Structures of Reversible and Irreversible Complexes of Thymidylate Synthetase and Fluorinated Pyrimidine Nucleotides†

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ABSTRACT: Binding studies have shown that the nucleotide and cofactor are bound in a 1 : 1 ratio in the covalent thymidylate synthetase–FdUrd-5′-P–CH_2H_4folate complex (Langenbach *et al.*, *Biochem. Biophys. Res. Commun. 48*, 1565 (1972b)). When the double-labeled complex is trypsinized, both [^{3}H]-inhibitor and [^{14}C]cofactor are attached to the same peptide. The methylene group of CH_2H_4folate is required for formation of the covalent complex. The difference spectrum resulting from formation of the complex displays a decrease in absorbance at 270 nm, presumably due to saturation of the 5,6 double bond of FdUrd-5′-P. In addition, there is a large optical density decrease at 290 nm, which suggests that changes also occur in the H_4folate chromophore. 10-MeH_4-folate, a potent enzyme–inhibitor competitive with CH_2H_4-folate, induces a type of binding of FdUrd-5′-P which allows isolation of the enzyme–inhibitor complex by Sephadex filtration, but does not involve stable covalent bonds. In the presence of 10-MeH_4folate, the enzyme catalyzes exchange with water of the C-5 proton of dUrd-5′-P. A difference spectrum using 10-MeH_4folate showed only the decrease in absorption at 270 nm. The data indicate that the binding of FdUrd-5′-P in the presence of the cofactor analog is due to the reversible addition of a nucleophile to the 5,6 double bond of the pyrimidine ring. 5-MeH_4folate, a weak noncompetitive inhibitor, allows 90% less binding of FdUrd-5′-P than does 10-MeH_4folate and also causes very little change in ultraviolet absorption. The amino acid residue in the active site essential for enzyme activity and inhibitor binding has been shown to be cysteine. These observations support a methylene-bridged structure for the enzyme–FdUrd-5′-P–cofactor complex (*loc. cit.*).

The fluorinated pyrimidine nucleosides FdUrd[1] and F_3dThd, synthesized by Heidelberger and coworkers (Heidelberger *et al.*, 1957, 1964), have been shown to possess clinical antitumor and antiviral activities (Heidelberger *et al.*, 1958; Heidelberger and Anderson, 1964; Heidelberger, 1970; Wellings *et al.*, 1972). Subsequent experiments showed that these compounds are chemotherapeutically effective as a result of their conversion *in vivo* to the corresponding nucleotides, FdUrd-5′-P and F_3dThd-5′-P, which are powerful inhibitors of thymidylate synthetase (methylenetetrahydrofolate: dUrd-5′-P-C-methyltransferase, EC 2.1.1.) (Cohen *et al.*, 1958; Heidelberger *et al.*, 1960; Hartmann and Heidelberger, 1961; Reyes and Heidelberger, 1965). This enzyme catalyzes a unique reaction in which a one-carbon fragment is first transferred from CH_2H_4folate to the 5 position of dUrd-5′-P and then is reduced to a methyl group, resulting in the release of dThd-5′-P. Thymidylate synthetase, thus, has an essential role in DNA biosynthesis and has been postulated to be a rate-limiting enzyme for this process (Blakley, 1969). By blocking the production of dThd-5′-P, the aforementioned fluorinated analogs ultimately function as inhibitors of DNA synthesis. In view of the importance of these compounds in cancer chemotherapy, a major direction of research in this laboratory has been to discover their mode of interaction with thymidylate synthetase.

Recent studies have demonstrated that the binding of FdUrd-5′-P to thymidylate synthetase is covalent (Langenbach *et al.*, 1972a,b; Santi and McHenry, 1972). Furthermore, results from this laboratory have shown that a molecule of CH_2H_4folate is also covalently bound in the enzyme–FdUrd-5′-P complex (Langenbach *et al.*, 1972b). It is reasonable to postulate that FdUrd-5′-P proceeds through several initial steps of the enzymatic mechanism; thus, formation of the ternary covalent complex provides a unique opportunity to study what may be a "frozen" transition state or central complex of an enzyme reaction. In this report we describe evidence that the structure of the ternary enzyme–FdUrd-5′-P–CH_2H_4folate complex involves a covalent bond from the methylene group of CH_2H_4folate to the 5 position of the pyrimidine ring and a bond from the pyrimidine to the enzyme resulting from addition of a nucleophile to the 5,6 double bond. Supporting this structure are binding studies of FdUrd-5′-P in the presence of methylated H_4folate analogs, isotope exchange at the 5 position of [5-^{3}H]dUrd-5′-P in the presence of 10-MeH_4folate, and identification of a catalytically essential residue in the active site as cysteine. Difference spectra of the enzyme–inhibitor–cofactor complexes are consistent with the methylene-bridged structures, and suggest that the H_4folate moiety may be attached to the pyrimidine ring through the N-10 position.

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[1] Abbreviations used are: FdUrd, 5-fluoro-2′-deoxyuridine; F_3dThd, 5-trifluoromethyl-2′-deoxyuridine; dUrd-5′-P, 2′-deoxyuridylic acid; dThd-5′-P, thymidylic acid; FdUrd-5′-P, 5-fluoro-2′-deoxyuridylic acid; F_3dThd-5′-P, 5-trifluoromethyl-2′-deoxyuridylic acid; H_4folate, tetrahydrofolic acid; CH_2H_4folate, 5,10-methylenetetrahydrofolic acid; 10-MeH_4folate, 10-methyltetrahydrofolic acid; 5-MeH_4folate, 5-methyltetrahydrofolic acid; H_4-homofolate, homotetrahydrofolic acid.

Materials and Methods

[^{14}C]CH_2H_4folate (specific activity 55 Ci/mol) was prepared as described previously (Langenbach *et al.*, 1972b). 5-MeH_4folate and 10-MeH_4folate were synthesized and purified according to the procedures of Gupta and Huennekens (1967). We are grateful to Dr. S. F. Zakrzewski for a sample of H_4-homofolate. Chromatography was carried out with Whatman DE81 paper, using the solvent system 4 N formic acid–0.1 N ammonium formate. [^{14}C]Iodoacetamide was obtained from New England Nuclear.

An amethopterin-resistant strain of *Lactobacillus casei* was kindly provided by Dr. F. M. Huennekens and was grown according to Dunlap *et al.* (1971). Isolation of the thymidylate synthetase was accomplished with a slight modification of the procedure of Dunlap *et al.* (1971). After ammonium sulfate fractionation and dialysis, the enzyme preparation was applied directly to the CM-Sephadex column. The tubes with enzyme activity were pooled and the pH was adjusted to 7.5 prior to chromatography on DEAE-Sephadex. This two-step procedure resulted in a preparation that was more than 90% pure (specific activity 2.3 units/mg) as indicated by polyacrylamide sodium dodecyl sulfate disc gel electrophoresis (Langenbach *et al.*, 1972b; Laemmli, 1970). (One unit of enzyme activity is defined as 1 μmol of tetrahydrofolate converted to dihydrofolate per min.) Subsequently, we have developed a method for purification of thymidylate synthetase by affinity chromatography (Danenberg *et al.*, 1972). Fraction VI enzyme preparation (Fridland *et al.*, 1971), with a specific activity of 62 nmol/min per mg, was used in all studies with the Ehrlich ascites enzyme. All experiments were carried out with the *L. casei* enzyme, except where otherwise indicated.

Enzyme assays were carried out as described previously (Fridland *et al.*, 1971), except that a Gilford Model 2400S recording spectrophotometer was used. Incubation of the enzyme with [^{14}C]FdUrd-5′-P or [^{14}C]F_3dThd-5′-P was carried out at 25° for periods of time described for the individual experiments. Enzyme-bound and unbound analogs were separated by Sephadex G-25 chromatography as previously described (Langenbach *et al.*, 1972b). Enzyme–inhibitor complexes were heated at 100° for 3 min in 3% sodium dodecyl sulfate to denature the protein. Membrane filtrations were carried out in an Amicon PM-10 apparatus.

Preparation of Radioactive Inhibitors. An enzymatic procedure was utilized for the synthesis of the [^{14}C]nucleotides. An equal volume of each of the following solutions was used in 30-min incubations at 37°; the 105,000*g* supernatant fraction of Ehrlich ascites cells homogenized in three volumes of 0.154 N KCl, 0.25 mM Tris-HCl (pH 8.3), 45 mM $MgCl_2$, 0.1 mM [^{14}C]FdUrd, 35 mM 3-phosphoglyceric acid (pH 5.5), and 25 mM ATP (pH 5.5). The reaction was terminated by boiling for 2 min and the protein was removed by centrifugation. The supernatant fluid was then applied to a Dowex 1 column (1 × 5 cm, acetate form) with a gradient made from 4 M ammonium acetate and 4 M acetic acid in the mixing chamber. The tubes containing the nucleotide were combined, lyophilized, and dissolved in 10^{-3} M HCl prior to passage through a Dowex 50 column (1 × 6 cm, Cl^- form). The purity of the nucleotide was checked by paper chromatography using the upper phase of ethyl acetate–formic acid–water (65:5:30). The specific activity of [^{14}C]FdUrd-5′-P was 10.1 Ci/mol, and that of [^{14}C]F_3dThd-5′-P was 7.5 Ci/mol.

[6-^{3}H]FdUrd-5′-P with a specific activity of 0.35 Ci/mmol was synthesized from the nucleoside by the method of Yoshikawa *et al.* (1967).

Proteolysis of the Thymidylate Synthetase–[6-^{3}H]FdUrd-5′-P–[^{14}C]CH_2H_4Folate Complex. Thymidylate synthetase (1 mg) which had been incubated with [^{14}C]CH_2H_4folate and [6-^{3}H]FdUrd-5′-P was denatured with 8 M urea for 4 hr. After removal of the urea by repeated ultrafiltrations, the protein was carboxymethylated by a 4-hr treatment with 10 mM iodoacetic acid (pH 8.0). Excess iodoacetic acid was similarly removed by repeated ultrafiltrations using 0.1 M phosphate (pH 7.0), containing 5 mM β-mercaptoethanol. The volume was adjusted to 1.0 ml, and 2 mg of crystalline trypsin was added. The mixture was then incubated at 37° for 12 hr to digest the enzyme.

Determination of the Loss of Tritium from [5-^{3}H]dUrd-5′-P. Thymidylate synthetase from *L. casei* (0.8 mg) was combined with 0.3 μmol of 10-MeH_4folate, 0.03 μmol (1.4 μCi) of [5-^{3}H]dUrd-5′-P in 1.0 ml of 0.1 M phosphate buffer (pH 7.0), containing 5 mM β-mercaptoethanol. The incubation was carried out at 25°, and 200-μl aliquots were removed periodically. The aliquots were added to 1 ml of 0.01 N hydrochloric acid containing 50 mg of charcoal. The charcoal suspensions were shaken thoroughly on a Vortex mixer for 2 min and then allowed to stand for 10 min. The charcoal was then removed by filtration, and 0.5 ml of the filtrate was removed for counting in 10 ml of Scintisol (Isolab). Appropriate controls were carried out deleting enzyme and 10-MeH_4folate; no exchange was observed in these cases for as long as 5 hr at 25°.

Labeling of the Active Site. The enzyme from *L. casei* (0.8 mg) purified by affinity chromatography (Danenberg *et al.*, 1972) was dialyzed against 0.1 M phosphate buffer (pH 7.1) to remove β-mercaptoethanol, and then treated with 10 mM iodoacetamide in the presence of 1 mM dUrd-5′-P in a total volume of 2 ml for 12 hr at 25°. The reagent was removed by five ultrafiltrations against 5 ml of 0.1 M phosphate buffer (pH 7.1), containing 1 mM β-mercaptoethanol. The enzyme solution (1.0 ml) was then treated with 1 ml of 10 mM [^{14}C]iodoacetamide (specific activity 8.6 μCi/mmol) and allowed to react at 25° for 18 hr. The radioactive iodoacetamide was removed by five ultrafiltrations using 5 ml of 0.1 M phosphate buffer (pH 7.1). The carboxymethylated protein was then hydrolyzed with 6 N hydrochloric acid for 48 hr at 120°. The resulting hydrolysate was evaporated to dryness, and the residue dissolved in 0.2 M citrate buffer (pH 2.2). Carrier *S*-carboxymethylcysteine and carboxymethylhistidines were added. The solution was applied to a Dowex 50W-X12 (200–400 mesh) column (1 × 30 cm), and washed with 0.2 M citrate buffer (pH 2.2), until nonadhering radioactivity had decreased to background values. Then the column was washed with 0.2 M citrate buffer (pH 3.25) until all of the carboxymethylcysteine had been removed. The elution of amino acids was monitored by spotting some of the solution from each tube on a piece of paper and spraying with ninhydrin. Carboxymethylhistidines were removed from the column by washing with 0.2 M citrate buffer (pH 3.75). The contents of each tube were then quantitatively analyzed for amino acid content by the ninhydrin method, and 1.0-ml samples were removed and counted in Scintisol (Isolab).

Difference spectra were obtained using the expanded scale (0–0.1 optical density) of a Cary Model 15 spectrophotometer. Split cuvettes (1 cm) with two compartments were employed. The solutions were prepared under argon and equal volumes were placed in compartments of the sample and reference cuvette. The cuvettes were then sealed under argon. For each spectrum a single cuvette contained 0.5 ml of a solution of 4×10^{-6} M bacterial enzyme and 10^{-5} M CH_2H_4folate in one compartment, and 0.5 ml of 10^{-5} M FdUrd-5′-P in the other

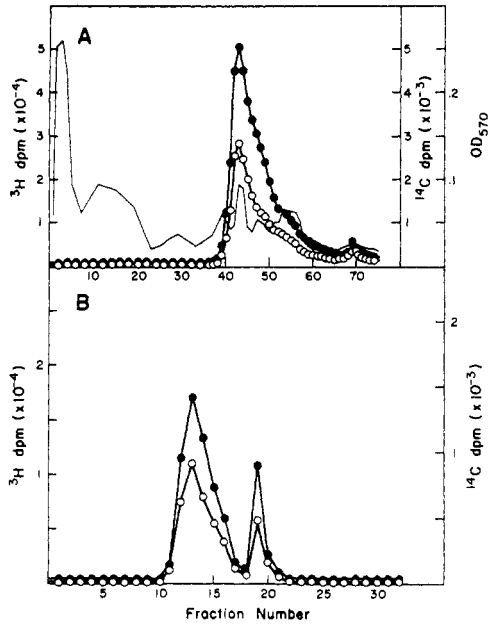

FIGURE 1: Chromatographic elution patterns of a trypsinized thymidylate synthetase–[6-^{3}H]FdUrd-5′-P–[^{14}C]CH_2H_4 folate complex. (A) The digest was applied to a Dowex 1-X8 (200–400 mesh) column (1 × 16 cm) and eluted with a gradient of 0.2 M pyridine–acetic acid buffer (pH 6.5) in the mixing chamber and 2 M pyridine–acetic acid (pH 5.0) in the reservior. Fractions of 4 ml were collected: ^{3}H (●), ^{14}C (○), OD_{570} (ninhydrin analysis) (–). (B) Sephadex G-25 chromatography of tubes 37–60 from the Dowex 1 column. Elution was carried out with 0.1 M phosphate buffer, on a 0.5 × 75 cm column and 2-ml fractions were collected: ^{3}H (●), ^{14}C (○).

compartment. All solutions were made up with deoxygenated 0.1 M potassium phosphate (pH 6.8), containing 5 mM β-mercaptoethanol. The spectrum was scanned between 350 and 240 nm at room temperature to determine the base line. The difference spectrum was then obtained by inverting the sample cell to mix the components and immediately rescanning the spectrum.

Results

Binding Studies. In an earlier report from this laboratory (Langenbach *et al.*, 1972a,b) we proposed that both FdUrd-5′-P and CH_2H_4folate are bound in a covalent complex with thymidylate synthetase. The covalent nature of the complex was inferred from denaturation experiments. Treatment of the enzyme with 8 M urea and heating in 3% sodium dodecyl sulfate did not release the radioactively labeled inhibitor or cofactor from the protein. Precipitation with 10% trichloroacetic acid also resulted in retention of radioactivity with the protein. To exclude the possibility that radioactive FdUrd-5′-P or cofactor are coprecipitated with the protein, the trichloroacetic acid precipitated protein was redissolved. Radioactivity still remained with the protein, as determined by nitrocellulose membrane filtration (Santi and McHenry, 1972).

Table I shows the results of experiments that were done in order to: (1) quantitate the amounts of each component in the complex, and (2) determine the necessity of the methylene group of CH_2H_4folate for formation of the covalent complex. Experiments 1 and 2, in which FdUrd-5′-P and CH_2H_4folate were incubated separately with enzyme from both Ehrlich ascites cells and *L. casei*, showed that each compound alone is not capable of binding tightly enough to form complexes that can be isolated. However, when both FdUrd-5′-P and CH_2H_4folate were incubated with the enzyme from both

TABLE I: Irreversible Binding of [^{14}C]FdUrd-5′-P and [^{14}C]-CH_2H_4folate to Thymidylate Synthetase.[a]

Reactants Thymidylate Synthetase +	Quantity Bound: Ehrlich Ascites (pmol/mg of Protein)	Quantity Bound: *L. casei* (nmol/nmol of Enzyme)
1. [^{14}C]CH_2H_4folate	3.7	0.0
2. [^{14}C]FdUrd-5′-P	0.0	0.0
3. [^{14}C]CH_2H_4folate + FdUrd-5′-P	76	0.75
4. CH_2H_4folate + [^{14}C]FdUrd-5′-P	76	0.85
5. H_4folate + [^{14}C]FdUrd-5′-P	35	0.35
6. H_4folate + [^{14}C]FdUrd-5′-P + 5 mM dimedone	0.0	0.06

[a] Reaction mixtures contained 0.05 mg of bacterial enzyme or 12.4 nmol/min (0.2 mg) of mammalian enzyme activity, 10^{-5} M FdUrd-5′-P and/or 10^{-5} M CH_2H_4folate, 5 mM β-mercaptoethanol, in 0.25 ml of 0.2 M phosphate buffer (pH 7.1). After incubation for 1 hr at 25°, the mixtures were applied to a Sephadex G-25 column, and the protein peak was isolated. The protein was then denatured as described in Methods, and five rapid membrane filtrations were carried out with 10 volumes of 0.1 M phosphate buffer (pH 7.1).

sources (expt 3 and 4), a ternary complex was formed which was stable to denaturation and could be isolated. This result confirms and extends our earlier observations (Langenbach *et al.*, 1972a,b) with the mammalian enzyme. Furthermore, we calculate that FdUrd-5′-P and CH_2H_4folate are bound in an approximate 1:1 ratio in the enzyme complex. Experiment 5 shows that deletion of formaldehyde from the cofactor solutions decreased the extent of complex formation by only 50%. Since the possibility of contamination of H_4folate solutions by traces of formaldehyde has been mentioned by Lomax and Greenberg (1967), reaction mixtures were first treated with dimedone, a formaldehyde sequestering reagent (Fieser and Fieser, 1967). Experiment 6 shows that this procedure prevents binding of the nucleotide to both enzymes.

Chromatography of Trypsinized Thymidylate Synthetase–[6-^{3}H]FdUrd-5′-P–[^{14}C]CH_2H_4folate Complex. The double-labeled ternary complex was treated with trypsin as described in Materials and Methods. We wished to determine whether the radioactive FdUrd-5′-P and cofactor were both bound to the proteolytic fragements and to the same peptide, a necessary condition of our proposed structure for the complex, which requires that the cofactor be attached to the nucleotide (Figure 4B). The trypsin digest was applied to a Dowex 1 column, and eluted with a gradient of pyridine–acetic acid buffer. From the elution profile (Figure 1A) it is apparent that both isotopes migrate identically. The same result was obtained by rechromatographing the peak tubes of Figure 1A on a Sephadex G-25 column (Figure 1B). The peak of radioactivity was eluted at a location intermediate between the native enzyme and small, unbound molecules. These observations provide compelling evidence that the FdUrd-5′-P and the cofactor are covalently bound to the protein and that both are bound to the same peptide fragments, since the isotope ratios are identical throughout the profiles.

Kinetics of Inhibition by H_4Folate Analogs. Neither 10-

TABLE II: Inhibition Data for Several H_4folate Analogs.

Compound	Inhibn *vs.* CH_2H_4folate	$(I/S)_{50}$[a]	Replot of Slopes *vs.* Inhibitor Concn
10-Me_4H_4folate	Competitive	0.5	Linear
5-MeH_4folate	Noncompetitive	110	Linear
H_4Homofolate	Noncompetitive	2.0	Parabolic

[a] The ratio of inhibitor to substrate necessary to achieve 50% inhibition of the enzyme.

MeH_4folate nor 5-MeH_4folate displayed any substrate properties at concentrations up to 1 mM. H_4homofolate, however, was found to be a weak substrate, permitting 1.5% of the enzyme activity of CH_2H_4folate at concentrations of 0.25 mM. This is unlikely to be due to contamination with 5,10-CH_2H_4-folate because of the method of synthesis.

The inhibitory properties of the above compounds were studied by varying the cofactor CH_2H_4folate at different levels of the inhibitor. The data were analyzed by means of reciprocal (Lineweaver–Burk) plots, and are summarized in Table II. Since the mode of binding was not the same for all analogs, the relative inhibition produced by each compound is expressed as the inhibitor to substrate ratio necessary to achieve 50% inhibition $[(I/S)_{50}]$. 10-MeH_4folate was a good inhibitor, competitive with CH_2H_4folate, and with a K_i value of 3.8×10^{-5} M. By contrast, the inhibition produced by the weak inhibitor 5-MeH_4folate was noncompetitive. Replots of slopes from the reciprocal plots were linear in both cases. Surprisingly, the inhibition by H_4homofolate was also noncompetitive, even though it is a good inhibitor (Table II) and has weak substrate properties. A Hill plot (Atkinson *et al.*, 1965) of the inhibition data for H_4-homofolate gave a slope of 1.75, and the replot of slopes *vs.* inhibitor concentration was parabolic, indicating that more than one molecule may be bound (Cleland, 1963). The noncompetitive inhibition observed with 5-MeH_4folate and H_4homofolate suggests that there is an alternate binding site for some H_4folate analogs. This idea was first postulated by Kalman and Fung (1972), who found that 10-methylfolate was a noncompetitive inhibitor which did not induce FdUrd-5′-P binding.

Our results with 10-MeH_4folate and 5-MeH_4folate are similar to those of Slavik and Zakrzewski (1967) who used a thymidylate synthetase from *Escherichia coli*. However, H_4-homofolate is a less potent inhibitor of the enzyme from *L. casei* than of that from *E. coli*, since Goodman *et al.* (1964) found that in the latter case, H_4homofolate bound approximately 100-fold better than the substrate.

Binding of FdUrd-5′-P in the Presence of H_4Folate Analogs. The binding of FdUrd-5′-P to thymidylate synthetase was assayed in the presence of 10-MeH_4folate, 5-MeH_4folate, and H_4homofolate to determine whether these compounds could induce the same type of covalent binding of FdUrd-5′-P as the substrate CH_2H_4folate. Table III shows that 10-MeH_4-folate and H_4homofolate caused an identical quantity of FdUrd-5′-P to migrate with the protein peak on Sephadex G-25 as does the cofactor CH_2H_4folate. By contrast, the weak noncompetitive inhibitor, 5-MeH_4folate, induced only 10% as much binding. Although isolation of enzyme–inhibitor complexes by passage through Sephadex G-25 is indicative of binding, it is not diagnostic of the formation of a stable covalent bond, since the enzyme is not denatured by this procedure. Indeed, when the above enzyme–FdUrd-5′-P complexes isolated by Sephadex filtration were denatured, only FdUrd-5′-P bound to the enzyme in the presence of the cofactor CH_2H_4folate remained with the protein (Table IV). Binding induced by all three of the H_4folate analogs was destroyed by denaturation, since the FdUrd-5′-P that had been bound initially was then readily removable by dialysis.

TABLE III: Binding of [^{14}C]FdUrd-5′-P to Thymidylate Synthetase in the Presence of H_4folate Analogs, as Measured by Chromatography on Sephadex G-25.[a]

Reactants Thymidylate Synthetase + [^{14}C]FdUrd-5′-P +	[^{14}C]FdUrd-5′-P Migrating with Protein Peak[b]
1. CH_2H_4Folate	19.4
2. 10-MeH_4folate	19.9
3. 5-MeH_4folate	2.1
4. H_4Homofolate	16.9

[a] The experimental procedure was identical with that described in the legend to Table I, except that H_4folate analogs were substituted for CH_2H_4folate where appropriate, and incubations were for 5 min. The protein peak obtained from the Sephadex G-25 column was not denatured, but was isolated and the radioactivity was determined. [b] Dpm/mg of protein $\times 10^{-5}$.

Exchange of the C-5 Proton of dUrd-5′-P. In the previous section we described that 10-MeH_4folate induces an unstable binding of FdUrd-5′-P to the enzyme. If this binding is due to addition of a nucleophile to the 5,6 double bond (see Discussion) then the corresponding effect on dUrd-5′-P should be to labilize the C-5 proton and cause exchange with protons from the medium. When [5-^{3}H]dUrd-5′-P was incubated with the enzyme and 10-MeH_4folate, we observed loss of tritium from the 5 position at a rate of 1.0 nmol/min per mg of protein. The rate was linear with time up to 5 hr.

Isolation of [^{14}C]S-Carboxymethylcysteine from a Hydrolysate of [^{14}C]Iodoacetamide-Labeled Enzyme. We have previously reported (Langenbach *et al.*, 1972a) that iodoacetamide

TABLE IV: Covalent Binding of [^{14}C]FdUrd-5′-P in the Presence of H_4Folate Analogs.[a]

Reactants Thymidylate Synthetase + [^{14}C]FdUrd-5′-P +	[^{14}C]FdUrd-5′-P Bound to Protein after Denatn of the E–I Complex[b]
1. CH_2H_4Folate	17.8
2. 10-MeH_4folate	0.04
3. 5-MeH_4folate	0.13
4. H_4Homofolate	0.03

[a] The experimental procedure was identical to that described under Table III. After isolation of the enzyme–[^{14}C]-FdUrd-5′-P complex, the volume of the solution was reduced by membrane filtration to 1 ml and then heated at 100° in the presence of 3% sodium dodecyl sulfate for 3 min. Five rapid membrane filtrations were carried out with 10 volumes of 0.1 M phosphate buffer (pH 7.1) and the radioactivity remaining with the protein was counted. [b] Dpm/mg of protein $\times 10^5$.

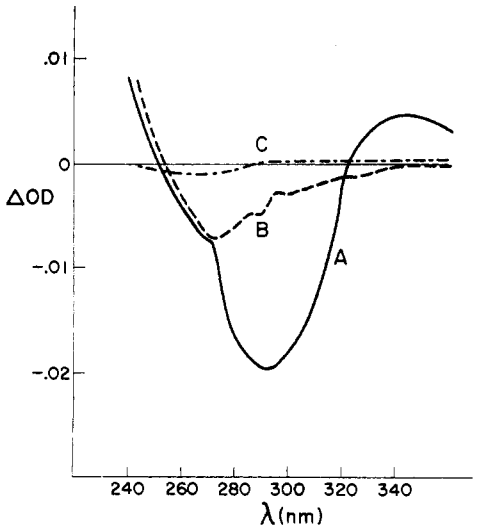

FIGURE 2: Difference spectra for the combination of thymidylate synthetase with (A) CH_2H_4folate, (B) 10-MeH_4folate, (C) 5-MeH_4-folate. The procedure is described in Materials and Methods.

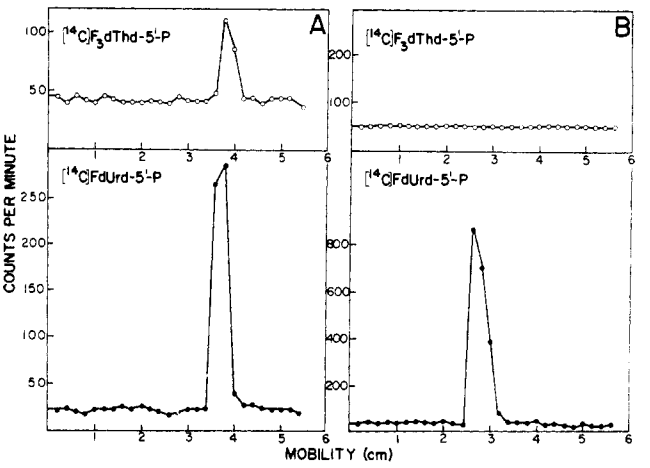

FIGURE 3: Gel electrophoretic analysis of the binding of fluorinated pyrimidine nucleotides to thymidylate synthetase from *L. casei*. The reaction was carried out as described in the legend to Table I, except that [^{14}C]F_3dThd-5'-P was used in some experiments. The reaction mixtures were then applied to the gels: (A) native gel electrophoresis; (B) sodium dodecyl sulfate gel electrophoresis (13, 17).

inactivates thymidylate synthetase from Ehrlich ascites cells. We assumed that the reactive residue was located within the active site because both dUrd-5'-P and the cofactor protect completely against this inactivation, but when they are removed, complete loss of activity occurs within 30 min. This property of the enzyme was utilized in an experiment designed to selectively label the catalytically essential amino acid.

Initial enzyme activity of an aliquot of the reaction mixture was 3.1 nmol/min. Residues outside the active site were first carboxymethylated with nonradioactive iodoacetamide in the presence of dUrd-5'-P, with no decrease in activity (3.05 nmol/min). Then the substrate was removed and the enzyme treated with [^{14}C]iodoacetamide, causing enzyme activity to decrease by 90% to 0.3 nmol/min. Subsequent acid hydrolysis of the enzyme and chromatographic analysis as described in Materials and Methods, disclosed that [^{14}C]*S*-carboxymethylcysteine was the major radioactive product. Unidentified minor products were obtained in quantities of less than 1% of the carboxymethylcysteine. Furthermore, it was calculated, from the amount of radioactivity recovered, that 0.9 mol of cysteine had reacted per mol of enzyme. This result is consistent with the data of Dunlap *et al.* (1971) who found complete loss of enzyme activity when 1 mol of *p*-chloromercuribenzoate had reacted per mol of enzyme.

Difference spectra were obtained for the combination of thymidylate synthetase with FdUrd-5'-P, F_3dThd-5'-P, CH_2H_4folate, and its methylated analogs in order to determine whether any changes occurred in the chromophores of these molecules upon binding to the enzyme. Fluctuations in the baselines across the wavelength range of 245–350 nm were quite small (0.005 ODU) and reproducible. The spectra in Figure 1 were corrected for these slight fluctuations. Below 245 nm, a spectrum could not be obtained because high absorbance from β-mercaptoethanol and other reaction components caused the slit width on the uv spectrophotometer to increase beyond the maximum limit of the instrument.

No change in any chromophore was observed upon incubation of the enzyme with each component individually. However, when FdUrd-5'-P, CH_2H_4folate, and the enzyme were all present there was a decreased absorption in the region of 275–320 nm with a minimum occurring at 293 nm (curve A, Figure 2). There was also an additional spectral decrease indicated by the shoulder in the region of 260–270 nm with its minimum at 270 nm. There was also a peak of increased absorbance centered at 335 nm, which has also been observed by Sharma and Kisliuk (1973). The magnitude of this peak increases with time and reaches its maximum at 15 min, suggesting a slow oxidation of H_4folate to H_2folate. However, the amount is only 30% of that expected for stoichiometric formation based on reaction at one active site per enzyme molecule. The difference spectrum, therefore, appears to reflect changes in at least two chromophores when the enzyme–FdUrd-5'-P–CH_2H_4folate complex is formed. Incubation of 10-MeH_4folate with the enzyme and FdUrd-5'-P produced only the decrease in absorption centered at 270 nm (curve B, Figure 2), corresponding to the shoulder on curve A, and with no spectral changes in the H_4folate absorption region of 280–300 nm. No difference spectrum was observed upon incubation of 5-MeH_4folate with thymidylate synthetase and FdUrd-5'-P (curve C, Figure 2). In cases where a difference spectrum was produced, mixing of the components in the reference cuvette caused cancellation of the spectrum.

Incubation of F_3dThd-5'-P and the enzyme produced no changes in the difference spectrum in spite of the ability of this nucleotide to bind in the absence of cofactor. The mode of binding of F_5dThd-5'-P therefore probably does not involve the addition of a nucleophile to the 5,6 double bond. A difference spectrum of enzyme and F_3dThd-5'-P in the presence of CH_2H_4folate produced no decreases in absorption; however, a slight increase in absorption centered at 330 nm was observed.

Binding of F_3dThd-5'-P. The thymidylic acid analog, F_3-dThd-5'-P, has previously been found to be a very potent inhibitor of thymidylate synthetase (Reyes and Heidelberger, 1965). In view of the abnormally high reactivity of its trifluoromethyl group (Heidelberger *et al.*, 1964), we wished to determine whether the analog inhibited the enzyme irreversibly as had been suggested (Reyes and Heidelberger, 1965; Santi and Sakai, 1971). Preliminary studies with the enzyme from Ehrlich ascites cells showed that when the enzyme–[^{14}C]F_3-dThd-5'-P complex was subjected to sodium dodecyl sulfate disc gel electrophoresis, no radioactivity could be detected on the gel (Langenbach *et al.*, 1972b). This result has been verified with the *L. casei* enzyme. Figure 3A shows that when the enzyme–F_3dThd-5'-P complex was electrophoresed on a nondenaturing gel, the radioactivity corresponded to the

location of the covalent enzyme–FdUrd-5′-P cofactor complex. However, when the same complex was denatured by sodium dodecyl sulfate and applied to a sodium dodecyl sulfate gel, no radioactivity was detectable on the gel (Figure 3B) whereas the [^{14}C]FdUrd-5′-P still remains bound to protein. To examine the possibility that the trifluoromethyl group of F_3-dThd-5′-P reacts with the a water molecule while bound to the enzyme to generate 5-carboxy-dUrd-5′-P and that the resulting potential charge interaction is responsible for the tight binding, the enzyme–F_3dThd-5′-P complex was isolated by Sephadex G-25 chromatography and denatured. The released radioactivity was found to migrate identically (R_F 0.6) with an authentic sample of F_3dThd-5′-P on DEAE-cellulose paper.

By contrast to FdUrd-5′-P, the noncovalent binding of F_3dThd-5′-P to the enzyme does not require CH_2H_4folate; identical quantities are bound with or without the cofactor. In the presence of the cofactor, a noncovalent ternary complex of enzyme–F_3dThd-5′-P–CH_2H_4folate can form as measured by Sephadex filtration, although this complex contains only 50% as much CH_2H_4folate as the analogous complex formed with FdUrd-5′-P. Since binding of the cofactor in the presence of F_3dThd-5′-P is noncovalent, partial dissociation of the complex probably occurs as it passes through the Sephadex G-25 column.

Discussion

Friedkin (1959) postulated a mechanism for the reaction catalyzed by thymidylate synthetase which involves a methylene bridge between the deoxyuridylate and H_4folate moieties as a means of transferring the one-carbon fragment. Thymidylate is then generated by a subsequent hydride transfer from the 6 position of H_4folate to the methylene group (Pastore and Friedkin, 1962). Other possibilities that have been considered are a methylated enzyme intermediate or conversion of the methylene group of CH_2H_4folate into a reactive methylonium species.

Lomax and Greenberg (1967) observed that the 5-hydrogen of dUrd-5′-P exchanges with protons from the solvent during the enzyme-catalyzed reaction. They favored reversibility of the methylene-bridge intermediate to account for this observation. Recently, it has been discovered that nucleophiles, especially thiol compounds, can also induce isotope exchange at the 5 position of 1-substituted uracil derivates (Heller, 1968; Santi and Brewer, 1968; Santi *et al.*, 1970; Kalman, 1971). The evidence indicates that attack of the nucleophile at the 6 position and intermediate formation of an unstable adduct across the 5,6 double bond activates the 5 position of the pyrimidine ring toward electrophilic substitution by protons. It is reasonable to extend these observations to the enzyme-catalyzed proton exchange and to suggest that a preliminary step in the enzymatic mechanism is attack of a nucleophile at the 6 position of dUrd-5′-P in order to render the 5 position more susceptible to attack by the activated methylene species, momentarily giving rise to an intermediate in which both the nucleotide and cofactor are covalently bound to the enzyme (structure A, Figure 4) through the nucleophilic group.

In a preliminary communication (Langenbach *et al.*, 1972a,b) we reported that thymidylate synthetase forms a ternary covalent complex with CH_2H_4folate and FdUrd-5′-P, when this powerful inhibitor is substituted for dUrd-5′-P. We have sought to investigate the nature of this complex and to determine the identity of the nucleophilic species in the active site. Since FdUrd-5′-P closely approximates the structure and

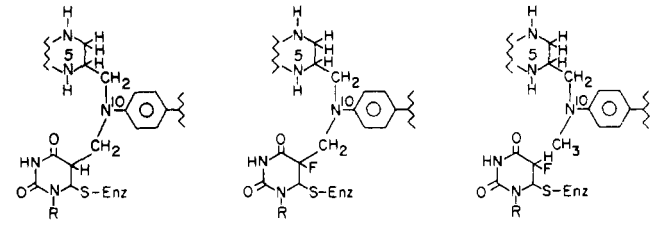

FIGURE 4: Proposed structures (A) of the transition state in the enzymatic mechanism, (B) of the covalent thymidylate synthetase–FdUrd-5′-P complex, and (C) of the enzyme–FdUrd-5′-P complex induced in the presence of 10-MeH_4folate.

dimensions of the natural substrate, a likely working hypothesis is that the fluorinated analog is capable of proceeding through the initial two steps of the enzymatic mechanism, *i.e.*, that addition of a nucleophile occurs to the 5,6 double bond, and that the methylene bridge is formed to the cofactor molecule by alkylation of the 5 position, resulting in structure B (Figure 4). At this stage, the transition state analog is stabilized, however, and reduction of the methylene group to a methyl group does not occur, owing either to subtle differences in the positions of the pertinent groups, or perhaps to electronic effects arising from the greater electronegativity of the fluorine atom.

The data presented in this paper appear to be consistent with structure B (Figure 4) for the covalent enzyme–FdUrd-5′-P cofactor complex, and, by analogy, with structure A (Figure 4) for the enzymatic intermediate. As necessary conditions for the existence of structure B (Figure 4), we established the following. (1) The nucleotide and cofactor are present in a one-to-one ratio in the enzyme complex; and if the enzyme is close to 100% purity as indicated by electrophoretic gels, there is only one complex per enzyme molecule. (2) Treatment of the reaction mixtures with dimedone, which ensures that the 5,10-methylene group of the cofactor will not form due to removal of residual formaldehyde, prevents formation of the covalent complex. (3) The analogs 10-MeH_4folate and 5-MeH_4folate, which are chemically incapable of alkylating the nucleotide to form a methylene bridge, do not form a complex with FdUrd-5′-P and the enzyme that is stable to denaturing conditions; however, an unstable complex is formed with 10-MeH_4folate. (4) The presence of a cysteine residue in the active site has been demonstrated by isolation of [^{14}C]*S*-carboxymethylcysteine from the hydrolysate of enzyme which had been treated with [^{14}C]iodoacetamide in the absence of dUrd-5′-P. The latter was found to protect the enzyme against iodoacetamide inactivation. We have recently observed (P. V. Danenberg and C. Heidelberger, unpublished results) that treatment of enzyme–[6-^{3}H]FdUrd-5′-P–[^{14}C]CH_2H_4folate complexes with Raney nickel results in simultaneous detachment of both isotopes from the protein. This experiment provides convincing evidence that binding of both molecules occurs *via* a single sulfide linkage. Full details of this method and results will be published separately. We have also obtained pH *vs.* log V and log V/K profiles for the mammalian enzyme that support the involvement of a sulfhydryl group in the catalytic sequence (R. Langenbach, P. V. Danenberg, and C. Heidelberger, unpublished results). (5) Chromatographic analysis of proteolytic fragments of enzyme–[6-^{3}H]FdUrd-5′-P–[^{14}C]CH_2H_4folate indicates that both labels are bound to the same peptide, since they migrate identically in several systems. (6) The difference spectrum generated by formation of the FdUrd-5′-P cofactor–enzyme complex is consistent with

the disappearance of the pyrimidine chromophore. In addition to the large absorbance decrease at 290 nm, there is a shoulder at 270 nm which may be interpreted as loss of the FdUrd-5′-P chromophore upon saturation of the 5,6 double bond. This observation is in accord with the data of Santi and McHenry (1972), who measured an absorption decrease at 270 nm, but did not publish the full difference spectrum. We wished to verify that there was actually a peak at 270 nm and that the absorbance decrease there was not the tailing off of a larger peak at another wavelength. It should be noted that for any spectral changes to occur, both the nucleotide and cofactor must be present, which correlates with the observation that no binding of FdUrd-5′-P occurs in the absence of cofactor (Table I) (Langenbach *et al.*, 1972b).

Santi and McHenry (1972) have suggested that the binding of FdUrd-5′-P to the enzyme involves only the first step of the sequence, *i.e.*, addition of a nucleophile across the 5,6 double bond, with no role for CH_2H_4folate other than perhaps to bring the enzyme into a favorable conformation. With that model it is difficult to rationalize the covalent binding of CH_2H_4folate in the presence of FdUrd-5′-P unless we assume that the reactive methylene group actually alkylates the enzyme, and that the pteridine portion subsequently remains attached to the methylene group. If a "methylated enzyme" is indeed the methyl group transferring species in the reaction, one might expect that the formation of this intermediate would be quite independent of the nature of the nucleotide, and therefore the H_4folate moiety should not remain attached. Moreover, this type of adduct (similar to structure C, Figure 4), without the stabilizing factor of a methylene bridge, would dissociate readily once the enzyme is denatured; under normal conditions, a 5,6 double bond adduct formed in a bimolecular reaction is not the favored equilibrium form (Kalman, 1971). For example, it is not known that β-mercaptoethanol by itself adds appreciably to this double bond. However, the formation of the 6,5-cyclonucleoside from 5′-thiouridine (Bannister and Kagan, 1960) demonstrates that a sulfhydryl group can form a stable adduct with the 5,6 double bond if the correct intramolecular proximity exists. This situation probably occurs with 10-MeH_4folate and H_4homofolate, which induce a reversible binding of the nucleotide that enables the complex to be isolated by Sephadex G-25 filtration, but is destroyed upon denaturation of the enzyme. The enzyme could stabilize structure C (Figure 4) by properly positioning the nucleotide in the active site relative to the functional nucleophilic group as long as the integrity of the protein structure is maintained. If this hypothesis is correct, it may be predicted that the tendency for an enzymatic nucleophilic group to add to the 5,6 double bond of the nucleotide in the presence of 10-MeH_4folate will cause proton exchange to occur at the 5 position of dUrd-5′-P. Our data bear out this prediction although the rate of exchange is about 100-fold lower than in the presence of the normal cofactor (Lomax and Greenberg, 1967). However, this considerable difference in exchange rates would undoubtedly be reduced severalfold if isotope effects were taken into account. Structure C (Figure 4) is further supported by the difference spectral data (curve B, Figure 2) for 10-MeH_4folate, which shows the decrease in absorption at 270 nm to be expected if saturation of the 5,6 double bond of FdUrd-5′-P occurs. The lack of a difference spectrum in the presence of 5-MeH_4folate is also consistent with this model; the latter induces little binding of the nucleotide and consequently there should be minimal change in the uv absorption.

It is of interest to compare the binding behavior of 5-MeH_4folate and 10-MeH_4folate. The ability of 10-MeH_4folate to bind in the active site in contrast to the weak noncompetitive inhibition of 5-MeH_4folate, and the greater ability of 10-MeH_4folate to induce binding of FdUrd-5′-P indicate that the enzyme–10-MeH_4folate–FdUrd-5′-P (structure C, Figure 4) complex more closely resembles the methylene-bridged intermediates A and B (Figure 4) than does the corresponding complex in the presence of 5-MeH_4folate. These data suggest that the methylene bridge may connect the 5 position of the pyridimine moiety with the N-10 of H_4folate (Figure 4A,B). The ease of oxidation of N-10-substituted H_4folate derivatives would facilitate the reductive step in the enzyme catalyzed reaction, whereas N-5-substituted compounds are quite stable in comparison (Gupta and Huennekens, 1967). In addition, formation of an N-10-substituted intermediate provides an explanation for at least a large part of the decrease in absorbance at 290 nm seen in the difference spectrum (curve A, Figure 4). Since H_4folate derivatives absorb strongly around 290 nm, the decrease at this wavelength is most likely attributable to some change in CH_2H_4folate when the covalent complex is formed; in the case of 10-MeH_4folate no covalent binding of H_4folate occurs, and consequently there is no absorbance decrease in this region (curve B, Figure 4). Comparison of ϵ_{max} values of various H_4folate compounds show that derivatives substituted on N-10 have 30% lower ϵ_{max} than the corresponding N-5-substituted derivatives; the latter have about the same ϵ_{max} values as CH_2H_4folate or perhaps slightly larger (Blakley, 1969). Detachment of the methylene group from N-5 of H_4folate upon alkylation of the pyrimidine ring would therefore result in a substantial loss in absorbance. Sharma and Kisliuk's (1973) suggestion that H_2folate is generated during formation of the ternary complex could account for a portion of the decrease at 290 nm, although we cannot at present accommodate such a reaction within our proposed structure for the complex.

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Guanosine Triphosphate and Guanosine Diphosphate as Conformation-Determining Molecules. Differential Interaction of a Fluorescent Probe with the Guanosine Nucleotide Complexes of Bacterial Elongation Factor Tu†

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ABSTRACT: Tritium exchange studies have recently provided evidence that conformational differences between EFTu–GTP and EFTu–GDP may account for the differential binding of AA-tRNA by EFTu–GTP (Printz, M. P., and Miller, D. L. (1973), *Biochem. Biophys. Res. Commun. 53*, 149). These conformational differences have been further characterized by studying the interaction of the fluorescent dye 1-anilino-8-naphthalenesulfonate with EFTu–GTP and EFTu–GDP. EFTu–GTP enhances the fluorescence of 1-anilino-8-naphthalenesulfonate to a greater extent than does EFTu–GDP. When EFTu–GTP is complexed with Phe-tRNA, however, its interaction with 1-anilino-8-naphthalenesulfonate increases the fluorescence of the dye only as much as EFTu–GDP does. Titration of a solution of the dye with excess protein shows that both EFTu–GTP and EFTu–GDP produce the same fluorescence enhancement, about 200-fold, for the tightest bound dye. Equilibrium dialysis binding measurements indicate that EFTu–GTP binds three molecules of the sulfonate dye with an apparent $K_{diss} \simeq 2 \times 10^{-5}$ M, whereas EFTu–GDP binds two molecules with an apparent $K_{diss} \simeq 5\text{–}8 \times 10^{-5}$ M. Both complexes have at least one other population of more weakly bound dyes. It would appear from these data that differences in conformation between EFTu–GTP and EFTu–GDP are centered chiefly in a region of EFTu–GTP sensitive to AA-tRNA binding. However, further analysis of the fluorescence data indicates that somewhat more extensive conformational differences exist between the two nucleotide complexes of EFTu. Slope changes in the curve of the titration of 1-anilino-8-naphthalenesulfonate by EFTu–GTP and in Scatchard plots of the fluorescence data indicate cooperativity in the fluorescence yield and thus interaction of the dye binding sites on EFTu–GTP. EFTu–GDP gives no evidence of site interaction.

Nucleoside triphosphates perform three distinct functions in organisms. They may be reagents or intermediates in the synthesis or degradation of cellular components, where the formation of a phosphate ester intermediate is a favorable pathway for removing or adding the elements of water. The synthesis and degradation of glycogen are examples of this function. In contrast to these reactions, nucleoside triphos-

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