Hydrodynamic Behavior of Human and Bacterial Thymidylate Synthetases and Thymidylate Synthetase–5-Fluoro-2′-deoxyuridylate–5,10-Methylenetetrahydrofolate Complexes. Evidence for Large Conformational Changes during Catalysis†

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ABSTRACT: The conformations of thymidylate synthetases from CCRF-CEM human leukemic cells and *Lactobacillus casei* were studied by hydrodynamic methods. Although the human enzyme has a molecular weight of 70 000–72 500, somewhat smaller than that of the *L. casei* enzyme, it has a larger Stokes radius and a lower sedimentation coefficient, indicating that the human enzyme is less spherical than the bacterial enzyme. Thymidylate synthetases from the human leukemic cells and the bacterial source both undergo substantial conformational changes upon the formation of a covalent ternary complex with the mechanism-based inhibitor 5-fluoro-2′-deoxyuridylate and 5,10-methylenetetrahydrofolate. The Stokes radius of both proteins decreases by 3.5% when the ternary complex is formed in spite of the 1.8% increase in molecular weight, and the sedimentation coefficient increases by 3.5% after appropriate corrections for the bound ligands. Ternary complex formation results in a more compact structure for both enzymes, with approximately the same reduction in the frictional ratio. Experiments with the bacterial enzyme indicate that ~70% of the total conformational change occurs upon binding of 1 mol of ligands/mol of enzyme. These results demonstrate that human and bacterial thymidylate synthetases undergo marked structural changes upon forming a ternary complex which is probably very similar to an activated complex formed with both substrates. These investigations also provide evidence for fundamental similarities in the mechanism of ternary complex formation with 5-fluoro-2′-deoxyuridylate and 5,10-methylenetetrahydrofolate, despite the marked differences in amino acid composition and the dissimilar conformations of these two enzymes obtained from widely divergent sources.

It is now well established that enzymes and other proteins can undergo conformational changes in response to the binding of small molecules. These structural alterations have been studied by a variety of techniques (Citri, 1973) and can be observed directly with physical methods such as X-ray crystallography. The concept of "flexible" proteins is indispensible in order to explain such phenomena as allosteric control of enzyme reactions, cooperativity effects, and the ordered binding of substrates to an enzyme.

K_{cat} inhibitors of enzyme reactions, also known as suicide or mechanism-based inhibitors (Rando, 1974; Abeles & Maycock, 1976), offer an approach for gaining some idea of the magnitude of conformational transitions during enzyme catalysis. These inhibitors are compounds which resemble the substrate sufficiently to undergo a partial catalytic conversion by the enzyme. In some cases, a stable covalent complex is formed which is a "frozen" analogue of a normal catalytic event. Thus, the enzyme in such a complex would be trapped in a conformation closely resembling a transitory state occurring during catalysis.

Among the most widely studied mechanism-based inhibitors is FdUMP,[1] which in the presence of the 5,10-CH_2H_4PteGlu cofactor forms a tightly bound covalent complex with thymidylate synthetase (EC 2.1.1.45). This ternary complex is considered analogous to a steady-state intermediate of the normal enzymatic reaction (Danenberg, 1977). (Thymidylate synthetase catalyzes the reductive methylation of dUMP by 5,10-CH_2H_4PteGlu to form dTMP and H_2PteGlu.) Ternary complex formation results in perturbations characterized by changes in the absorption spectrum, fluorescence quenching, and the circular dichroism spectrum of the enzyme (Danenberg et al., 1974; Santi et al., 1974a; Sharma & Kisliuk, 1975; Leary et al., 1975; Galivan et al., 1975; Donato et al., 1976). It was of interest therefore to determine if ternary complex formation led to alterations of shape of sufficient magnitude to detect by hydrodynamic methods.

Most of the recent mechanistic studies on thymidylate synthetase have been carried out with the enzyme isolated from methotrexate-resistant *Lactobacillus casei* (Danenberg, 1977). This enzyme is composed of two identical subunits and has a molecular weight of 73 176 established recently by amino acid sequencing (Maley et al., 1979). Although both subunits participate in ligand binding, apparently not all of the homogeneous enzyme is capable of binding 2 mol of FdUMP and cofactor/mol of enzyme. The maximum average molar ratio of these ligands bound to the enzyme is 1.7–1.8 (Santi et al., 1974a; Aull et al., 1974a; Galivan et al., 1976).[2] The ternary complex containing 2 mol of FdUMP and cofactor (2:2:1 complex) can be separated from that containing 1 mol of each ligand (1:1:1 complex) by nondenaturing gel electrophoresis or ion-exchange chromatography (Aull et al., 1974a,b).

Identification and characterization of the ternary complex formed with the *L. casei* enzyme provided supportive evidence for earlier conclusions that FdUMP inhibition of thymidylate

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[1] Abbreviations used: FdUMP, 5-fluoro-2′-deoxyuridylate; 5,10-CH_2H_4PteGlu, L(+)-5,10-methylenetetrahydrofolate; dUMP, 2′-deoxyuridylate; dTMP, 2′-deoxythymidylate; H_2PteGlu, 7,8-dihydrofolate.

[2] In our experiments, the 2:2:1 ternary complex refers to the highest average molar ratio of ligands bound without physical separation of the two forms of the ternary complex, i.e., 1.7–1.8 mol of FdUMP and 5,10-CH_2H_4PteGlu/mol of enzyme.

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synthetase is the major factor responsible for the chemotherapeutic activity of the well-known anticancer drug 5-fluorouracil (Heidelberger, 1975). Because human enzymes are the targets of FdUMP inhibition in cancer chemotherapy, we have been interested in studying the interaction of these enzymes with FdUMP and comparing these results with those obtained with the bacterial enzyme.

Human thymidylate synthetases are normally present in low amounts in tissues and are apparently less stable than the bacterial counterparts, and, therefore, intensive investigations of these enzymes have awaited development of improved purification procedures. We have used affinity chromatography to achieve a facile purification to homogeneity of high specific activity human thymidylate synthetase isolated from CCRF-CEM human lymphoblastic leukemia cells (Lockshin et al., 1979). This enzyme is composed of two apparently identical subunits and also binds 1.7 mol of FdUMP/mol of enzyme in the presence of 5,10-CH_2H_4PteGlu. However, the amino acid composition of the human enzyme is markedly different from that of the *L. casei* enzyme. Urea (6 M) causes an apparent release of FdUMP from the human enzyme ternary complex, but it has no such effect on the *L. casei* ternary complex. In this paper, we present evidence demonstrating that both the bacterial and human enzymes exhibit similarly large changes in their hydrodynamic properties upon complex formation.

Materials and Methods

Materials. 5,10-CH_2H_4PteGlu (Lockshin et al., 1979) and FdUMP (Danenberg & Heidelberger, 1976) were synthesized as previously described. [^{3}H]FdUMP (20 Ci/mmol) and [^{14}C]FdUMP (50 mCi/mmol) were purchased from Moravek Biochemicals (City of Industry, CA).

CCRF-CEM human lymphoblastic leukemic cells (Foley et al., 1965) were grown in suspension (Lockshin et al., 1979) and provided by the Los Angeles County/University of Southern California Cell Culture Core Facility.

Sephadex G-100 was obtained from Pharmacia. Preformed 10% polyacrylamide gels containing sodium dodecyl sulfate were obtained from Bio-Rad Laboratories, as were molecular-weight markers for electrophoresis (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme).

Thymidylate Synthetase. Homogeneous enzyme was obtained from CCRF-CEM human leukemic cells (Lockshin et al., 1979) and methotrexate-resistant *L. casei* (Dunlap et al., 1971; Danenberg et al., 1974). The human enzyme was routinely stored in liquid nitrogen in the buffer from which it was eluted from the affinity column (0.05 M Tris-HCl, pH 7.4–1.0 M KCl–20 mM dithiothreitol–10% glycerol) plus 0.5 mg/mL bovine serum albumin. Under these conditions, the enzyme retained over 80% of its original activity for at least 10 months.

The radiolabeled FdUMP ternary complexes of the CCRF-CEM and *L. casei* enzymes were formed during 2-h incubation at 20 °C in 0.05 M Tris-HCl, pH 7.4, and 8 mM dithiothreitol. The 2:2:1 complex[2] of both enzymes was formed with excess radiolabeled FdUMP (at least 2 mol/mol of enzyme) and saturating cofactor (at least 30 μM). The 1:1:1 complex of the *L. casei* enzyme was formed by using a large excess of enzyme (20-fold or greater) over [^{3}H]FdUMP plus saturating cofactor. Unless otherwise indicated, ternary complex will refer to the 2:2:1 complex[2] for both enzymes.

Enzyme Assays. The spectrophotometric assay for thymidylate synthetase was performed as previously described (Wahba & Friedkin, 1961; Lockshin et al., 1979). Ternary-complex formation with radiolabeled FdUMP was assayed by filtration through nitrocellulose disks (Santi et al., 1974b; Lockshin et al., 1979) or by separation of protein-bound from unbound FdUMP with a 1:5 dilution of activated charcoal containing bovine serum albumin and dextran (Moran et al., 1979). Radioactivity was determined with a counting error of <2% for the dual isotope experiments and <4% for the other experiments.

Sephadex G-100 Gel Filtration. Protein mixtures contained in a volume of 400 μL were chromatographed at 4 °C on a Sephadex G-100 column (0.9 × 100 cm) with 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M KCl and 8 mM dithiothreitol, unless otherwise noted. Blue dextran was included in each sample applied to the column to determine the void volume. Marker proteins were detected by determining the optical density at 280 nm. Elution volumes were obtained by extrapolating the graphed slopes of each peak to a maximum (Andrews, 1965).

For cochromatography of the native and the radiolabeled FdUMP ternary complex forms of thymidylate synthetase, the following procedure was employed. The ternary complex was formed by incubating enzyme with 0.1 mM 5,10-CH_2H_4PteGlu and a 3:1 molar ratio of radiolabeled FdUMP to enzyme. A charcoal–albumin–dextran slurry (7.5 volumes) was then added to absorb unbound ligands. After centrifugation to remove the charcoal, an aliquot of the supernatant containing the ternary complex was added to native enzyme and blue dextran, and the mixture was chromatographed. For dual-label experiments with both the 2:2:1 and the 1:1:1 forms of the *L. casei* enzyme ternary complex, the same procedure was followed except that some of the [^{3}H]FdUMP 1:1:1 complex was added with native enzyme to the [^{14}C]FdUMP 2:2:1 complex.

For the determination of the amount of enzyme (native plus [^{3}H]FdUMP ternary complex) in column fractions, duplicate aliquots of each fraction and assay controls were incubated with 0.03 mM 5,10-CH_2H_4PteGlu and [^{3}H]FdUMP (13 or 250 nM for the CCRF-CEM and the *L. casei* enzymes, respectively) in a total volume of 140 μL. After a 20-min incubation at 20 °C, 1.12 mL of the charcoal slurry was added, and 900-μL aliquots of the supernatant obtained after centrifugation were removed for the determination of radioactivity. The amount of preformed ternary complex in each fraction was determined by similar charcoal treatment of duplicate aliquots, omitting the incubation with extra cofactor and [^{3}H]FdUMP. The quantity of native enzyme in each fraction was determined by subtracting the radioactivity of the preformed [^{3}H]FdUMP ternary complex from the total radioactivity obtained in the assays with excess cofactor and [^{3}H]FdUMP.

The Stokes radius, r, of the native thymidylate synthetases and the ternary complexes was determined by comparing the relative elutions from a Sephadex G-100 column of these proteins with those of marker proteins (Laurent & Killander, 1964). For ovalbumin and bovine serum albumin, r values are reported as 27.3 (Laurent & Killander, 1964) and 35 Å (Siegel & Monty, 1966), respectively. For bovine pancreas trypsinogen, an r value of 22 Å was calculated from the diffusion coefficient, $D_{20,w}$, of 9.7 × 10^{-7} (Tietze, 1953) by the equation

$$r = kT/(6\pi\eta D)$$

where k is the Boltzman constant, T is the absolute temperature, and η is the viscosity of the system. A temperature of 20 °C and the viscosity of water at 20 °C were used in the calculations. A similar calculation for rabbit muscle aldolase

with $D_{20,w} = 4.63 \times 10^{-7}$ (Taylor & Lowry, 1956) gave an r value of 46.3 Å for this protein.

Sucrose Density Gradient Centrifugation. Sedimentation experiments were performed according to the method of Martin & Ames (1961). Linear sucrose gradients were obtained by mixing 8 and 25% sucrose solutions buffered with 0.05 M Tris-HCl, pH 7.4, containing 8 mM dithiothreitol. Samples (125 or 150 μL) containing the native enzymes and/or ternary complexes plus marker proteins were layered onto 3.8-mL gradients, which were centrifuged in a Beckman SW 60 rotor with a Beckman Model L5-75 ultracentrifuge at 4 °C for 20 h at 56000 rpm or 18 h at 57000 rpm. Fractions (~140 μL) were collected with a Density Gradient Fractionator (ISCO Model 185). The marker proteins used were as follows: horse heart cytochrome *c* (80 μg), sedimentation coefficient $s_{20,w}$ = 1.80 S (Stewart & Margoliash, 1965); horseradish peroxidase (1 μg), $s_{20,w}$ = 3.48 S (Cecil & Ogston, 1951); bovine hemoglobin (600 μg), $s_{20,w}$ = 4.3 S (Svedberg & Pedersen, 1940); horse liver alcohol dehydrogenase (30 μg), $s_{20,w}$ = 5.11 S (Ehrenberg & Dalziel, 1958).

For the sedimentation of the native and [^{3}H]FdUMP ternary complex forms of thymidylate synthetase on the same gradient, the ternary complexes were formed with 0.1 mM 5,10-CH_2H_4PteGlu and a 2:1 molar excess of [^{3}H]FdUMP. Native enzyme was added prior to centrifugation. Assays of fractions for the native and complexed enzyme were similar to those conducted for the analogous gel filtration experiments. Aliquots of each fraction (32 μL for the CCRF-CEM enzyme and 30 μL for the *L. casei* enzyme) were incubated with 5,10-CH_2H_4PteGlu and [^{3}H]FdUMP, where added, in a total volume of 100 μL. The amount of protein-bound [^{3}H]FdUMP was determined by adding 800 μL of charcoal slurry and counting the radioactivity of 620 μL of the supernatant obtained after centrifugation.

The partial specific volumes, $\bar{v}$, of the enzymes were calculated (Cohn & Edsall, 1943) from the amino acid compositions. For the ternary complexes, $\bar{v}$ values were estimated by assuming additivities of weights and volumes of the enzymes and ligands (Kirschner & Schachman, 1971); $\bar{v}$ values of the latter were estimated by the method of Traube (1899).

The change in the $s_{20,w}$ of thymidylate synthetase to be expected from addition of ligands to form the ternary complex was calculated by numerically integrating eq 3 of Martin & Ames (1961) using the trapezoidal approximation. The integration was performed by Dr. Jorge Garcia-Peña using an IBM 3031 computer (FORTRAN IV program with 200 intervals in X).

The molecular weight was calculated from $s_{20,w}$, $D_{20,w}$, and $\bar{v}$ according to the equation

$$M = \frac{s}{D} \frac{RT}{(1 - \rho\bar{v})}$$

where ρ is the density of the solvent (water). The frictional ratio (f/f_0) was calculated from the equation

$$f/f_0 = r\left(\frac{3\bar{v}M}{4\pi N}\right)^{-1/3}$$

where N is Avogadro's number.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Protein samples were usually concentrated with deoxycholate and trichloroacetic acid prior to electrophoresis (Bensadoun & Weinstein, 1976; Lockshin et al., 1979). Samples were dissolved in 0.01 M sodium phosphate buffer, pH 7.0, containing 1 or 3% sodium dodecyl sulfate, 0.1 M dithiothreitol, 10% glycerol, and 0.01% bromophenol blue and were denatured by heating at 100 °C for 4–5 min. Electrophoresis was conducted in 10% gels in 0.025 M Tris–0.19 M glycine buffer, pH 8.3, containing 0.1% sodium dodecyl sulfate and 1 mM EDTA at a current of 2.5 mA/gel for 3 h at 20 °C (Bio-Rad Laboratories, 1978). All gels were stained with 0.05% Coomassie brilliant blue R in a 5:1:5 mixture of methanol, acetic acid, and water. The apparent mobilities of the protein bands were calculated as described by Weber & Osborn (1975). Radioactivity in gels was located by slicing them into uniform sections and extracting the radiolabeled protein (Lockshin et al., 1979).

Results

Sephadex G-100 Chromatography. A Sephadex G-100 column was standardized with protein mixtures containing blue dextran and (a) bovine serum albumin (4 mg) and trypsinogen (4 mg) or (b) aldolase (2.5 mg) and ovalbumin (4 mg). Samples of native CCRF-CEM thymidylate synthetase or the preformed [^{3}H]FdUMP enzyme ternary complex were chromatographed with mixture (a), and similar experiments were carried out with the *L. casei* enzyme. Because the elution volume of bovine serum albumin was very close to that of both the human and bacterial thymidylate synthetases and their ternary complexes, this marker protein served as a useful standard to compare differences in the elution of the enzymes under investigation.

Repeated experiments showed that the human enzyme ternary complex eluted just ahead of bovine serum albumin (0.19 ± 0.05 mL), indicating greater exclusion from the Sephadex beads and therefore a slightly larger Stokes radius for the ternary complex compared to bovine serum albumin. The native human enzyme eluted earlier than the ternary complex (1.25 ± 0.1 mL before the albumin), even though the molecular weight of the native enzyme is 1.8% less than that of the complex due to the addition of ~1300[3] from the covalently bound ligands. Cochromatography of the native human enzyme and the preformed ternary complex showed unequivocally that the two protein forms eluted differently on the Sephadex G-100 column (Figure 1A).

In contrast to the human thymidylate synthetase, the *L. casei* native enzyme and ternary complex both eluted *after* bovine serum albumin on the Sephadex column, indicating more nearly spherical structures (smaller Stokes radii) for the bacterial enzyme forms compared to the albumin and to the human enzyme forms. However, the order of elution of the native and complexed bacterial enzyme was the same as that found for the CCRF-CEM enzyme. The native enzyme eluted 1.3 ± 0.3 mL and the ternary complex eluted 2.3 ± 0.2 mL after bovine serum albumin. Simultaneous chromatography on the same column of the native bacterial enzyme and the [^{3}H]FdUMP ternary complex also confirmed that there were significant differences in their elution volumes (Figure 1B). The same result was found when the native *L. casei* enzyme was cochromatographed with its saturated [^{14}C]FdUMP ternary complex (not shown). In this experiment also, the native enzyme was located by complexing it with [^{3}H]FdUMP. Cochromatography followed by assay using both radiolabels allowed direct comparison in each fraction of the native enzyme and the ternary complex, thereby cancelling out differences in relative elution which might arise from sampling errors. No effect on elution volumes was found when 0.05 M Tris-HCl, pH 7.4, was substituted for potassium phosphate in the column buffer. Cochromatography of the [^{3}H]FdUMP

[3] Based on a maximum average of 1.7 mol of ligands bound/mol of enzyme.

Table I: Hydrodynamic Properties of CCRF-CEM and *L. casei* Native Thymidylate Synthetases and Enzyme–FdUMP–5,10-CH_2H_4PteGlu Ternary Complexes

	CCRF-CEM		*L. casei*	
	native enzyme	ternary complex	native enzyme	ternary complex
r (A)[a]	37.2 ± 0.2	36.1 ± 0.3	33.8 ± 0.1	32.7 ± 0.2
$D_{20,w} \times 10^{-7}$ (cm^2 s^{-1}) (calcd)	5.76	5.94	6.34	6.56
$s_{20,w}$ (S)	4.4 ± 0.1[b]	4.7 ± 0.05[b]		5.2[c]
app $\bar{\nu}$ (mL g^{-1}) (calcd)	0.734[d]	0.733[e]	0.736[d]	0.735[e]
M_r from s and D	70000	72500		73300
frictional ratio	1.36	1.31	1.22	1.17
subunit M_r from gel electrophoresis	36000 ± 700	36000 ± 700	37000 ± 700	

[a] Mean values and standard errors of three to seven determinations. [b] Mean values and standard errors of three determinations. [c] Approximate value based on comparison with the CCRF-CEM enzyme ternary complex. [d] Calculation based on the amino acid composition of the CCRF-CEM enzyme (Lockshin et al., 1979) and the *L. casei* enzyme (Maley et al., 1979). [e] $\bar{\nu}$ of the bound ligands was calculated to be 0.66 (Traube, 1899).

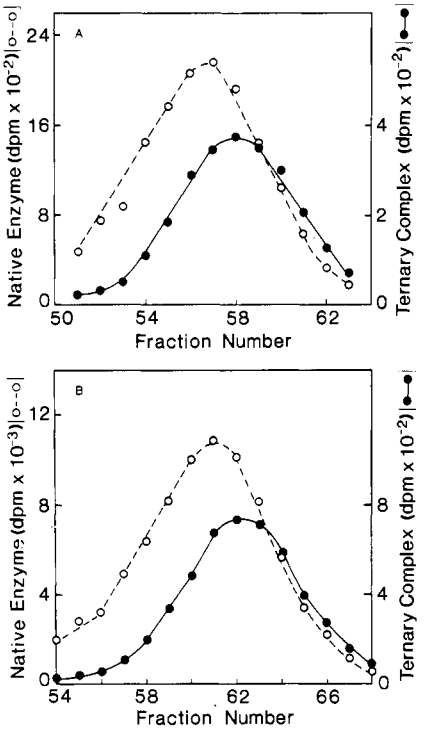

FIGURE 1: (A) Cochromatography on Sephadex G-100 of native CCRF-CEM thymidylate synthetase and the [^{3}H]FdUMP CCRF-CEM thymidylate synthetase ternary complex. Native enzyme (3.2 pmol) and ternary complex (0.52 pmol) were chromatographed, and 80-μL aliquots of the 640-μL fractions were assayed for native and complexed enzyme as described under Materials and Methods. (B) Cochromatography on Sephadex G-100 of native *L. casei* thymidylate synthetase and the [^{3}H]FdUMP *L. casei* thymidylate synthetase ternary complex. Native enzyme (25 pmol) and ternary complex (1.8 pmol) were chromatographed, and 40-μL aliquots of the 640-μL fractions were assayed for native and complexed enzyme.

CCRF-CEM ternary complex and the [^{14}C]FdUMP *L. casei* ternary complex (not shown) provided confirmation that the human enzyme ternary complex eluted considerably earlier than did the bacterial enzyme counterpart.

The following values of the Stokes radius for the native and complexed enzymes were calculated by comparing their elution volumes on Sephadex G-100 with those of the marker proteins: 37.2 ± 0.2 Å for native CCRF-CEM thymidylate synthetase (three determinations) and 36.1 ± 0.3 Å for the CCRF-CEM ternary complex (three determinations); 33.8 ± 0.1 Å for the native *L. casei* enzyme (four determinations) and 32.7 ± 0.2 Å for the *L. casei* ternary complex (seven determinations) (Figure 2). The diffusion coefficients calculated from these values are given in Table I.

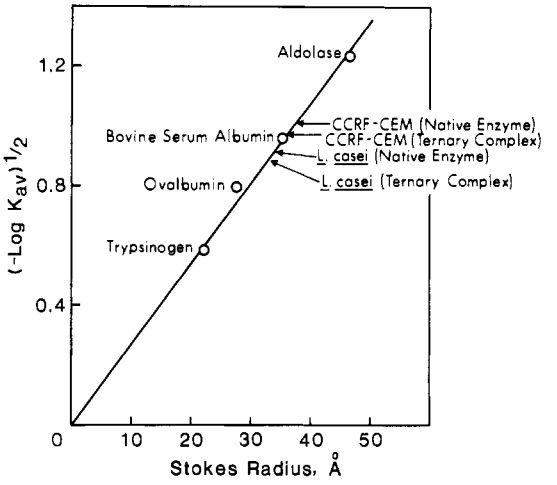

FIGURE 2: Stokes radii of CCRF-CEM and *L. casei* native thymidylate synthetases and ternary complexes as determined by Sephadex G-100 gel filtration. The void volume of the column and the peaks of the proteins and their elution constants (K_{av}) were determined as described under Materials and Methods. The K_{av} for each protein was obtained as the mean value from repeated determinations on a single Sephadex G-100 column.

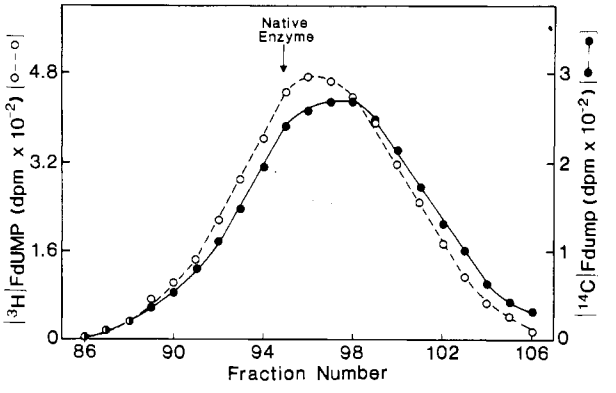

FIGURE 3: Cochromatography on Sephadex G-100 of native *L. casei* thymidylate synthetase, the [^{3}H]FdUMP *L. casei* thymidylate synthetase 1:1:1 ternary complex, and the [^{14}C]FdUMP *L. casei* thymidylate synthetase 2:2:1 ternary complex.[2] Native enzyme (360 pmol), the 1:1:1 ternary complex (1.8 pmol), and the 2:2:1 ternary complex (220 pmol) were chromatographed, and 400-μL fractions were collected. Triplicate 40-μL aliquots of each fraction were assayed for native and complexed enzyme.

Cochromatography of the [^{14}C]FdUMP *L. casei* 2:2:1 ternary complex[2] and the [^{3}H]FdUMP *L. casei* 1:1:1 ternary complex demonstrated detectable differences in their elution volumes on the Sephadex G-100 column (Figure 3), with the

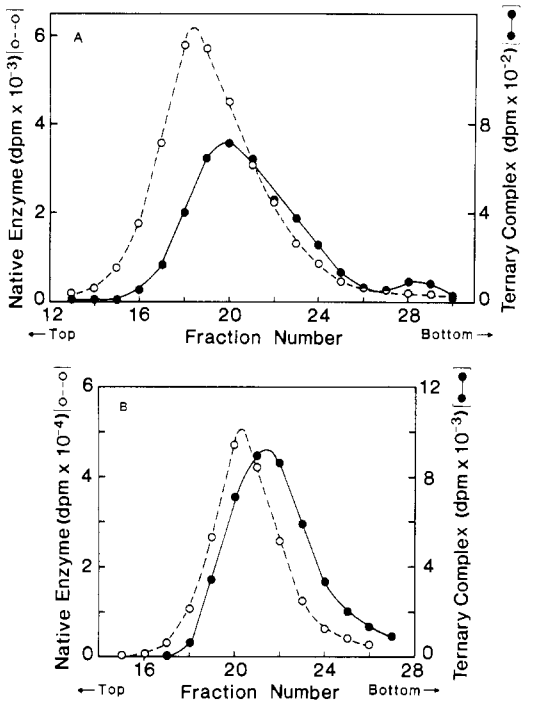

FIGURE 4: (A) Sedimentation of native CCRF-CEM thymidylate synthetase and the [^{3}H]FdUMP CCRF-CEM thymidylate synthetase ternary complex on a single sucrose density gradient. Native enzyme (2.2 pmol) and ternary complex (0.33 pmol) were centrifuged at 57 000 rpm for 18 h, and 115-μL fractions were collected. The amount of [^{3}H]FdUMP bound to the preformed ternary complex and after assays for the native enzyme was determined by the charcoal method. (B) Sedimentation of native *L. casei* thymidylate synthetase and the [^{3}H]FdUMP *L. casei* thymidylate synthetase ternary complex (2:2:1) on a single sucrose gradient. Native enzyme (24 pmol) and ternary complex (5.0 pmol) were centrifuged at 57 000 rpm for 18 h. The amount of [^{3}H]FdUMP bound to the preformed ternary complex and after assays for the native enzyme was determined by the charcoal method.

1:1:1 ternary complex eluting after the native enzyme but somewhat ahead of the 2:2:1 ternary complex. The same order of elution was observed when the [^{3}H]FdUMP 2:2:1 ternary complex was chromatographed with the [^{14}C]FdUMP 1:1:1 ternary complex. As expected, the [^{3}H]FdUMP and [^{14}C]-FdUMP 2:2:1 ternary complexes of the *L. casei* enzyme eluted simultaneously from the Sephadex G-100 column. Approximately 70% of the total observed shift in the elution volume occurred upon the binding of 1 mol of ligands. Similar experiments could not be performed with the CCRF-CEM enzyme, owing to the relatively low specific radioactivity of the [^{14}C]FdUMP.

Sucrose Density Gradient Centrifugation. This technique was also employed to detect conformational differences in thymidylate synthetase after complex formation with [^{3}H]-FdUMP and 5,10-CH_2H_4PteGlu. Comparison of the sedimentation of the CCRF-CEM thymidylate synthetase ternary complex with marker proteins indicated an $s_{20,w}$ value of 4.7 ± 0.05 S (three separate gradients). Similar analysis of three separate gradients showed that the native CCRF-CEM enzyme had an $s_{20,w}$ of 4.4 ± 0.1 S. The two enzyme forms were centrifuged on a single gradient and the fractions were assayed to verify that the native human enzyme sedimented at a slower rate than its ternary complex. Figure 4A shows the result of one such gradient. The ternary complex sedimented at a rate substantially greater (6%) than can be accounted for by the increased molecular weight due to the bound ligands (1.8%). Similar experiments comparing the *L. casei* enzyme and its ternary complex showed more than a 5% increase in the sedimentation rate upon complex formation (Figure 4B).

Cosedimentation of the [^{3}H]FdUMP CCRF-CEM ternary complex and the [^{14}C]FdUMP *L. casei* ternary complex (not shown) also showed that the bacterial enzyme ternary complex sedimented considerably faster than did the CCRF-CEM ternary complex and, taken together with the Sephadex gel filtration experiments, confirmed that the bacterial enzyme behaved as a more symmetrical and/or compact entity than did the human enzyme.

The amount of enzyme used in the gel filtration and sedimentation experiments was always small (a maximum of 45 μg of all forms of the *L. casei* enzyme and 0.8 μg of the CCRF-CEM enzyme in any single experiment), and no effect of protein concentration on the hydrodynamic parameters was evident at these low concentrations.

The tightly bound ternary complexes did not dissociate appreciably during either the gel filtration or sedimentation experiments. Recovery of the radiolabeled ternary complexes was usually 90–100%. If fractions were kept cold and were assayed within a day after completion of an experiment, the peaks of radioactivity coincided whether the radioactivity of fraction aliquots was determined directly or after nitrocellulose filtration or charcoal treatment, indicating that significant dissociation of the ternary complexes had not occurred. On occasion, higher molecular weight aggregates of the ternary complexes were evident for the enzymes (e.g., Figure 4A), but these aggregates amounted to only a small proportion of the total radioactivity and were far removed from the major peak.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the denatured CCRF-CEM enzyme and six marker proteins was conducted on a single 10% gel (not shown). A molecular weight of 36 000 was found, or about $^1/_2$ that of the holoenzyme (Table I), indicating that the enzyme is composed of two subunits of identical or nearly identical molecular weight. No significant difference was found in the relative mobilities of the native human enzyme and its ternary complex. An earlier report of 33 000 for the molecular weight of the CCRF-CEM enzyme subunit (Lockshin et al., 1979) was based on the comparison of the relative mobility with a different set of standard proteins (bovine serum albumin, ovalbumin, porcine stomach mucosa pepsin, bovine pancreas trypsinogen, and bovine milk β-lactoglobulin) run on a parallel 7.5% gel. The value obtained for the CCRF-CEM enzyme subunit with the 10% gel is probably more accurate because the molecular weight found for the *L. casei* enzyme subunit with this system was 37 000, in close agreement with the value determined by amino acid sequencing (Maley et al., 1979), whereas a molecular weight of 35 000 [also reported previously by Dunlap et al. (1971)] was obtained for the bacterial enzyme subunit with the 7.5% gel system.

When the radiolabeled ternary complexes of both enzymes were denatured with 3% sodium dodecyl sulfate and subjected to electrophoresis on the same gel (either 7.5 or 10%), the [^{3}H]FdUMP CCRF-CEM ternary complex subunit migrated somewhat faster than did the [^{14}C]FdUMP *L. casei* complex, indicating a molecular weight ~500 lower for the human enzyme subunit (Figure 5).

Denaturation of the gels by heating in 1% sodium dodecyl sulfate did not completely dissociate the subunits of either complexed enzyme, as evidenced by small peaks of radioactivity corresponding to double the molecular weight of the subunits. When the proteins were denatured in 3% sodium dodecyl sulfate, these small peaks of radioactivity disappeared. The radiolabeled ternary complexes showed the same relative

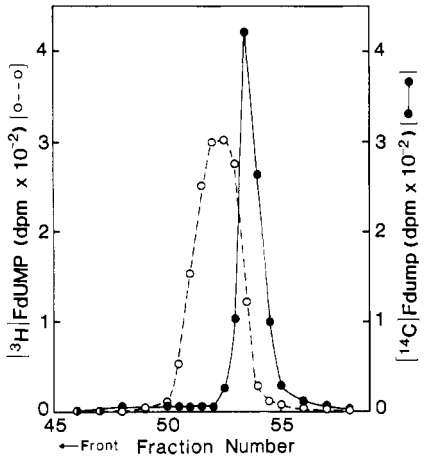

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the [^{3}H]FdUMP CCRF-CEM thymidylate synthetase ternary complex and the [^{14}C]FdUMP *L. casei* thymidylate synthetase ternary complex on a 10% gel. The ternary complexes were prepared and denatured separately with 3% sodium dodecyl sulfate and were then combined for electrophoresis on a single gel. After fixation and staining, the gel (total length 9.6 cm) was sliced into uniform 1-mm sections, except in the area of the radioactivity peaks, where 0.5-mm sections were obtained. The radiolabeled proteins then were extracted, and the radioactivity was measured.

mobility whether or not the samples were pretreated with deoxycholate and trichloroacetic acid (see Materials and Methods).

Summary of Observed Properties. Ternary-complex formation resulted in an ~3.5% decrease of the Stokes radius (Figure 1 and Table I) and an ~6% increase in the sedimentation coefficient (Figure 4 and Table I) for both enzymes. The partial specific volumes of native CCRF-CEM and *L. casei* thymidylate synthetases were calculated to be 0.734 and 0.736, respectively (Table I). Upon ternary-complex formation, the calculated $\bar{v}$ values decrease very slightly for both enzymes.

The increases in the $s_{20,w}$ values of the ternary complexes which are attributable to the additional weight of the ligands and to the decreased $\bar{v}$ values (Martin & Ames, 1961) are 1.8 and 0.3%, respectively. Therefore, the net difference in the sedimentation coefficients for both enzymes due to conformational change is ~3.5%.

The molecular weight of the CCRF-CEM enzyme, calculated from the hydrodynamic parameters, of 70 000–71 000 (the latter value obtained from the ternary complex after substraction of the bound ligands) compares well with the dimeric enzyme molecular weight of 72 000 obtained from gel electrophoresis. The data show that both the human and bacterial thymidylate synthetases undergo approximately the same reduction in their frictional ratio upon covalently bonded complex formation with FdUMP and 5,10-CH_2H_4PteGlu.

Discussion

These studies were undertaken as part of an investigation of human thymidylate synthetases and their interaction with FdUMP. Most of the detailed studies of thymidylate synthetase have been carried out with the *L. casei* enzyme, which has been well characterized as a dimeric enzyme, containing identical subunits and a cysteine residue at each subunit's nucleotide binding site. The bacterial enzyme has an ordered mechanism of ligand binding and release (Danenberg & Danenberg, 1978) and covalently binds FdUMP in the presence of the 5,10-CH_2H_4PteGlu cofactor with a maximum average ratio of ligands to enzyme of somewhat less than 2:1. In these respects, the CCRF-CEM enzyme appears to be similar (Lockshin et al., 1979; Lockshin & Danenberg, 1979a). However, there are also substantial differences in some properties of the *L. casei* and CCRF-CEM thymidylate synthetases, including the amino acid composition, the apparent stability of the ternary complex to 6 M urea (Lockshin et al., 1979), as well as the relative stability of ternary complexes formed with folate analogues (unpublished experiments). From the present studies, it is also evident that the shapes of the two proteins are significantly different. The CCRF-CEM enzyme, whether in native or complexed form, has a higher frictional ratio than the bacterial enzyme counterpart.

The results obtained by repeated gel filtration and sedimentation experiments of marker proteins plus the native enzyme or the ternary complex indicated that by complex formation significant changes are brought about in the Stokes radius (and thus the diffusion coefficient) of both enzymes and in the sedimentation coefficient of the human enzyme. These results were verified by conducting experiments in which both forms of the enzymes were simultaneously chromatographed or centrifuged, thereby eliminating the small variabilities encountered in experiments where each enzyme form was treated separately. The latter experiments unequivocally confirmed the conclusions drawn from the experiments using marker proteins. These results differ from those of Aull et al. (1974a), who were unable to detect any differences in the elution from Sephadex G-100 of the two forms of *L. casei* thymidylate synthetase, leading them to conclude that there are no gross differences in the tertiary structures of the native and complexed enzyme.

Two methods of hydrodynamic analysis were used in order to ensure that the observed effects were due to conformational change and not due to conceivable artifacts of one or the other method. For instance, the relative retardation of the heavier ternary complexes on Sephadex cannot be accounted for by partial dissociation of the ligands, followed by rebinding of radiolabeled FdUMP, because this process should also cause similar apparent retardation during sedimentation. In fact, the ternary complexes sedimented *faster* than the native enzymes. Moreover, the dissociation rates of these tightly bound ternary complexes at 4 °C are extremely slow, and the concentration of available 5,10-CH_2H_4PteGlu in the columns or gradients would be too low for rebinding. If the greater elution volumes were due to ligand-induced dissociation of the enzyme subunits, this phenomenon should also result in relative retardation of the ternary complex during sucrose gradient centrifugation, contrary to what was actually observed. There is no evidence that either the native *L. casei* enzyme or the 1:1:1 or the 2:2:1 complexes dissociate detectably into subunits under nondenaturing conditions (Aull et al., 1974a,b; Donato et al.; Danenberg & Danenberg, 1979). We also observed that even hot 1% sodium dodecyl sulfate does not entirely dissociate the ternary complexes of either enzyme (see Results). The data obtained with both analytical methods argue against preferential interaction of the ternary complex with the Sephadex matrix, since this interpretation is also incompatible with the results of the sedimentation experiments. We have also observed that the *L. casei* enzyme ternary complex which was denatured with hot sodium dodecyl sulfate is not retarded relative to similarly denatured native enzyme on Sephadex G-100, providing additional evidence that ligand binding does not induce interaction with the matrix.

Although the $s_{20,w}$ values for the native and complexed *L. casei* enzyme were not determined by cosedimentation with marker proteins, comparison of the CCRF-CEM and *L. casei*

enzyme ternary complexes indicates that the *L. casei* $s_{20,w}$ values are substantially higher (~0.5 S) than the CCRF-CEM enzyme counterparts, or ~5.2 S for the *L. casei* enzyme ternary complex. Other workers have recently reported an $s_{20,w}$ value of 4.15 S for the native *L. casei* enzyme (and a higher frictional ratio of 1.35) based on sedimentation velocity experiments (Reinsch et al., 1979). However, their studies were conducted in very high ionic strength buffer (0.5 M phosphate), which might account for some of the discrepancy.

Maximum binding of the ligands results in a 1.8% increase[3] in the particle weight of thymidylate synthetase and a 0.2% decrease in the partial specific volume. If changes in sedimentation and elution profiles were due to these factors alone, there should be a 2.1% increase in $s_{20,w}$ and a small decrease in *D*. However, the $s_{20,w}$ increases ~6% for both enzymes, and *D also increases* ~3.5% when the ternary complexes are formed. These results clearly indicate that the small increases in particle weight and density are not the major causes of the observed effects. The apparently higher molecular weight of the complexed CCRF-CEM enzyme (71 000 after subtracting the molecular weight of the ligands, compared to 70 000 for the native enzyme) is within the experimental error of the determinations for $s_{20,w}$ and $D_{20,w}$.

The reduction in the frictional ratio for both thymidylate synthetases upon ternary-complex formation indicates gross alterations in the protein structure, which could be due to lessened dissymmetry of the protein, a decrease in hydration, or the formation of a more compact structure. These results provide a striking example of the degree to which enzymes may manifest structural changes during catalysis, which in this case is demonstrated by the formation of a structure probably very similar to an activated complex of the enzymatic reaction.

A change of similar magnitude (but of opposite direction) was observed for the multisubunit allosterically regulated enzyme aspartate transcarbamylase in the presence of effector ligands (Gerhart & Schachman, 1968). A large conformational change might be expected in this case because the binding of these molecules is a signal that must be transmitted to a distant site in another subunit.

Conformational changes accompanying the binding of substrates to free enzyme have been conveniently studied by using close analogues of the substrate to form dead-end complexes or by using one or more, but not all, of the substrates of a multisubstrate reaction to form stable enzyme–substrate complexes. However, formation of the initial enzyme–substrate complex is only the first step of the catalytic sequence and does not answer the question of whether additional conformational changes take place during catalysis. Koshland (1958) introduced the concept of a flexible active site in which binding of the substrate causes proper alignment of catalytic groups (the "induced fit"). This theory suggests that the enzyme conformation changes continually in a dynamic manner throughout the course of the reaction (Hammes, 1964). The actual scale of conformational change in an enzyme during catalysis is difficult to predict or to measure, and so there is little direct evidence to indicate what it may be. One might expect that the greatest difference among conformational states would be between the free enzyme and the enzyme–transition state complex. The transition state of a substrate is considered to bind to an enzyme many orders of magnitude more tightly than the substrate in the ground state (Pauling, 1946; Wolfenden, 1972; Lienhard, 1973). Such a powerful attractive force between an enzyme and substrate(s) could well require substantial realignment of the domains of the protein. Because transition states cannot normally be isolated, one is limited to studies of complexes formed between an enzyme and a transition-state analogue in order to obtain indications of changes occurring during normal catalysis. However, most transition-state analogues that have been designed to date fall far short of the binding theoretically predicted for the transition state, illustrating thereby their imperfect structural resemblance to the actual transition state of the enzymatic reaction.

FdUMP (which differs from dUMP only by the replacement of a hydrogen atom with fluorine at the 5 position) reacts with thymidylate synthetase and the cofactor to form a very tight binding complex with a K_D of 10^{-11}–10^{-12} M (Santi et al., 1974a; Santi, 1980), and several lines of evidence strongly indicate that the ternary complex so formed is an analogue of an activated complex formed by the enzyme and both substrates [for a review of this topic, see Danenberg & Lockshin (1980)]. Our results indicate that K_{cat} inhibitors such as FdUMP, which mimic substrates sufficiently to undergo partial catalysis by an enzyme, may offer an approach to estimating the magnitude of conformational transitions during enzymatic catalysis.

At least two major factors may be responsible for the marked conformational change brought about by ternary complex formation with FdUMP and 5,10-CH_2H_4PteGlu. The binding of the two ligands may induce (require) alterations of the tertiary structure of each enzyme subunit in order to bring about the proper alignment of ligands and catalytic groups necessary for ternary complex formation. In addition, the ligand-induced opening of the binding site in the second subunit upon binding in the first subunit (Danenberg & Danenberg, 1979) probably requires substantial alterations in enzyme geometry. It is likely that both factors contribute to the observed conformation change. It is interesting that both thymidylate synthetases undergo very similar conformational changes upon complex formation in spite of marked differences in amino acid composition, shape, and other properties.

Although the presumed transient enzyme–dUMP–5,10-CH_2H_4PteGlu intermediate has not been isolated, additional indirect evidence suggests that enzyme conformational change(s) take(s) place in the course of this interaction. Pteroylmonoglutamates and dUMP together provide substantial protection against proteolysis of thymidylate synthetase, whereas the two ligands separately offer no protection (Galivan et al., 1977). Incubation of the enzyme with dUMP and pteroyltriglutamate also leads to the formation of a tightly bound (albeit noncovalent) ternary complex (Lockshin & Danenberg, 1979b), which is accompanied by shifts in the ultraviolet–visible spectrum (unpublished experiments) that are indicative of an enzyme conformational change.

Previous studies have shown that ternary-complex formation of *L. casei* thymidylate synthetase with FdUMP and 5,10-CH_2H_4PteGlu results in changes in the absorption, fluorescence, and circular dichroic spectra, which could be caused by perturbations of the ligand chromophores, the protein microenvironment, or the tertiary structure (conformation). Interpretation of these spectral shifts has been complicated by difficulties in determining which changes are attributable to the ligands and which to the protein. For example, it was suggested that an increase in light absorbance at 330 nm upon ternary-complex formation was caused by the oxidation of the tetrahydrofolate portion of the ternary complex to H_2PteGlu (Sharma & Kisliuk, 1973), which was later shown not to occur (Santi et al., 1976). The change in the Stokes radius of the *L. casei* enzyme upon the formation of the 1:1:1 and 2:2:1

ternary complexes correlates approximately with previously reported studies that measured fluorescence quenching as a function of FdUMP binding (Sharma & Kisliuk, 1975; Donato et al., 1976). This observation provides evidence that these fluorescence changes reflect major alterations of the tertiary structure and do not arise solely from modifications of the ligands or from their interaction with individual aromatic residues of the enzyme, although further investigation is required to firmly establish this relationship.

An additional advantage of studying the hydrodynamic properties of thymidylate synthetases (native and complexed) is that investigations of conformational changes can be carried out on picamole quantities of this enzyme obtained from human tissues or other sources which do not contain the high amounts of thymidylate synthetase needed for the spectral studies. This approach should also be applicable to the investigation of other enzymes which form very tight bonds with specific ligands.

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