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Biphasic Binding of 5-Fluoro-2′-deoxyuridylate to Human Thymidylate Synthase

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SUMMARY

Thymidylate synthase (TS) is a homodimeric enzyme that catalyzes the reductive methylation of dUMP by N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolic acid, to form dTMP. Inhibition of TS by the dUMP analog 5-fluoro-dUMP (FdUMP) occurs through the formation of a covalent ternary complex containing the nucleotide analog, N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolic acid, and the enzyme; this complex is termed the inhibitory ternary complex (ITC). In the present report, the kinetics of FdUMP binding into an ITC with purified preparations of human TS were examined. Rapid chemical-quench techniques, as well as steady state binding methods, showed that the enzyme contains two distinct FdUMP binding sites with different affinities for the nucleotide analog. Binding to the first, or high affinity, site was rapid and reached a maximum stoichiometry of 1.0 mol of FdUMP/mol of dimer; binding to the second, or low affinity, site was much slower and reached a stoichiometry of 1.7 mol of FdUMP/mol of dimer. Rate constants for FdUMP binding to and dissociation from the ITC (k_{on} and k_{off}, respectively) were determined, as were equilibrium dissociation constants (K_d). A naturally occurring mutant form of TS, which contains a tyrosine to histidine substitution at residue 33 and renders cells relatively resistant to fluoropyrimidines, exhibited a lower affinity for FdUMP specifically at the second binding site, with little or no change at the first. Hill coefficients were <1.0, with the His-33 enzyme having a significantly lower coefficient than the wild-type enzyme. The results, in total, indicate that the two FdUMP binding sites on the TS dimer are nonequivalent. We suggest that such nonequivalence may be due to negative cooperativity, where nucleotide binding to the first subunit elicits a conformational change that results in reduced affinity for ligand at the second subunit. This negative cooperativity may be stronger for the His-33 mutant. Thus, the relative fluoropyrimidine resistance conferred by the His-33 substitution may be due to enhanced negative cooperative effects on FdUMP binding into the ITC, thereby reducing the effectiveness of the pyrimidine analog as an inhibitor of thymidylate biosynthesis.

TS catalyzes the reductive methylation of dUMP by CH_2H_4PteGlu, to form dTMP and H_2PteGlu. The enzyme, which has been purified from a number of organisms, is composed of two identical subunits, has a total molecular mass of 63–74 kDa, and has a relatively well conserved primary structure (1–3). The crystal structures of TS from *Lactobacillus casei* and *Escherichia coli* indicate that the two subunits are arranged in a back-to-back fashion, with the active site cleft of each extending almost to the subunit interface (4–8). The active site of each subunit is lined by approximately 30 conserved amino acids, two of which are contributed by the other subunit.

Because it catalyzes an indispensable step in the biosynthesis of dTMP, TS is critical in the replication of DNA and has traditionally been an attractive target to which antineoplastic agents, particularly folate and pyrimidine analogs, are directed (9, 10). One such agent, 5-fluorouracil, is metabolized in the cell to FdUMP, which acts as a mechanism-based inhibitor of TS by forming a stable covalent complex with CH_2H_4PteGlu and the enzyme; this complex is referred to as the ITC. X-ray structural studies of the ITC (5) indicate that FdUMP binds at the base of the active site cleft of the enzyme; the pyrimidine and ribose moieties of the nucleotide analog provide a docking surface for binding of the folate cosubstrate. The ITC is held together by two covalent linkages, one between the methylene group of CH_2H_4PteGlu and C5 of FdUMP and the other between a cysteine sulfhydryl group of the enzyme and C6 of FdUMP. The carboxyl-terminal region of TS, which is disordered in the ligand-free en-

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ABBREVIATIONS: TS, thymidylate synthase; CH_2H_4PteGlu, N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolic acid; H_2PteGlu, 7,8-dihydrofolic acid; FdUMP, 5-fluoro-dUMP; FdUrd, 5-fluoro-2′-deoxyuridine; ITC, inhibitory ternary complex; MES, 2-(*N*-morpholino)ethanesulfonic acid; BSA, bovine serum albumin.

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zyme and extends into the solvent, closes down over the active site upon the binding of ligands, effectively sequestering them from the bulk solvent. The large conformational changes associated with ligand binding are reflected in a 3.5% reduction in the Stokes radius of the enzyme (11).

Studies of variations in fluoropyrimidine response among human colonic tumor cell lines have led to the identification of a naturally occurring mutant TS form that is associated with relative resistance to FdUrd (12, 13). The altered form, which contains a histidine residue in place of tyrosine at position 33 (14), confers a 3–4-fold level of resistance to FdUrd in both mammalian and bacterial cells (15). Recent analysis of the wild-type and mutant enzymes purified directly from human colonic tumor cells showed that the mutant TS has a specific activity that is 10-fold lower than that of the wild-type enzyme, whereas the Michaelis constants for both dUMP and CH_2H_4PteGlu are only slightly changed (16). Analysis of ITC formation revealed that the K_d for FdUMP binding into an ITC with the His-33 form is 4-fold higher than that with the wild-type (i.e., Tyr-33) form (16). Introduction of a phenylalanine residue at position 33 does not alter the properties of TS (16), indicating that the functional effects of the His-33 substitution are due to the presence of the imidazole ring of histidine, rather than to the absence of the ζ-hydroxyl of tyrosine.

In the present report, we describe kinetic analysis of FdUMP binding into an ITC with the purified Tyr-33, His-33, and Phe-33 forms of human TS. We provide evidence indicating that FdUMP binds the two subunits of TS in a nonequivalent fashion. This may be due to negative cooperativity of nucleotide binding, which is enhanced in the His-33 enzyme, compared with the Tyr-33 or Phe-33 form. Such enhancement may explain the FdUrd resistance of cells expressing the His-33 form, implicating a role for residue 33 in the conformational changes underlying the putative cooperative effects of ligand binding to TS.

Experimental Procedures

Chemicals. [6-^{3}H]FdUMP (18 Ci/mmol) and [5-^{3}H]dUMP (15 Ci/mmol) were purchased from Moravek Biochemicals. Folic acid, FdUMP, dUMP, BSA, Triton X-100, glycerol, and 2-mercaptoethanol were obtained from Sigma. Tris-OH, MES, and dithiothreitol were products of Research Organics. CH_2H_4PteGlu was prepared from 5,6,7,8-tetrahydrofolic acid, as described elsewhere.[2]

Bacteria. Strain χ2913 of *E. coli*, which carries plasmid pDHTS-S1 expressing the Tyr-33 form of human TS (17), was obtained from Dr. D. Santi (Dept. of Biochemistry, University of California, San Francisco, CA). Derivatives of pDHTS-S1 encoding the His-33 and Phe-33 forms were produced by site-directed mutagenesis and have been described previously (15, 16). Bacteria were maintained in standard L-broth, and were cultured at 37° in a shaking incubator (15, 16).

Enzyme purification. An overnight culture (10 ml) of the appropriate bacterial strain was inoculated into 1 liter of L-broth and grown at 37° for 36–48 hr. The cells were harvested by centrifugation at 3000 × *g* for 30 min and washed with phosphate-buffered saline. Cells (3–5 g) were placed in a sonication vessel containing 100–150 ml of Blue A buffer (50 mM Tris-OH, pH 7.5, 0.2% 2-mercaptoethanol, 10 mM dithiothreitol) and were sonicated on ice (five cycles, 5 min/cycle) using a Branson Sonifier 450 (power level, 6; duty cycle, 60%). Cell debris was removed by centrifugation for 30 min at 10,000 × *g* at 4°, and the cell-free extract was loaded onto a column of Cibacron Blue-Sepharose (total column volume, 60 ml) that had been equilibrated in Blue A buffer. The column was eluted with a 0–1 M KCl gradient; fractions containing TS, which eluted at about 1 M KCl, were pooled, brought to 250 μM dUMP, and loaded onto a methotrexate-Sepharose affinity column (18) equilibrated in buffer containing 50 mM Tris-OH, pH 7.5, 250 μM dUMP, and 0.2% 2-mercaptoethanol (total column volume, 10 ml). The column was washed thoroughly with 50 mM Tris-OH, pH 7.5, 1 M KCl, 250 μM dUMP, 0.2% 2-mercaptoethanol, and TS was eluted with dUMP-free buffer; fractions containing enzyme activity were pooled, diluted with an equal volume of glycerol, and stored in 1-ml aliquots at −20°. All buffers for enzyme purification were adjusted to the appropriate pH at 4°, filtered through 0.45-μm Versapor acrylic copolymer filters (Gelman), degassed with helium for approximately 45 min, and used immediately after addition of reducing agent. Protein concentrations were determined by the method of Bradford (19).

Enzyme activity measurements. TS activity was assayed spectrophotometrically by measuring H_2PteGlu production during conversion of dUMP to dTMP. Reaction mixtures (1.0 ml) contained enzyme, 100 μM dUMP, and 100 μM CH_2H_4PteGlu in Morrison buffer (20), which consists of 120 mM Tris, 60 mM MES, and 60 mM acetic acid, pH 7.5; mixtures were incubated at 25°, and absorbance at 340 nm due to the production of H_2PteGlu was measured. One unit of activity is defined as an amount of enzyme necessary to catalyze production of 1 μmol of H_2PteGlu/min; the extinction coefficient for H_2PteGlu at 340 nm was taken to be 6400 cm^{-1} M^{-1} (21).

TS activity was also measured by the tritium exchange assay, which monitors the production of [^{3}H]water from [5-^{3}H]dUMP (22). Reaction mixtures (0.3 ml) contained the enzyme, 100 μM dUMP (2 Ci/mmol), 200 μM CH_2H_4PteGlu, 500 μg/ml BSA, and 0.1% Triton X-100 in Morrison buffer, pH 7.5; mixtures were incubated at 37°. [^{3}H]Water was measured after charcoal extraction (22); 1 unit of activity is an amount of enzyme required to catalyze production of 1 μmol of [^{3}H]water/min.

Nucleotide binding assays and kinetic analyses. ITC formation was quantitated by trichloroacetic acid precipitation of complexes made in the presence of [6-^{3}H]FdUMP (22). Reaction mixtures (0.5 ml) contained enzyme, 100 μM [6-^{3}H]FdUMP (2–18 Ci/mmol), 100 μM CH_2H_4PteGlu, 500 μg/ml BSA, and 0.1% Triton X-100 in Morrison buffer, pH 7.5. The mixtures were incubated at 7°; ITCs were precipitated and washed in 5% trichloroacetic acid, and radioactivity in the pellets was quantitated by scintillation counting.

Kinetic analysis of FdUMP binding into the ITC was based upon the following ordered sequential scheme (23, 24):

$$\mathrm{TS + FdUMP} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{TS\text{-}FdUMP} \underset{k_4}{\overset{k_3\,\mathrm{CH_2H_4PteGlu}}{\rightleftarrows}} \mathrm{ITC}$$

In this scheme, TS-FdUMP represents the binary complex between the enzyme and FdUMP; k_1, k_2, k_3, and k_4 are individual rate constants. FdUMP binding into the ITC can be described by a rate constant k_{on}, which is defined by the following equation:

$$k_{on} = \frac{k_1 k_3[\mathrm{CH_2H_4PteGlu}]}{k_2 + k_3\,[\mathrm{CH_2H_4PteGlu}]} \quad (1)$$

The k_{on} value is an apparent rate constant, because ITC formation is not strictly a bimolecular reaction (24). Under conditions where the CH_2H_4PteGlu concentration is in excess, it is the case that $k_3[CH_2H_4PteGlu] \gg k_2$, so that k_{on} approximates k_1 (24).

The k_{on} values were determined by measuring the initial rate of [^{3}H]FdUMP incorporation into the ITC in the presence of excess

[2] W. Deng, R. J. Cisneros, R. T. Reilly, W. C. Cooper, L. F. Johnson, and R. B. Dunlap. The mechanism of covalent inhibitory ternary complex formation between recombinant mouse thymidylate synthase and 5-fluoro-2′-deoxyuridylic acid, manuscript in preparation.

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$CH_2H_4PteGlu$. Under these conditions, k_{on} is calculated from the following second-order equation (24):

$$\frac{1}{[E_0] - [FdUMP_0]} \ln \frac{[FdUMP_0]([E_0] - [X])}{[E_0]([FdUMP_0] - [X])} = k_{on}t \qquad (2)$$

where $[E_0]$ is the initial concentration of enzyme binding sites, $[FdUMP_0]$ is the initial FdUMP concentration, and [X] is the concentration of ITC at any time t. The slope of a straight line plot of eq. 2 represents k_{on}.

Reaction mixtures for determination of k_{on} contained 0.21 μM purified TS and various concentrations of [6-^{3}H]FdUMP (0.21–110 μM); they were incubated at 7° for various times (25 msec to 2 hr) and quenched with trichloroacetic acid. For incubation times of <10 sec, quenching was performed in a PQ-53 preparative quencher (Hi-Tech Scientific); for incubation times of >10 sec, quenching was done manually. After addition of the trichloroacetic acid, ^{3}H-labeled complexes were precipitated and washed, and radioactivity was measured by scintillation counting.

Equilibrium dissociation constants (K_d values) for FdUMP binding to TS were calculated from measurements of nucleotide binding under steady state conditions. Reaction mixtures, containing 2 nM purified TS and various concentrations of [6-^{3}H]FdUMP (0.1 nM to 25 μM), were incubated for 6 hr at 7°; ^{3}H-labeled complexes were precipitated and washed with trichloroacetic acid, and radioactivity in the pellets was measured by scintillation counting. The rate constant for FdUMP dissociation from the ITC, k_{off}, was calculated using the equation $K_d = k_{off}/k_{on}$ (16, 24).

Results

Purification of human TS from recombinant *E. coli*. The Tyr-33, His-33, and Phe-33 forms of human TS were purified from recombinant *E. coli* carrying and expressing human TS cDNA plasmids. The normal, or Tyr-33, form of human TS is expressed in *E. coli* χ2913 cells containing plasmid pDHTS-S1 (17); derivatives of pDHTS-S1 expressing the His-33 and Phe-33 forms were generated by site-directed mutagenesis (15, 16). TS levels in the bacteria were found to increase markedly as cells entered the stationary phase and were maximal approximately 24–36 hr after the initial inoculation (data not shown). As measured by [^{3}H]FdUMP binding, extracts of stationary-phase cells typically contained 100–500 pmol of TS/mg of soluble protein, which represents about 0.8–4% of total protein in the extracts.

Purification was accomplished using a two-step protocol that involved Cibacron Blue chromatography followed by methotrexate affinity chromatography (see Experimental Procedures for details), a procedure that could be completed in <9 hr. A typical purification profile is presented for each TS form in Table 1. The purity of the enzyme preparations was estimated by [^{3}H]FdUMP binding activities, which were found to be 22,000–23,000 pmol of nucleotide/mg of protein for each enzyme (Table 1). This is the value expected from the known molecular mass of the human TS dimer (72 kDa) and the fact that the maximum stoichiometry for FdUMP binding is 1.7 mol of nucleotide/mol of dimer (25). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that each TS preparation was at least 95% pure (data not shown).

The specific catalytic activities of the purified TS forms, as assayed by H_2PteGlu formation (see Experimental Procedures), were 1.2 units/mg, 0.093 units/mg, and 1.2 units/mg for the Tyr-33, His-33, and Phe-33 enzymes, respectively. The k_{cat} values, calculated from the specific activities, were 1.5 sec^{-1}, 0.12 sec^{-1}, and 1.5 sec^{-1}, respectively. A low k_{cat} for the His-33 enzyme was noted in previous studies (15, 16) and reflects an intrinsic property of this TS form, rather than an artifact of the purification process. The Michaelis constants for dUMP, as determined by the tritium exchange assay, were 1.0 μM for the Tyr-33 and Phe-33 forms and 2.1 μM for the His-33 form. The Tyr-33 and Phe-33 enzymes could be stored in 50% glycerol at −20° for several months without loss of activity, whereas the His-33 form retained full activity for only about 2–3 weeks under these conditions. Studies aimed at establishing conditions for long term storage of the enzymes are ongoing.

Biphasic binding of FdUMP into the ITC. The three purified TS forms were compared with respect to the kinetics of nucleotide binding into the ITC by using rapid chemical-quench techniques, as outlined in Experimental Procedures. ITC formation was measured as a function of time at various concentrations of [^{3}H]FdUMP. Data for the Tyr-33 enzyme, shown in Fig. 1, indicated that the enzyme binds FdUMP in a biphasic manner. A rapid phase, reaching a maximum binding stoichiometry of 1.0 mol of nucleotide/mol of TS dimer, occurred within 4–6 sec at the highest FdUMP concentrations (Fig. 1A). This was followed by a second, much slower, phase that took place over about 90 min and reached a maximum stoichiometry of 1.7 mol of nucleotide/mol of TS dimer (Fig. 1B). Similar biphasic kinetics were obtained with the purified Phe-33 and His-33 enzyme forms (data not shown). Thus, purified TS exhibits two distinct FdUMP binding sites that form ITCs at very different rates. A similar phenomenon has been observed for the purified mouse TS.[2]

TABLE 1
Purification profiles for the Tyr-33, Phe-33, and His-33 forms of TS

Fraction	Volume	Protein	Binding units	Specific binding	Recovery	Purification
	ml	*mg*	*nmol of FdUMP*	*pmol/mg*	*%*	*fold*
Tyr-33						
Crude extract	100	350	360	1,000	100	1
Blue-Sepharose	38	28	240	8,600	67	8.6
Methotrexate-Sepharose	40	8.3	180	22,000	50	22
Phe-33						
Crude extract	110	300	190	630	100	1
Blue-Sepharose	82	22	125	8,500	66	14
Methotrexate-Sepharose	18	4.1	95	23,000	50	36
His-33						
Crude extract	110	260	260	1,000	100	1
Blue-Sepharose	62	20	170	8,500	65	8.5
Methotrexate-Sepharose	18	5.1	110	22,000	42	22

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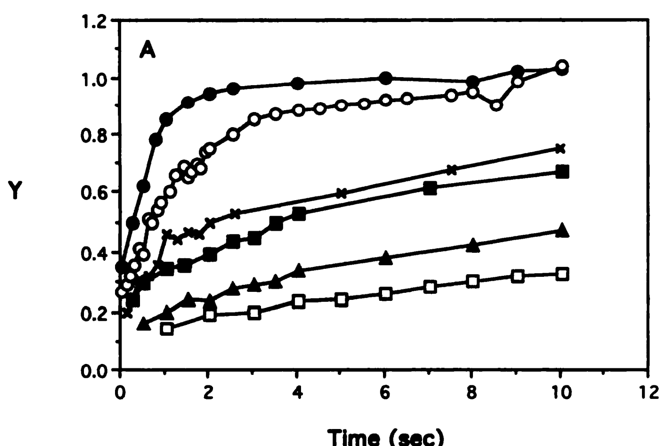


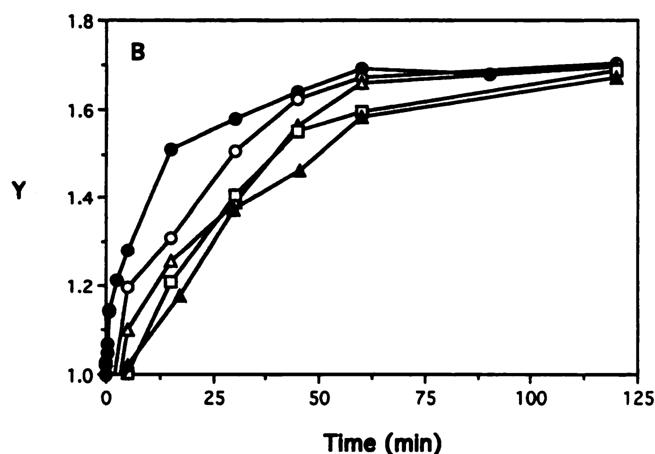
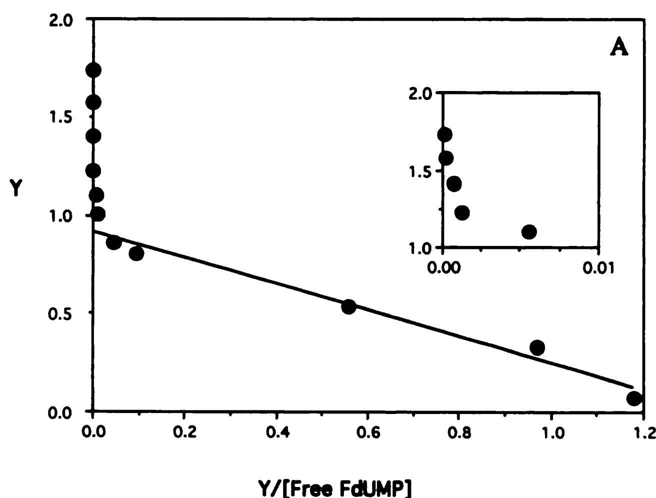


Fig. 1. Kinetics of FdUMP binding into the ITC. Reaction mixtures containing the purified Tyr-33 form of TS, $CH_2H_4PteGlu$, and a range of [6-^{3}H]FdUMP concentrations were incubated for various times at 7°. ITC formation was measured by trichloroacetic acid precipitation (see Experimental Procedures for details). *Y* is the binding ratio, i.e., the ratio of ITC to total enzyme. A, Complex formation as a function of time for the first site; [6-^{3}H]FdUMP concentrations were 210 nM (□), 420 nM (▲), 1.1 μM (■), 2.1 μM (×), 4.2 μM (○), and 42 μM (●). B, Complex formation as a function of time for the second site; [6-^{3}H]FdUMP concentrations were 5.3 μM (▲), 11 μM (□), 21 μM (△), 32 μM (○), and 42 μM (●).

Determination of the k_{on} for FdUMP association into the ITC. The k_{on} was determined at each of several FdUMP concentrations for both binding sites on each of the three TS enzyme forms. The Tyr-33 and Phe-33 enzymes were very similar at both ligand binding sites. At the first (high affinity) site, the k_{on} values were 1.44×10^6 M^{-1} min^{-1} and 1.34×10^6 M^{-1} min^{-1} for the Tyr-33 and Phe-33 enzymes, respectively; at the second (low affinity) binding site, values were 1.59×10^3 M^{-1} min^{-1} and 1.40×10^3 M^{-1} min^{-1}, representing about a 1000-fold reduction, relative to the first site. The His-33 mutant was similar to the other two forms in displaying a biphasic FdUMP binding pattern; however, the k_{on} values (1.90×10^7 M^{-1} min^{-1} for the first site and 2.44×10^4 M^{-1} min^{-1} for the second) were increased by about 13–17-fold.

Thus, human TS contains two distinct FdUMP binding sites that differ in k_{on}. Furthermore, the Tyr-33 to His-33 substitution causes a significant increase in the rate constant for FdUMP binding at each site. This was a rather unexpected finding, because earlier studies had shown that the His-33 form has a reduced affinity for FdUMP (13, 15, 16). The fact that the Phe-33 mutant is essentially indistinguishable from the Tyr-33 enzyme makes it unlikely that the functional effects of the His-33 substitution are a consequence of the absence of the ζ-hydroxyl group of tyrosine; rather, as suggested earlier (15, 16), the effects must be due to the presence of the imidazole ring of histidine.

Determination of the K_d for FdUMP binding. The K_d for the interaction between FdUMP and TS was measured for both the Tyr-33 and His-33 forms under steady state conditions. Scatchard plots of the data were nonlinear for both enzymes, indicating that the two FdUMP binding sites on the TS dimer have different affinities for the nucleotide analog (Fig. 2). The first, or high affinity, site was described by a linear segment of the plot that extrapolated to the ordinate at a point corresponding to a maximum binding ratio of 1



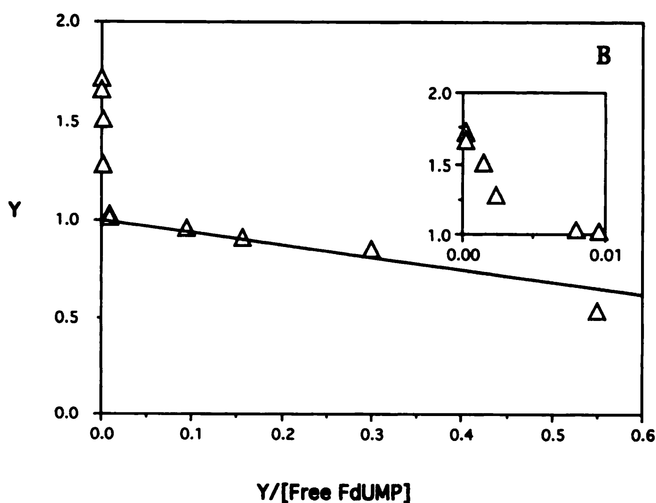


Fig. 2. Scatchard plots of FdUMP binding into the ITC. Samples containing purified TS forms and various concentrations of [6-^{3}H]FdUMP were incubated for 6 hr at 7°. ITC formation was measured by trichloroacetic acid precipitation, as described in Experimental Procedures. *Y* is the binding ratio, i.e., the ratio of ITC to total enzyme. *Insets*, expansion of portions of the curves corresponding to low *Y*/free FdUMP concentration values. A, Tyr-33 form; B, His-33 form.

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FdUMP molecule/TS dimer. The slope of this segment, representing the K_d for the high affinity site, was 0.64 nM for the Tyr-33 enzyme and 0.91 nM for the His-33 form. The second, or low affinity, site, which reached a maximum stoichiometry of 1.7 FdUMP molecules/dimer, was described by the nonlinear portion of the plot. To calculate the K_d for this site, we used the method of Conway and Koshland (26), which assumes that the K_d for the second site is significantly larger than that for the first and is based upon the following equation:

$$\frac{[\mathrm{FdUMP}_b] - [\mathrm{E}_t]}{[\mathrm{E}_t]} = 1 - K_d \frac{[\mathrm{FdUMP}_b] - [\mathrm{E}_t]}{[\mathrm{E}_t][\mathrm{FdUMP}_f]} \quad (3)$$

In this equation, $[\mathrm{FdUMP}_b]$ is the concentration of bound FdUMP, $[\mathrm{FdUMP}_f]$ is the concentration of free FdUMP, and $[\mathrm{E}_t]$ is the total enzyme concentration (26). A plot of eq. 3 results in a straight line with slope $-K_d$ (26). Fig. 3 shows the results of such a plot in the range of 1–1.7 molecules of FdUMP/TS dimer and indicates K_d values of 0.46 μM and 1.5 μM for the Tyr-33 and His-33 enzymes, respectively.

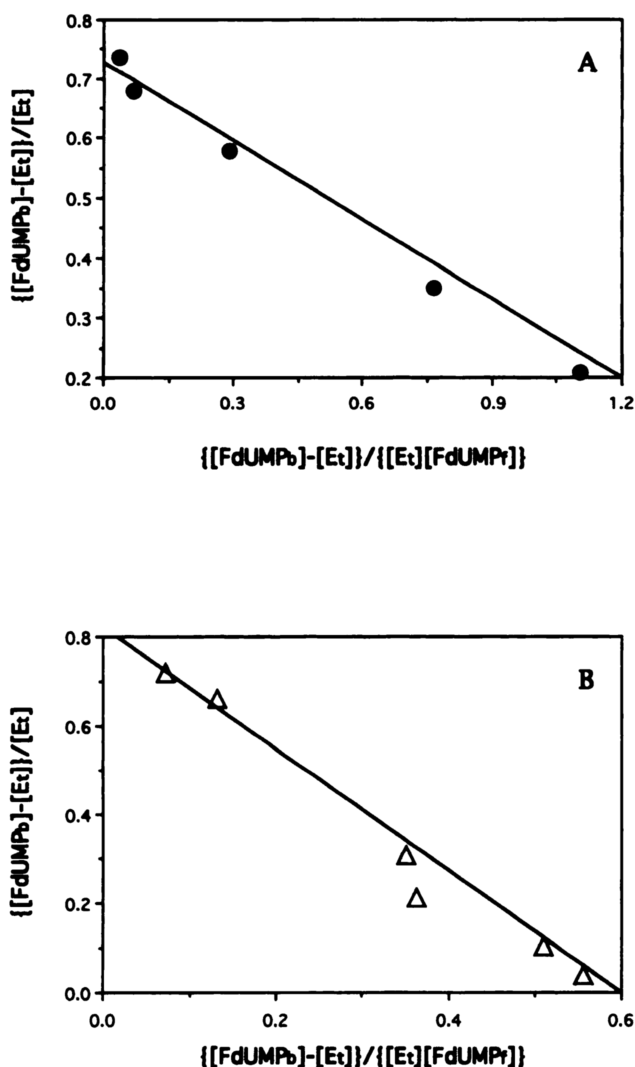

Fig. 3. Determination of K_d values for the second FdUMP binding sites. The nonlinear portions of the Scatchard plots in Fig. 2 (i.e., the segments corresponding to the second binding site, representing binding ratios between 1.0 and 1.7 molecules of FdUMP/molecule of TS dimer) were replotted according to the scheme of Conway and Koshland (26) (see text for details). A, Tyr-33 form; B, His-33 form. Abbreviations are defined in the text.

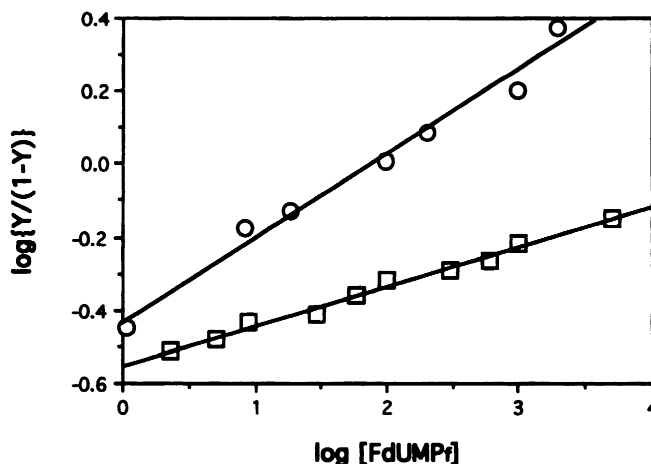

Fig. 4. Hill plots for FdUMP binding into the ITC. The data in Fig. 2 were used to construct Hill plots for the Tyr-33 (○) and His-33 (□) forms. *Y* is the binding ratio, i.e., the ratio of ITC to total enzyme; $[\mathrm{FdUMP}_f]$ is the concentration of free, or unbound, FdUMP.

Thus, the K_d difference between the two sites on the TS dimer is about 700-fold for the Tyr-33 enzyme and about 1600-fold for the His-33 enzyme. The two enzyme forms showed little, if any, difference at the high affinity site (0.64 nM versus 0.91 nM) but were about 3-fold different at the second, or low affinity, site (0.46 μM versus 1.5 μM).

Hill plots generated from the binding data are shown in Fig. 4. Hill coefficients, calculated from the slopes of the resulting straight lines, were 0.24 and 0.10 for the Tyr-33 and His-33 enzymes, respectively (Fig. 4). The fact that these coefficients are <1.0 is consistent with negative cooperativity (26, 27), which appears to be more pronounced for the His-33 enzyme, because its coefficient was significantly lower than that for the Tyr-33 form.

Estimation of k_{off}. Rates of FdUMP dissociation from the ITC, described by k_{off}, were calculated from the relationship $K_d = k_{off}/k_{on}$ (16, 24). As summarized in Table 2, the Tyr-33 enzyme had k_{off} values of 9.22×10^{-4} min^{-1} at the first site and 7.47×10^{-4} min^{-1} at the second; the His-33 enzyme exhibited k_{off} values of 1.73×10^{-2} min^{-1} at the first site and 3.66×10^{-2} min^{-1} at the second. Thus, whereas k_{off} values were much higher for the His-33 enzyme, relative to the Tyr-33 enzyme, at both binding sites, the difference between the two enzymes was more pronounced at the second site.

The k_{off} can be directly determined by measuring FdUMP exchange from the ITC (16, 24). Unfortunately, the purified TS preparations were not stable over a sufficiently long period of time to allow such measurements.[3]

Discussion

In the present study, we have examined the kinetics of FdUMP binding to highly purified preparations of both wild-type and mutant forms of human TS. The purification scheme described here required only two steps and was accomplished in <9 hr, with an overall recovery of 50%; this represents a significant improvement over previously pub-

[3] R. T. Reilly, unpublished observations.

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TABLE 2

Kinetic analysis of FdUMP binding into the ITC

The k_{on} values were determined at several FdUMP concentrations, using the rapid chemical-quench data shown in Fig. 1. The K_d values were determined from the Scatchard plots depicted in Fig. 2. The k_{off} values were calculated from the relationship $K_d = k_{off}/k_{on}$ (16, 24).

	Tyr-33	Phe-33	His-33
First site			
k_{on} (M^{-1} min^{-1}	1.44×10^6	1.34×10^6	1.90×10^7
k_{off} (min^{-1})	9.22×10^{-4}	ND[a]	1.73×10^{-2}
K_d (M)	6.4×10^{-10}	ND	9.1×10^{-10}
Second site			
k_{on} (M^{-1} min^{-1})	1.59×10^3	1.40×10^3	2.44×10^4
k_{off} (min^{-1})	7.47×10^{-4}	ND	3.66×10^{-2}
K_d (M)	4.7×10^{-7}	ND	1.5×10^{-6}

[a] ND, not determined.

lished protocols (16–18, 28–30). Several criteria indicated that the enzymes were pure and fully active. First, the FdUMP binding activities of the preparations were 22,000–23,000 pmol of nucleotide/mg of protein; this is exactly the activity expected for a dimer that has a molecular weight of 72,000 and that binds 1.7 molecules of FdUMP/dimer (25). Second, the specific activities (1.2 μmol of product/min/mg of protein) and k_{cat} values (1.5 sec^{-1}) for the purified Tyr-33 and Phe-33 enzymes were equal to or higher than those reported previously (16–18, 28–30). The His-33 mutant form had a lower specific activity and k_{cat}, reflecting an intrinsic property of this form, rather than an artifact of the purification process (15, 16). Third, the K_m values for dUMP were consistent with published values (16, 17, 28). Finally, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the enzymes were indeed pure (data not shown).

Three kinetic parameters describing the interaction between FdUMP and the enzyme were measured for each of the purified TS preparations. The rate constant k_{on}, which describes FdUMP association into the ITC, was determined by rapid chemical-quench methods. The equilibrium dissociation constant K_d was calculated from steady state binding measurements and Scatchard analyses. Finally, the rate constant k_{off}, describing dissociation of FdUMP from the ITC, was calculated from the relationship $K_d = k_{off}/k_{on}$ (16, 24). The results clearly showed that FdUMP binding is biphasic, indicating the presence of nonequivalent binding sites for the nucleotide analog. The first binding phase occurred rapidly and reached a maximum stoichiometry of 1.0 FdUMP molecule/dimer; the second, which was much slower, reached a stoichiometry of 1.7 FdUMP molecules/dimer (Fig. 1). The K_d was much lower at the first binding site than at the second, primarily reflecting a large difference in k_{on} (i.e., about 1000-fold) between the two sites (Table 2).

The His-33 enzyme, like the Tyr-33 and Phe-33 forms, exhibited biphasic binding kinetics; however, its rate constants were significantly different from those of the other two. The k_{on} was about 13–15-fold higher for the His-33 form at both the high and low affinity sites (Table 2). The k_{off} was increased by about 19-fold for the first site and by about 49-fold for the second site (Table 2). These differences in rate constants between the Tyr-33 and His-33 forms result in similar K_d values at the first site (i.e., 0.64 nM versus 0.91 nM) but very distinct K_d values at the second (i.e., 0.47 μM versus 1.5 μM). The fact that the Tyr-33 and Phe-33 enzymes had similar k_{on} values indicates that the functional changes exhibited by the His-33 enzyme are not explained by loss of the hydrogen bond between the hydroxyl group of Tyr-33 and the main-chain keto group of Met-216. This is consistent with earlier evidence suggesting that substitution of phenylalanine at residue 33 does not detectably alter enzyme function (16).

Several explanations for the nonequivalence of FdUMP binding sites on the TS dimer are possible. Some sort of enzyme heterogeniety may be present in the purified TS preparations. We consider this to be unlikely. FdUMP binding at the high affinity site reached a maximum stoichiometry of 1 FdUMP molecule/dimer, as measured either by rapid kinetic means (Fig. 1) or by steady state analysis (Fig. 2). This was the case for a number of preparations of each of the three TS forms. To postulate enzyme heterogeniety as responsible for the nonequivalence of FdUMP binding sites would require that the purified TS preparations contain one species that is consistently 59% of the total (1.0/1.7, the ratio of maximal binding stoichiometries at the two sites). It seems unlikely that this would invariably be the case for multiple preparations of each of the three TS forms.

A second possibility is that the two FdUMP binding sites have intrinsically different conformations and bind FdUMP with distinct kinetics. This cannot be formally excluded at the present time. Although X-ray studies of the *E. coli* and *L. casei* enzymes seem to suggest that the ligand-free TS dimer is symmetrical (4–8), it is possible that subtle conformational differences between the two active sites have not been detected.

A third explanation, and one we consider most likely, is negative cooperativity, where the binding of FdUMP into the ITC at one subunit (the high affinity site) elicits a conformational change that lowers the affinity for nucleotide at the other subunit (the low affinity site). The fact that Hill coefficients calculated from the steady state data were <1 (Fig. 4) is consistent with negative cooperativity. Interestingly, such negative cooperativity may be enhanced for the His-33 enzyme, as indicated by the observation that the K_d difference between the high and low affinity sites was about 1600-fold for the His-33 form but 700-fold for the Tyr-33 form (Table 2). In addition, the Hill coefficient was significantly lower for the His-33 form, compared with the Tyr-33 form (Fig. 4), consistent with a stronger negative cooperativity for the former. This may explain the 3–4-fold level of FdUrd resistance in cells expressing the His-33 form of the enzyme (15).

Nonequivalent ligand binding sites on TS have been noted previously. Heterogeneous binding sites for dUMP (31, 32) and FdUMP (33) have been observed for the *L. casei* enzyme. Recently, Dev *et al.* (34) postulated negative cooperativity in the binding of folate analogs to human TS. Finally, on the basis of experiments that are similar to those reported here, Deng *et al.*[2] invoked subunit nonequivalence to explain the kinetics of FdUMP and CH_2H_4PteGlu binding to purified preparations of mouse TS.

Not all workers have observed subunit heterogeniety for the TS molecule (for example, see Refs. 16, 24, and 35). Possible explanations for the discrepancies may relate to the specific methods used in the analyses. For example, Santi *et al.* (35) conducted their studies under conditions where FdUMP maximally binds 1 site/dimer; thus, no heterogeniety was observed. The kinetic studies of Hughey *et al.* (16) and

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Lockshin and Danenberg (24) were conducted at 25° and 37°, respectively, conditions where the K_d difference between the two FdUMP binding sites is significantly decreased.[4] Furthermore, both Hughey *et al.* (16) and Lockshin and Danenberg (24) measured ITC formation by charcoal extraction, rather than by trichloroacetic acid precipitation; charcoal extraction does not distinguish between covalent and noncovalent complexes, so that binding measurements using this method may be very different from those using trichloroacetic acid precipitation.

The fact that the Tyr-33 to His-33 substitution caused an amplification of the differences between the two subunits implies that residue 33 must play a role in the conformations of the two ligand binding sites on the TS dimer. Tyr-33 is invariant among the TS molecules sequenced to date and is located within an amphipathic α-helix (termed helix A) that is near the active site cavity (4, 36). The ζ-hydroxyl group of Tyr-33 forms a hydrogen bond with the main-chain carbonyl oxygen of Met-216, at the base of another α-helix (termed helix J) that comprises one wall of the active site cavity.[5] The properties of the Phe-33 enzyme indicate that this hydrogen bond is not required either for enzyme activity or for negative cooperativity. However, perturbation of the relationship between helices A and J, such as that caused by the His-33 substitution, does have an impact upon the subunit interactions that are responsible for negative cooperativity.

EPR studies have led Carreras *et al.* (37) to suggest that ligand-free TS exists in an equilibrium between "open" and "closed" conformational states. In the open state, the carboxyl terminus is freely moving and the active site is accessible to ligands; in the closed state, the carboxyl terminus is tightly positioned over the active site in such a way as to prevent ligand binding. The addition of ligands causes a shift in the equilibrium toward the closed conformation (37). These findings are in agreement with X-ray crystallographic analyses, which indicate that binding of ligands induces the carboxyl-terminal region of the TS polypeptide to close down over the active site and sequester the bound ligands from the solvent (4–8). It is interesting to speculate that the two subunits of the ligand-free TS dimer are continually alternating between the two conformations. At any given instant, one subunit may be in the open conformation, while the other is closed; averaged over time, the two subunits are essentially equivalent. It is possible that, upon the binding of FdUMP and CH_2H_4PteGlu to the open site and formation of an ITC at that site, contraction of the carboxyl terminus around the bound ligands changes the conformation of the active site on the second subunit. This would allow the binding of ligands at the second site, albeit with reduced affinity. The constraints upon complex formation in the second subunit that may be brought about by ligand binding at the first may lead to the observed negative cooperativity.

The binding of dUMP, the normal substrate for TS, has not been examined in the present report. However, in light of previous studies (31–33), it is reasonable to expect that, like FdUMP, it interacts with the active sites of the enzyme in a negatively cooperative fashion. It is interesting to speculate on the role of the proposed negative cooperativity in control of TS activity and regulation of intracellular dUMP and dTMP levels. Several studies have indicated that abnormal dUMP/dTMP ratios can be detrimental to cells. In yeast, high levels of dTMP are mutagenic, whereas low levels are recombinogenic (38). High dUMP/dTMP ratios lead to DNA strand breaks, due to misincorporation of dUTP into DNA (39). It is essential, therefore, that substrate flow through TS be regulated. Control of TS activity by product inhibition (40–42) is one mechanism for regulation. Negative cooperativity provides another. At low dUMP levels, the availability of the high affinity site on the TS molecule would promote the most efficient substrate flow through the enzyme, allowing maintenance of basal dTTP levels and preventing excessive dUTP accumulation. With rising dUMP levels, negative cooperativity would inhibit catalysis at the lower affinity site, thereby attenuating the accumulation of dTTP and depletion of dUTP. Thus, negative cooperativity allows the cell to effectively maintain proper dUTP/dTTP ratios.

[4] R. T. Reilly, unpublished observations.

[5] R. Stroud, personal communication.

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