemic cell line CCRF-CEM (22, 24). For the His-33 form, k_{on} was 0.48 × 10^8 M^{-1} min^{-1} (Table 2), representing about a 2.5-fold reduction, relative to the Tyr-33 form.

FdUMP dissociation from the ternary complex (k_{off}) can be determined by measuring the half-life (k_{off} = ln 2/$t_{½}$) of preformed complexes containing [^{3}H]FdUMP (21, 22). Because the dissociation of $CH_2H_4PteGlu$ from the complex precedes the dissociation of FdUMP (see scheme 1 above), observed k_{off} values are a function of the folate co-substrate concentration, as well as k_2, k_3, and k_4. The relationship between k_{off} and the concentration of $CH_2H_4PteGlu$ can be described by the following equation (21, 22):

$$1/k_{off} = (k_3/k_2k_4)[CH_2H_4PteGlu] + 1/k_4 \quad (3)$$

Thus, a plot of $1/k_{off}$ versus the concentration of $CH_2H_4PteGlu$ results in a straight line having slope k_3/k_2k_4 and intercept $1/k_4$. As expected, for both the Tyr-33 and His-33 enzymes the $t_{½}$ for FdUMP dissociation from the ternary complex was observed to increase linearly as a function of the $CH_2H_4PteGlu$ concentration. At each concentration of the folate co-substrate, the k_{off} was significantly greater for the His-33 form than for the Tyr-33 form (Fig. 3). For example, in the presence of 150 μM $CH_2H_4PteGlu$, which is the standard folate concentration used in TS analyses, k_{off} values were 0.0094 min^{-1} for the Tyr-33 enzyme and 0.021 min^{-1} for the His-33 enzyme (Table 2). Bapat *et al.* (24) measured a k_{off} of 0.0088 min^{-1} for the enzyme from the human leukemic cell line CCRF-CEM; this compares quite favorably with the k_{off} value for the Tyr-33 form in HCT116 cells.

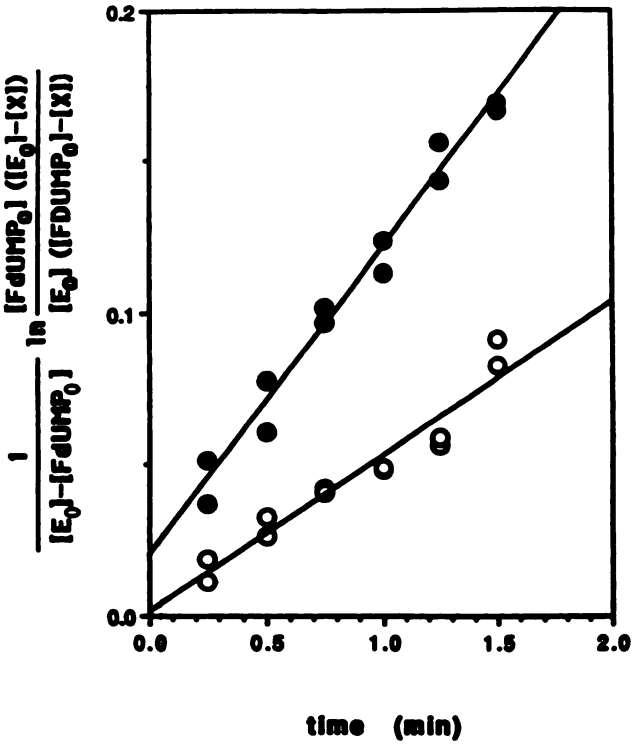

Fig. 2. Determination of the k_{on} for FdUMP binding into the ternary complex. Purified Tyr-33 (●) or His-33 (○) enzyme (1.5 nM ligand binding sites) was incubated for various times with 150 μM $CH_2H_4PteGlu$ and 0.5–1.5 nM [6-^{3}H]FdUMP. The level of bound FdUMP was measured (18), and the data were plotted according to eq. 2 (22) (see text). Each *point* represents the average of three independent determinations.

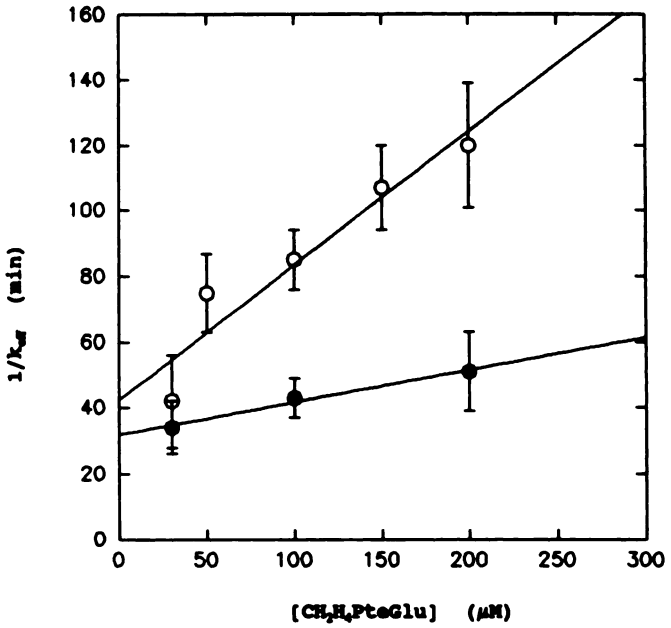

Fig. 3. Determination of k_{off} for FdUMP binding into the ternary complex. Ternary complex, labeled with [6-^{3}H]FdUMP and containing the Tyr-33 (○) or His-33 (●) form of TS, was prepared as described in Experimental Procedures; the isolated complex was incubated in the presence of various concentrations of $CH_2H_4PteGlu$ and 100 μM unlabeled FdUMP. At each concentration of folate co-substrate, the $t_{½}$ for FdUMP dissociation from the complex was measured (18), and k_{off} was calculated. Each *point* represents the average of two or three independent measurements, vertical lines, standard errors.

The two straight lines in Fig. 3 extrapolated to nearly the same point on the ordinate, indicating k_4 values of 0.024 min^{-1} for the Tyr-33 form and 0.031 min^{-1} for the His-33 form. The k_3/k_2 ratios, which measure the tendency of the binary complex to either bind $CH_2H_4PteGlu$ and form the ternary complex or dissociate (see scheme 1), were determined from the slopes of the lines in Fig. 3. Values of 9.87×10^3 M^{-1} and 3.06×10^3 M^{-1} were observed for the Tyr-33 and His-33 enzymes, respectively; this 3.2-fold difference between the two forms suggests that the Tyr-33 to His-33 substitution has caused an alteration in k_2, k_3, or both.

The k_{off}/k_{on} ratio provides an estimate of the equilibrium dissociation constant (K_d) for FdUMP binding into the ternary complex. The K_d determined in this manner was found to be almost 6-fold higher for the His-33 enzyme than for the Tyr-33 enzyme (Table 3). The K_d for each enzyme form was also determined experimentally by equilibrium binding studies. A Scatchard plot of the binding data (Fig. 4) showed that both TS forms contain a single class of FdUMP binding sites. The K_d values were similar to those calculated from k_{on} and k_{off} determinations, with the His-33 form showing about a 3-fold higher K_d, relative to the Tyr-33 form (Table 3).

It is clear from the data presented above that replacement of tyrosine by histidine at residue 33 of human TS causes an increase in the K_d for FdUMP; this increase results from both a reduction in k_{on} and an increase in k_{off}. The reduction in k_{on} is due to a decrease in the rate of binding of FdUMP into the binary complex (i.e., k_1). The increase in k_{off} reflects either higher rates of dissociation of FdUMP from binary complexes (i.e., k_2) or reduced rates of $CH_2H_4PteGlu$ binding into the ternary complex (i.e., k_3), or both.

Temperature and pH effects on the reaction catalyzed by the Tyr-33 and His-33 forms. The effects of temperature on enzyme catalysis were compared for the Tyr-33 and His-33 forms by measuring activities in the temperature range of 29–41°. An Arrhenius plot of the data (Fig. 5A) showed that below 35° the activation energy was about 15.8 kcal/mol for both enzymes; this is similar to earlier studies, where activation energies of 16.9 and 20.9 kcal/mol, at temperatures between 27° and 35°, were measured (25, 26). Above 35° the enzymes underwent significant inactivation (Fig. 5A). This contrasts sharply with earlier analyses that indicated the existence of a transition to lower activation energies at the higher temperatures (25, 26). The reasons for the discrepancy between the present results and earlier work are not known.

The pH/activity profiles for the two TS forms are shown in Fig. 5B. Of interest was the observation that the His-33 form appeared to be more sensitive to pH values below 7. Between pH 6.5 and 5.5, the activity of the Tyr-33 form decreased by 2.7-fold, whereas the activity of the His-33 enzyme decreased by 16.4-fold (Fig. 5B). Such marked pH sensitivity for the His-33 form was also observed for enzyme expressed in *E. coli* (see Fig. 6) and most likely derives from protonation of the imidazole nitrogen of histidine.

TABLE 3

Dissociation constants (K_d) for FdUMP binding to TS forms

Method	K_d	
	Tyr-33 enzyme	His-33 enzyme
	M	
k_{off}/k_{on} ratio[a]	0.78×10^{-10}	4.4×10^{-10}
Experimental[b]	$1.1 \pm 0.1 \times 10^{-10}$	$3.1 \pm 0.3 \times 10^{-10}$

[a] k_{off} and k_{on} are taken from Table 2.
[b] Experimental determination of K_d was by Scatchard analysis (Fig. 4); values represent the averages ± standard deviations of three experiments.

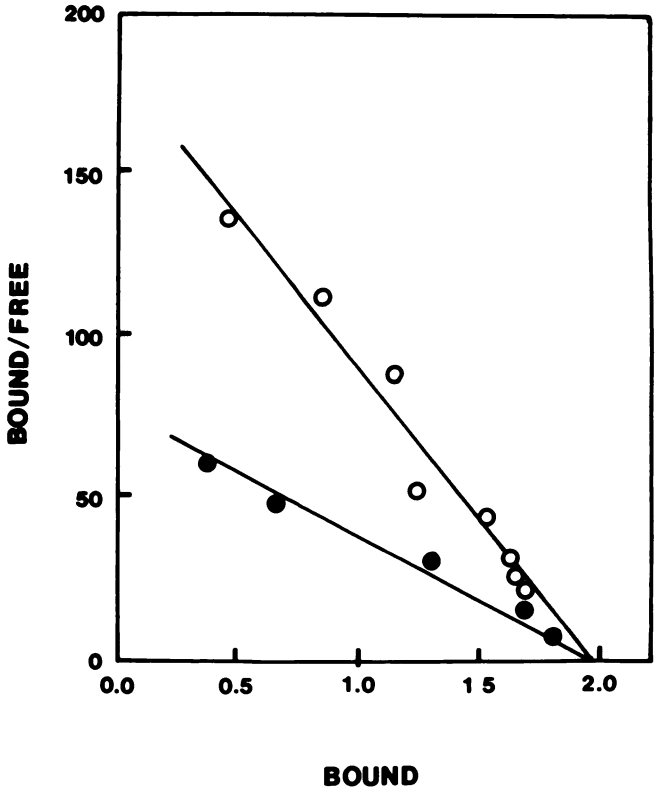

Fig. 4. Determination of the K_d for FdUMP binding into the ternary complex. Purified Tyr-33 (○) or His-33 (●) enzyme was incubated with 150 μM $CH_2H_4PteGlu$ and 0.5–32 nM [6-^{3}H]FdUMP. The level of bound FdUMP was measured (18), and the data were plotted according to the Scatchard equation (23); each *point* represents the average of three separate determinations.

Mechanisms underlying the functional effects of the Tyr-33 to His-33 substitution. X-ray structural studies have shown that in human TS the hydroxyl group of Tyr-33, which is located within an amphipathic helix, forms a hydrogen bond with the main-chain carbonyl oxygen of Met-219, which is located at the base of a second helix that forms part of the active site cavity (27–29).[2] Two mechanisms might be invoked to explain the effects of the His-33 substitution on TS function. The absence of the tyrosine residue, and its hydrogen bond, may destabilize the relative positions of the two helices, resulting in a change in active site structure and a perturbation of ligand binding. Alternatively, the presence of the imidazole ring of histidine may alter active site conformation either through steric effects or through unique interactions with other amino acid side-groups in the vicinity.

To determine whether the functional alterations exhibited by the His-33 form result from the absence of tyrosine or the presence of histidine, the properties of a TS form containing phenylalanine at residue 33 were studied. The Phe-33 form of TS was generated by site-directed mutagenesis of plasmid pDHTS-S1, which contains a human TS cDNA in an *E. coli* expression vector (19). The mutant plasmid supported growth of TS-negative *E. coli* cells in thymine-free medium, indicating

[2] C. Schiffer and R. Stroud, personal communication.

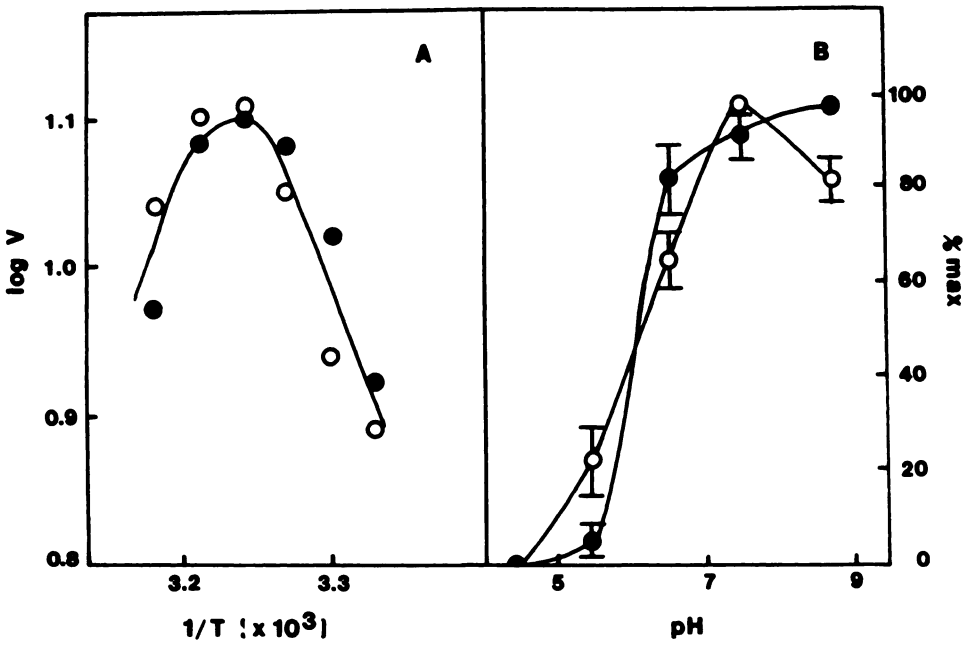

Fig. 5. Temperature and pH effects on TS activity. Enzyme activity was measurerd for the Tyr-33 (○) or His-33 (●) form of TS, under standard assay conditions (see Experimental Procedures). A, Assays were conducted at various temperatures, and an Arrhenius plot of the data was constructed; *v* is the reaction velocity (in milliunits) and *T* is the temperature (in °K). Each *point* represents the average of two or three separate determinations. B, Activities were measured at different pH values. The maximum activity for each TS form was set at 100%, and activities were expressed relative to the maximum. Each *point* represents the average of three separate measurements; *vertical lines*, standard errors.

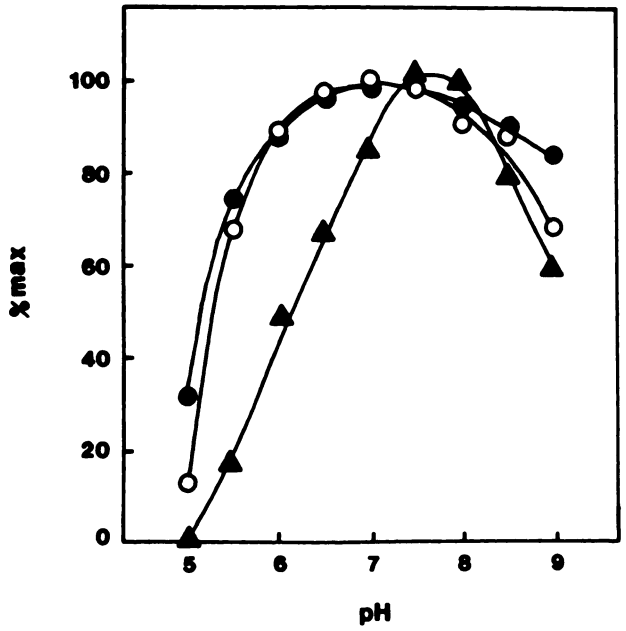

Fig. 6. Effects of pH on TS enzymes expressed in *E. coli*. Enzyme activities in crude extracts of bacteria expressing the Tyr-33 (○), His-33 (▲), or Phe-33 (●) form of TS were measured at several pH values. For each form, the maximum activity was set at 100%, and activities were expressed relative to the maximum. Each *point* represents the average of two or three separate determinations.

that the Phe-33 form is catalytically active. Bacterial cells expressing the Phe-33 enzyme were as responsive to FdUrd as cells expressing the Tyr-33 form (data not shown); this contrasts with cells expressing the His-33 form, which were 4-fold more resistant to the fluoropyrimidine analog (16). Thus, the Phe-33 enzyme behaves similarly to the Tyr-33 enzyme, indicating that the absence of the hydroxyl group at residue 33 does not, by itself, have an impact upon TS function.

This conclusion is supported by a comparative analysis of k_{cat} values and pH/activity profiles. The k_{cat} for each of the three TS forms was determined in crude extracts of bacterial cells transformed with the appropriate plasmid. The Tyr-33 and Phe-33 enzymes exhibited nearly identical k_{cat} values of 3.0–4.0 sec^{-1}, which are similar to the k_{cat} for the purified Tyr-33 form of TS (see Table 2). The k_{cat} for the His-33 enzyme was 0.2–0.4 sec^{-1}, which is also close to that determined for the purified enzyme (Table 2). The pH/activity profiles for the Tyr-33 and Phe-33 forms were essentially indistinguishable, whereas the His-33 form was markedly sensitive to pH values below 7.5 (Fig. 6); this pH sensitivity was noted for the purified His-33 enzyme (see Fig. 5B) and probably results from protonation of the imidazole nitrogen.

Discussion

Earlier studies had shown that the variant structural form of TS, which differs from the normal form by a tyrosine to histidine substitution at residue 33, confers resistance to FdUrd in both mammalian and bacterial cells; drug resistance was presumed to be a consequence of reduced affinity between the enzyme and ligands (FdUMP and/or $CH_2H_4PteGlu$), which decreases both the formation and the stability of the inhibitory ternary complex (12, 14–16). In the present study, the biochemical properties of purified preparations of the Tyr-33 and His-33 forms have been compared in detail. The results show that the two enzymes differ in a number of parameters related to ligand binding and catalytic function, indicating that the amino acid substitution significantly alters TS function.

Apparent K_d values for FdUMP binding into the ternary complex were 3–4-fold higher for the His-33 form, compared with the Tyr-33 form (Table 3). This was shown to derive from changes in both k_{on} and k_{off}. k_{on}, which describes the rate of FdUMP association into the ternary complex, was observed to be about 2.5-fold lower for the His-33 form (Fig. 2). In the presence of excess $CH_2H_4PteGlu$, k_{on} is essentially an estimate of the rate of formation of the binary complex between FdUMP and the enzyme (see scheme 1). k_{off}, which is a measure of the rate of dissociation of FdUMP from the ternary complex, was greater for the His-33 enzyme (Fig. 3). k_{off} is dependent upon the concentration of $CH_2H_4PteGlu$, as well as upon several rate

constants describing individual steps in the formation of the ternary complex (i.e., k_2, k_3, and k_4 in scheme 1) (21, 22). The analysis in Fig. 3 indicates that the increased k_{off} for the His-33 enzyme results from either a higher rate of dissociation of FdUMP from the binary complex (described by k_2) or a lower rate of association of CH_2H_4PteGlu into the ternary complex (described by k_3), or both. The current data do not allow independent measurement of k_2 and k_3, so ascertaining the relative contributions of these rate constants to the k_{off} difference between the two TS forms must await further analysis.

The Tyr-33 to His-33 substitution also affects the catalytic properties of the TS molecule. The K_m values for dUMP and CH_2H_4PteGlu were not significantly different between the two forms. However, an 8-fold lower k_{cat} was observed for the His-33 form. This alteration is not an artifact of the purification process, because similar differences in catalytic efficiency can be measured in crude extracts of mammalian or bacterial cells expressing one or the other of the TS enzymes (data not shown). The k_{cat} value is therefore diagnostic of the structural difference between the two TS forms and is independent of the state of enzyme purity.

Because the enzymes used in these studies were purified from cells expressing one form of TS in about 20-fold excess, relative to the other (12), it is possible that each enzyme is contaminated with small amounts of the alternative form; as much as 5% contamination might be expected for each preparation, potentially complicating the results of the kinetic studies. However, there are several reasons why we do not believe such contamination exists. First, highly sensitive isoelectric focusing analyses of [^{32}P]FdUMP-containing ternary complexes showed no evidence of the alternative form in either enzyme preparation,[3] indicating that the two forms may have been separated during the purification procedure. At present, we do not know at which step(s) in the protocol this separation occurred. Second, crude preparations of the Tyr-33 and His-33 forms expressed independently in *E. coli* displayed an 8-fold difference in k_{cat}, as do the enzymes purified from the colonic tumor cell lines (see Results); recent preliminary experiments indicate that a similar difference exists for partially purified TS forms from these bacteria.[4] Thus, at least with regard to k_{cat}, the TS forms purified from enzyme-overproducing colonic tumor cells are quite similar to the enzymes produced in *E. coli*. There appears, therefore, to be little or no contamination of cell line-derived TS preparations by alternative enzyme forms. Current efforts are focusing on the purification and kinetic analysis of TS forms expressed in *E. coli*.

Cellular sensitivity to tight-binding enzyme inhibitors, such as FdUMP, can be defined by the following equation (30):

$$ID_{50} = 0.5E_t + K_d \quad (4)$$

where ID_{50} is the concentration of inhibitor required for 50% inhibition of cell growth, E_t is the enzyme concentration, and K_d is the dissociation constant for enzyme-inhibitor complexes. This equation predicts that when enzyme levels are low, i.e., when $K_d > E_t$, the ID_{50} will be directly proportional to K_d. A direct relationship between the ID_{50} for FdUrd and the K_d for FdUMP has been observed in mammalian and bacterial cells expressing the Tyr-33 or His-33 TS forms (12, 16).

Several examples of fluoropyrimidine resistance associated with perturbations in TS structure have been documented in model cell lines. A FdUrd-resistant line of Ehrlich ascites carcinoma cells expressed a TS that differed significantly from the enzyme in parental cells (26). Purified enzyme from the resistant line had a higher k_{cat}, a lower affinity for FdUMP as measured by enzyme inhibition studies, and a lower activation energy at temperatures above 36° (26). In another study, a human leukemic cell line resistant to FdUrd was found to produce a TS enzyme with 23-fold higher K_d for FdUMP; k_{on} was reduced by 14-fold, whereas k_{off} was increased by 1.7-fold. It was concluded that k_1 and k_2 were altered in the mutant TS molecule, whereas k_3 and k_4 were not (24). Most recently, comparisons of colonic tumor cell lines RCA and HCT-C showed that the former is about 10-fold resistant to FdUrd, relative to the latter (6, 10). TS purified from cell line RCA exhibited an increase in K_d for FdUMP, which was a consequence of a reduction in k_{on} (10). Direct measurements of FdUMP binding into the binary complex verified that k_1 is reduced in the TS from the drug-resistant line (10). In none of the examples cited above has the nature of the structural change responsible for the functional alterations in the TS molecule been determined.

Kinetic alterations in the TS molecule have been observed among the TS enzymes purified directly from tumor tissues (31). Tumors of 11 patients exhibited K_d values that varied by as much as 150-fold; this was primarily associated with alterations in k_{on} and little change in k_{off} (31). These results implicate k_{on} as a major variable in clinically occurring tumors. It will be of interest to determine whether there are any associations between k_{on} values and response to fluoropyrimidine chemotherapy.

Tyr-33 of human TS is one of 40 amino acid residues that are invariant among the enzymes from a large variety of organisms, including viral, prokaryotic, and eukaryotic species (17). Such a high degree of conservation suggests that this residue must play a critical role in TS function. The three-dimensional structures of the TS molecules from T4 phage, *E. coli*, *Lactobacillus casei*, and human have been solved by X-ray crystallographic studies (17, 27–29).[5] These studies show that Tyr-33 (Tyr-4 in T4 phage and *E. coli* and Tyr-6 in *L. casei*) is located within an amphipathic α-helix that corresponds to helix A in the *L. casei* enzyme (27) and is near the active site. The hydroxyl group of Tyr-33 forms a hydrogen bond with the main-chain carbonyl oxygen of Met-219 (Val-138 in T4 phage, Val-170 in *E. coli*, and Ile-222 in *L. casei*), which is located at the base of a second helix, corresponding to helix J in *L. casei* (27); this helix lines the active site cavity and contains several residues that directly participate in ligand binding (27–29). Hydrogen bonding between helices A and J probably plays a role in stabilizing their relative positions, thereby contributing to the conformation of the active site. It might be predicted, therefore, that amino acid substitutions at residue 33 would perturb the structure of the active site through loss of the hydrogen bond donated by the tyrosine residue. However, this explanation is likely to be too simple, because a mutant form of TS containing phenylalanine at residue 33 had properties very similar to those of the Tyr-33 enzyme (see Results and Fig. 6). Thus, the loss of the tyrosine hydroxyl group, and its

[3] C. T. Hughey, unpublished observations.
[4] T. Riley, unpublished obeservations.
[5] C. Schiffer and R. Stroud, personal communication.

hydrogen bond, does not account for the phenotype of the His-33 form, indicating that the functional alterations exhibited by this form result from the presence of histidine, rather than from the absence of tyrosine.

The precise mechanism by which the histidine residue alters TS function is not known. Based upon the considerations discussed above, it is likely that the imidazole moiety directly perturbs the relative positions of helices A and J, resulting in a change in the conformation of the active site. These effects may occur as a consequence of unique interactions with other amino acid groups. For example, the imidazole nitrogen, like the tyrosine hydroxyl in the Tyr-33 form, may enter into a hydrogen bond with the main-chain carbonyl of Met-219. Computer modeling has shown that such a hydrogen bond could be accomodated with a slight (i.e., 1–2-Å) shift in the relative orientations of helices A and J[6]; this could conceivably cause a conformational change, affecting the catalytic and ligand-binding properties of the His-33 enzyme. Another possibility relates to the observation that the TS polypeptide undergoes large segmental changes in conformation upon the binding of ligands (29); these conformational shifts are most likely important determinants of active site function and may be affected by the presence of histidine at residue 33. Obviously, additional studies will be necessary to define the precise mechanism(s) by which the Tyr-33 to His-33 substitution affects TS function.

Acknowledgments

We thank Dr. Daniel Santi for providing plasmid pDHTS-S1 and host bacterial strain χ2913.

References

1. Heidelberger, C., P. V. Danenberg, and R. G. Moran. Fluorinated pyrimidines and their nucleosides. *Adv. Enzymol. Relat. Areas Mol. Biol.* **54**:57–119 (1983).
2. Parker, W. B., and Y. C. Cheng. Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol. Ther.* **48**:381–395 (1990).
3. Santi, D. V., C. S. McHenry, and H. Sommer. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry* **13**:471–481 (1974).
4. Santi, D. V., C. S. McHenry, R. T. Raines, and K. M. Ivanetich. Kinetics and thermodynamics of the interaction of 5-fluoro-2′-deoxyuridylate with thymidylate synthase. *Biochemistry* **26**:8606–8613 (1987).
5. Washtien, W. Thymidylate synthetase levels as a factor in 5-fluorodeoxyuridine and methotrexate cytotoxicity in gastrointestinal tumor cells. *Mol. Pharmacol.* **21**:723–728 (1982).
6. Berger, S. H., C.-H. Jenh, L. F. Johnson, and F. G. Berger. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol. Pharmacol.* **28**:461–467 (1985).
7. Umeda, M., and C. Heidelberger. Comparative studies of fluorinated pyrimidines with various cell lines. *Cancer Res.* **28**:2529–2538 (1968).
8. Grem, J. L., and P. H. Fischer. Alteration of fluorouracil metabolism in human colon cancer cells by dipyridamole with a selective increase in fluorodeoxyuridine monophosphate levels. *Cancer Res.* **46**:6191–6199 (1986).
9. Keyomarsi, K., and R. G. Moran. Mechanism of the cytotoxic synergism of fluoropyrimidines and folinic acid in mouse leukemic cells. *J. Biol. Chem.* **263**:14402–14409 (1988).
10. Davis, S. T., and S. H. Berger. Modulation of 5-fluoro-2′-deoxyuridine response by folinic acid in human colonic tumor cell lines: the role of thymidylate synthase. *Mol. Pharmacol.* **35**:422–427 (1989).
11. Zhang, Z.-G., and Y. M. Rustum. Effects of diastereoisomers of 5-formyltetrahydrofolate on cellular growth, sensitivity to 5-fluoro-2′-deoxyuridine, and methylenetetrahydrofolate polyglutamate levels in HCT-8 cells. *Cancer Res.* **51**:3476–3481 (1991).
12. Berger, S. H., K. W. Barbour, and F. G. Berger. A naturally occurring variation in thymidylate synthase structure is associated with a reduced response to 5-fluoro-2′-deoxyuridine in a human colon tumor cell line. *Mol. Pharmacol.* **34**:480–484 (1988).
13. Houghton, P. J., A. Raman, C. L. Will, B. J. Dolnick, and J. A. Houghton. Mutation(s) of the thymidylate synthase gene of human adenocarcinoma cells causes a thymidylate synthase-negative phenotype that can be attenuated by exogenous folates. *Cancer Res.* **52**:558–565 (1992).
14. Berger, S. H., and F. G. Berger. Thymidylate synthase as a determinant of 5-fluoro-2′-deoxyuridine response in human colonic tumor cell lines. *Mol. Pharmacol.* **34**:474–479 (1988).
15. Barbour, K. W., S. H. Berger, and F. G. Berger. Single amino acid substitution defines a naturally occurring genetic variant of human thymidylate synthase. *Mol. Pharmacol.* **37**:515–518 (1990).
16. Barbour, K. W., D. K. Hoganson, S. H. Berger, and F. G. Berger. A naturally occurring tyrosine to histidine replacement at residue 33 of human thymidylate synthase confers resistance to 5-fluoro-2′-deoxyuridine in mammalian and bacterial cells. *Mol. Pharmacol.* **42**:242–248 (1992).
17. Perry, K. M., E. B. Fauman, J. S. Finer-Moore, W. R. Montfort, G. F. Maley, F. Maley, and R. M. Stroud. Plastic adaptation toward mutations in proteins: structural comparison of thymidylate synthases. *Proteins* **8**:315–333 (1990).
18. Davis, S. T., and S. H. Berger. Variation in human thymidylate synthase is associated with resistance to 5-fluoro-2′-deoxyuridine. *Mol. Pharmacol.* **43**:702–708 (1993).
19. Davisson, V. J., W. Sirawaraporn, and D. V. Santi. Expression of human thymidylate synthase in *Escherichia coli*. *J. Biol. Chem.* **264**:9145–9148 (1989).
20. Ellis, K. G., and J. F. Morrison. Buffers of constant ionic strength for studying pH-dependent processes. *Methods Enzymol.* **87**:405–426 (1982).
21. Danenberg, P. V., and K. D. Danenberg. Effect of 5,10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2′-deoxyuridylate from thymidylate synthetase: evidence for an ordered mechanism. *Biochemistry* **17**:4018–4024 (1978).
22. Lockshin, A., and P. V. Danenberg. Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding to human thymidylate synthase. *Biochem. Pharmacol.* **30**:247–257 (1981).
23. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
24. Bapat, A. R., C. Zarow, and P. V. Danenberg. Human leukemic cells resistant to 5-fluoro-2′-deoxyuridine contain a thymidylate synthetase with lower affinity for nucleotides. *J. Biol. Chem.* **258**:4130–4136 (1983).
25. Dolnick, B. J., and Y.-C. Cheng. Human thymidylate synthetase derived from patients with acute myelocytic leukemia. *J. Biol. Chem.* **25**:7697–7703 (1977).
26. Jastreboff, M. M., B. Kedzierska, and W. Rode. Altered thymidylate synthetase in 5-fluorodeoxyuridine-resistant Ehrlich ascites carcinoma cells. *Biochem. Pharmacol.* **32**:2259–2267 (1983).
27. Hardy, L. W., J. S. Finer-Moore, W. R. Montfort, M. O. Jones, D. V. Santi, and R. M. Stroud. Atomic structure of thymidylate synthase: target for rational drug design. *Science (Washington D. C.)* **235**:448–455 (1987).
28. Matthews, D. A., K. Appelt, S. J. Oatley, and N. H. Xuong. Crystal structure of *Escherichia coli* thymidylate synthase containing bound 5-fluoro-2′-deoxyuridylate and 10-propargyl-5,8-dideazafolate. *J. Mol. Biol.* **214**:923–936 (1990).
29. Montfort, W. R., K. M. Perry, E. B. Fauman, J. S. Finer-Moore, G. F. Maley, L. Hardy, F. Maley, and R. M. Stroud. Structure, multiple site binding, and segmental accomodation in thymidylate synthase on binding dUMP and an anti-folate. *Biochemistry* **29**:6964–6977 (1990).
30. Cha, S. Tight-binding inhibitors. I. Kinetic behavior. *Biochem. Pharmacol.* **24**:2177–2185 (1975).
31. Danenberg, P. V., and A. R. Bapat. Thymidylate synthases isolated from human colon tumors have varied affinity towards 5-fluoro-2′-deoxyuridylate. *Reg. Cancer Treat.* **2**:1–4 (1989).

[6] L. Lebeioda and F. Berger, unpublished observations.

Send reprint requests to: Sondra H. Berger, Department of Basic Pharmaceutical Sciences, University of South Carolina, Columbia, SC 29208.