Trapping and Partial Characterization of an Adduct Postulated To Be the Covalent Catalytic Ternary Complex of Thymidylate Synthase†

Mark A. Moore, Faizy Ahmed, and R. Bruce Dunlap*
Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208
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ABSTRACT: The proposed mechanism of action of thymidylate synthase envisages the formation of a covalent ternary complex of the enzyme with the substrate dUMP and the cofactor 5,10-methylenetetrahydrofolate (CH_2H_4folate). The proposed structure of this adduct has been based by analogy on that of the covalent inhibitory ternary complex thymidylate synthase–FdUMP–CH_2H_4folate. Our recent success in using the protein precipitant trichloroacetic acid to trap the latter complex and covalent binary complexes of the enzyme with FdUMP, dUMP, and dTMP led to the use of this technique in attempts to trap the transient putative covalent catalytic ternary complex. Experiments performed with [2-^{14}C]dUMP and [3′,5′,7,9-^{3}H]CH_2H_4folate show that both the substrate and the cofactor remained bound to the protein after precipitation with trichloroacetic acid. The trapped putative covalent catalytic complex was subjected to CNBr fragmentation, and the resulting peptides were fractionated by reverse-phase high-pressure liquid chromatography. The isolated active site peptide was shown to retain the two ligands and was further characterized by a limited sequence analysis using the dansyl Edman procedure. The inhibitory ternary complex, which was formed with [^{14}C]FdUMP and [^{3}H]CH_2H_4folate, served as a control. The active site peptide isolated from the CNBr-treated inhibitory ternary complex was also subjected to sequence analysis. The two peptides exhibited identical sequences for the first four residues from the N-terminus, Ala-Leu-Pro-Pro-, and the fifth amino acid residue was found to be associated with the labeled nucleotides and the cofactor. Sequence analysis of the active site CNBr peptide derived from native enzyme, which had been denatured and treated with iodoacetate, confirmed both the sequence of the first four residues and the fact that the fifth amino acid residue was the carboxymethyl derivative of Cys-198, the active site nucleophilic covalent catalyst. These results conclusively show that the putative covalent catalytic ternary complex of thymidylate synthase has been isolated. This may represent only the second instance in which a covalent catalytic ternary complex intermediate of an enzyme-catalyzed reaction has been trapped.

Thymidylate synthase catalyzes the reductive methylation of dUMP[1] to dTMP, utilizing CH_2H_4folate as the source of the methyl group. In an initial attempt to rationalize the mechanism of this reaction, Friedkin (Friedkin, 1957, 1959; Wahba & Friedkin, 1962), and later Huennekens (1963), proposed an intermediate that involved a covalent linkage between dUMP and CH_2H_4folate. In the proposed adduct, dUMP retains its double bond between C-5 and C-6 and is linked to the N-5 position of the folate via a methylene bridge (Figure 1A). The proposed intermediate *was not* envisaged as being covalent bound to the enzyme. In order to evaluate the viability of the proposed intermediate, a number of groups over the last 20 years have synthesized structurally related chemical models and have determined their tendency to yield thymine on decomposition. Gupta and Huennekens (1967) prepared 5-thyminyl-H_4folate as well as 10-thyminyl-H_4folate and 5,10-dithyminyl-H_4folate. When heated at 100 °C for 30 min, 5-thyminyl-H_4folate was found to yield no thymine and 10-thyminyl-H_4folate decomposed to folate and (hydroxymethyl)uracil. Wilson and Mertes (1972, 1973) tested 1,2-dihydro-*N*-thyminylquinoline (Figure 1B) as a model for the proposed intermediate and found that heating a neat sample to 205 °C resulted in quinoline (49%) and thymine (42%). Charlton and Young (1980) prepared 5,10-dithyminyl-H_4folate and 5-thyminyl-10-methyl-H_4folate and showed that their pyrolysis yielded 39% and 47% yields of thymine, respectively. Finally, van der Meij et al. (1985) have shown that the reaction of a 6-aminouracil derivative with imidazolidines (models of CH_2H_4folate) (Figure 1C) can react under acidic conditions to yield an exocyclic methylene intermediate that can be trapped with nucleophiles or reduced to a thymine derivative.

In a related approach structural analogues of the proposed intermediate (Figure 1A), termed multisubstrate inhibitors, were tested as inhibitors of thymidylate synthase. Park et al. (1979) observed that a tetrahydroquinoxalyl derivative of dUMP (Figure 1D) was a potent inhibitor (K_i = 0.75 μM) and was 50 times more effective than the piperazinyl derivative with the *Lactobacillus casei* enzyme. Maggiora et al. (1983) studied 5-*p*-benzoquinonyl-2′-deoxyuridine 5′-phosphate and found it to be a potent inhibitor (K_i = 2 μM) of the latter enzyme. Recently, Srinivasan et al. (1984) synthesized an 8-deaza-H_4folate derivative of dUMP (Figure 1E) and demonstrated that it was an excellent inhibitor of the thymidylate synthase from HeLa cells. The compound showed competitive inhibition with respect to both dUMP (K_i = 0.06 μM) and CH_2H_4folate (K_i = 0.25 μM). Although these synthetic analogues of the proposed reaction intermediates interact tightly with thymidylate synthase, it is not known if the enzyme binds them covalently or if the enzyme can catalyze their

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[1] Abbreviations: dUMP, deoxyuridylate; FdUMP, 5-fluoro-2′-deoxyuridylate; dTMP, thymidylate; dU, deoxyuridine; FdU, 5-fluorodeoxyuridine; dT, thymidine; H_4folate, (±)-tetrahydrofolate; CH_2H_4folate, 5,10-methylenetetrahydrofolate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; Gdn-HCl, guanidine hydrochloride; PITC, phenyl isothiocyanate; EtOAc, ethyl acetate; CNBr, cyanogen bromide; CmCys, (carboxymethyl)cysteine; Tris, tris(hydroxymethyl)aminomethane; NADPH, reduced nicotinamide adenine dinucleotide phosphate; HPLC, high-pressure liquid chromatography.

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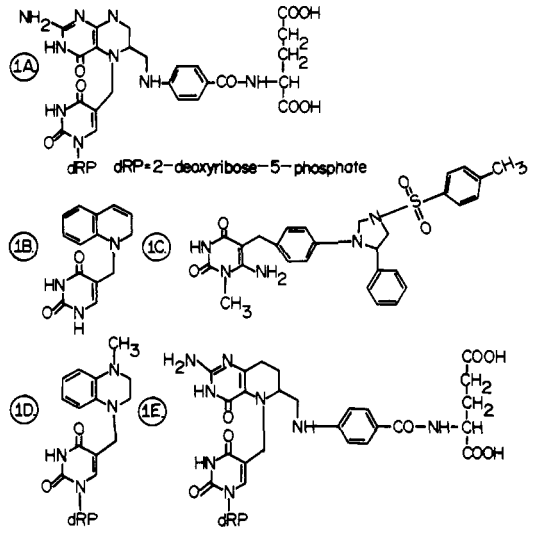
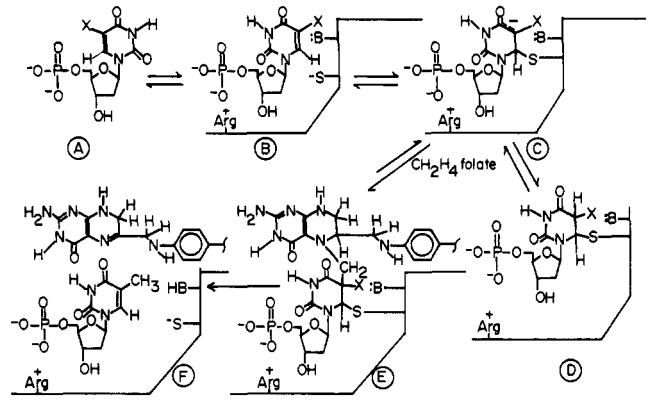


FIGURE 1: Proposed intermediate in the thymidylate synthase reaction and analogues thereof: (1A) Friedkin intermediate; (1B) 1,2-dihydro-*N*-thyminylquinoline; (1C) a C-5 adduct of 1-methyl-6-aminouracil with derivatized imidazolidine; (1D) 5-[(4-methyl-1,2,3,4-tetrahydroquinoxalyl)methyl]deoxyuridylate; (1E) *N*-5-thyminyl-8-deazatetrahydrofolate.

breakdown to form products. While the model system studies and investigations with multisubstrate inhibitors have enabled greater understanding of this system, the assumption inherent in this approach is that the Friedkin intermediate exists as a free-standing entity in the mechanistic pathway of thymidylate synthase. However, as developed below, thymidylate synthase is known to interact covalently with a number of nucleotides that are mechanism-based inhibitors, thus raising the question of the involvement of covalent catalysis in the enzyme-catalyzed synthesis of thymidylate. Therein lies a serious mechanistic question: that is, in the course of catalysis is the enzyme covalently associated with an adduct of dUMP and CH_2H_4-folate that resembles the Friedkin intermediate?

In addressing this question, it is important to review studies of the interactions of thymidylate synthase with various nucleotides. The inhibition of thymidylate synthase has been observed [for review, see Santi (1981) and Lewis and Dunlap (1981)] with a variety of dUMP analogues, bearing the following substituents at C-5: CH_2OH, I, Br, Cl, CF_3, CHO, NO_2, CN, or F. The evidence points toward the covalent interaction of these inhibitors via attack of the active site cysteine-198 at the C-6 position of these nucleotides (see structure D of Scheme I) [for reviews, see Santi (1981), Lewis and Dunlap (1981), and Maley and Maley (1981)]. In fact, ^{19}F NMR studies (Lewis et al., 1980) have demonstrated that FdUMP can bind to the enzyme in a covalent manner in the absence of any folate. Recent studies (Moore et al., 1984) (see structure D of Scheme I) involving the trichloroacetic acid dependent isolation of this covalent binary complex have provided chemical confirmation of this result. Also, this method has been used to isolate and to determine the stoichiometry of the covalent binary complexes between thymidylate synthase and either dUMP or dTMP, again in the absence of any folate (Moore et al., 1986). Sequence analysis of the corresponding CNBr active site peptides showed that the nucleotides were attached to the active site cysteine (Moore et al., 1986). In fact, the C-6 position of the pyrimidine nucleotides has been shown to be susceptible to nucleophilic attack by bisulfite or glutathione [for reviews, see Santi (1981) and Lewis and Dunlap (1981)]. Accordingly, the modification of sulfhydryl groups on thymidylate synthase inactivates the enzyme (Plese & Dunlap, 1977).

Scheme I



The interaction of folates with the binary complexes of enzyme with nucleotides has been documented [for review, see Lewis and Dunlap (1981)]. ^{19}F NMR studies (Lewis et al., 1981), equilibrium dialysis experiments (Galivan et al., 1976), filter binding assays (Santi et al., 1974), and the TCA precipitation method (Moore et al., 1986) have all demonstrated the enhancement of FdUMP binding to thymidylate synthase in the presence of folates. The equilibrium dialysis studies also indicate a greater affinity of the enzyme for folate in the presence of nucleotide. The enhancement of dUMP binding to the enzyme in the presence of folates has been shown by a filter binding assay (Lockshin & Danenberg, 1979), equilibrium dialysis (Galivan et al., 1976), and the TCA precipitation method (Moore et al., 1986).

The most structurally significant enzyme adduct examined to date is the covalent inhibitory complex between CH_2H_4-folate, FdUMP, and thymidylate synthase (Santi, 1981; Danenberg, 1977; Lewis & Dunlap, 1981; Maley & Maley, 1981). The structure of this complex (structure E in Scheme I, where X is F) has been shown to involve the covalent attachment of Cys-198 of the enzyme to the 6-position of FdUMP and also a methylene bridge between FdUMP and the cofactor (Maley et al., 1979; Bellisario et al., 1979). This complex is widely believed to be structurally analogous to the proposed catalytically competent covalent ternary complex between CH_2H_4folate, dUMP, and thymidylate synthase (structure E in Scheme I, where X is H). Thus, in effecting the conversion of dUMP to dTMP, thymidylate synthase would be covalently linked to dUMP which in turn is covalently bound to CH_2H_4folate.

Presumably, the proposed transient nature of such an intermediate has discouraged attempts to isolate and study it. The recent development of the trichloroacetic acid precipitation method (Moore et al., 1984) for the isolation of covalent complexes of thymidylate synthase, however, has provoked such an attempt. We report herein the isolation and partial characterization of a covalent ternary complex between CH_2H_4folate, dUMP, and thymidylate synthase that we postulate to be the catalytically competent intermediate shown in Scheme I (structure E, where X is H).

Experimental Procedures

Materials. Thymidylate synthase was purified from *L. casei* in the presence of exogenous thiol (10 mM β-mercaptoethanol) according to the method of Lyon et al. (1975). Prior to use, the enzyme was activated by dialysis for at least 18 h at 5 °C against a buffer containing 100 mM Tris, 100 mM KCl, and 50 mM β-mercaptoethanol, pH 7.3.

[6-^{3}H]FdUMP, [2-^{14}C]FdUMP, [2-^{14}C]dUMP, and [3′,5′,7,9-^{3}H]folic acid were obtained from Moravek Biochemicals. [6-^{3}H]dUMP was obtained from Schwarz-Mann.

The radiolabeled compounds were normally diluted with the corresponding unlabeled nucleotide or nucleoside prior to use (obtained from Sigma). Folic acid and CNBr were also obtained from Sigma Chemical Co. TCA was obtained from J. T. Baker Chemical Company. Materials employed for sequence analysis, including trifluoroacetic acid, dansyl chloride, phenyl isothiocyanate, pyridine, 6 N HCl, and polyamide sheets, were obtained from Pierce Chemical Co. Tritiated (−)-H_4folate was prepared following the procedure of Horne et al. (1977). This method involves the addition of NADPH and ascorbate to a [3′,5′,7,9-^{3}H]folic acid solution, followed by the addition of dihydrofolate reductase. Formaldehyde was included in the reaction mixture if (+)-CH_2H_4folate was desired. After a designated reaction time, the mixture was applied to a Bio-Gel TSK-DEAE-5-PW HPLC column for purification using a linear gradient (1 mL/min) from 5 mM Tris and 50 mM β-mercaptoethanol, pH 7.2, at injection to 500 mM NaCl, 5 mM Tris, and 50 mM β-mercaptoethanol, pH 7.2, at 50 min.

Trichloroacetic Acid Precipitation Procedure. Reaction mixtures (0.5 mL) were prepared in microcentrifuge tubes to contain 1 μM thymidylate synthase, 100 mM Tris, and 100 mM KCl, pH 7.3. Also added were the appropriate quantities of nucleotide and folate. After the reaction mixture was incubated for the appropriate time at room temperature, a TCA solution was added to make the final concentration 10% in TCA. The addition of TCA both denatured and precipitated the protein. The tubes were then placed in an Eppendorf microfuge and centrifuged for 2–3 min to pellet the precipitated protein on the side of the tube. The precipitate was then rinsed at least 4 times with a 10% TCA solution to remove any nucleotide or folate that was not covalently attached to the enzyme. The precipitate was solubilized by using 3 × 100 μL of a 0.2 N solution of NaOH in 50% ethanol and transferred to a scintillation vial. Ten milliliters of Ready-Solv HP scintillation fluid (Beckman) was added, and the samples were counted in a Beckman LS 7500 scintillation counter. The resulting dpm values permitted calculation of the quantity of bound ligand, which was then divided by the amount of protein in the original reaction mixture to determine the covalent binding stoichiometry.

The procedure described here is similar to that reported in previous articles (Moore et al., 1984, 1986). In those articles we noted several control experiments for the assay. No detectable binding of FdUMP, dUMP, or dTMP was observed in the presence of *N*-ethylmaleimide-modified enzyme. Also, no binding of FdU, dU, or dT was noted with unmodified thymidylate synthase in the presence or absence of folates. These controls suggest that only covalently bound forms of the nucleotides are precipitated with the enzyme. This was confirmed by protein cleavage and separation and identification of the active site peptide to which radiolabeled nucleotide was bound (Moore et al., 1986).

Isolation of Ternary Complexes and Cleavage with CNBr. The inhibitory ternary complex was generated by incubating enzyme (1 mg, 13.7 nmol) with a 10-fold excess of [2-^{14}C]-FdUMP (137 nmol) and a 20-fold excess of [3′,5′,7,9-^{3}H]-(+)-CH_2H_4folate (274 nmol) for 1 h at 25 °C in a 0.5-mL reaction mixture containing 100 mM KCl and 50 mM β-mercaptoethanol, pH 7.3. The reaction was quenched by the addition of 0.5 mL of 20% TCA, and the precipitated protein was isolated by centrifugation for 10 min at 15 000 rpm. The precipitate was then dissolved in a minimal amount of 70% formic acid, and 2 mL of 6 M Gdn-HCl and 1 M Tris-HCl, pH 8.0, was added. The pH was adjusted to 8.0 before the addition of 14.3 mg (70.8 μmol) of tryptophan and 7.1 mg (40.34 μmol) of iodoacetic acid. After 20 min the reaction was stopped by the addition of 0.1 mL of β-mercaptoethanol. A TCA solution was added to make the final concentration 10% in TCA. The solution was centrifuged for 10 min at 15 000 rpm and the supernatant decanted. The precipitate was washed 3 times with water to remove any Gdn-HCl. The *remaining protein* precipitate was dissolved in 70% formic acid containing 0.58 mg of CNBr (5.5 μmol, a 400-fold excess with respect to protein). The reaction was allowed to proceed at room temperature for 18 h whereupon 10 mL of water was added, and the mixture was lyophilized.

In an effort designed to trap an adduct of the enzyme with its substrates, enzyme (1 mg, 13.7 nmol) was incubated with 100-fold excesses of [2-^{14}C]dUMP (1.37 μmol) and [3′,5′,7,9-^{3}H]-(+)-CH_2H_4folate (1.37 μmol) in 100 mM Tris, 100 mM KCl, and 50 mM β-mercaptoethanol, pH 7.3 at 25 °C. After 2 s of incubation, the reaction mixture (0.5 mL) was quenched with 0.5 mL of 20% TCA and was centrifuged to isolate the precipitated protein. The same procedure used to generate CNBr fragments of the inhibitory ternary complex was applied to the isolated protein.

In order to prepare a carboxymethylated enzyme derivative in the absence of ligands, native enzyme (1 mg) was chromatographed on a 2.5 × 30 cm Sephadex G-25 column equilibrated and eluted with 100 mM Tris and 100 mM KCl, pH 7.3, in order to remove β-mercaptoethanol. The dethiolated enzyme was subjected to modification with iodoacetic acid in the presence of 4 M Gdn-HCl, and the carboxymethylated protein was precipitated with TCA and processed as described above to yield CNBr fragments. The carboxymethylation and cyanogen bromide cleavage procedures described here were adaptations of methods cited in Maley et al. (1979).

Separation of CNBr Cleavage Products by Reverse-Phase HPLC. The lyophilized CNBr fragments were extracted twice with 1 mL of 50% acetic acid, and the extracts were dialyzed against 0.1% TFA in water. After the complete removal of acetic acid, which takes about 1 h, the extract was loaded on a 30 × 0.4 cm Varian Protein-Pak C_{18} column. Separation of the CNBr fragments was effected by using two Waters 510 pumps interfaced with a Waters automated gradient controller. A nonlinear gradient of 0.1% TFA in water to 70% CH_3CN/30% water containing 0.05% TFA was employed as shown in Figure 3 to ensure the separation of the labeled peptide. Aliquots of the separated peptides were counted to identify the labeled peptide.

Amino Acid Sequence Analysis of the Active Site Peptide (CNBr-4). The nomenclature used to identify the active site peptide as CNBr-4 is based on the work of Maley et al. (1979), who found that this peptide was the fourth CNBr peptide from the N-terminus of the protein. The dansyl-Edman procedure was employed to sequence the first five N-terminal amino acids of the purified CNBr-4 (Burton & Hartley, 1970; Gray & Smith, 1970). About 5 nmol of the peptide was dissolved in 0.5 mL of deionized water and 100 μL dispensed into each of five Pyrex glass reaction tubes (0.8 × 7 cm) which were numbered 0–4. After drying the samples in a SpeedVac (Savant), tube 0 was set aside for dansylation. To each of the remaining tubes was added 50 μL of a freshly prepared coupling mixture (30 μL of 5% v/v PITC in pyridine and 5 μL of trimethylamine in 20 μL of deionized water). The tubes were flushed with nitrogen, covered with Teflon tape (Dupont), and incubated at 50 °C for 1 h. The tubes were then transferred to a heat block (Pierce) contained in a heated desiccator

(70 °C) and dried under vacuum. After ensuring that the samples were thoroughly dried, 50 μL of anhydrous TFA was added to each tube, after which the tubes were incubated at 70 °C for 15 min without covering. Following this cleavage reaction, the TFA was removed under vacuum. At the end of this first cycle of coupling and cleavage, tube 1 was set aside for dansylation. The remaining three tubes in order were subjected to 2, 3, and 4 cycles of coupling/cleavage, respectively. The residues remaining in the four tubes (1–4) were dissolved in 40 μL of water from the aqueous layer of a water/EtOAc mixture (1:1 v/v) and extracted with 200 μL of EtOAc 3 times from the same mixture. The EtOAc extracts were pooled and counted. To the remaining aqueous phase in tubes 1–4 and tube 0, 15 μL of 0.2 M $NaHCO_3$ was added, and the samples were dried under vacuum. The residues were redissolved in 15 μL of deionized water, and the pH of the solutions was checked on 1-μL aliquots by using pH indicator paper. After ensuring that the pH of the solutions was >7.5, 15 μL of a solution of dansyl chloride in acetone (3 mg/mL) was added to each tube, and the tubes were covered with Teflon tape and incubated at 50 °C for 1 h. After the reaction mixture was dried under vacuum, 100 μL of constant boiling HCl, 1 μL of thioglycolic acid, and 20 μL of a 5% solution of phenol were added to each, the tubes were sealed and incubated at 105 °C for 18 h. At the end of hydrolysis, HCl was removed under vacuum, and the residue was extracted twice with 200 μL of EtOAc. The combined extracts (400 μL) from each sample were evaporated to dryness and redissolved in 50 μL of EtOAc. The dansylated end-group amino acid in each tube was characterized by two-dimensional TLC on polyamide sheets, (7.5 × 7.5 cm) using (1) water/90% formic acid (200:3 v/v) and (2) benzene/acetic acid (9:1 v/v) (Woods & Wang, 1967). The dansyl amino acid spots were visualized under UV light and identified by comparison with standard dansyl amino acids. The spots were then cut out, extracted with 200 μL of EtOAc, and counted in 10 mL of scintillation fluid by using a Beckman LS7500 liquid scintillation counter.

In a second set of experiments about 5 nmol of the CNBr-4 peptide was subjected to four cycles of Edman degradation. After extraction with water/EtOAc at the end of four cycles, the remaining peptide was subjected to one more Edman cycle and extracted. The EtOAc extracts of the first four cycles and the fifth cycle were counted separately in 10 mL of scintillation fluid.

Results

Trichloroacetic Acid Precipitation Assay for the CH_2H_4-folate–FdUMP–Thymidylate Synthase Inhibitory Ternary Complex. In order to determine if the thymidylate synthase used in these studies would exhibit typical binding characteristics, an assay was performed for the well-characterized inhibitory ternary complex of the enzyme with FdUMP and CH_2H_4folate. All of the enzyme used in this work uniformly gave a binding stoichiometry of 1.5–1.7 mol of FdUMP bound/mol of enzyme for this complex, which is consistent with the results of other methods (Santi, 1981; Danenberg, 1981; Lewis & Dunlap, 1981; Maley & Maley, 1981; Moore et al., 1984).

Trichloroacetic Acid Precipitation of a Putative Covalent CH_2H_4folate–dUMP–Thymidylate Synthase Catalytic Ternary Complex. In an initial attempt to trap a covalent catalytic intermediate, an experiment was designed that utilized [6-^{3}H]dUMP, unlabeled CH_2H_4folate, and thymidylate synthase. The enzyme and a 100-fold excess of labeled dUMP were incubated for 15 min, allowing the formation of a binary

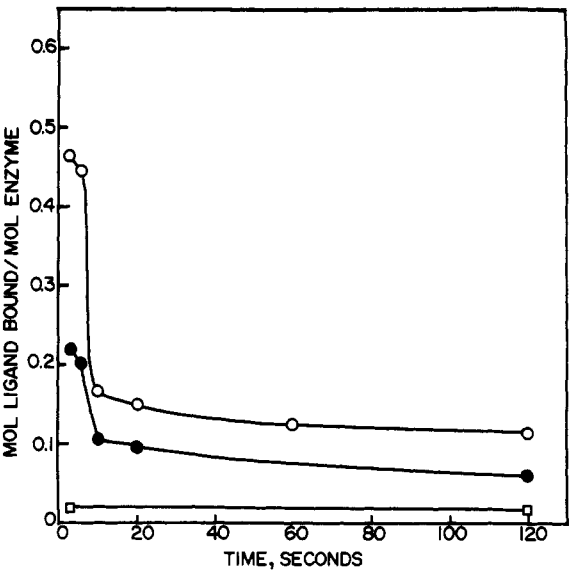


FIGURE 2: Plot of moles of covalently bound ligand per mole of enzyme as a function of time of incubation. In addition to enzyme, reaction mixtures contained either (1) 100-fold excesses of [6-^{3}H]dUMP and CH_2H_4folate (○), (2) 100-fold excesses of dUMP and [3′,5′,7,9-^{3}H]-(+)-CH_2H_4folate (●), or (3) 100-fold excesses of dUMP and [3′,5′,7,9-^{3}H]H_4folate (□).

complex, thereby "loading the system" for catalysis. At the point just before CH_2H_4folate addition, the *covalent* binary complex ratio for dUMP was 0.15 mol/mol of enzyme as determined by a TCA assay. At this point, a 100-fold excess of CH_2H_4folate was added to the various separate binary complex reaction mixtures, and the reactions were allowed to proceed for the designated times (Figure 2). The reactions were stopped by the addition of TCA, and the samples were worked up as described under Experimental Procedures. As seen in Figure 2, the amount of dUMP covalently attached to the enzyme a few seconds after the addition of CH_2H_4folate is nearly 0.5 mol/mol of enzyme (as previously mentioned, control experiments without enzyme or with nucleoside and enzyme show insignificant nonspecific binding in the presence of CH_2H_4folate). This ratio rapidly decreases to a value of less than 0.2 within 10 s after mixing and levels off at about 0.1 within 2 min. The radiolabeled nucleotide observed to be attached to the enzyme could most probably reflect the presence of one or more of at least three different native enzyme complexes: (A) a covalent dUMP–thymidylate synthase complex (either by itself or in association with noncovalently bound folate), (B) a covalent enzyme–dUMP–CH_2H_4folate catalytic ternary complex, or (C) a covalent enzyme–dTMP complex or its exocyclic methylene form (either by itself or in association with a noncovalently bound folate). In fact, according to the proposed mechanism (Scheme I) and on the basis of work on covalent dUMP and dTMP binding (Moore et al., 1986), it would seem likely that the reaction mixture would contain varying levels of *each* of these complexes at different times. The amount of each of these complexes present at any instant after mixing is a complex function of the pertinent microscopic equilibrium constants, rate constants, and concentrations of the various components. It is especially pertinent to note here the results of an experiment (Moore et al., 1986) in which the presence of H_2-folate or H_4folate produced a 10–15-fold enhancement in the stoichiometry of covalent binding of dUMP to thymidylate synthase after equilibration with the folate for 1 h. Similarly, in the results of the experiment reported here in Figure 2, the amount of dUMP covalently attached to the enzyme increases

Table I: Comparison of the Isolation and Sequence Analysis of CNBr-4 Derived from Doubly Labeled Inhibitory and Catalytic Ternary Complexes

	inhibitory ternary complex (total dpm × 10^{-3})			catalytic ternary complex (total dpm × 10^{-3})		
	^{3}H cofactor	[^{14}C]-FdUMP	molar ratio[a] CH_2H_4-folate/FdUMP	^{3}H cofactor	[^{14}C]-dUMP	molar ratio[a] CH_2H_4-folate/dUMP
suspension of CNBr fragments in 50% AcOH	322	457	0.81	67	117	0.57
50% AcOH, extract	326	465	0.806	39	73	0.63
after dialysis vs. 0.1% TFA	202	416	0.53	30	46	0.65
CNBr-4	139	330	0.49	21	33	0.64
Edman extracts 1–4	38	67	0.63	6	6	0.83
Edman extract 4	99	208	0.55	14	20	0.66

[a] The moles of covalently bound ligand were computed from the specific radioactivities of the individual radiolabeled ligands.

dramatically on the addition of CH_2H_4folate (the ratio was 0.15 before its addition) and then, in contrast, decreases rapidly. *The low stoichiometry of nucleotide binding after 10 s is most easily explained by the conversion dUMP to the product, dTMP*, which does not bind as well at the nucleotide to enzyme ratios used. The initial high stoichiometries reported here (nearly 0.5) clearly reflect the enhancement of covalent dUMP binding produced by the presence of folates (Moore et al., 1986). At this point what is uncertain is whether at least some of the covalently bound dUMP is also covalently attached to the cofactor in accordance with the mechanism proposed for the formation of the catalytic ternary complex (see structure E in Scheme I, where X is H).

To test whether the proposed covalent catalytic *ternary* complex could be isolated, the experiment was repeated with 100-fold excesses of radiolabeled (+)-CH_2H_4folate and unlabeled dUMP. Also, to avoid any question on "loading" the enzyme with dUMP prior to the addition of CH_2H_4folate, the enzyme was added last. The *inhibitory* ternary complex formed with FdUMP and radiolabeled CH_2H_4folate rapidly formed and maintained a high binding stoichiometry (1.5) as expected (data not shown). Figure 2 shows the results of a set of controls using dUMP and radiolabeled H_4folate which demonstrated insignificant nonspecific retention of the folate by the protein. It should be noted here that, while unbound nucleotide is easily washed away by the TCA washes of the precipitated protein, folates tend to precipitate under these conditions and are more difficult to solubilize. In the data points that represent mixtures of dUMP, enzyme, and labeled CH_2H_4folate (Figure 2), however, a substantial amount of radiolabel is retained in the TCA precipitates when compared to the controls, thus indicating that the CH_2H_4folate is covalently bound to the enzyme in the presence of dUMP. A control experiment was performed whose results showed that CH_2H_4folate did not exhibit significant binding to the enzyme in the absence of dUMP or in the presence of dU or dTMP (data not shown).

The results of the experiments described, when taken together, provide evidence that a covalent ternary complex between thymidylate synthase, the substrate, dUMP, and the cofactor, CH_2H_4folate, has been isolated. If the isolated complex is, in fact, the proposed covalent catalytic intermediate (structure E in Scheme I, where X is H), then sequence analysis of the appropriate CNBr peptide should demonstrate that the radiolabeled cofactor and dUMP are covalently attached to Cys-198.

In an effort to obtain further evidence for the existence of the proposed catalytic ternary complex, 1 mg of enzyme was incubated with 100-fold excesses of [2-^{14}C]dUMP and [3′,5′,7,9-^{3}H]-(+)-CH_2H_4folate, and the mixture was quenched with TCA after 2 s. Also, double-labeled *inhibitory* ternary complex was formed from a mixture of 1 mg of enzyme and 10-fold excesses of [2-^{14}C]FdUMP and tritiated cofactor and isolated by the TCA method. As described under Experimental Procedures, both doubly labelled complexes were then carboxymethylated and subjected to CNBr cleavage.

Separation and Sequence Analysis of CNBr Peptides. The lyophilized CNBr peptide were found to be largely insoluble in 0.1% TFA and hence unsuitable for direct HPLC separation. Previous work by Maley et al. (1979) demonstrated the differential solubility of the peptides in varying concentrations of acetic acid. Accordingly, as shown in Table I, extraction of the lyophilized CNBr fragments with 50% acetic acid led to the appearance of between 60% and 100% of the radiolabel (^{14}C and ^{3}H) in the extracts derived from the inhibitory and proposed catalytic ternary complexes. Analysis of the dpm in these extracts revealed a folate cofactor to nucleotide ratio of about 0.8 for the extracts derived from the inhibitory ternary complex while a ratio of 0.5 was found for the catalytic ternary complex. As discussed above, these ratios are not unexpected. Prior to HPLC separation, the extracts were dialyzed against 0.1% TFA. At this point it was noticed that there was a substantial decrease in the dpm associated with both dUMP and CH_2H_4folate (see Table I), thus reflecting a loss of both ligands from the peptide fragments. Perhaps the lengthy exposure of the complexes and their peptide fragments to the acidic conditions of CNBr cleavage, extraction, and dialysis may result in the release of the ligands. Since in both ternary complexes, the enzyme is covalently attached to a nucleotide which is in turn covalently bound to the cofactor and because the tritium label is found at carbons 3′, 5′, 7, and 9 in the reduced folate molecule, in the event of breakdown of these ternary complexes, it is likely that tritium label from the folate component or some segment thereof would be lost initially, thus lowering the ratio of ligands.

The dialyzed CNBr fragments obtained from the doubly labeled inhibitory ternary complex were separated on a reverse-phase HPLC column, yielding the profile shown in Figure 3. As reported in Table I, the CNBr peptide identified by the arrow in Figure 3 contained ~70% of the injected radiolabels. The remaining radiolabels eluted in peaks A and B as shown in Figure 3. Further, separation of the dialyzed CNBr peptides resulting from the trapped catalytic ternary complex bearing ^{14}C and ^{3}H labels yielded a similar pattern with the radiolabels (~70%) eluting at the same position as described above (see Figure 3). Finally, the HPLC separation of the dialyzed CNBr peptides resulting from cleavage of denatured and carboxymethylated protein gave a pattern quite similar to those obtained for the ternary complexes.

The CNBr-4 peptide derived from denatured and carboxymethylated enzyme (indicated by the arrow in Figure 3) was subjected to dansyl Edman analysis and yielded a sequence

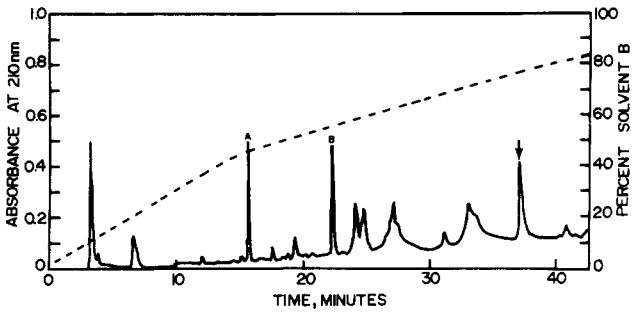


FIGURE 3: Typical profile of separation of the CNBr fragments of thymidylate synthase. A reverse-phase Protein-Pak C_{18} column, 300 × 4 mm (Varian), was used. A nonlinear gradient starting with 0.1% TFA (solvent A) to 70% CH_3CN/30% water in 0.05% TFA (solvent B) was employed, as indicated by the dashed line. Flow rate was 1 mL/min with a back pressure of 700 psi. The peak indicated by the arrow contained CNBr-4. Scintillation counting of each of the peaks revealed that this peptide contained most of the radiolabeled cofactor (^3H) and nucleotide (^{14}C) following CNBr cleavage. The remaining ^{14}C counts (associated with the nucleotide) were found in peak A, and the ^3H counts (derived from the cofactor) eluted in peak B.

of Ala-Leu-Pro-Pro-CmCys for the first five residues from the N-terminus. These results are identical with those reported by Bellisario et al. (1976) and Maley et al. (1979) and confirmed that the peptide under investigation was CNB-4 which contains the active site Cys-198 as the fifth residue from the N-terminus. Similarly, corresponding CNBr-4 peptides isolated from the cleavage of the inhibitory and catalytic ternary complexes were subjected to the dansyl Edman procedure and yielded a N-terminal sequence of Ala-Leu-Pro-Pro-(X). Again, this sequence is identical with that reported by Bellisario et al. (1976) and Maley et al. (1979) for the analysis of the active site CNBr peptide derived from CNBr cleavage of the inhibitory ternary complex. Unfortunately, it was not possible to recover the dansylated fifth amino acid with the radiolabeled ligands still attached to it. The drastic conditions of hydrolysis employed to cleave the dansylated end-group amino acid residue are also very likely to cleave the bound ligands. To overcome this problem and to test whether the fifth N-terminal residue (Cys-198) is attached to the ligands, we subjected the CNBr-4 peptides derived from the two ternary complexes to four continuous Edman cycles followed by extraction of the products. The peptide resulting from this procedure was subjected to a fifth Edman cycle and extraction. The extracts of the first four cycles, which would correspond to the PITC derivatives of the first four amino acid residues, contained 20–27% of the radiolabel (^{14}C and ^3H) initially present in CNBr-4 (see Table I). The extract of the fifth cycle, which should correspond to the PITC of Cys-198 bearing the attached ligands, exhibited 60–70% of the total radioactivity present before sequence analysis was initiated (Table I). The results of this experiment clearly indicated that the fifth N-terminal amino acid residue (Cys-198) indeed contained the bound ligands. The release of about 20% of the radioactivity in the early cycles of Edman degradation may be due to the high temperature and acid conditions employed in the cleavage process. Similar observations were reported by Bellisario et al. (1976) in the sequence analysis of CNBr-4 derived from the inhibitory ternary complex of enzyme, FdUMP, and cofactor.

Discussion

This paper provides chemical and physical evidence supporting the idea that thymidylate synthase forms a covalent catalytic ternary complex with its substrates, dUMP and CH_2H_4folate. Rapid quenching of ternary complex mixtures (dUMP + CH_2H_4folate + enzyme) containing radiolabeled dUMP and/or radiolabeled CH_2H_4folate with TCA provided data supporting the isolation of an enzyme ternary complex incorporating covalently bound dUMP and CH_2H_4folate. Subsequent isolation and sequence analysis of the active site CNBr-4 peptide demonstrated that both ligands were covalently associated with Cys-198, whose sulfhydryl group is known to function as a nucleophile in the active site of the enzyme. Although our present results do not permit the complete assignment of the structure of the ternary complex derived from enzyme, dUMP, and CH_2H_4folate, they are completely consistent with the structure of the putative covalent catalytic ternary complex shown in structure E in Scheme I (where X is H) which was predicted by analogy with the structure of the inhibitory ternary complex (structure E in Scheme I, where X is F). Further characterization of the structure of the isolated complex utilizing spectroscopic and chemical techniques is now being performed. We are also in the process of optimizing the conditions for isolation of the putative catalytic ternary complex. The data presented in Figure 2 suggest that a higher covalent binding ratio of dUMP and CH_2H_4folate to enzyme may be obtained at shorter reaction times, thus necessitating studies using chemical quenching under stop-flow mixing conditions. Parameters such as temperature and levels of substrates must also be optimized. With regard to the rapid decrease in covalent binding of dUMP or CH_2H_4folate to the enzyme observed within a few seconds after mixing (Figure 2), it is important to note that only enough substrate and cofactor molecules were present for at most 100 catalytic cycles per enzyme dimer or actually only 50 per active site if both sites on the enzyme dimer were fully catalytically active under these conditions. Perhaps much larger excesses of substrates will facilitate the isolation of catalytic ternary complexes with substantially higher covalent binding stoichiometries.

In his thought-provoking book, Spector (1982) has suggested that all enzymes employ covalent catalysis in their reaction mechanisms and thus interact through a covalent bond in at least one of the intermediates on the reaction pathway. It is thus especially interesting to note that, among the hundreds of known enzymes that feature a ternary complex in the reaction pathway, only one other example of the isolation of a *covalent* ternary catalytic complex has been reported, namely, that involving the enzyme, 3-hydroxy-3-methylglutarylcoenzyme A synthetase (Miziorko et al., 1976; Miziorko & Lane, 1977).

Registry No. 1E (isomer 1), 92008-00-9; 1E (isomer 2), 92008-01-0; dUMP, 964-26-1; CH_2H_4folate, 3432-99-3; thymidylate synthase, 9031-61-2.

References

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

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

Burton, C. J., & Hartley, B. S. (1970) *J. Mol. Biol. 52*, 165–178.

Charlton, P. A., & Young, D. W. (1980) *J. Chem. Soc., Chem. Commun.*, 614–616.

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

Friedkin, M. (1959) in *The Kinetics of Cellular Proliferation* (Stohlman, F., Jr., Ed.) p 97, Grune and Stratton, New York.

Freidkin, M., & Kornberg, A. (1957) in *A Symposium on the Chemical Basis of Heredity* (McElroy, W. D., & Glass, B., Eds.) p 609, Johns Hopkins University Press, Baltimore.

Galivan, J. H., Maley, G. F., & Maley, F. (1976) *Biochemistry 15*, 356–362.
Gray, W. R., & Smith, J. F. (1970) *Anal. Biochem. 33*, 36–42.
Gupta, V. S., & Huennekens, F. M. (1967) *Biochemistry 6*, 2168–2177.
Horne, D. W., Briggs, W. T., & Wagner, C. (1977) *Anal. Biochem. 83*, 615–621.
Huennekens, F. M. (1963) *Biochemistry 2*, 151–159.
Lewis, C. A., Jr., & Dunlap, R. B. (1981) in *Topics in Molecular Pharmacology* (Burgen, A. S. V., & Roberts, G. C. K., Eds.) Vol. 1, pp 169–219, Elsevier/North-Holland Biomedical Press, Amsterdam.
Lewis, C. A., Jr., Ellis, P. D., & Dunlap, R. B. (1980) *Biochemistry 19*, 116–123.
Lewis, C. A., Jr., Ellis, P. D., & Dunlap, R. B. (1981) *Biochemistry 20*, 2275–2285.
Lockshin, A., & Danenberg, P. V. (1979) *J. Biol. Chem. 254*, 12285–12288.
Lyon, J. A., Polard, A. L., Loeble, R. B., & Dunlap, R. B. (1975) *Cancer Biochem. Biophys. 1*, 121–128.
Maggioria, L., Chang, C. T.-C., Hasson, M. E., Bigge, C. F., & Mertes, M. P. (1983) *J. Med. Chem. 26*, 1028–1036.
Maley, F., & Maley, G. (1981) in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Sartorelli, A. C., Lazo, J. S., & Bertino, J. R., Eds.) pp 265–283, Academic Press, New York.
Maley, G. F., Bellisario, R. L., Guarino, D. U., & Maley, F. (1979) *J. Biol. Chem. 254*, 1288–1295.
Miziorko, H. M., & Lane, M. D. (1977) *J. Biol. Chem. 252*, 1414–1420.
Miziorko, H. M., Shortle, D., & Lane, M. D. (1976) *Biochem. Biophys. Res. Commun. 69*, 92–98.
Moore, M. A., Ahmed, F., & Dunlap, R. B. (1984) *Biochem. Biophys. Res. Commun. 124*, 37–43.
Moore, M. A., Ahmed, F., & Dunlap, R. B. (1986) *J. Biol. Chem.* (in press).
Park, J. S., Chung, C. T.-C., & Mertes, M. P. (1979) *J. Med. Chem. 22*, 1134.
Plese, P. C., & Dunlap, R. B. (1977) *J. Biol. Chem. 252*, 6139–6144.
Santi, D. V. (1981) in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents*, pp 285–300, Academic Press, New York.
Santi, D. V., McHenry, C. S., & Sommer, H. (1974) *Biochemistry 13*, 471–481.
Spector, L. B. (1982) in *Covalent Catalysis by Enzymes*, Springer-Verlag, New York.
Srinivasan, A., Amarnath, V., Broom, A. D., Zou, F. C., & Cheng, Y.-C. (1984) *J. Med. Chem. 27*, 1710–1717.
van der Meij, P. F. C., Lohmann, R. D., de Waard, E. R., Chen, T. B. R. A., & Pendit, U. K. (1985) *J. Chem. Soc., Chem. Commun.*, 1229–1230.
Wahba, A. J., & Friedkin, M. (1962) *J. Biol. Chem. 237*, 3794–3801.
Wilson, R. S., & Mertes, M. P. (1972) *J. Am. Chem. Soc. 94*, 7182–7183.
Wilson, R. S., & Mertes, M. P. (1973) *Biochemistry 12*, 2879–2886.
Woods, K. R., & Wang, K.-T. (1967) *Biochim. Biophys. Acta 133*, 369–370.