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Human thymidylate synthase with loop 181–197 stabilized in an inactive conformation: Ligand interactions, phosphorylation, and inhibition profiles

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Abstract: Thymidylate synthase (TS) is a well-validated cancer target that undergoes conformational switching between active and inactive states. Two mutant human TS (hTS) proteins are predicted from crystal structures to be stabilized in an inactive conformation to differing extents, with M190K populating the inactive conformation to a greater extent than A191K. Studies of intrinsic fluorescence and circular dichroism revealed that the structures of the mutants differ from those of hTS. Inclusion of the substrate dUMP was without effect on M190K but induced structural changes in A191K that are unique, relative to hTS. The effect of strong stabilization in an inactive conformation on protein phosphorylation by casein kinase 2 (CK2) was investigated. M190K was highly phosphorylated by CK2 relative to an active-stabilized mutant, R163K hTS. dUMP had no detectable effect on phosphorylation of M190K; however, dUMP inhibited phosphorylation of hTS and R163K. Studies of temperature dependence of catalysis revealed that the E_{act} and temperature optimum are higher for A191K than hTS. The potency of the active-site inhibitor, raltitrexed, was lower for A191K than hTS. The response of A191K to the allosteric inhibitor, propylene diphosphonate (PDPA) was concentration dependent. Mixed inhibition was observed at low concentrations; at higher concentrations, A191K exhibited nonhyperbolic behavior with respect to dUMP and inhibition of catalysis was reversed by substrate saturation. In summary, inactive-stabilized mutants differ from hTS in thermal stability and response to substrates and PDPA. Importantly, phosphorylation of hTS by CK2 is selective for the inactive conformation, providing the first indication of physiological relevance for conformational switching.

Keywords: thymidylate synthase; energy of activation; conformational plasticity; protein phosphorylation; casein kinase 2; circular dichroism; allosteric inhibition

Abbreviations: $K_{m,app}$; apparent K_m, CD, circular dichroism; CK2, casein kinase 2; E_{act}, energy of activation; FdUMP, 5-fluoro-2′-deoxyuridylate; hTS, human thymidylate synthase; mTHF, 5,10-methylenetetrahydrofolic acid; RTX, raltitrexed or ZD1694, N-(5-[N-(3,4-dihydro-2-methyl-4-oxyquinazolin-6-ylmethyl)-N-methyl-amino]-2-thenoyl)-l-glutamic acid; PDPA, propylene diphosphonate.

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Introduction

Thymidylate synthase (TS) catalyses the reaction that forms dTMP from dUMP, using 5,10-methylenetetrahydrofolate (mTHF) as a cosubstrate. Analogs of the nucleotide and folate substrates are used as antimetabolites in the therapy of cancer due to the requirement for dTMP in DNA synthesis and repair.[1,2] TS is highly conserved among prokaryotes and eukaryotes; however, an N-terminal extension is species specific and two insertions at positions 117 (12 residues) and 146 (8 residues) occur in eukaryotes only.[3] High-resolution structures provided evidence for the existence of the native human enzyme in an inactive conformation based on the position of loop 181–197 containing Cys195, the nucleophile involved in catalysis.[4–6] In this conformation, loop 181–197 is rotated approximately 180°, positioning Cys195 approximately 10 Å from the active site.[4] In crystal structures of hTS complexes with dUMP and the folate analog, ZD1694 (RTX) or hTS mutants with either a deletion of 23 residues in the N-terminus or substitution of a lysine residue at position 163, Cys195 was located in the active site and positioned for nucleophilic attack on dUMP.[5,7,8] Collectively, the data show that loop 181–197 of hTS populates at least two major conformations, active and inactive.

Structural modeling led to predictions regarding the roles of residues in stabilization of active and inactive structures. One residue that plays an important role in stabilization of the inactive conformation of hTS is Arg163. In rodent TSs, conservative substitution with lysine occurs at this relative position.[3] Murinized hTS (R163K) exhibited an increase in catalytic activity and is the first full-length hTS to crystallize in an active conformation in the native state.[8] Two residues were predicted by modeling studies to contribute to the formation of a critical hydrophobic cavity that stabilizes loop 181–197 in an active conformation. These residues, Met190 and Ala191, are invariant in TSs from prokaryotes and eukaryotes. Mutants were created to introduce charged residues at these sites. The resulting mutants, M190K and A191K, exhibited $<1\%$ and approximately 25% of the catalytic activity of hTS, with A191K exhibiting higher activity.[9] The structure of eukaryotic-specific insert 1, which is correlated with the conformation of loop 181–197, differed in crystal structures of the native proteins. The conformation of insert 1 was ordered in M190K, with loop 181–197 trapped in an inactive conformation. In contrast, insert 1 in A191K is disordered, similar to that observed in crystal structures of hTS.[4,9]

The catalytic activity of TS is essential for cell proliferation and viability; thus, the existence of an inactive conformer is counterintuitive. In the inactive conformation, three to four phosphate/sulfate ions are bound with geometry that may reflect enzyme binding to its own mRNA. This mRNA binding was proposed to be a regulatory mechanism that leads to autoinhibition of enzyme synthesis.[10] Second, the catalytic nucleophile Cys195 and Cys180 become buried in the dimer interface in the inactive conformation.[4] This may serve to protect these thiols from oxidation, for example, during periods of the cell cycle in which there is diminished demand for dTMP. Regardless of physiological relevance, stabilization of the inactive conformer is a novel and attractive approach for inhibition of hTS activity. Current inhibitors utilized clinically bind to the active site, interfering with processes that control enzyme levels, including translational repression and protein turnover.[10,11] Perturbation of these regulatory processes leads to elevation in the steady-state levels of TS, increasing the potential for drug resistance.[12,13]

The major objective of this investigation was to analyze the biochemical effects of stabilization of hTS in an inactive conformation. The data revealed that M190K is unresponsive to the binding of dUMP or the allosteric inhibitor, phosphate. This enzyme, with structural and kinetic characteristics of strong stabilization in an inactive conformation, is highly susceptible to phosphorylation by an ubiquitous kinase, CK2, providing the first evidence for altered physiological behavior of hTS in an inactive conformation. The second mutant, A191K, which is largely superimposable with the crystal structure of the inactive conformation of hTS, exhibited a structure upon dUMP binding that differs from hTS and unique behavior in the presence of an allosteric inhibitor that is reported to stabilize the inactive conformation of hTS.

Results

Intrinsic fluorescence studies

The indole ring of a conserved tryptophan (Trp182) in loop 181–197 occupies positions that are 5 Å apart in the active and inactive conformations. Studies of intrinsic fluorescence provide an approach for investigation of enzyme structure in solution and of the effect of ligand binding on enzyme conformation. Studies of crystal structures and intrinsic fluorescence of hTS provided strong evidence that phosphate binding stabilizes an inactive conformation.[4,14] Titration of hTS with phosphate resulted in a dose-dependent increase in the intrinsic fluorescence of hTS. In contrast, the substrate dUMP resulted in a dose-dependent decrease in intrinsic fluorescence of hTS.[4,15] In the present investigation, the intrinsic fluorescence of A191K was increased by both phosphate and dUMP (Fig. 1). In the absence of ligands, the intrinsic fluorescence of A191K was 1.4-fold lower than that of hTS.[16] Intrinsic fluorescence of M190K was unaffected by either phosphate or dUMP. The lack of response to dUMP is consistent

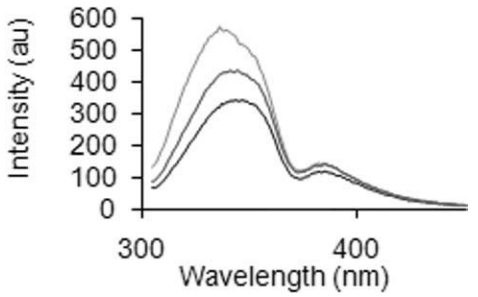

Figure 1. Intrinsic fluorescence of A191K mutant. Intrinsic fluorescence was measured as described in the Methods utilizing 1 μM TS alone (lower trace) or with 1 mM dUMP (middle trace) or 250 mM potassium phosphate (upper trace).

with an enzyme strongly stabilized in an inactive state (Supporting Information Fig. 1). Relative to hTS, the intrinsic fluorescence of unliganded M190K was lower by 1.8-fold.[16]

Secondary structure analysis by CD

CD spectra of M190K and A191K were analyzed in the far UV range. Both mutant enzymes exhibited a significant negative molar ellipticity in the range 205–243 nm relative to hTS (Fig. 2). Estimation of secondary structure was conducted by K2D2 analysis. Human TS has α-helix and β-strand contents of 61% and 4%, respectively. Estimated α-helix and β-strand contents of M190K and A191K are similar, 84% and 1%, respectively. The effect of high concentrations of dUMP and phosphate on secondary structure was analyzed [Fig. 3(A,B)]. Phosphate had minimal effect on the secondary structure of either protein. On dUMP binding, the molar ellipticity of A191K decreased significantly but was essentially unaltered in M190K.

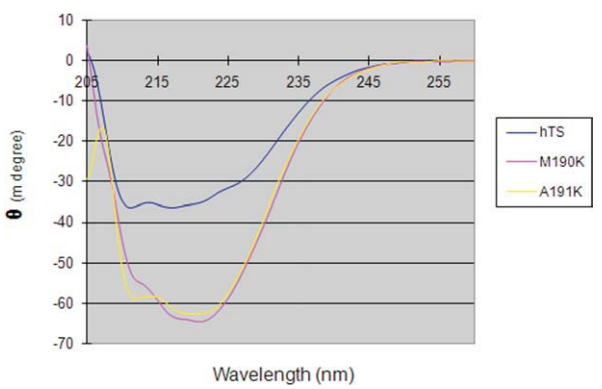

Figure 2. Circular dichroism spectra of human TSs. Circular dichroism spectra in the far UV were obtained as described in Methods. Composite spectra of hTS, M190K, and A191K (4 μM) from two to three separate enzyme preparations are shown.

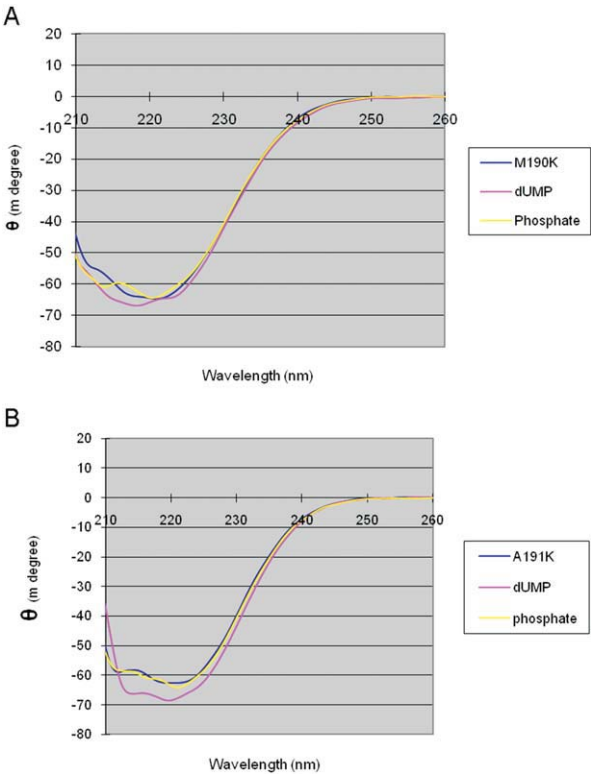

Figure 3. Effect of dUMP and phosphate on CD spectra of mutant hTSs. CD spectra were obtained as described in Methods. The effect of 100 μ*M* dUMP or 100 m*M* phosphate on molar ellipticity of M190K (A) or A191K (B) at 4 μ*M* concentration is shown.

Effect of temperature on catalysis by A191K

The catalytic activity of A191K is more than 25-fold higher than M190K.[9] Thus, the effect of temperature on the catalytic activity of A191K was analyzed (Fig. 4). The E_{act} for catalysis was higher than hTS (12.2 and 10.3 kcal mol^{-1}, respectively), indicative

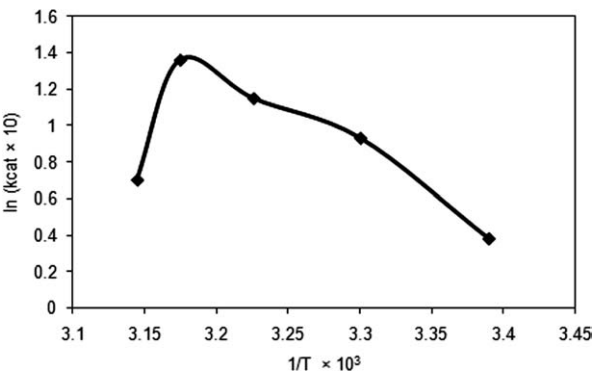

Figure 4. Arrhenius plot for catalysis by A191K. A191K (200 n*M*) and reaction mixtures were pre-incubated at indicated temperatures for 3 min. Catalysis was initiated by addition of reaction mixtures to TS and product formation monitored spectrophotometrically as described in Methods. The data are the means of three separate determinations.

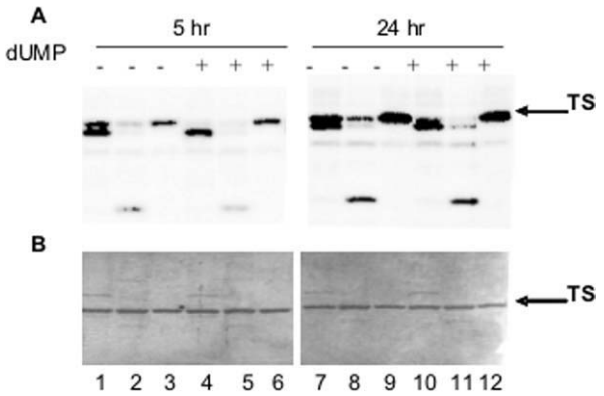

Figure 5. Phosphorylation of TS by CK2. Purified TS was incubated with CK2 for 5 or 24 hr in the presence or absence of 100 μ*M* dUMP as described in Methods. Proteins were resolved by SDS-PAGE followed by either (A) autoradiography or (B) Coomassie blue staining. Lanes: 1, 4, 7, 10 (hTS); 2, 5, 8, 11 (R163K); 3, 6, 9, 12 (M190K). Nonspecific bands represent minor co-purifying proteins that are apparent substrates for CK2 and autophosphorylated CK2 subunits.

of higher energy barriers for catalysis.[15] The temperature optimum (42°C) was shifted to higher temperatures relative to hTS (37°C) indicating that A191K has higher thermal stability.

Phosphorylation of hTS

Superpositioning of the structures of native hTS and the hTS/dUMP/ZD1694 (RTX) complex revealed that the conformation of loop 181–197 is correlated with two conformational states of an eukaryote-specific insert in the protein, insert 1.[4,7] Insert 1 is predicted by phosphorylation site algorithms to contain a high-probability consensus sequence for the serine–threonine kinase casein kinase (CK) 2.[17] CK2-mediated phosphorylation of M190K was examined and compared with that of hTS and the active conformation-stabilized mutant R163K. The data, shown in Figure 5(A), indicated that M190K is highly phosphorylated by CK2. In contrast, phosphorylation was significantly reduced in R163K, which was more resistant to phosphorylation than hTS. Inclusion of the substrate dUMP in CK2 reactions had no significant effect on phosphorylation of M190K. In contrast, phosphorylation of R163K and hTS was inhibited by dUMP. The data are consistent with structural studies that reveal that insert 1 differs among active- and inactive-stabilized hTS and indicate that inactive-stabilized enzymes are more susceptible to phosphorylation.

Inhibition profiles of hTS inhibitors

Inhibition profiles of A191K with orthosteric and allosteric inhibitors of hTS were analyzed. RTX is a structural analog of the folate substrate that binds to the active site of hTS and stabilizes an active conformation.[18] The inhibition profile of A191K with RTX was right-shifted relative to hTS [Fig. 6(A)], with IC_{50}s of 11.3 ± 1.8 nM and 5.9 ± 0.1 nM, respectively. PDPA is a mixed competitive–noncompetitive inhibitor of hTS with respect to dUMP (Supporting Information Fig. 2).[14] At concentrations between 0.1 and 10 μ*M*, PDPA exhibited mixed competitive–noncompetitive inhibition of A191K versus dUMP (Supporting Information Fig. 3). The K_i, determined from $K_{m,\mathrm{app}}$ values was 0.34 μ*M* [Supporting Information Fig. 4(A)]. In contrast, the K_i value for hTS was 1.44 μ*M* at concentrations ≤3 μ*M* [Supporting Information Fig. 4(B)]. A191K exhibited nonhyperbolic behavior in the presence of PDPA at concentrations above 10 μ*M* (Supporting Information Fig. 5). At saturating concentrations of dUMP and mTHF, PDPA modestly increased the activity of A191K. This pattern was also observed at concentrations of mTHF near the K_m (75 μ*M*). This response was distinct to that of hTS, which exhibited a concentration-dependent decrease in catalytic activity [Fig. 6(B)].

Discussion

A major aim of this study was to examine the structures and functionality of mutant hTSs, selected as models of enzymes that differentially populate the inactive state. M190K is strongly stabilized in an inactive conformation, as suggested by k_{cat} decreased

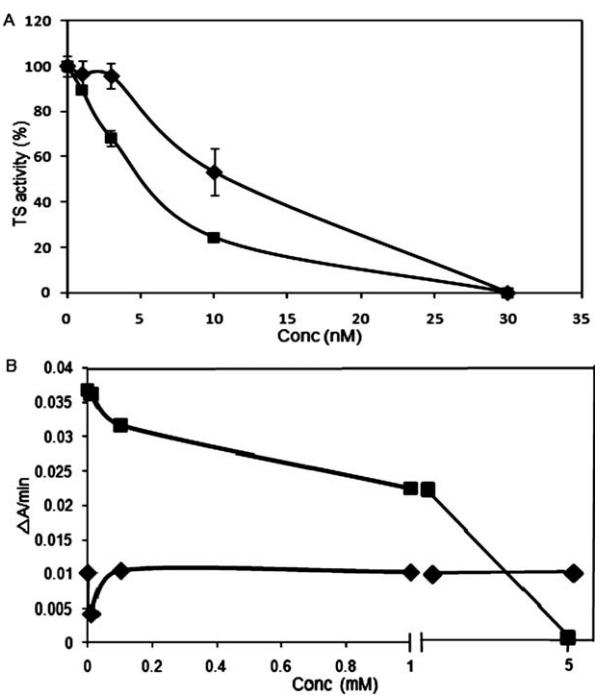

Figure 6. Inhibitory potencies of RTX and PDPA on TS catalytic activity. Purified TS (hTS, -■-; A191K, -◆-) was incubated with varying concentrations of RTX (A) or PDPA (B). Reactions were conducted as described in Methods and contained 200 nM TS and 100 μ*M* dUMP. The data are the mean of six to nine separate determinations.

by 100-fold relative to hTS and loop 181–197 in an inactive conformation, and with unique conformational changes within the loop that hinder its mobility.[9] A191K exhibits higher catalytic activity than M190K and crystal structures of native hTS and A191K are largely superimposable, consistent with a mutant enzyme that populates the inactive state but retains the ability to undergo conformational switching.[9] Although studies of crystal structures provide insight into the structure of thermodynamically stable conformers, they cannot provide quantitative information regarding the ratio of conformers in solution. Intrinsic fluorescence was utilized as a tool to monitor movement of loop 181–197 since the environment of Trp182 differs significantly in active and inactive conformations. CD studies in the far UV provide information on secondary structure content of the mutant proteins. Both studies indicated that unliganded structures differ in solution from hTS, consistent with the prediction that the equilibrium is shifted toward the inactive conformation in solution. M190K and A191K exhibited lower intrinsic fluorescence and more negative ellipticities relative to hTS. Inclusion of dUMP or phosphate had no significant effect on the intrinsic fluorescence or CD spectrum of M190K, which is consistent with strong stabilization of this enzyme in an inactive conformation. In contrast, ligand-mediated conformational changes were observed in A191K in solution. On ligation to A191K, phosphate, which is predicted to stabilize an inactive state, induced upward shifts in fluorescence, similar to its effects on hTS.[4,14] In contrast to ligation with phosphate, A191K and hTS exhibited inverse responses to dUMP. dUMP mediated an upward shift in fluorescence in A191K and a downward shift in fluorescence in hTS. A191K is unique among all TSs examined to date in exhibiting an increase in intrinsic fluorescence on dUMP binding.

Analysis of the effect of temperature on the catalytic activity of A191K revealed that the E_{act} is 12.2 kcal mol^{-1}, which falls within the range of values (11.5–12.9 kcal mol^{-1}) reported for mutant hTSs that have a slower rate of isomerization to a catalytically competent ternary complex.[15] These mutants have substitutions at glutamine 214 and retain $\geq$25% of the catalytic activity of hTS.[19] Glutamine 214 is located at a β-bulge that is predicted by structural and biochemical studies to function in ligand-induced conformational changes.[15,20] This suggests that A191K, like the Q214 mutants, resides in an energy landscape with steep barriers to the transition state. Although similar in E_{act}, the kinetic properties of A191K and the Q214 mutants differ. The K_m of A191K for dUMP (0.85 μ*M*)[9] is lower than hTS (2.59 μ*M*)[9], whereas the K_m for mTHF is significantly higher, 75 μ*M*. In contrast, the K_m of the Q214 mutants for dUMP (11–34 μ*M*) is significantly higher than hTS, whereas the K_m for mTHF (11–24 μ*M*) is increased slightly (hTS, 6.8 μ*M*).[19] The data suggest that the binding of mTHF is decreased in an enzyme stabilized in an inactive conformation. Moreover, catalytic activity remains compromised at saturating concentrations of mTHF. This leads to the interpretation that loop 181–197 in an inactive conformation perturbs the productive binding of mTHF. As mTHF binding is an essential step in isomerization to a catalytically competent complex, this perturbation may contribute to the higher E_{act}.

A191K exhibited a higher temperature optimum than hTS, suggesting that it has higher thermal stability.[15] This leads to the interpretation that unfolding at temperatures up to 42°C is less likely to induce irreversible inactivation in the inactive conformation. Alternatively, protein dynamics are slower in the inactive conformation. The data indicate that the inactive conformation is optimized for stability, perhaps at the expense of flexibility.

Phosphorylation site algorithms predict overlapping CK2 recognition sites in hTS, which occur in insert 1.[17] Recently, studies of phosphorylation of hTS by CK2 have been reported and a phosphorylation site identified as Ser124.[21] In the recognition sequence (STREE), n+3 is glutamate for Ser124 and Thr125; phosphorylation of Thr125 generates an acidic residue at n+1 and is expected to increase CK2-mediated phosphorylation of Ser124.[22] Because of structural data that support not only an association between the conformation of insert 1 and loop 181–197 but also strong stabilization of M190K in an inactive conformation, the phosphorylation of M190K by CK2 was examined. M190K was phosphorylated by CK2 and the extent of phosphorylation was much higher relative to the active-stabilized R163K mutant. Consistent with the lack of effect of dUMP on M190K structure observed in biophysical studies, addition of dUMP had no effect on phosphorylation of M190K. This is in contrast to the inhibitory effects of dUMP on phosphorylation of hTS and R163K. The relative extents of phosphorylation, M190K> R163K, in concert with a dUMP-mediated decrease in phosphorylation of hTS and R163K, provide evidence that CK2-mediated phosphorylation of hTS is conformation dependent, with the inactive conformation, the preferred CK2 target. Additional studies are warranted to determine the site(s) of phosphorylation and the role of the conformation assumed by insert 1 in M190K in recognition by CK2. CK2 is a promiscuous kinase, raising questions about the physiological relevance of phosphorylation of hTS.[22] In preliminary studies, phosphorylation of hTS was observed in a human tumor cell line and an activator of CK2 increased the level of the phosphorylated protein (M. Yousef, in preparation).

TS is an important chemotherapeutic target for the therapy of cancers of the gastrointestinal tract,

breast, pancreas, and head and neck.[1,2] Drugs that are utilized clinically to target TS, including 5-fluorouracil, capecitabine, RTX, and pemetrexed, bind to the active site, resulting in the formation of inhibitory complexes that are structural analogs of the catalytic complex.[3] Higher concentrations of RTX were required to inhibit the catalytic activity of A191K relative to hTS. Inhibition of A191K by PDPA, an allosteric inhibitor that binds to one of the phosphate binding sites in the inactive conformation of hTS, was concentration dependent. At concentrations $\leq$10 μM, the K_i of A191K for PDPA was lower, relative to hTS. At concentrations above 10 μM, A191K exhibited nonhyperbolic behavior with respect to dUMP. The apparent K_m for dUMP was increased significantly by PDPA; however, no inhibition of catalysis was observed at saturating dUMP concentrations. The data indicate that PDPA binding to A191K is multimodal. In previous studies, hTS exhibited an increase in hydrodynamic radius, consistent with tetramerization, in solution with PDPA.[14] Thus, stabilization of hTS in an inactive conformation may alter quaternary structure, which, in turn, alters subunit interactions and binding sites for substrates and inhibitors.

Paradoxically, exposure to active site inhibitors is associated with elevation in TS levels, presumably due to perturbation of pathways that regulate the steady-state levels of TS.[10–12] Elevation in TS after exposure to inhibitors is observed clinically and is postulated to contribute to the resistance that is reported in patients receiving TS-targeted chemotherapy.[23,24] In an effort to surmount the resistance associated with active site-targeted therapy, alternative approaches, such as stabilization of an inactive conformation, are attractive. Collectively, biophysical data and inhibitory profiles with RTX and PDPA are consistent with the prediction that A191K exists predominantly in the inactive conformation in solution. Unlike M190K, A191K exhibits the biophysical properties of an enzyme that undergoes conformational switching in the presence of ligands. This property will facilitate identification of conformation-selective, allosteric inhibitors of hTS. In addition, A191K, by retaining catalytic activity, is an invaluable tool for investigation of the physiological relevance of phosphorylation of hTS.

Materials and Methods

Creation, expression, and purification of mutant hTSss

The *Escherichia coli* strain TX61 (thyA$^-$) containing kanamycin resistance and the pTS080 plasmid containing tetracycline and ampicillin resistance, expressing hTS, were generously provided by W. S. Dallas (Glaxo Wellcome, Research Triangle Park, NC), and have been described previously.[25,26] TX61 was created by transposon-mediated mutagenesis and lacks detectable TS activity.[25] Creation of mutants with substitutions at amino acid positions 190 and 191 of hTS by site-directed mutagenesis has been reported previously.[9] TX61 transformants were grown and TSs purified by AKTA® FPLC system (Pharmacia, Piscataway, NJ) using Blue-Sepharose and Q-Sepharose columns as described previously.[27]

Catalytic assays

Enzyme activity was measured spectrophotometrically by monitoring the absorbance change accompanying the conversion of mTHF to DHF (ε_{340} = 6.4 mM^{-1} cm^{-1}) using a Shimadzu UV 1601 spectrophotometer (Shimadzu Corporation, Japan) as described previously.[27] For determination of kinetic constants, initial velocities were measured at 22°C by utilizing 200 nM of purified protein, 0.05–0.5 mM of mTHF, and 1.0–100 μM dUMP in buffer A (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% β-mercaptoethanol). For analysis of enzyme inhibition and the effect of temperature on catalysis, initial velocities were measured by utilizing 200 nM of purified protein, 0.5 mM mTHF, and 100 μM dUMP. The inhibitors raltitrexed (RTX) and propylene diphosphonate (PDPA) were added at varying concentrations and dUMP concentrations varied from 1.0 to 100 μM. In studies of the effect of temperature on activity, enzyme and reaction mixtures were preincubated for 3 min at indicated temperatures. Kinetic constants and initial rates were analyzed with the software package *Prism* (v.5 GraphPad Software, La Jolla, CA).

Intrinsic fluorescence studies

Fluorescence data were obtained by excitation at 295 nm and emission scanning from 305 to 450 nm at a rate of 5 nm s^{-1} at 21°C (SLM-Aminco 8100 Spectrofluorometer, Urbana, IL). Protein concentrations were 1–4 μM in buffer A; in titration studies, ligands (potassium phosphate, pH 7.4 or dUMP) were added in increments not exceeding 10% of the total volume. No significant effect of ionic strength on intrinsic fluorescence was observed.

Secondary structure determination by circular dichroism

Circular dichroism spectra were obtained between 190 and 260 nm with 0.2-nm resolution (JASCO J-600 Spectropolarimeter, Tokyo, Japan). Final concentration of TS was 4 μM and 16 scans were accumulated with a scan rate of 100 nm min^{-1} at a time constant of 0.125 s at 21°C. Spectral analysis was preformed by comparing experimental spectra with published reference spectrum[28] and analyzed using K2D2 secondary structure prediction tool (http://www.ogic.ca/projects/k2d2/).[29] The effects of ligand

binding were determined by titrating the proteins in increments not exceeding 10% of the total volume. Data were corrected by subtraction of the spectra for buffer and ligands.

In vitro *phosphorylation of hTS by CK2*

Purified hTSs (50 nM) were incubated with 250 units of recombinant CK2 (New England Biolabs, Beverly, MA) in reaction mixtures containing 20 m*M* Tris, 50 m*M* KCl, 10 m*M* $MgCl_2$, 250 μ*M* ATP and [γ-^{32}P] ATP (Perkin-Elmer Life Sciences, Boston, MA), to a final specific activity of 200 μCi μmol^{-1}. The reactions were stopped either with 10 m*M* ATP or by boiling with SDS-loading buffer for 5 min. The reactions were loaded on 12% SDS-polyacrylamide gels, stained, dried, and exposed to Kodak X-Omat film at −70°C.

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