Fluorine-19 Nuclear Magnetic Resonance Studies of Binary and Ternary Complexes of Thymidylate Synthase Utilizing a Fluorine-Labeled Folate Analogue†

Thomas J. Connick, R. Todd Reilly, R. Bruce Dunlap,* and Paul D. Ellis

Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208

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ABSTRACT: Traditional fluorine-19 nuclear magnetic resonance (^{19}F NMR) studies of thymidylate synthase (TS) have utilized the fluorine substituent of 5-fluorodeoxyuridine 5′-monophosphate (FdUMP), a mechanism-based inhibitor of the enzyme, in complexes with various folate and folate analogues in order to establish a paradigm for the formation of binary and ternary complexes. In order to extent and confirm this paradigm, complexes of thymidylate synthase (TS) and the *N*-10-(fluoroethyl)quinazolinylfolate analogue CB3731 with either deoxyuridine 5′-monophosphate (dUMP), deoxythymidine 5′-monophosphate (dTMP), or FdUMP were examined from the perspective of the folate analogue using ^{19}F NMR. The spectrum of the free folate analogue gave rise to a multiplet centered at −57.0 ppm, which was broadened by approximately 50% upon incubation with the enzyme. The use of FdUMP with CB3731 afforded us the opportunity to compare the spectrum obtained for the folate with that of the nucleotide. This comparison led to the assignment of the resonance at −53.5 ppm as representing the noncovalent TS:FdUMP:CB3731 ternary complex, while a new resonance at −52.0 ppm corresponded to the species in which the nucleotide is covalently attached to the enzyme and the folate is noncovalently associated. Ternary complexes consisting of TS, CB3731, and either dUMP or dTMP displayed a resonance at −53.5 ppm which represented the noncovalent TS–nucleotide adduct. None of the TS:nucleotide:CB3731 ternary complexes, however, was stable to native polyacrylamide gel electrophoresis.

The enzyme thymidylate synthase (TS)[1] (EC 2.1.1.45) is a dimer of identical subunits, each with an active site and a molecular weight of 32000–37000, depending on the source (Lewis & Dunlap, 1981). TS catalyzes the reductive methylation of dUMP to dTMP, utilizing the coenzyme CH_2H_4-folate as a reductant as well as the methyl donor. The fact that this reaction represents the sole pathway for the *de novo* synthesis of dTMP, together with the observation that the enzyme is normally present in cells at very low levels except during periods of rapid division, such as in fetal and regenerating tissues, suggested it to be a control point for the rate of DNA synthesis and therefore of cell division (Santi & Danenberg, 1984). This insight has been exploited for the control of accelerated cell division in cancerous tissues by the development of a number of 5-substituted pyrimidines as possible chemotherapeutic agents (Lewis & Dunlap, 1981). The best known of these are 5-fluorouracil and 5-(trifluoromethyl)-2′-deoxyuridine, which are precursors of mechanism-based inhibitors of TS (Santi & McHenry, 1972; Heidelberger *et al.*, 1983; Santi, 1980; Lewis & Dunlap, 1981).

Complexes of TS with FdUMP and various folates were first examined by ^{19}F NMR by Santi and co-workers in 1976, who found that an active-site peptide fragment, isolated after proteolysis of the inhibitory ternary complex of *Lactobacillus casei* TS with CH_2H_4folate and FdUMP, contained covalently bound FdUMP. From the spectra obtained, the researchers determined that the enzyme fragment and cofactor occupied *trans* pseudoequatorial positions in the active-site peptide fragment. Studies from this laboratory of the binary and ternary complexes in their native conformations using intact TS demonstrated that the nucleotide is bound with its pyrimidine ring in the *syn* conformation in the binary complex and that the stereochemistry of the ternary complex is such that Cys 146 and the methylene group occupy pseudoaxial positions in the native complex, but isomerize to the pseudoequatorial positions upon denaturation (Byrd *et al.*, 1977, 1978). In 1981, Lewis and co-workers utilized folate analogues which resembled the natural substrate, product, or a reaction intermediate to study their effects on the covalent and noncovalent complexes formed between TS and FdUMP via ^{19}F NMR. From these studies it was determined that, while the TS:FdUMP binary complex exists in an equilibrium mixture which favors their noncovalent interaction (Lewis *et al.*, 1978, 1980), the addition of certain folates to the binary complex shifts that equilibrium to favor covalent attachment of the nucleotide (Lewis *et al.*, 1981). The dissociation constants for these pseudoternary complexes were shown to be significantly lower than those of either the nucleotide of folate alone, and the extent of covalent bond formation between the enzyme and nucleotide varied depending on the nature of the folate.

The partitioning of covalent and noncovalent associations observed exclusively in the ^{19}F spectra of the binary and pseudoternary complexes prompted us to develop a chemical method to isolate and quantitate these covalent TS–nucleotide adducts. Using a trichloroacetic acid precipitation assay, we were able to isolate not only covalent complexes of TS with FdUMP in the presence and absence of folates (Moore *et al.*,

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[1] Abbreviations: TS, thymidylate synthase; FdUMP, 5-fluorodeoxyuridine 5′-monophosphate; CH_2H_4folate, (±)-5,10-methylene-5,6,7,8-tetrahydrofolate; CB3731, *N*-[4-[[*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]-*N*-(fluoroethyl)]amino]benzoyl]-L-glutamic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TCA, trichloroacetic acid.

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1984; Cisneros & Dunlap, 1990) but also covalent catalytic complexes of TS and dUMP or dTMP, with and without folates (Moore et al., 1986). Adaptation of this technology has led to the analysis of the pre-steady-state kinetics of inhibitory ternary complex formation by the stopped-flow chemical quench procedure (Deng *et al.*, 1993). A number of other techniques, such as nitrocellulose filter binding assays (Santi *et al.*, 1974; Ahmed *et al.*, 1985) and equilibrium dialysis (Galivan *et al.*, 1976), have been used to quantitate the sum of the covalent and noncovalent nucleotide binding to TS. However, these techniques are limited in that they cannot distinguish between covalent and noncovalent complexes, indicating only the sum of the two interactions.

The three-dimensional structure of TS obtained by X-ray crystallography of the ternary complex between TS, FdUMP, and CH_2H_4folate (Matthews *et al.*, 1990) gave support for the stereochemistry predicted by ^{19}F NMR. The crystal data showed that the nucleotide sits at the bottom of the TS active site with the folate cofactor stacking parallel to and above the pyrimidine ring of the nucleotide. The addition of Cys 146 and the methylene group of the cofactor occurs in such a way that these two entities do, in fact, occupy *trans* diaxial positions in the ternary complex crystal.

The covalent inhibitory ternary complex has proven to be a very useful paradigm in the study of the TS catalytic mechanism, yielding a great deal of kinetic and structural information which is relevant as an approximation of the catalytic mechanism. However, the majority of the data collected to date, and specifically the NMR results, have focused on FdUMP and its interactions with TS in the binary, pseudoternary, and inhibitory ternary complexes. In order to fully validate and extend these previous studies, we sought to investigate the process of ternary complex formation from the perspective of the folate. To this end, we have employed the folate analogue *N*-10-(fluoroethyl)-5,8-dideazafolate (CB3731) as an NMR probe with which to study complex formation, not only with FdUMP but with dUMP and dTMP as well. In this article, we expand the ^{19}F NMR studies initiated by Lewis *et al.* (1980, 1981) by examining various TS:CB3731:nucleotide complexes by ^{19}F NMR, utilizing the fluorine nucleus of the folate analogue as well as that of the nucleotide, FdUMP.

EXPERIMENTAL PROCEDURES

Enzyme Preparation and Characterization. Thymidylate synthase was purified from amethopterin-resistant *Lactobacillus casei* in the presence of 10 mM 2-mercaptoethanol as described by Lyon *et al.* (1975). Polyacrylamide gel electrophoresis was performed on the pooled enzyme as described previously (Lyon *et al.*, 1975). The enzyme was usually pure at this stage and could be used in experiments in which bound phosphate was not a problem (Lewis *et al.*, 1980). However, for most of the NMR experiments, it was desirable to remove any phosphate. Tightly bound phosphate was extracted from the enzyme with a DEAE–Sephadex column (2 × 50 cm). The enzyme was dialyzed against 0.1 M Tris Cl buffer (pH 7.3) containing 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol and loaded onto the column. A linear gradient was used for elution: mixing chamber, 500 mL of dialysis buffer; reservoir chamber, 500 mL of 0.1 M Tris Cl (pH 6.8) containing 150 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The protein eluted in the first 500 mL at a flow rate of 0.3–0.5 mL/min.

Protein homogeneity was again determined by polyacrylamide gel electrophoresis of the native enzyme and the enzyme—FdUMP—CH_2H_4folate ternary complex. The enzyme activity slowly decreased while the sample was stored at 4 °C, and a minimum activity of 2.5 units/mg protein was established for use of the enzyme. Pure, fully active TS usually exhibits a specific activity of 2.5–3.5 units/mg at 25 °C. The enzymatic reaction was assayed by monitoring the change in absorbance at 340 nm as described by Dunlap *et al.* (1971). One unit of enzyme catalyzes the formation of 1 μmol of thymidylate per minute. Enzyme was activated before each experiment by dialysis for at least 8 h against 0.1 M Tris Cl buffer (pH 7.3) containing 1 mM EDTA and 50 mM 2-mercaptoethanol.

Ligand Preparation. The quinazoline antifolates, PDDF, and its *N*-10-(fluoroethyl) analogue, CB3731, were gracious gifts of Dr. Terrence Jones (Agouron Pharmaceuticals Corporation, La Jolla, CA) and were used, without further purification, to prepare stock solutions in 0.1 M Tris Cl buffer (pH 7.3) containing 30% D_2O. Concentrations were determined by analysis of the UV spectra of the stock solutions, employing the following molar extinction coefficients: $A_{301.5}$ for PDDF = 26 600 cm^{-1}; A_{307} for CB3731 = 29 200 cm^{-1} (Jones *et al.*, 1981).

5,6,7,8-H_4folate was prepared by the catalytic hydrogenation of folic acid (Calbiochem) by the method of Hatefi *et al.* (1960), and the lyophilized powder was stored at −70 °C. The cofactor solution containing 5,10-CH_2H_4folate was obtained by the addition of formaldehyde to H_4folate as previously described by Lyon (1975). FdUMP, dUMP, and dTMP were purchased from Sigma Chemical Company, and the weighed powders were dissolved into 0.1 M Tris Cl buffer (pH 7.3) containing 1 mM EDTA and 30% D_2O. Values of pH remain uncorrected for the deuterium isotope effect. Concentrations of stock solutions (20–40 mM) were determined by analysis of their UV spectra.

NMR Methods. For NMR samples, the activated enzyme was concentrated using PM-10 Diaflo ultrafiltration membranes and concentration cells from Amicon Corporation. At a concentration of approximately 50 μM enzyme, the solution was diluted by 30% with an aliquot of the same buffer prepared with 99.98% D_2O, and the volume reduction was resumed until a TS concentration of 100–200 μM was reached as determined by its extinction coefficient, A_{278} = 105 000 M^{-1}. The final sample contained 30% D_2O, which provided an internal lock reference for the NMR experiments.

Fluorine-19 NMR at 188.2 MHz was performed on a Bruker WP 200 spectrometer. Spectra were recorded in a 5- or 15-mm fluorine probe with the temperature regulated at 4 °C. For acquisition of the NMR signals from FdUMP and CB3731, quadrature detection was employed, and unless otherwise specified, a sweep width of 31 kHz was used for the observation of protein-containing samples. A 90° pulse width of 4.5 and 16.5 μs was determined for the 5- and 15-mm probes, respectively. Spectra were typically acquired using an 8K data table and an acquisition time of 0.13 s with a 45° pulse width.

Preparation of Enzyme Complexes for NMR Samples. The complexes used in the NMR studies were formed by the addition of the ligands, in small aliquots, to the enzyme solution contained in the NMR tube. In the case of the ternary complexes, the first ligand was allowed to incubate with the enzyme for at least 30 min prior to the addition of the second ligand.

TS:FdUMP:Folate Complexes. For the titration of TS with the folates, 0.3 mL of an enzyme solution containing 125

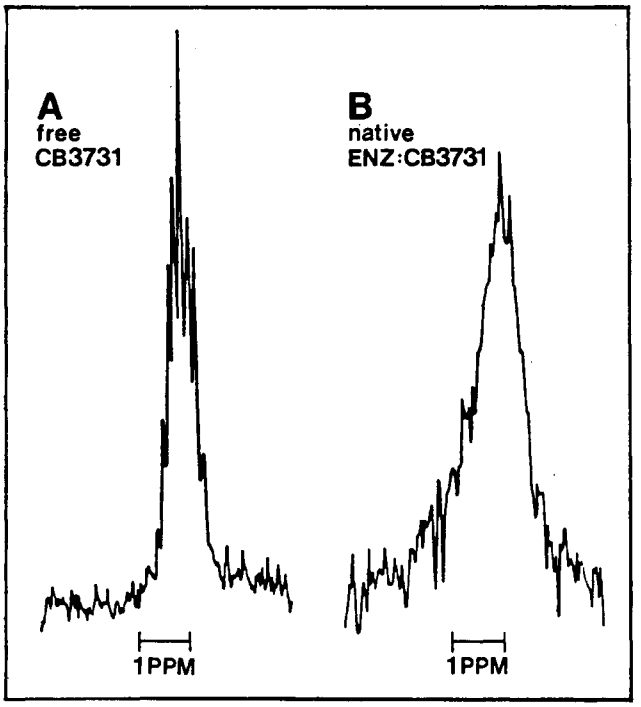

FIGURE 1: Interaction of TS with the quinazoline folate CB3731: (A) CB3731; (B) TS with added CB3731 (4-fold over the enzyme). The spectra were recorded with a 2600-Hz sweep width using an 8K data table, and 5 Hz of line broadening was applied prior to Fourier transformation.

$\times 10^{-6}$ M enzyme was placed in a 5-mm NMR tube, to which the desired folate was then added to the protein solution using stock solutions of 20 mM. The stoichiometries of the complexes for each spectrum plotted are given in the text. FdUMP was also added in small increments from a 20 mM stock solution with a micropipet.

TS:Nucleotide:CB3731 Ternary Complex. For the titration of TS with FdUMP, dUMP, and dTMP, 0.3 mL of an enzyme solution containing 125×10^{-6} M enzyme was first placed in a 5-mm NMR tube. The desired nucleotide was then added to the protein solution using 20 mM stock nucleotide solutions and a calibrated microcapillary pipet. Finally, the CB3731 was added in small increments from a 20 mM stock solution with a micropipet. The stoichiometries of the complexes for each spectrum plotted are given in the text.

Denatured TS:dUMP:CB3731 Complex. For the spectra seen in Figure 4, the ternary complex was formed by incubation of the enzyme with a 5-fold molar excess of dUMP and a 3-fold excess of CB3731 mixed in the NMR tube. After acquisition of the spectrum of the native complex, the solution was made 1.2% in sodium dodecyl sulfate (SDS) by the addition of an aliquot from a 25% stock solution.

Polyacrylamide Gel Electrophoresis. The procedure for disc gel electrophoresis described by Aull *et al.* (1974) was employed to examine binary and ternary complexes of TS utilizing the ligands employed in the NMR studies described above. Samples having a total volume of 100 μL were prepared by premixing the ligands and enzyme and incubating the mixture for 30 min at room temperature. The samples were loaded onto 7.5% polyacrylamide gels and run at 3 mA per gel until the dye front reached the end of the gel. A 2 mM solution of Coomassie blue in 10% acetic acid was employed to fix and stain the protein bands.

The gels were loaded with the following mixtures: (1) 0.14 nmol of TS alone; (b) 0.14 nmol of enzyme preincubated with a 100-fold excess of FdUMP, dTMP, or dUMP; (c) enzyme preincubated with a 100-fold excess of the *N*-10-(fluoroethyl)-quinazolinylfolate in the presence and absence of a 100-fold excess of either FdUMP, dTMP, or dUMP.

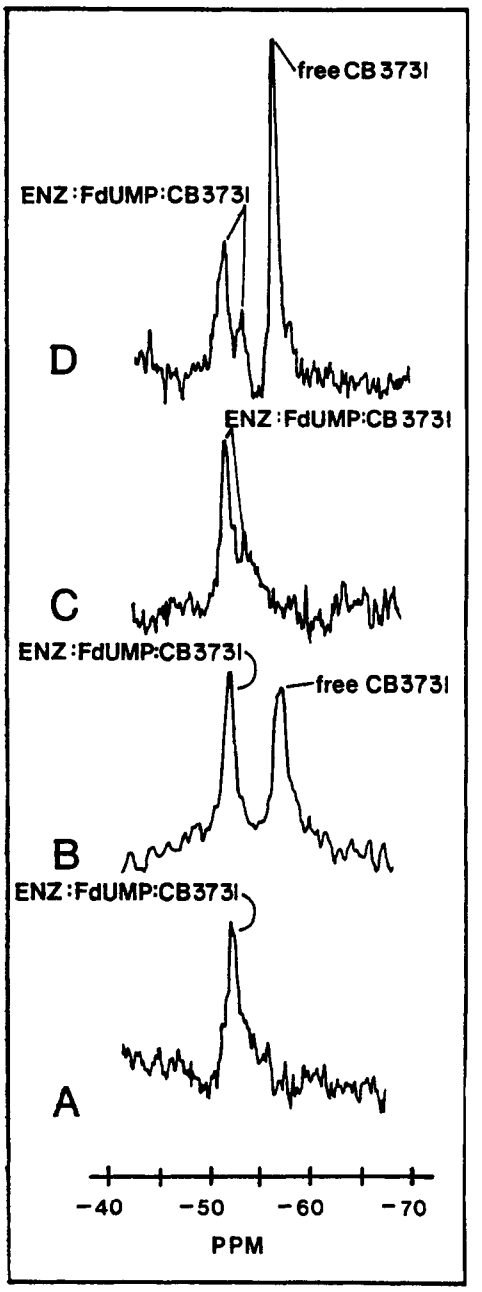

FIGURE 2: ^{19}F spectra of the interaction of the quinazoline folate CB3731 with TS and FdUMP. Spectra A–D correspond to protein samples with the following molar ratios of enzyme to FdUMP to CB3731: (A) 1:0.5:0.5; (B) 1:0.5:1.2; (C) 1:1.5:1.2; and (D) 1:5:5. Excess folate appears at –57.0 ppm. Various titration experiments indicate that the peaks at –53.5 and –52.0 ppm belong to folate residing in the active site with noncovalently and covalently bound nucleotides, respectively.

RESULTS AND DISCUSSION

TS:CB3731 Binary Complex. CB3731 was directly observable by fluorine NMR due to its *N*-10-(fluoroethyl) substituent. A 4:1 molar ratio of ligand to enzyme was used throughout, and the enzyme concentration varied between 100 and 130 μM. The experiments were conducted in the absence of proton decoupling because of a severe decrease in S/N resulting from the presence of a negative nuclear Overhauser effect. This effect has been reported previously

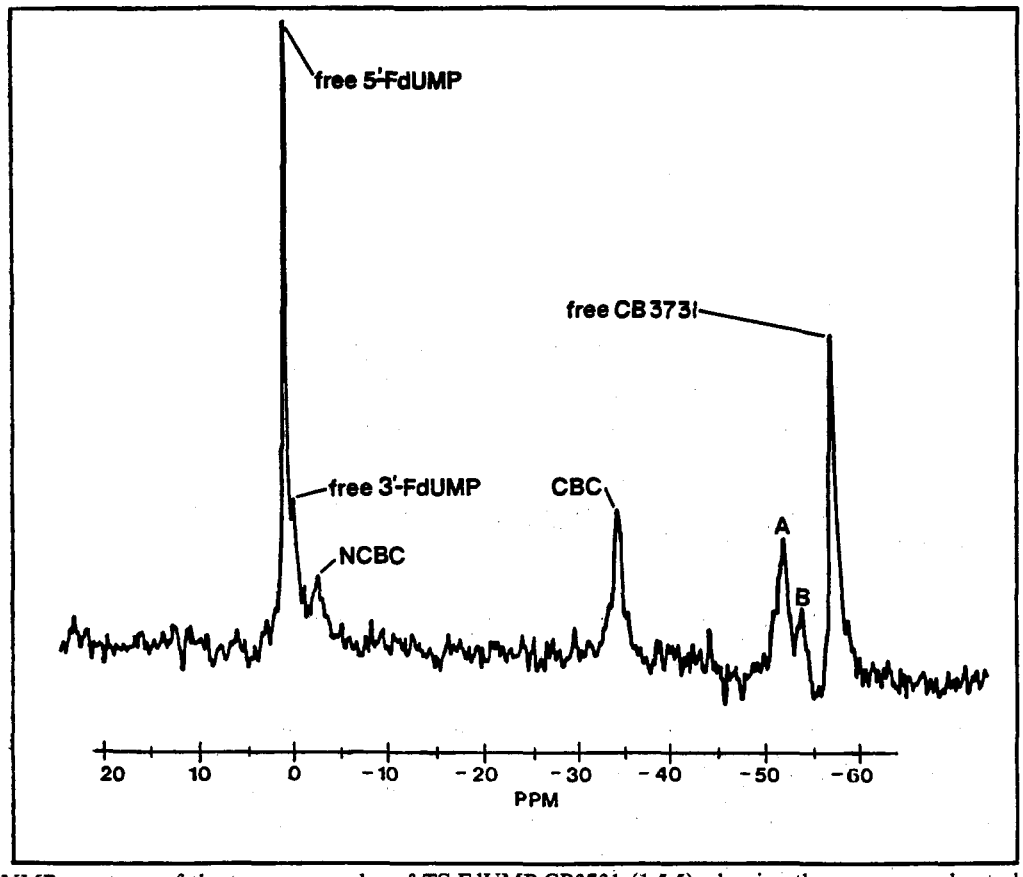

FIGURE 3: ^{19}F NMR spectrum of the ternary complex of TS:FdUMP:CB3731 (1:5:5), showing the resonances due to both FdUMP and CB3731. Peak A corresponds to the TS—FdUMP- - -CB3731 complex and arises from the fluorine of CB3731, while the resonance for this complex arising from the fluorine of FdUMP is labeled CBC. Similarly, the resonance labeled B corresponds to the TS- - -FdUMP- - -CB3731 complex and arises from CB3731, while that arising from FdUMP in this complex is labeled NCBC.

by groups performing protein NMR studies (Bothner-By, 1973; Gerig, 1977; Hull & Sykes 1975) and is a consequence of a long correlation time for the spin system which, in the present study, is the fluoroethyl group of CB3731.

The proton-coupled spectrum of CB3731 contained a single resonance centered at –57.0 ppm in the form of an apparent septet (Figure 1A). The multiplet was actually composed of nine lines due to the coupling of the fluorine to the two adjacent methylene groups, with J_{HF} coupling constants of 50 and 26 Hz for the 2-bond and 3-bond couplings, respectively. Incubation of CB3731 with TS at a molar ratio of 4:1 resulted in a 50% broadening of the lines in the septet observed for the fluoroquinazoline analogue at –57.0 ppm (Figure 1B). No new resonances became visible upon addition of the enzyme. Tight binding of CB3731 by the enzyme would have imposed a slow rate of exchange, and therefore, the on and off states of the ligand would have been sufficiently long-lived to be detected as separate resonances. As the spectrum of the sample of CB3731 with native enzyme shows (Figure 1B), only one set of resonances is visible. The observed broadening of the signal, therefore, indicates a rapid exchange of the ligand on and off of the enzyme, demonstrating that the folate analogue was weakly bound. Covalent modification of the enzyme with DTNB, as well as denaturation with SDS, resulted in spectra which were identical to that of free CB3731 (data not shown). These results suggest that under these conditions there is only weak interaction between the enzyme and the *N*-10-(fluoroethyl)quinazolinylfolate.

TS:CB3731:FdUMP Ternary Complexes. The ternary complexes formed with enzyme, FdUMP, and CB3731 were characterized by the resonances from the fluorine nucleus of both fluorinated ligands. One or both ligands could be monitored by proper selection of the spectral window. For this discussion, we have taken a series of spectra from the titration of one sample containing enzyme with both FdUMP and CB3731 and obtained the ^{19}F NMR spectrum of the folate analogue (Figure 2), as well as that of the nucleotide (Figure 3).

In Figure 2 are shown the spectra for points in the titration of TS with both FdUMP and CB3731. Spectra A–D correspond to protein samples with the following molar ratios of enzymes to FdUMP to CB3731: (A) 1:0.5:0.5; (B) 1:0.5;1.2; (C) 1:1.5:1.2; and (D) 1:5:5. The spectrum from the first titration point (Figure 2A) contains a distinct resonance at –52.0 ppm. We also note that a small spike lies to just higher shielding of this resonance at what appears to be –53.5 ppm, but we cannot assign any large importance to it because of the similar size of the noise in the spectrum. It is worth noting only in view of its position in relation to the peaks in spectra C and D and could be considered to be a real signal in light of that similarity. The signal at –52.0 ppm is the result of the binding of CB3731 in an enzyme active site with FdUMP. In order to determine the nature of the TS:CB3731:FdUMP complex, it was necessary to add more of the folate analogue.

Spectrum B in Figure 2 shows that, for an increase in the concentration of CB3731 to 1.2 molar equiv at constant levels of enzyme and FdUMP, all of the additional CB3731 remains unbound. This result could indicate either saturation of the folate binding sites on the enzyme or the inability of CB3731 to bind in the same manner in the absence of FdUMP. Both of these possibilities were addressed by increasing the concentration of FdUMP to a 1.5 molar excess over the enzyme in the next experiment.

An interesting spectrum resulted upon the addition of FdUMP to give the sample with molar ratios of 1:1.5:1.2 enzyme to FdUMP to CB3731 (Figure 2C). Not only did the additional FdUMP cause the resonance at −57.0 ppm to disappear completely, but at that point a new resonance was observed at −53.5 ppm. The presence of *two* deshielded resonances is of great importance. Because 1.2 molar equiv of CB3731 had been added to the sample, and no resonance appeared at −57.0 ppm for free ligand, it appeared that 1.2 mol of the ligand was bound per enzyme molecule. If the chemical shift changes observed were the result of specific binding, then a maximum of one of each type of ligand could be bound per active site, and the two resonances would indicate two distinct binding interactions. This was tested by further titration of the enzyme with both ligands.

The levels of both ligands were raised to a final concentration which was 5 times the TS concentration. The sample gave rise to the spectrum shown in Figure 2D, with a total binding of approximately 1.8 CB3731 molecules (and, by inference, FdUMP molecules) per dimer. At the molar ratio of 1:5:5, the resonances at −52.0 and −53.5 ppm corresponded to 1.5 and 0.3 equiv of CB3731 per dimer, respectively. Maximum binding was indicated by the leveling off of the growth of the resonances at −52.0 and −53.5 ppm and the reappearance of the signal at −57.0 ppm. The relative areas of the peaks in the spectrum of the sample indicate that most of the bound ligand (83%) in the active sites was associated with the resonance exhibiting the greatest deshielding. The possibility remained that some of the quinazoline derivative was bound in an active site which did not contain FdUMP. The answer to this question was provided by analysis of the resonances which arose from the fluorine atom at the 5-position of the pyrimidine ring of FdUMP, since FdUMP was, of course, present in the sample. The following discussion presents the data for the interaction of FdUMP with TS and CB3731 from the perspective of the nucleotide.

Correlation between the ^{19}F NMR Resonances of FdUMP and CB3731. The three resonances from the fluorine nucleus of FdUMP in the enzyme—FdUMP---CB3731[2] ternary complex are mirrored by the three resonances observed for the fluorine nucleus of CB3731 in the same complex. The spectrum containing the resonances due to both ligands in the ternary complex is shown in Figure 3 for the 1:5:5 mixture discussed above. The FdUMP resonances have been well characterized (see the Introduction). Again, they correspond to free ligand, noncovalently bound ligand, and covalently bound ligand at 0.0, −3.4, and −34.5 ppm, respectively (for the ternary complex containing CB3731 as the second ligand). Close examination of Figure 3 reveals a quantitative correlation between the three sets of fluorine resonances for the TS:FdUMP:CB3731 complexes as seen by FdUMP and *via* the fluorine-containing folate. The resonances at 0.0 and −57.0 ppm are known to arise from excess FdUMP and CB3731, respectively. The magnitude of the resonance at −52.0 ppm changes in concert with that of the peak for covalently bound FdUMP. Thus, the deshielding of the resonance at −52.0 ppm must be the result of the binding of CB3731 in the same enzyme active site which simultaneously contains covalently bound FdUMP.

By the same reasoning, the resonance at −53.5 ppm reflects simultaneous occupation of an active site with CB3731 and *noncovalently* bound FdUMP. In Figure 3, the peaks at −3.4 and −53.5 ppm, then, are directly related quantitatively in a one-to-one stoichiometry and qualitatively in that the shift in the frequency by which each peak is produced represents the mutual perturbation experienced by each on account of the other.

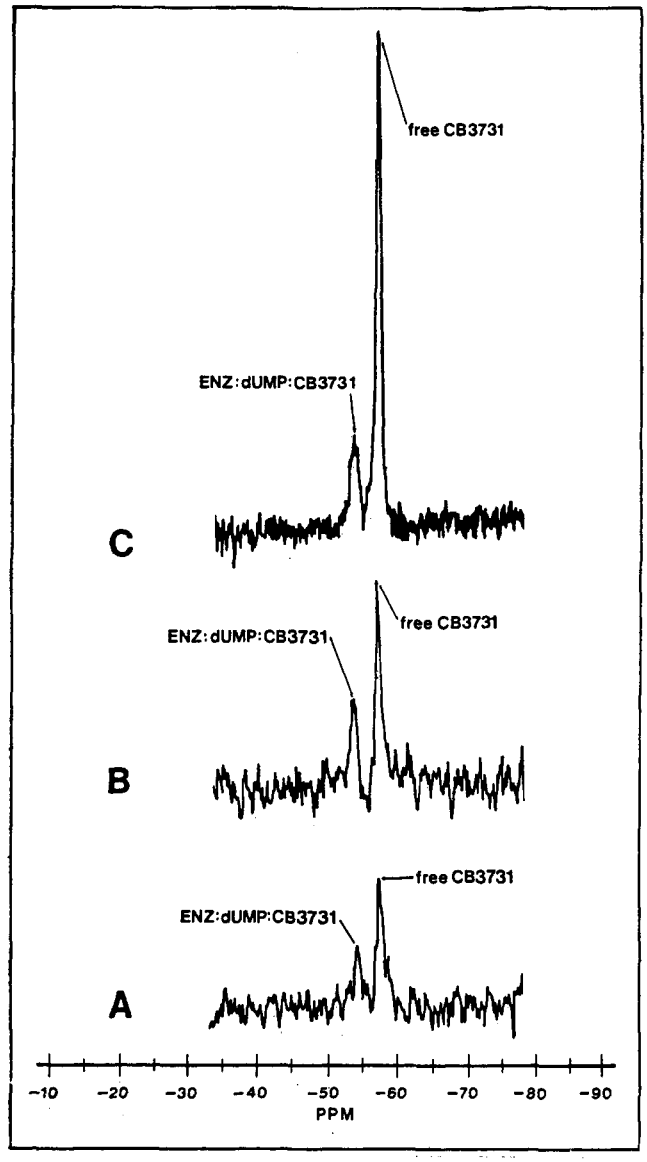

FIGURE 4: Titration of the TS:dUMP binary complex with CB3731 as seen by fluorine NMR of the latter. Complexes were formed using the following molar ratios: (A) 1:4:1.2 (TS:dUMP:CB3731); (B) 1:2:2.2; (C) 1:4:8.

These results extend the paradigm established with FdUMP for nucleotide binding with various folates and folate analogues and allow the interpretation of ^{19}F NMR analyses of ternary complexes of TS, CB3731, and various nucleotides. To this end, we proceeded to examine ternary complex formation between TS and its natural substrate, dUMP, as well as the product, dTMP, utilizing CB3731 as an NMR probe.

TS:CB3731:dUMP Ternary Complexes. The titration of TS was performed by incubation of a 4-fold excess of dUMP with the enzyme for 30 min, followed by successive additions of CB3731 (Figure 4A–C). For the sample containing a mixture of enzyme, dUMP, and CB 3731 in a molar ratio of 1:4:1.2 (Figure 4A), two resonances appeared in the fluorine spectrum with chemical shifts of −57.0 and −53.0 ppm. A comparison of the spectrum of CB3731 in Figure 2A to that of the quinazoline derivative alone (Figure 1A), or together

[2] Bonds in binary and ternary complexes are indicated by dotted lines when noncovalent or by a solid line when covalent (*i.e.*, enzyme—FdUMP---CB3731 signifies covalent attachment of FdUMP to the enzyme, but noncovalent interaction of CB3731).

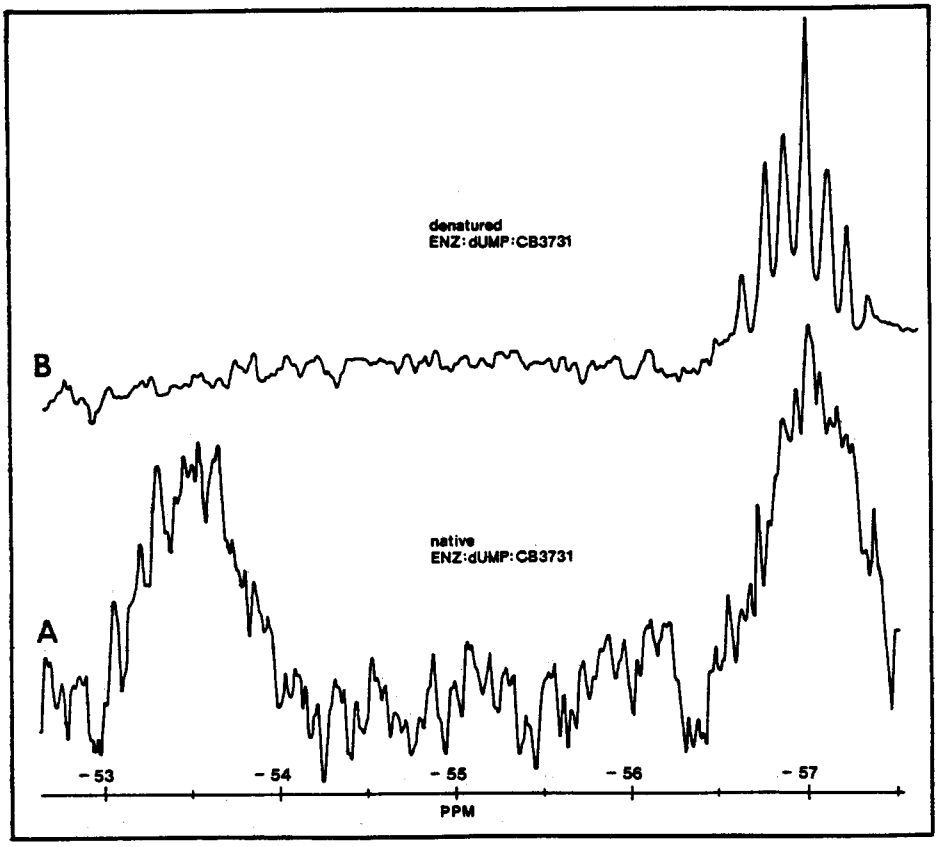

FIGURE 5: Effect of denaturation (1.2% SDS) upon the ^{19}F NMR spectrum of the TS:dUMP:CB3731 ternary complex (1:5:3). A shows the ternary complex before the addition of SDS, while B is of the denatured complex.

with the enzyme (Figure 1B), shows that the resonance at −57.0 ppm for the titration sample is due to either free or weakly bound quinazoline folate.

The resonance at −53.5 ppm is analogous to that observed in the TS:CB3731:FdUMP ternary complex (discussed above) and corresponds to the *N*-10-(fluoroethyl)quinazolinylfolate in a complex with TS and noncovalently bound dUMP. If we assume that the total area of the two resonances in spectrum A corresponds to 1.2 molar equiv of CB3731 with respect to the enzyme (1.2-fold excess of CB3731 added), the peak at −53.5 ppm represents 0.2 equiv of CB3731 and dUMP noncovalently associated with the enzyme and that seen at −57.0 ppm represents 1.0 equiv of free or weakly bound CB3731.

For the second titration point, the concentrations of TS and dUMP were maintained, while that of CB3731 was raised to give molar ratios of 1:4:2.2 in enzyme, nucleotide, and quinazoline, respectively. The spectrum for this sample (Figure 4B) differed from that of the first titration only in the relative magnitudes of the resonances it contained. As a result of the higher concentration of CB3731, over 0.3 mol of CB3731 per enzyme dimer was bound together with noncovalently bound dUMP, as estimated by analysis of the resonance at −53.5 ppm. At a concentration of CB3731 4 times that of the protein (spectrum not shown), approximately 0.6 equiv of CB3731 was associated in a complex with the protein and noncovalently bound dUMP, appearing at −53.5 ppm in the fluorine spectrum.

Maximum binding was achieved in the third titration point, using relative concentrations of 1:4:8 (Figure 4C). The 8-fold excess of CB3731 resulted in 1.6 equiv of bound ligand. Excesses of folate in this range have been shown to be sufficient to stimulate maximum FdUMP binding, but levels 10–100 times this have been proven necessary to observe increased levels of dUMP binding when measured by the TCA precipitation technique. Raising the concentration of dUMP to a 100-fold excess did not alter the appearance of the spectrum shown in Figure 2C. Byrd *et al.* (1977, 1978) reported a 2.4 ppm shift to higher shielding for the 2′-fluorine of 5,2′-difluoro-2′-deoxyuridylate, reflecting the change in its microenvironment. Conversely, the 5-fluoro substituent exhibits a shift of 11.9 ppm to higher shielding upon covalent bond formation at the C-6 position. Therefore, the 3.5 ppm shift to lower shielding upon formation of the complex reflects the extent to which the environment of the fluorine nucleus differs in the active site from its environment in solution, not the formation of a covalent complex with the folate analogue.

Denaturation of the enzymatic complex containing CB3731 and dUMP resulted in the loss of the resonance at −53.5 ppm and a narrowing of the lines in the resonance at −57.0 ppm. The spectrum shown in Figure 5A represents a sample containing TS, dUMP, and CB3731 at a 1:5:3 molar ratio. The spectrum in Figure 5B shows the same sample following the addition of SDS. In the latter spectrum, the resonance at −57.0 ppm is an apparent septet with 10-Hz lines, as seen earlier for the spectrum of the TS:CB3731 complex and for the spectrum of CB3731 alone (Figure 1). These results suggest the breakdown of the complex and release of the fluoroquinazoline into the solution. This was shown to be the case by the loss of the fluorine signal after dialysis of the sample against fresh buffer which did not contain CB3731 or dUMP.

TS:CB3731:dTMP Ternary Complexes. The binding between TS and the reaction product, dTMP, was studied in the same manner as the binding of the substrate dUMP. Once again, two resonances were observed at −53.5 and −57.0 ppm. The spectrum from a point in the titration of a sample containing 140 μM TS and a 5-fold excess of both dTMP and

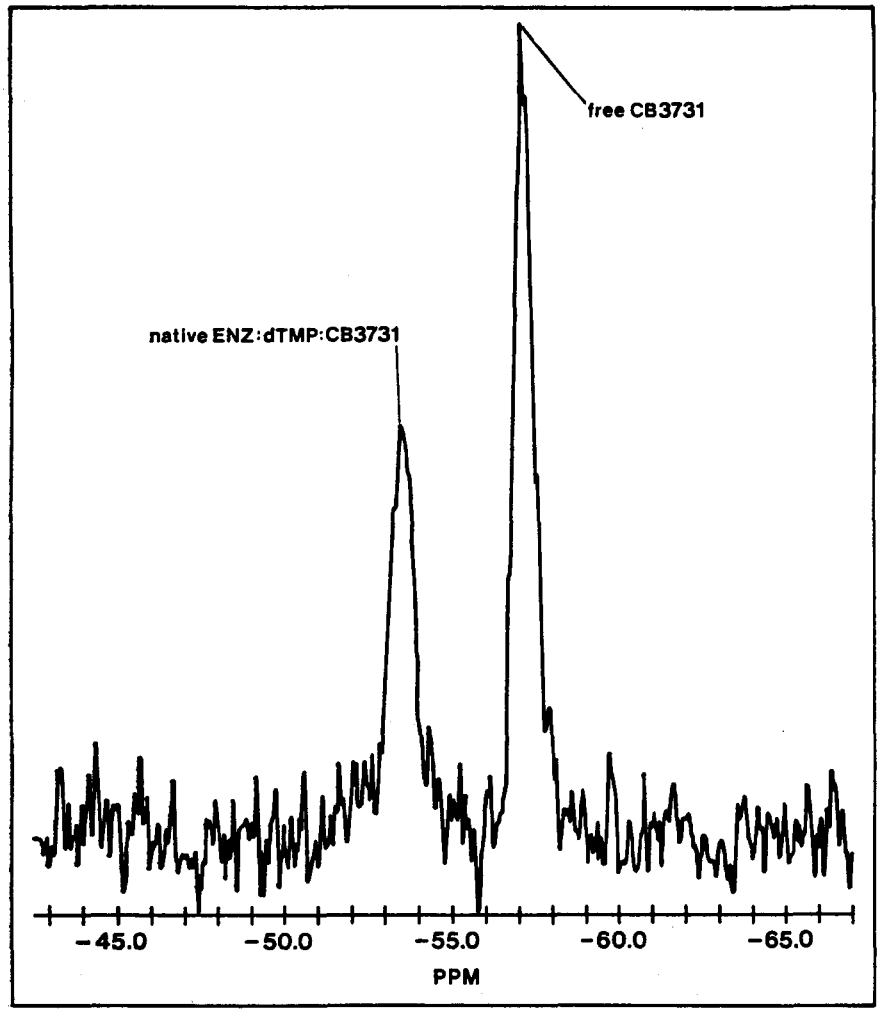

FIGURE 6: Interaction of TS with the reaction product dTMP and the antifolate CB3731. The expanded spectrum of enzyme:nucleotide:CB3731 is shown for a sample prepared with molar ratio 1:3:5.

CB3731 is shown in Figure 6. The resonances had the same chemical shifts as those in the spectra for the titration with dUMP, and the relative areas of the peaks observed at this titration point represented roughly 1.5 bound nucleotides.

Polyacrylamide Gel Electrophoresis of TS Complexes. Once evidence was detected for ligand binding in the above complexes by NMR spectroscopy, disc gel electrophoresis was performed on several complexes with the idea of trapping the enzyme-bound ligands. The native enzyme ran as a single band on 7.5% polyacrylamide gels when no ligands were present. When FdUMP and CH_2H_4folate were incubated in a 100-fold excess with TS and the mixture was run on 7.5% gels, up to three bands were formed. Aull *et al.* (1974) determined that the bands denote three forms of TS. These forms, designated forms I, II, and III, represent enzyme:nucleotide:folate ratios of 1:0:0, 1:1:1, and 1:2:2, respectively. Pure preparations of TS isolated in the presence of thiols and incubated with excesses of FdUMP and CH_2H_4folate form a 30:70 mixture of forms II and III.

For the binary and ternary complexes examined in this work by ^{19}F NMR, we saw only a single band when polyacrylamide gel electrophoresis was performed using 7.5% polyacrylamide gels. The gel containing native enzyme alone served as the control, for which a single band (form I) was detected which was found to migrate identically with the single band generated by all of the above mixtures (retention coefficient $K_d = 0.57$). Thus, the noncovalently bound CB3731 could not form a complex that was strong enough to survive polyacrylamide gel electrophoresis. Only complexes of TS, FdUMP, and CH_2H_4folate, which also served as a control for complex formation, survived native gel electrophoresis.

CONCLUSIONS

The work of Lewis *et al.* (1980, 1981) led to the establishment of a paradigm for the study of TS complexes by ^{19}F NMR utilizing the mechanism-based inhibitor FdUMP as an NMR probe. As stated in the introduction, the fluorine-containing nucleotide was an ideal choice as probe because its binding to the enzyme had already been well characterized by a number of different assays. ^{19}F NMR analysis of TS complexes led to the identification of unique resonances corresponding to both covalent and noncovalent binary and ternary complexes of TS with FdUMP and a wide spectrum of folates and folate analogues. While much information pertaining to nucleotide binding was obtained using FdUMP, the binding state of the cofactor could not be definitively established by this method. For this reason, it was necessary to investigate TS complexes from the point of view of the folate cofactor.

This was accomplished by the use of the fluorine-containing folate analogue CB3731. By incubating the enzyme with both FdUMP and CB3731, we were able to observe simultaneously the NMR signals arising from each substrate. Using the ^{19}F NMR spectrum arising from FdUMP as a standard, it was possible not only to assign resonances for free and bound CB3731, but we were also able to distinguish between

covalently and noncovalently bound nucleotide by the chemical shifts of the resonances in the *cofactor's* signal. These results then allowed the analysis of TS complexes with CB3731 and either dUMP or dTMP. Again, we were able to distinguish between covalently and noncovalently bound nucleotide.

The data presented here confirm and extend the TS binding paradigm established with the ^{19}F NMR studies conducted by Lewis *et al.* (1980, 1981) by allowing the observation of TS complexes with the fluorine-containing folate analogue CB3731 with FdUMP, dUMP, or dTMP from the perspective of the cofactor.

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