Modification of *Escherichia coli* Thymidylate Synthase at Tyrosine-94 by 5-Imidazolylpropynyl-2′-deoxyuridine 5′-Monophosphate†

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ABSTRACT: Evidence is presented that 5-imidazolylpropynyl-2′-deoxyuridine 5′-monophosphate (IP-dUMP) is a mechanism-based, irreversible inactivator of *Escherichia coli* thymidylate synthase (TS), which covalently modifies Tyr94 at the active site of the enzyme. The inactivation of TS was time and concentration dependent and did not require the folate cofactor. Due to the rapidity of the inactivation process, accurate kinetic parameters could be determined only in the presence of saturating concentrations ($1000K_M$) of the competing substrate, dUMP. Under these conditions, a K_I of 0.36 ± 0.09 μM and an inactivation rate constant (k_{inact}) of 0.53 ± 0.15 min^{-1} were obtained from Kitz−Wilson plots. Electrospray ionization−mass spectrometry (ESI-MS) determined a 412 amu mass increase of TS after inhibition by IP-dUMP with no mass difference being detected for the TS mutants Tyr94Phe or Cys146Ala, thus indicating the importance of these residues for complex formation. The change in WT-TS mass was consistent with covalent modification by IP-dUMP, which was confirmed by proteolytic digestion of the modified protein followed by ESI-MS. By these means, a 43-residue trypsin peptide (residues 54−96), a 16-residue endoAspN peptide (residues 89−104), and an 8-residue endoAspN/endoLysC peptide (residues 89−96), each containing the IP-dUMP adduct, were observed. MS/MS analysis of the IP-dUMP-endoAspN peptide identified a modified 3-residue daughter ion, YGK (residues 94−96). A mechanistic scheme requiring the participation of Cys146 is proposed for the covalent modification of IP-dUMP by Tyr94, which, unlike an earlier proposal [Kalman, T. I., Nie, Z., and Kamat, A. (2001) *Nucleosides Nucleotides Nucleic Acids 20,* 869−871], does not require the release of imidazole for the activation of the inhibitor.

Thymidylate synthase (TS)[1] (EC 2.1.1.45) catalyzes the last step of the sole de novo pathway for the synthesis of thymidine 5′-monophosphate (dTMP). Because it is essential for cell division and survival due to its critical role in DNA synthesis, this highly conserved enzyme (*1*) has been identified as an important target for the development of clinically useful antineoplastic agents. Since first being described by Friedkin and Kornberg (*2*), TS has been extensively studied, and in particular, X-ray structures of this enzyme in both its free and analogue-bound forms from multiple species have been determined (*3*−*8*). From these studies a unique mechanism has been proposed by which TS transfers a methylene group from 5,10-methylene-6(*R*)-tetrahydrofolate (CH_2H_4PteGlu) to 2′-deoxyuridine 5′-monophosphate (dUMP) accompanied by the reduction of the methylene to a methyl group to yield dTMP and 7,8-dihydrofolate (H_2PteGlu).

Thus, formation of a ternary complex between the enzyme, dUMP, and CH_2H_4PteGlu aligns a completely conserved active site cysteine residue (Cys146 in *Escherichia coli* TS) for Michael addition to C6 of dUMP (*1*). The resultant enolic C5 of dUMP forms a covalent bond with the methylene group of CH_2H_4PteGlu, which had been activated previously by the addition of a proton to form an iminium ion at nitrogen-5 of CH_2H_4PteGlu. Abstraction of the C5 proton by Tyr94 of *E. coli* TS (Tyr146 of *Lactobacillus casei* TS) (*7*, *9*) with the possible participation of ordered water molecules within a hydrogen-bonded network involving Glu58 of *E. coli* TS (Glu60 of *L. casei* TS) (*10*, *11*) enables the formation of an exocyclic methylene-dUMP intermediate from CH_2H_4PteGlu. Hydride ion transfer from the C6 position of the pterin ring (*12*) of H_4PteGlu to the exocyclic methylene intermediate followed by re-formation of the 5,6 double bond of the pyrimidine ring with elimination of Cys146 of the enzyme completes the synthesis of dTMP.

On the basis of the extensive structural and mechanistic information known about TS (*1*) and the characterization of structurally related analogues of dUMP (*13*−*16*), 5-propy-

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[1] Abbreviations: TS, thymidylate synthase; H_2PteGlu, 7,8-dihydrofolate; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EdUMP, 5-ethynyl-2′-deoxyuridine 5′-monophosphate; ESI-LC-MS/MS, electrospray ionization−liquid chromatography−mass spectrometry/mass spectrometry; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IP-dUMP, 5-imidazolylpropynyl-2′-deoxyuridine 5′-monophosphate; CH_2H_4PteGlu, 5,10-methylene-6(*R*,*S*)-tetrahydrofolate; ^{1}H NMR, proton nuclear magnetic resonance ; $Pd(PPh_3)_4$, tetrakis(triphenylphosphine)palladium(0); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; dTMP, thymidine 5′-monophosphate; TPCK, tosylphenyl chloromethyl ketone; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; dUMP, 2′-deoxyuridine 5′-monophosphate.

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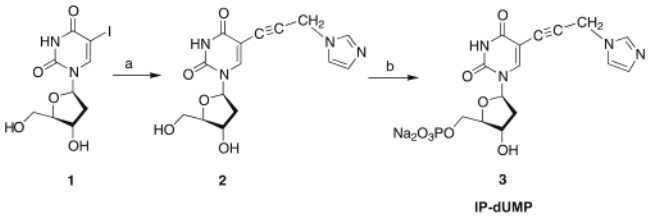
Scheme 1: Outline of the Synthesis of IP-dUMP



(a) 5-Iodo-2′-deoxyuridine (**1**) was coupled with *N*-propynylimidazole in the presence of $Pd(PPh_3)_4$, CuI, and TEA in DMF at 50 °C to yield 5-imidazolylpropynyl-2′-deoxyuridine (**2**). (b) Phosphorylation of **2** by $POCl_3$ was carried out in $(EtO)_3PO_4$ in the presence of a catalytic amount of pyridine at −10 °C, followed by hydrolysis. IP-dUMP was isolated as its disodium salt (**3**).

nylpyrimidine derivatives were rationally designed as irreversible, mechanism-based inactivators of TS, which do not require the presence of the cofactor, $CH_2H_4PteGlu$ (*17*−*19*). A representative member of this inhibitor family, 5-imidazolylpropynyl-2′-deoxyuridine 5′-monophosphate (IP-dUMP) (Scheme 1), was found to be an irreversible inactivator of *L. casei* TS as established by dialysis experiments (*19*). Kalman and Nie (*17*) proposed that the inactivation of TS by the 5-propynylpyrimidines involved enzyme-generated, chemically reactive cumulene derivatives covalently linked to the catalytic cysteine residue, which may subsequently alkylate another active site nucleophile. Since the cumulene has three reaction centers, multiple reactions are possible. Through the use of molecular modeling it was postulated that Tyr94, which is about 3 Å from one of the reactive carbon atoms of the cumulene side chain, was the alkylated residue. In this paper we present unambiguous evidence for covalent modification of Tyr94 by IP-dUMP, and since the results do not support the involvement of a cumulene intermediate as hypothesized originally (*17*), an alternative inactivation mechanism is presented.

MATERIALS AND METHODS

Chemicals and Reagents. Ammonium sulfate obtained from ICN Pharmaceuticals (Costa Mesa, CA) and Tris·HCl from United States Biochemicals (Clevland, OH) were of ultrapure grade. Oligonucleotides were synthesized by the Wadsworth Center Oligonucleotide Synthesis Core Facility. Restriction, ligation, and other DNA modification enzymes were purchased from New England Biolabs (Beverly, MA) and Boehringer Mannheim (Indianapolis, IN), while Taq polymerase was obtained from Stratagene (La Jolla, CA). Electrophoresis reagents were from Bio-Rad (Hercules, CA). DE-52 and phenyl-Sepharose were obtained from Schleicher and Schuell, Inc. (Keene, NH). TPCK-treated trypsin was a generous gift from Dr. Thomas Plummer of our laboratory. Endoprotease LysC was purchased from Boehringer Mannheim and endoprotease AspN from Roche Diagnostics (Indianapolis, IN). Chromatography-grade acetonitrile was purchased from Baker-Mallinckrodt, Inc. (Paris, KY). Iodoacetamide was purified by recrystallization and stored at −70 °C. All other materials were of reagent grade and were obtained from standard commercial sources.

Chemistry. IP-dUMP was synthesized (Scheme 1) by Pd(0)/CuI-catalyzed coupling (*20*) of 5-iodo-2′-deoxyuridine (**1**) and *N*-propynylimidazole. The latter compound was obtained by alkylation of imidazole with propargyl bromide (*21*). The resulting 5-imidazolylpropynyl-2′-deoxyuridine (**2**) was phosphorylated to the 5′-monophosphate using $POCl_3$ in triethyl phosphate (*22*).

5-[3-(Imidazol-1-yl)propyn-1-yl]-2′-deoxyuridine (2). Triethylamine (819 μL, 6 mmol) was added to a mixture of 1.04 g (3 mmol) of 5-iodo-2′-deoxyuridine (**1**), 170 mg (0.15 mmol) of $Pd(PPh_3)_4$, and 56 mg (0.3 mmol) of CuI in 5 mL of DMF followed by the slow addition of 530 mg (5 mmol) of *N*-propynylimidazole. The resulting green solution was then stirred at 50 °C for 2−4 h, followed by evaporation of the solvent. The dark brown residue was directly applied onto a silica gel flash column and eluted using CH_2Cl_2−MeOH (5:1). After being allowed to stand overnight, the eluate yielded 523 mg of crystals. A second crop, obtained after concentration, increased the yield to a total of 628 mg (64%) of pure **2**: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.11 (m, 2H, 2′- + 2″-H), 3.56 (m, 2H, 5′- + 5″-H), 3.78 (m, 1H, 4′-H), 4.21 (m, 1H, 3′-H), 5.12 (br s, overlap, 3H, α-CH_2 + 5′-OH), 5.24 (d, J = 4.0 Hz, 1H, D_2O exchangeable, 3′-OH), 6.08 (t, J = 6.8 Hz, 1H, 1′-H), 6.92 (s, 1H, imidazole 4-H), 7.22 (s, 1H, imidazole 5-H), 7.71 (s, 1H, imidazole 2-H), 8.26 (s, 1H, 6-H), 11.67 (br s, 1H, D_2O exchangeable, NH). Anal. Calcd for $C_{15}H_{16}N_4O_5$: C, 54.22; H, 4.85; N, 16.86. Found: C, 54.38; H, 4.90; N, 16.94.

5-[3-(Imidazol-1-yl)propyn-1-yl]-2′-deoxyuridine 5′-Monophosphate Disodium Salt (3). Compound **2** (33.2 mg, 0.1 mmol) was coevaporated with 2 mL of pyridine three times and dissolved in 1 mL of triethyl phosphate. To the above solution was added 20 μL (0.2 mmol) of phosphorus oxychloride at −10 °C, under argon protection. After 4 h, 1 mL of water was added to hydrolyze the intermediate. The reaction mixture was concentrated to an oil, which was dissolved in 1 mL of H_2O. The pH of the solution was adjusted to 8−9 with dilute NaOH. Reverse-phase HPLC purification (0−100% MeOH/H_2O) followed by lyophilization gave 10 mg (22%) of **3** as the disodium salt. Anal. Calcd for $C_{15}H_{15}N_4Na_2O_8P\cdot 2.5H_2O$: C, 35.94; H, 4.02; N, 11.18. Found: C, 36.10; H, 3.88; N, 11.00.

Preparation of Wild-Type and Mutant TS Enzymes. Highly purified TS was prepared using an expression system which produced the enzyme to 50−60% extent of the cellular protein of *E. coli* (*23*). The mutants of TS were prepared from the *thyA* gene (*24*) with the appropriate oligonucleotides using the Quick-Change kit from Stratagene. The mutant proteins were purified as above for WT-TS (*23*). A phenyl-Sepharose chromatography purification step (*25*) after the DE-52 column was added when necessary. Enzyme assay and SDS−PAGE were used for low activity and inactive mutants, respectively, to determine the fractions to be pooled. The enzyme pools were concentrated in an Amicon ultrafiltration device (W. R. Grace & Co., Beverly, MA) with a 19 kDa cutoff filter. The proteins were stored as ammonium sulfate pellets at −70 °C. Prior to use, the proteins were dissolved in a small aliquot of 50 mM potassium phosphate, pH 7.5, 10% ethylene glycol, and 1 mM DTT and dialyzed against 2 × 500 mL of this buffer overnight. The dialyzed solutions were stored at −20 °C.

Measurement of Thymidylate Synthase Activity. TS activity was measured at 30 °C using a kinetic spectrophotometric assay (*26*) in the presence of 50 mM $MgCl_2$ due to the high Mg^{2+} requirement of the *E. coli* enzyme (*27*). The components of the reaction mixture were 0.25 mL from a cocktail

containing 10 mL of 0.25 M ascorbate (pH 7.5), 25 mL of 1 M 2-mercaptoethanol, 30 μL of 52 mM formaldehyde, 50 mL of 0.5 M Tris·HCl (pH 7.5), and 100 mg of 5,10-CH_2-6(*R*,*S*)-H_4PteGlu (Schircks Labs, Jona, Switzerland) brought to 125 mL, 50 μL of 1 M $MgCl_2$, and 50 μL of 10 mM dUMP. These reaction components were added to a 1 mm path, silica cuvette, brought to about 1.0 mL, and equilibrated to 30 °C in a model 7400 Beckman diode array spectrophotometer equipped with a Peltier temperature controller and Beckman's kinetic software package (Enzyme Mechanism). The kinetic assays were initiated by bringing the final volume of the reaction to 1.0 mL with the addition of 10−50 μL of enzyme solution. One unit of TS activity is defined as the amount of enzyme required to synthesize 1 μmol of dTMP/min at 30 °C under the conditions of the assay. The protein concentration of TS was calculated from the *thyA* TS amino acid sequence (*24*) using the method of Gill and von Hippel (*28*), which provided a molar extinction coefficient of 0.591×10^5 M^{-1} cm^{-1} at 280 nm. This value is equivalent to 1.71 A_{280} units/mg of protein.

Protein Modification. A 75-fold molar excess of IP-dUMP (5 mM) was added to TS (66 μM) in 50 mM potassium phosphate, pH 7.5. The reaction was incubated at 25 °C for 30 min or until all of the TS activity was inhibited, with progress of the reaction being followed by the kinetic spectrophotometric assay (*26*). When no remaining activity could be detected, the modified protein was dialyzed against excess 400 mM ammonium bicarbonate, pH 8.0. Aliquots of the protein were dried in a Savant (RT100) speedvac (Farmingdale, NY) and stored at −70 °C.

Kinetics of Inhibition. To determine the kinetics of inhibition, TS (0.9 μM) was incubated at 25 °C with IP-dUMP at concentrations from 15 to 200 μM in 50 mM potassium phosphate, pH 7.5, in the presence of 5 mM dUMP. At various times after addition of the inhibitor, 20 μL aliquots were removed and assayed for TS activity at 30 °C. The increase in absorbance at 340 nm, due to H_2PteGlu formation, was followed for 5−10 min with data points taken every 30 s. In the absence of IP-dUMP, the activity of WT-TS did not decrease during the course of the experiments. A molar extinction coefficient of 1.0×10^4 M^{-1} cm^{-1} at 278 nm, pH 7.0, was determined for IP-dUMP from the ratio of the absorbance at 278 nm to the concentration of phosphate, as determined by Elon phosphate analysis (Elon developing agent; Kodak, Rochester, NY) (*29*), after the release of the phosphate by calf intestine alkaline phosphatase (Boehringer Mannheim).

Protein Digestion. Lyophilized IP-dUMP-modified TS (100 μg, 1.7 nmol) and native protein (100 μg, 1.7 nmol) were taken up in a solution containing 300 μL of 8 M urea and 400 mM ammonium bicarbonate, pH 8.0, and incubated in the presence of 5 mM DTT at 50 °C for 15 min. After being cooled to 25 °C, the denatured proteins were incubated in the presence of 20 mM iodoacetamide at 25 °C for 15 min. The protein solution was then diluted to 2 mL with water and digested overnight at 25 °C with trypsin or endoAspN (1:50 w/w). The resulting peptides were separated by C18 reverse-phase HPLC with a 0−60% acetonitrile gradient and analyzed by nano ESI-LC-MS/MS and N-terminal sequencing. The IP-dUMP-modified endoAspN peptide was lyophilized, then taken up in 100 μL of 25 mM Tris·HCl and 1 mM EDTA (pH 7.8), and digested overnight at 25 °C with endoLysC (1:50 w/w). The resulting peptides were separated by C18 reverse-phase HPLC and further analyzed by ESI-MS and N-terminal sequencing.

HPLC Analysis. Enzyme samples, both modified and unmodified, were analyzed at room temperature by reverse-phase HPLC using a Waters HPLC system (Waters Corp., Milford, MA) (Model 625 LC, a Model 996 photodiode array detector, and a Model 717 plus autosampler). A Varian C18 column (4.6 mm × 15 cm) was eluted with a 0.1% TFA and acetonitrile solvent system (mobile phase A, 0.1% TFA/H_2O; mobile phase B, 0.1% TFA/100% acetonitrile). The peptides were eluted with a gradient of 0−60% as mobile phase B in 60 min at a flow rate of 1 mL/min; 0.5 mL fractions were collected using an injection loop of 200 μL or less. Protein was detected at both 220 and 240 nm. The data were collected and processed (i.e., peak retention time, height, area, etc.) using the Millennium version 2.00 software provided with the HPLC system. The HPLC fractions were analyzed by ESI-MS.

The isolated IP-dUMP-modified and native endoAspN peptides, from Asp89−Pro104, were further analyzed by HPLC after identification by ESI-MS and N-terminal sequencing. A 100 μL sample containing 15 μg of each peptide in 0.1% TFA/H_2O/30% acetonitrile was injected onto the column. The modified peptide eluted at 23.0 min, and the native peptide eluted at 23.8 min.

N-Terminal Sequencing. The sequence of the amino ends of the HPLC-purified IP-dUMP-modified and native TS peptides was determined with a model 477A protein/peptide sequencer from Applied Biosystems (Foster City, CA) using their blott cartridge.

Mass Spectrometry. ESI-MS spectra were obtained on a Finnigan MAT-TSQ 700 triple quad mass spectrometer or on a Finnigan LCQ-DECA ion trap mass spectrometer equipped with nano ESI-LC-MS/MS. The data were processed manually or with the aid of SEQUEST protein identification software. Before analysis salts were removed from whole protein solutions with a C4 zip tip from Millipore (Bedford, MA) after which the peptides were eluted from the tip with 65% acetonitrile/1% acetic acid.

Ternary Complex Formation. The ability of WT-TS and mutant TS proteins to form covalent ternary complexes was assessed by SDS−PAGE. The covalent ternary complexes were formed by incubating WT-TS (244 pmol, 19.5 μM) and its corresponding mutants Y94F, Y94A, Y94K, or E58A (all at 262 pmol, 21 μM) with [6-^{3}H]dUMP (70 μM, 14.2 Ci/mmol) and 0.67 mM (*R*,*S*)-CH_2H_4PteGlu in 16 mM HEPES, pH 7.0, and 4.0 mM $MgCl_2$, pH 7.0, at room temperature. Aliquots were removed and denatured by heat after the addition of an equal volume (12.5 μL) of SDS loading buffer at various times after initiation of the reaction. The time course of complex formation was analyzed subsequently by SDS−PAGE (12.5%) and visualized by Coomassie Blue stain and autoradiography after incubation with Amplify (Amersham, Piscataway, NJ) according to the manufacturer's instructions. The X-ray film at each time interval was photographed and overlayed to yield a composite (Figure 3). The ability of WT-TS to form covalent ternary complexes with either the (*R*) or (*S*) enantiomers of CH_2H_4PteGlu compared to the (*R*,*S*) racemic mixture was assessed similarly.

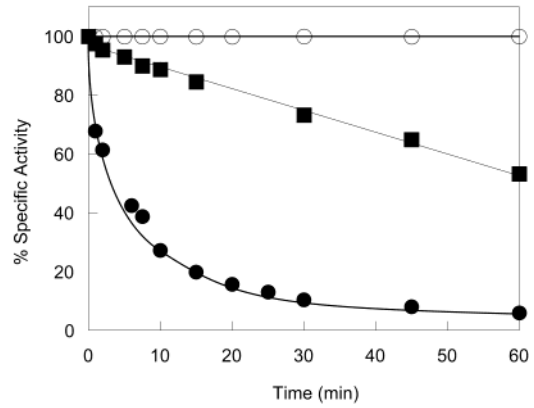


FIGURE 1: Time course of the inactivation of TS by IP-dUMP: percent of WT-TS specific activity in the absence (○), the presence of 15 μM IP-dUMP (●), and the presence of 15 μM IP-dUMP plus 5 mM dUMP (■) as described in Materials and Methods.

RESULTS

Kinetic Analysis of IP-dUMP Inactivation. The inactivation of *E. coli* WT-TS by IP-dUMP (Figure 1) was too rapid for accurate kinetic parameters to be obtained. However, the presence of a saturating concentration (1000K_M) of competing dUMP in the reaction solution slowed the inactivation rate to a measurable range (Figure 1), and as a consequence the time and concentration dependence of the inhibition kinetics were determined in its presence. As shown in Figure 2A, in the presence of 5 mM dUMP, the inactivation of TS by IP-dUMP followed pseudo-first-order kinetics, which enabled the half-time of inactivation ($t_{0.5}$) to be determined at each inhibitor concentration. At pH 8.5 ($t_{0.5}$ = 18.1 min) 15 μM IP-dUMP inactivated WT-TS more rapidly than at pH 7.5 ($t_{0.5}$ = 26.0 min), which in turn was faster than that at pH 6.5 ($t_{0.5}$ = 41.0 min) (data not shown).

The kinetic parameters describing the interaction of the inhibitor with TS were determined from the Kitz−Wilson plot (*30*) of $1/k_{obs}$ vs 1/[I] (Figure 2B) with each point representing the mean of three independent determinations from which the standard deviations of the means were calculated. It is assumed that an enzyme (E) initially binds to the inhibitor (I) in a reversible complex E·I, which becomes irreversible with time via formation of a covalent E−I complex at a rate equal to k_{inact} (eq 1). $K_{I\,app}$ (the apparent dissociation constant of E·I in the presence of dUMP) and k_{inact} are obtained from eq 2 by plotting the reciprocal of the observed pseudo-first-order rate constant k_{obs} against the inverse of each inhibitor concentration [I] (Figure 2B). Extrapolating from this plot, $1/k_{inact}$ was obtained from the intercept on the ordinate and K_I/k_{inact} from the slope of the line. The values thus obtained from eq 2 for the modification of TS by IP-dUMP in the presence of 5 mM dUMP were $K_{I\,app}$ = 360 ± 92 μM and k_{inact} = 0.53 ± 0.15 min^{-1} (Figure 2B). Considering that the K_M for dUMP approximates 5.0 μM (*26*)[2], using eq 3 (*31*), a K_I value of 0.36 ± 0.09 μM for IP-dUMP was calculated, which is comparable to that obtained for the *L. casei* TS (*19*) (K_I = 0.5 μM).

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_{inact}} E - I \qquad (1)$$

$$\frac{1}{k_{obs}} = \frac{1}{k_{inact}} + \frac{K_{I\,app}}{k_{inact}} \cdot \frac{1}{[I]} \qquad (2)$$

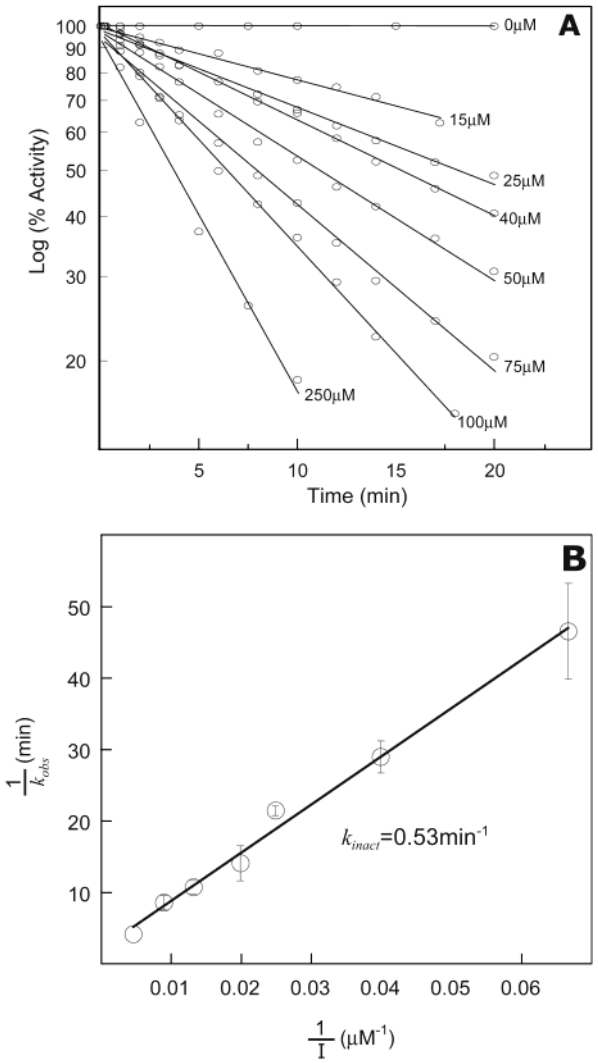


FIGURE 2: Kinetics of inactivation of TS by IP-dUMP. The reactions were initiated by the addition of varying concentrations of IP-dUMP to solutions containing TS in 25 mM potassium phosphate, pH 7.5, at 25 °C. At the indicated times, 20 μL aliquots were removed and assayed for TS activity as described in Materials and Methods. (A) Time and concentration dependence of the loss of activity of TS (0.88 μM) in the presence of the indicated concentrations of IP-dUMP. (B) Plot of $1/k_{obs}$ vs 1/[I] obtained according to Kitz and Wilson (*33*) to yield K_I and k_{inact}. Each data point represents the mean of three independently obtained data sets. The straight line was generated by linear regression (R = 0.996).

$$K_I = \frac{k_{-1}}{k_1} = \frac{K_{I\,app}}{1 + [dUMP]/K_M} \qquad (3)$$

Extent of IP-dUMP Incorporation. ESI-MS was used to identify the increase in mass of WT-TS upon IP-dUMP inhibition. Mass spectrometric analysis of the reaction identified a mass increase for a WT-TS monomer of 412 amu, which is the mass of a single IP-dUMP molecule (Table 1). In contrast, IP-dUMP did not change the mass of the TS

[2] The K_M value reported in this paper of 5 × 10^{-5} M for dUMP was in error and should be 5 × 10^{-6} M.

Table 1: Electrospray Ionization−Mass Spectroscopy of WT-TS, Y94F TS, C146A TS, C146S TS, Trypsin Peptide (54−96), EndoAspN Peptide (89−104), and EndoAspN/EndoLysC Peptide (89−96)

protein or peptide	mass (amu)[a]	mass (amu) + IP-dUMP	Δ mass (amu)[b]
WT-TS	30482	30894	+412[c]
Y94F	30467	30460	NSD[d]
C146A	30443	30445	NSD
C146S	30459	30461	NSD
trypsin (54−96)	4986	5398	+412
endoAspN (89−104)	1872	2284	+412
endoAspN/endoLysC (89−96)	848	1260	+412

[a] The determined masses are in atomic mass units (amu). [b] The mass differences (Δ) between the native and modified protein or peptide. [c] The theoretical mass of IP-dUMP is 412 amu. [d] No significant difference.

mutants Y94F, C146A, and C146S (Table 1). The mass of Y94F was 16 amu less than WT-TS, reflecting the loss of the Tyr hydroxyl group, while the mass of C146A was 32 amu less than the mass of WT-TS due to the loss of the sulfhydryl group, and the mass of C146S was 16 amu less than the mass of WT-TS, which is to be expected for the difference in mass of the sulfhydryl and hydroxyl groups (Table 1) of this mutant. The extent of incorporation of IP-dUMP into WT-TS, as determined by ESI-MS, correlated with the loss in TS activity (data not shown).

No change in the mass of the modified WT-TS was observed upon urea denaturation or DTT reduction. Secondary reaction of the denatured, reduced IP-dUMP-reacted protein with iodoacetamide resulted in the incorporation of 285 amu (data not shown). Since the acetamide group is 57 amu, this mass change represents the modification of all five cysteines in the WT-TS monomer, which eliminates this amino acid as the site of IP-dUMP addition. Because neither C146A nor Y94F can be modified by IP-dUMP, these results indicate that both Cys146 and Tyr94 have a role in the modification of TS by IP-dUMP. Together, the WT and mutant TS data support Tyr94 as the most probable site of modification.

Tyrosine Involvement in Ternary Complex Formation. On the basis of our earlier proposal (*7*) that Tyr94 is involved in the catalytic process in view of its proximity to the C5 hydrogen of dUMP, we present evidence to this effect in Figure 3. As shown in this figure, Tyr94 mutants of TS differed in their ability to form ternary complexes with [6-^{3}H]-dUMP and CH_2H_4PteGlu, as detected by SDS−PAGE. The amount and the stability of ternary complex visualized were dependent on the amino acid substituted for tyrosine (Figure 3), and as shown, the rate and extent of ternary complex formation varied with the amino acid substitution of the mutant proteins. Although a relatively small amount of complex formation for Y94A was seen at 30 s, the complex band density peaked by 30 min. Likewise, a complex band for Y94K, although faintly seen at 30 min, had dissipated by 3 h. No band was seen for the inactive mutant E58A (data not shown) regardless of the incubation time. In contrast, Y94F reacted almost immediately and did not dissipate in strength over the time course of the reaction. This finding differs from the transient complex band formation previously reported for the equivalent mutant from *L. casei* (Y146F) (*9*) or with the Glu60 mutants described earlier (*12*). The

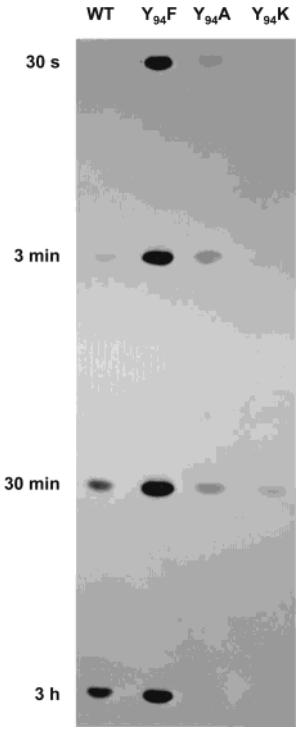


FIGURE 3: Time course of covalent ternary complex formation by WT-TS and its mutants, Y94F, Y94A, and Y94K, with [6-^{3}H]dUMP and CH_2H_4PteGlu. Autoradiograms of SDS−PAGE showing (lane 1) WT-TS, (lane 2) Y94F, (lane 3) Y94A, and (lane 4) Y94K. See Materials and Methods for details.

FIGURE 4: Covalent ternary complex formation with [6-^{3}H]dUMP and different enantiomers of CH_2H_4PteGlu. Autoradiogram of SDS−PAGE showing (lane 1) Y94F plus (*R*,*S*)-CH_2H_4PteGlu, (lane 2) WT-TS plus (*S*)-CH_2H_4PteGlu, (lane 3) WT-TS plus (*R*)-CH_2H_4-PteGlu, (lane 4) WT-TS plus (*R*)- and (*S*)-CH_2H_4PteGlu, and (lane 5) WT-TS.

corresponding Glu58 mutants in *E. coli* TS formed only weak complexes with [6-^{3}H]dUMP, which were even weaker with [5-^{3}H]dUMP due to the higher residual enzyme activity with the *E. coli* mutants. For similar reasons we could not detect the ternary complex of [5-^{3}H]dUMP and Y94F with CH_2H_4PteGlu (data not shown), indicating that while the mechanism of the catalytic process is identical for the *L. casei* and *E. coli* TSs, differences in the rate and extent of complex formation are but a matter of degree. The gradual appearance of an apparent ternary complex in the case of the WT-TS (Figure 3, lane 1) is possibly due to the formation of [6-^{3}H]dTMP-containing complexes that accumulate to a small extent during conversion of [6-^{3}H]dUMP by WT-TS in the course of its normal turnover. This suggests that covalent complex formation is possible between dTMP and *E. coli* TS under the experimental conditions. Indeed, covalent complex formation between dTMP and TS has previously been demonstrated in the case of the *L. casei* enzyme (*32*).

Stereospecificity of TS Folate Binding. Identification of the enantiomer of CH_2H_4PteGlu that binds to TS in the formation of the ternary complex is shown (Figure 4), and as indicated, only (*R*)- and (*R*,*S*)-CH_2H_4PteGlu interact with [6-^{3}H]dUMP and WT-TS or Y94F. No stable ternary complex was seen for the (*S*) enantiomer under the conditions

Table 2: Sequence of Isolated Peptides

Enzyme	Residues	Sequence					
		54	60	70	80		
Trypsin	54-96	SIIHEL	LWFLQGDTNI	AYLHENNVTI	WDEWADENGD	LGPV**Y**GK····	·····
*Endo*AspN	89-104	·····	·····	·····	·····D	LGPV**Y**GKQ$_{WR}$	AWPTP
*endo*AspN /*endo*LysC	89-96	·····	·····	·····	·····D	LGPV**Y**GK····	·····

of SDS−PAGE, although (*S*)-CH_2H_4PteGlu can inhibit (*33*) and quench the fluorescence of *L. casei* TS (*34*), as well as form a complex with TS that is observable by circular dichroism (*35*).

Identification of the IP-dUMP-Bound Residue. Proteolysis, reverse-phase HPLC, ESI-MS, and LC-ESI-MS/MS were used to identify the amino acid residue that was covalently bound to IP-dUMP. Trypsin digestion of IP-dUMP-modified WT-TS yielded a 43-residue peptide (residues 54−96) (Table 2) with a 412 amu mass increase relative to the corresponding native peptide (Table 1). Subsequent LC-ESI-MS/MS of endoAspN digestion of IP-dUMP-inhibited WT-TS and unmodified TS identified a 16-residue peptide (residues 89−104) (Table 2) whose mass was also increased by 412 amu in the former case (Table 1). Purified native and IP-dUMP-modified endoAspN peptides were injected on an analytical C18 reverse-phase column. The native peptide eluted at 23.8 min (Figure 5A), and the modified peptide eluted at 23.0 min (Figure 5B). That the native peptide had been converted to a faster moving peptide by modification was further confirmed by comparison to the elution profile of the co-injected peptides (Figure 5C). Further digestion of the modified endoAspN peptide by endoLysC yielded an 8-residue peptide (residues 89−96) (Table 2) whose mass was increased by 412 amu relative to the corresponding native peptide (Table 1). MS/MS of the endoAspN-modified peptide resulted in fragmentation of the modifier to a form without a phosphate, deoxyribose, and imidazole, before fragmentation of the peptide. Analysis of the modified peptide's daughter ion fragmentation pattern resulted in the identification of multiple b- and y-ions. The smallest mass assigned (*m*/*z* 551.3) correlated to the y-ion of the 3-residue peptide YGK (*m*/*z* 367.2) (residues 94−96) containing the remaining fragment of the modifier (*m*/*z* 148). The sequences of these modified peptides all contained Tyr94 (Table 2).

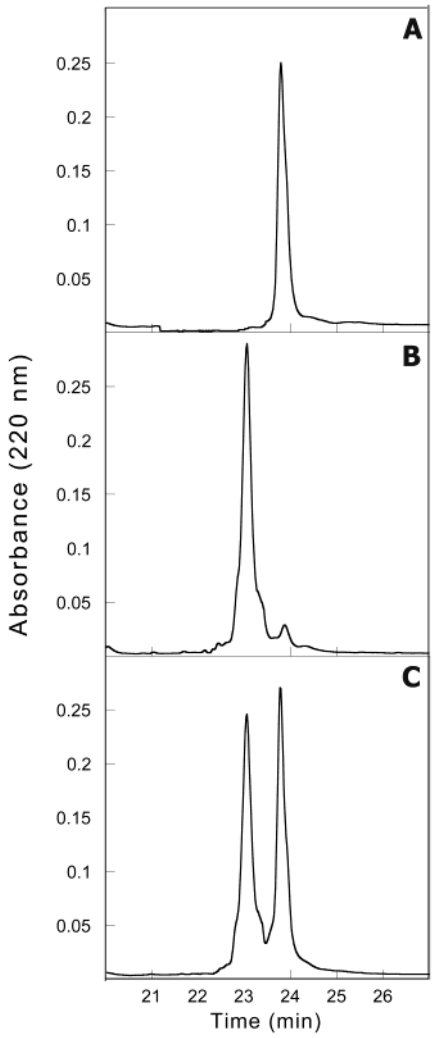


FIGURE 5: HPLC chromatograms of the isolated peptides (residues 89−104) from the endoAspN digest of WT-TS and IP-dUMP-modified WT-TS. (A) The unmodified peptide eluted at 23.8 min and (B) the modified peptide eluted at 23.0 min. (C) Co-injection of modified and unmodified peptide showing two peaks at 23.0 and 23.8 min, respectively.

DISCUSSION

Role of Tyr94 in the Catalytic Mechanism. The crystal structure of *E. coli* WT-TS bound to FdUMP and CH_2H_4-PteGlu suggested that the phenolate anion of Tyr94 functions as a base to abstract the proton from C5 of the dUMP pyrimidine ring either directly (*7*) or indirectly through the intervention of a buried water (*36*). However, it was acknowledged in the former study (*7*) that explaining how Tyr94 was ionized to a phenolate in the absence of a nearby basic residue presented somewhat of a dilemma. That Tyr94 can exist in the anionic form necessary for proton extraction is supported by the report of the covalent attachment of 5-(trifluoromethyl)-2′-deoxyuridylate (CF_3dUMP) to the equivalent tyrosine, Tyr146, in *L. casei* TS (*15*). A mutagenesis study of Tyr146 in *L. casei* further implicated this residue as the base that assists either directly or indirectly in the abstraction of the C5 proton of dUMP (*9*).

As indicated in Hyatt et al. (*7*), three potential bases lie near the 5-F in the FdUMP−TS−CH_2H_4PteGlu ternary complex, Wat_2Glu58, Wat_1Tyr94, and the phenolate anion of Tyr94. All have been proposed as bases capable of abstracting the proton from the 5 position of dUMP (*7*, *9*). In contrast to the structures presented by Liu et al. (*9*) for the corresponding *L. casei* TS ternary complex, the Tyr94 hydroxyl in *E. coli* TS is in van der Waals contact (3.2 Å) with 5-F (*7*). Since FdUMP can form an observable ternary

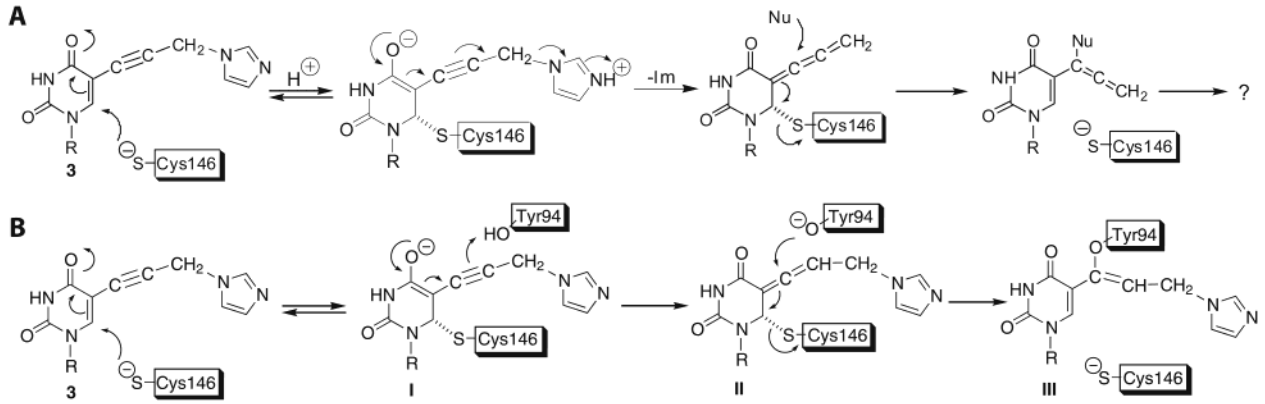


FIGURE 6: Hypothetical mechanisms for the covalent modification of wild-type *E. coli* TS by IP-dUMP. (A) Previously postulated mechanism for the inactivation of TS by IP-dUMP with imidazole release (*20*). (B) Mechanism proposed to account for the retention of the imidazole (obtained in this paper).

complex with WT-TS, similar to that of [5-^{3}H]- or [6-^{3}H]-dUMP with Y94F, it is probable that the reason for the accumulation of the ternary complexes of the substrate is the inability of the mutant proteins to cleave the C−H bond at the 5 position of dUMP. It is therefore likely that the tyrosine phenolate anion is the major base involved directly or indirectly in abstracting the 5-H from dUMP. The fact that replacement of Tyr94 with a Phe reduces *E. coli* TS activity by 500-fold, but does not eliminate it, suggests that the above-mentioned water molecules could substitute to some extent, although weakly, for Tyr94. Confirming the essential role of Tyr94 in promoting the TS reaction is the fact that specific dUMP derivatives can form covalent complexes with this amino acid, as shown in this paper and in an earlier one (*15*). Consequently, Tyr94 should be considered a potential target site in the design of new inhibitors of TS in view of tight binding inhibitors that can interact with this site to form irreversible complexes.

Additional support for the importance of Tyr94 in the catalytic process comes from the fact that mutation of Tyr94 enhances the ability of TS to be visualized as a ternary complex using SDS−PAGE (Figure 3) and that the rate and the extent of ternary complex formation vary with the amino acid substituted for Tyr94. Replacement of the phenolic tyrosine side chain with either an aliphatic alanine (Y94A) or a charged lysine (Y94K) greatly reduced the enzyme's ability to form a ternary complex, indicating that there is either a diminished capacity to form the complex or an enhanced rate of breakdown. In contrast, the conservative substitution of Tyr94 with a Phe (Y94F) enabled the modified enzyme to be trapped as a ternary complex poised for the next step, signifying the rate-determining step in the TS reaction. It was previously reported (*9*) that the ternary complex of *L. casei* Y146F diminished over time, whereas that reported in the present study for *E. coli* Y94F breaks down even more slowly. One difference is that in the earlier study (*9*) [5-^{3}H]dUMP was used for complex formation, whereas our study used [6-^{3}H]dUMP. When [5-^{3}H]dUMP was used with Y94F (*E. coli* TS), we could not detect the complex, probably because Y94F is more active than *L. casei* Y146F and the steady-state levels of [5-^{3}H]dUMP−TS−CH_2H_4PteGlu formed are much lower in the former case. It should be emphasized that Y94F does retain a small amount of activity as measured by tritium release from [5-^{3}H]dUMP (0.25% of WT-TS).

Uniqueness of This Interaction Compared to Other Compounds That Bind to Tyrosine TS. Analogues of dUMP with electron-withdrawing substituents at the 5 position are frequently good inhibitors of TS and often increase the extent of the nucleophilic addition of the catalytic cysteine SH to the pyrimidine ring (*15*, *16*, *37*−*39*). As a result, normally inert substituents at the 5 position have been designed to be converted to chemically reactive species following addition to the active site cysteine. Examples of such nucleotide analogues are 5-ethynyl-dUMP (*16*), (*E*)-5-(2-bromovinyl)-dUMP (*38*), and CF_3dUMP (*15*). The inactivation of TS by 5-ethynyl-dUMP, which has the closest structural resemblance to IP-dUMP, was found to require the presence of the cofactor, CH_2H_4PteGlu (*16*, *40*), but the formation of even this ternary complex was shown to be reversible (*19*, *41*, *42*). In the absence of the cofactor, 5-ethynyl-dUMP serves as a substrate and is converted by TS to reaction products with nucleophiles in the medium (*16*). Analogous alkylation products are formed with external nucleophiles in reactions of (*E*)-5-(2-bromovinyl)-dUMP catalyzed by TS, without inactivation of the enzyme (*38*). Only CF_3dUMP was shown to interact covalently with Tyr146 of *L. casei* TS. However, the resulting complexes were not stable and readily decomposed in the presence of thiols to 5-carboxy-dUMP and free enzyme (*15*), unlike IP-dUMP which forms a stable covalent complex with *E. coli* TS.

It was observed that the rate of inactivation of TS with 15 μM IP-dUMP increases with the pH, proceeding 1.4-fold faster at pH 8.5 than at pH 7.5 and 1.6-fold faster at pH 7.5 than at pH 6.5 (data not shown). The pH dependence of the inactivation rate may be due to the fact that the Tyr-OH is deprotonated to a greater extent at higher pH, enabling the inhibition reaction to proceed at a faster rate. However, it is noted that the pK_a of the imidazole ring of IP-dUMP is around 6.5, and since pH changes affect the protonation states of the functional groups of both the enzyme and the inhibitor, multiple interpretations are possible.

Proposed Mechanism for the Inactivation of TS by IP-dUMP. Acetylenic groups are usually inert toward nucleophiles, but enzymes have been shown to promote their own inactivation [suicide inhibition (*43*)] by generating a carbanion at the α-carbon leading to isomerization to an allene that can potentially alkylate nucleophilic residues at the active site (*44*). IP-dUMP was designed (*17*, *19*) to form the homologous cumulene with an additional reactive center,

which results from elimination of the imidazole as a leaving group, according to the mechanistic hypothesis outlined in Figure 6A. However, the mass spectral data (Table 1) showed that there was no change in the mass of IP-dUMP during the modification reaction, indicating that expulsion of the imidazole, which must accompany cumulene formation, does not occur. Therefore, an alternative mechanism is proposed to account for the results obtained as described in Figure 6B. According to this mechanism, nucleophilic addition of the thiol of Cys146 to the 5,6 double bond of IP-dUMP (**3**) initiates a rearrangement of the alkyne (**I**) to an allene intermediate (**II**), with the probable assistance of proton transfer from Tyr94-OH. The central α-C atom of the allene (**II**) is the most reactive and thus subject to nucleophilic attack by the phenolate of Tyr94. The electron donated by the phenolate ion permits elimination of Cys146 from C6 and simultaneous re-formation of the 5,6 double bond leading to the covalently modified, inactivated enzyme (**III**). Nucleophilic attack by Tyr94 at the *β*-carbon (not shown), which is less reactive, would place a hydrogen on the α-carbon resulting in retention of the double bond between the C5 and the α-carbon, preventing the elimination of Cys146. The ability of Cys146 to incorporate an acetamide after IP-dUMP modification contraindicates Tyr94 attack at the *β*-carbon. This mechanism is supported by the MS/MS identification of a 3-residue modified peptide ion that still retains tyrosine (data not shown). The precise nature of the IP-dUMP-TS adduct will have to await X-ray crystallography and NMR studies, which are currently in progress. These studies may also provide an explanation for the above-discussed intriguing difference between the way TS interacts with IP-dUMP and with the structurally related 5-ethynyl-dUMP.

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