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Elucidation of the Detailed Structures of the Native and Denatured Ternary Complexes of Thymidylate Synthetase via ^{19}F NMR

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Abstract: It is known that the inhibition of thymidylate synthetase by the substrate analogue FdUMP results from the formation of a tightly bound ternary complex in which both FdUMP and the cofactor ($5,10\text{-}CH_2H_4$folate) are simultaneously bound to the enzyme. It is believed that the ternary complex results because FdUMP is able to function in a manner identical with dUMP during the initial stages of catalysis, but gets "stuck" partway through. The stage at which no further reaction occurs is postulated to be a proton abstraction which, in the case of FdUMP, would require an unfavorable C–F bond cleavage. In this work we investigated the structure of the ternary complex by ^{19}F NMR to determine the structure of the proposed intermediates and to explore the possible mechanistic implications. The binding of FdUMP to form a ternary complex is accompanied by an 12.4-ppm shift toward increased shielding. With the aid of model compounds it is possible to interpret this shift to be the result of an attack at the pyrimidinyl 6 position by a nucleophile (presumably a cysteinyl SH) followed by attachment at the 5 position to the CH_2 of the cofactor. Verification of the latter point was obtained indirectly by loss of H–F coupling (unresolved) and directly by observing a ^{13}C–^{19}F coupling constant when ternary complex was formed from cofactor prepared with CD_2O and $^{13}CD_2O$ (90% ^{13}C enriched), respectively. Denaturation of the ternary complex causes an 10.5-ppm shift of the ^{19}F resonance toward decreased shielding. The ternary complex remains intact as evidenced by the retention of the ^{19}F–^{13}C coupling to the cofactor. By analogy to α-fluorocyclohexanone, this shift reveals that the C–F bond has moved relative to the plane of anisotropy of the adjacent carbonyl group, i.e., upon denaturation the pyrimidine ring undergoes a conformational change. A sharpening of the ^{19}F resonance upon denaturation concurs with the greater mobility of the denatured complex. Indirect measurement of the ^{1}H–^{19}F coupling constants to both the CH_2 of cofactor and H_6 of the pyrimidine ring (using deuterium differencing) and application of the Karplus-type relationship enable a detailed representation of the relative spatial orientations of the groups on the pyrimidine 5,6 bond to be derived. In native ternary complex one proton of the methylene group of 5,10-methylenetetrahydrofolate is trans to the fluorine while the other is gauche. The proton at C-6 of the nucleotide and the fluorine are in a pseudo-trans-diequatorial relationship (i.e., the cysteine and the methylene group must be trans diaxial). Denaturation alters this arrangement such that the fluorine and the C-6 proton are trans diaxial.

Thymidylate synthetase catalyzes the reductive methylation of 2′-deoxyuridylate via the coenzyme 5,10-methylene-tetrahydrofolate ($5,10\text{-}CH_2H_4$folate)[2] to form thymidylate and 7,8-dihydrofolate. The proposed involvement of de novo synthesis of thymidylate as a rate-determining factor in DNA synthesis and cell division has resulted in many attempts to elucidate the mechanism of action of thymidylate synthetase. Model studies suggested that the catalytic mechanism is initiated by attack of an active site nucleophile on carbon 6 of the pyrimidine ring to generate a carbanion which subsequently attacks $5,10\text{-}CH_2H_4$folate to yield a transient ternary complex (**1a**). Methylation is completed by the transfer of the proton from carbon 6 of tetrahydrofolate[4] to the methylene group and abstraction of the hydrogen from carbon 5 of the pyrimidine ring. Chemical modifications of the enzyme by various sulfhydryl reagents[5] have indicated the presence of cysteine in the active site, and it has been suggested that cysteine acts as the catalytic nucleophile.[3a–d,5a–d,g,h,6] Addition of the inhibitor

1a, X = H
b, X = F
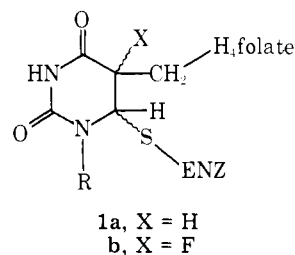
R = 5′-phospho-2′-deoxyribose

FdUMP to a mixture of the enzyme and $5,10\text{-}CH_2H_4$folate results in the formation of stable ternary complexes.[7] Formation of the ternary complexes was postulated to involve the same enzyme-bound nucleophile that participates in the catalytic mechanism; hence, the mechanism describing inhibition by FdUMP terminates with the formation of a stable covalent ternary complex whose proposed structure is **1b**. The utility

of comparing catalytic activity with ternary complex formation, as well as evidence for more than one active site per enzyme molecule, has been established by the direct correlation of the effect of sulfhydryl group modification on enzyme activity and ternary complex formation.[5g]

In order to fully understand the catalytic mechanism in terms of stereochemistry and to elucidate steps beyond the initial nucleophilic attack, detailed structural information is needed to verify the proposed ternary complex (**1b**). Formation of the ternary complex was observed to be coincident with a loss of optical density at 269 nm indicating nucleophilic attack and saturation of the pyrimidine 5,6 double bond.[7a] Spectroscopic studies (including UV–visible, fluorescence, and circular dichroism) of the intact ternary complexes have provided information on binding equilibria and macroscopic analytical features of the system;[7g,8] however, only inferences could be made with regard to the specific nature of the interactions. Danenberg et al.[5b] and Sommer and Santi[9] have reported procedures for the enzymatic degradation of the ternary complex to yield a small peptide fragment, believed to be derived from the active site, to which both FdUMP and 5,10-CH₂H₄folate moieties remain bound. These studies were based on radiolabeling techniques; hence, there was no specific bonding information available to account for the association. Bellisario et al.[10a] have presented evidence (confirmed by Pogolotti et al.[10b]) which suggests that the FdUMP is linked to a cysteinyl residue in a related peptide. Amino acid analysis of these peptides and treatment of the enzyme complex with Raney nickel[6] provide strong evidence that covalent attachment of FdUMP to thymidylate synthetase results from nucleophilic attack by a cysteine sulfhydryl group. Santi and co-workers[11] reported ¹⁹F NMR data obtained from their isolated peptide and interpreted the results as supportive of the structure **1b**. Based on their results a detailed stereochemical pathway was proposed for the catalytic mechanism. However, in a previous paper[12] we have reported the ¹⁹F NMR of the *native* ternary complex and found it to be substantially different from the data reported for the peptide. Consequently, the exact structure of the ternary complex and the inferences drawn with respect to the catalytic mechanism are still in question.

In the present report we present an analysis of the observed chemical shifts for both the native and denatured forms of the ternary complex by comparison with the chemical shifts of model compounds. Further, by utilizing isotopic labeling, direct evidence has been obtained for the linkage of the methylene carbon of the coenzyme to the 5 position of FdUMP in both the native and denatured states of the enzyme. The spin-coupling interaction between the hydrogen at carbon 6 and the fluorine at carbon 5 of FdUMP has been measured for both the native and denatured forms of the ternary complex. An analysis of this spin-spin coupling has been performed in terms of the dihedral angle between the C6–H6 and C5–F bonds. The change in chemical shift of the ¹⁹F resonance upon denaturation of the ternary complex is used, in conjunction with the spin-spin coupling data, to develop a detailed geometrical picture of the *native* ternary complex and to elucidate the relative stereochemistry of the first phase of the catalytic mechanism. The ramifications of these data with regard to previous reports and the mechanism of the thymidylate synthetase reaction are discussed.

Experimental Section

The materials used in the following experiments were obtained commercially as follows: urea and guanidine hydrochloride from Schwartz-Mann; sodium dodecyl sulfate (electrophoresis purity reagent) and hydroxylapatite (Biogel HT) from Bio-Rad Laboratories; acrylamide from Aldrich Chemical Co.; *N,N'*-methylenebisacrylamide and analytical cellulose thin layer chromatography plates with

fluorescent indicator from Eastman Chemical Co.; 2-mercaptoethanol and Dowex-50 ion exchange resin from Fisher Scientific; folic acid from Calbiochem; FdUMP from Terra Marine Biochemicals and Sigma Chemical Co.; dUMP, thymine, and 5-fluorouracil from Sigma Chemical Co.; 3-bromo-1,1,1-trifluoropropanone from PCR; Sephadex chromatography gels from Pharmacia Fine Chemicals; barium (¹³C) carbonate from Koch Isotopes; lithium aluminum tetradeuteride from Alfa-Ventron; 99.8% deuterium oxide from Stohler Chemical Co. and Thomson-Packard; and precision 18-mm NMR tubes from Wilmad Glass Co.

2′,5-Difluoro-2′-deoxyuridine was prepared by direct fluorination of 3′,5′-di-*O*-acetyl-2′-fluoro-2′-deoxyuridine with trifluoromethyl hypofluorite in methanol/fluorotrichloromethane at −78 °C using the procedure of Robins et al.[13] 5-Fluoro-5-methyl-6-methoxy-5,6-dihydrouracil[14] was prepared from the reaction of thymine with trifluoromethyl hypofluorite in methanol, via a procedure adopted from the work of Robins et al.[13] 5-Fluoro-2′-deoxyuridine-6-*d* was prepared from 5-fluoro-2′-deoxyuridine by a base-catalyzed (NaOD/D₂O) isotope exchange, essentially as reported by Cushley et al.[3j] Phosphorylation of the nucleosides was conducted according to the procedure of Dawson et al.[15] All compounds were characterized by ¹H, ¹⁹F, and ³¹P NMR and yielded spectra which agreed with those previously reported.

The isotopically labeled formaldehyde was prepared according to the procedure of Arnstein[16] for the oxidation of methanol to formaldehyde. However, a different catalyst was used. In place of the molybdenum oxide/iron oxide mixture used by Arnstein, a commercial catalyst, Formox, was used, which was a generous gift of J. Manlope of Reichold Chemicals, Inc. Using the apparatus described by Arnstein,[16] 0.5 g of perdeuteriomethanol, CD₃OD, was oxidized to yield 418 mg of dideuterioformaldehyde. Similarly, dideuterioformaldehyde-¹³C was prepared by oxidation of perdeuteriomethanol-¹³C. The perdeuteriomethanol was prepared from the lithium aluminum tetradeuteride reduction of carbon dioxide as described by Cox et al.[17] Carbon dioxide-¹³C was generated quantitatively from barium carbonate-¹³C by the careful addition of dilute perchloric acid. The formaldehyde solutions were analyzed and quantitated by iodometric titration and ¹³C NMR.

Purification and Analysis of Thymidylate Synthetase. Thymidylate synthetase was purified from amethopterin-resistant *L. casei* according to the method described by Lyon et al.[18] As a final step, enzyme employed in these studies was subjected to chromatography on DEAE-Sephadex by a method similar to that reported by Dunlap et al.[5a] The protein was dialyzed against 0.1 M Tris-Cl buffer, pH 7.3, containing 10 mM magnesium chloride, 10 mM 2-mercaptoethanol, and 1 mM EDTA and then applied to a DEAE-Sephadex column (2 × 25 cm) which had been equilibrated with the dialysis buffer. The column was eluted with a linear gradient composed of 500 mL of 0.1 M Tris-Cl buffer, pH 7.3, containing 10 mM MgCl₂, 10 mM 2-mercaptoethanol, and 1 mM EDTA and 500 mL of 0.1 M Tris-Cl, pH 6.8, containing 200 mM MgCl₂, 10 mM 2-mercaptoethanol, and 1 mM EDTA. A flow rate of approximately 0.5 mL/min was employed and 5-mL fractions were collected. The enzyme eluted after about 250 mL (fractions 72–80) of gradient had passed through the column. Polyacrylamide gel electrophoresis indicated that the enzyme was homogeneous and was separated from a small peak corresponding to fractions 50–55 which migrated just slightly slower than thymidylate synthetase in the gels. This chromatographic method was also used to recover pure thymidylate synthetase from impure fractions of the hydroxylapatite column used in the usual purification procedure. Enzyme isolated from the column exhibited specific activities of 3.0–3.6 units/mg of protein which is analogous to normal preparations.[18]

Concentrations of enzyme solutions were measured spectrophotometrically based on the extinction coefficient at 278 nm, determined by Lyon et al.[18] to be 105 000 M⁻¹ cm⁻¹. Thymidylate synthetase activity was monitored spectrophotometrically by measuring dihydrofolate production at 340 nm (ϵ 6400 M⁻¹ cm⁻¹) at 25 °C. The methods used for polyacrylamide gel electrophoresis of thymidylate synthetase and its ternary complexes are those described by Aull et al.[7f,g]

Trifluoroacetonylated Thymidylate Synthetase. Thymidylate synthetase, activated by dialysis against 25 mM 2-mercaptoethanol in 0.1 M sodium phosphate buffer, pH 7.3, and dethiolated by gel filtration chromatography on Sephadex G-25, was treated with a 500-fold molar excess of 3-bromo-1,1,1-trifluoropropane. Total in-
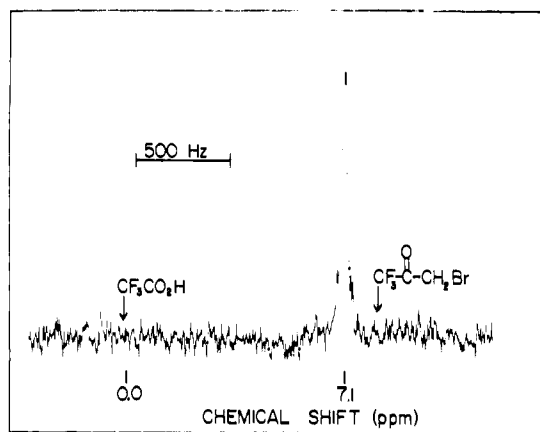
Figure 1. The 94.1-MHz ^{19}F NMR spectrum of 0.1 mM trifluoroace-tonylated thymidylate synthetase in 0.1 M phosphate buffer, pH 6.8. This spectrum represents 10 000 accumulations using 90° rf pulses, a spectral width of 2500 Hz, a recycle time of 0.4 s, and 1.6-Hz broadening due to exponential multiplication. The sample (5 mL) was prepared in an 18-mm tube. The frequencies for trifluoroacetic acid and the halogenated pro-panone are indicated by the arrows in the figure.

activation was found after 90 min of incubation. Studies to be described in a separate manuscript show that trifluoroacetonylation of thymidylate synthetase is limited to the catalytic sulfhydryl groups.

Preparation of Ternary Complex Samples for ^{19}F NMR. Thymidylate synthetase was activated by dialysis for 12 h against the desired buffer, either sodium phosphate, Tris-Cl, or Tris-sulfate, pH 6.8, containing 50 mM 2-mercaptoethanol. Only enzyme preparations exhibiting a specific activity of at least 3.0 units/mg of protein after activation were used for NMR experiments. The enzyme was concentrated in the range of 0.1-0.3 mM using ultrafiltration cells and PM10 Diaflo ultrafiltration membranes obtained from Amicon Corp.

Enzyme solutions of the requisite concentration were centrifuged at 4 °C in a Sorvall RC2-B refrigerated centrifuge, and the supernatant was dialyzed against the appropriate buffer containing 25 mM 2-mercaptoethanol and 1 mM EDTA made with a 70:30 mixture by volume of H_2O/D_2O. This procedure removes any precipitated protein as a result of the concentration process and introduces sufficient deuterium in the solvent to provide a stable field-frequency lock in the NMR experiment. The pH values of these buffer solutions were measured with a Corning Digital 109 pH Meter and are *uncorrected* for the deuterium isotope effect.

Ternary complex was formed by the incubation of 3.0 mL of thymidylate synthetase solution, FdUMP (or F_2dUMP), and 5,10-CH_2H_4folate at 25 °C for 15 min in the 18-mm NMR tube. The FdUMP is added in a molar ratio of 2.5:1 with respect to thymidylate synthetase concentration in order to provide excess inhibitor as an internal chemical shift reference. The cofactor is always present at a molar excess of 10:1 relative to the enzyme to ensure that the complex remains intact throughout the NMR experiment. In order to avoid excessive dilution of the thymidylate synthetase, the inhibitor was added either as a solid, in the case of FdUMP, or microliter quantities from a stock solution, as in the case of F_2dUMP and 6-^{2}H-FdUMP. To prepare the cofactor solution, an amount of *dl*-L-5,6,7,8-tetrahydrofolate, corresponding to a tenfold molar excess with respect to enzyme, is dissolved in 50 μL of 0.5 M sodium bicarbonate, and formaldehyde is added to achieve a formaldehyde:tetrahydrofolate molar ratio of 20:1. Dideuterioformaldehyde or the dideuteriofor-maldehyde-^{13}C was used in place of formaldehyde in order to generate 5,10-CD_2H_4folate and 5,10-$^{13}CD_2H_4$folate, respectively. Evidence of ternary complex formation is provided by the characteristic yellow color arising from the 375-nm absorption band reported by Donato et al.[8c] The extent of ternary complex formation was analyzed by polyacrylamide gel electrophoresis prior to and after the NMR experiment. Ternary complex samples were denatured by addition of sodium dodecyl sulfate solution (final concentration, 1%). This concentration of denaturant has been shown to completely denature the enzyme and ternary complex.[5b,7b]

NMR Methods. All NMR spectra were obtained on a Varian

XL-100 NMR spectrometer operating in the pulse Fourier transform mode. Ternary complex spectra were recorded using the multinuclear 18-mm probe.[19] Temperature was maintained at 20 ± 1 °C for all experiments using the Varian XL-100 variable temperature accessory. Chemical shifts were measured relative to internal FdUMP or external trifluoroacetic acid and are accurate to 0.1 ppm. All spectra were collected using a 5000-Hz sweep width and were processed by Fourier transformation of 8196 data points.

In some cases, isotopic substitution was used to estimate the magnitude of various spin coupling constants. The ^{19}F NMR lines shapes used in this procedure were not always Lorentzian in shape owing to the sensitivity enhancement factor applied to the free-induction decay. The line widths were, however, estimated in the usual manner, i.e., width at half peak height. The line widths were reproducible to 1 or 2 Hz, and this variation represents our experimental error.

Extreme care was taken to remove the ubiquitous paramagnetic ions from our solutions. However, of equal importance when differential line widths were determined between a pair of samples was the fact that both samples were prepared in nearly the same manner. Hence, if any paramagnetic ions were introduced, their effect would be essentially constant in both samples. Therefore, the paramagnetic impurity would have negligible effects on the differential *line width*.

Results

General Comments Concerning the ^{19}F NMR of Thymidylate Synthetase. The analysis of the ternary complexes of thymidylate synthetase via ^{19}F NMR spectroscopy posed several practical problems. These problems arise from the molecular weight of the enzyme ($\sim$70 000) and its relationship to the reorientational correlation time of the ^{19}F nucleus, and the low solubility of the enzyme ($\leq$0.3 mM). The most severe problem is the correlation time of the enzyme. That is, assuming that the fluorouracil ring is firmly bound within the ternary complex, the correlation time observed will be that of the backbone of the protein as opposed to that of a side chain moiety with segmental motion. Consequently, the resonance is expected to be broad owing to a long correlation time (50–100 ns).[20] Initial studies at 94 MHz with 5- and 12-mm NMR tubes proved to be fruitless, even with 2 days of signal averaging, and demonstrated graphically the severe sensitivity problems that this system presented.

To overcome this critical situation, we resorted to the use of our multinuclear 18-mm NMR system.[19] We further employed trifluoroacetonylated thymidylate synthetase as a model to develop the necessary experimental conditions needed to observe the ^{19}F NMR of the ternary complex. This CF_3 system, while retaining the same stoichiometry of bound groups as exists in the ternary complex,[7f,g] circumvents the motional problems associated with the complex, since the CF_3 group is expected to exhibit internal rotation. Also, the effective ^{19}F concentration is tripled, permitting a more rapid evaluation of experimental conditions. The ^{19}F NMR spectrum obtained from 0.1 mM trifluoroacetonylated thymidylate synthetase after only 1 h of signal averaging with an 18-mm sample tube is shown in Figure 1. The sensitivity achieved in this spectrum clearly demonstrates the feasibility of studying the ternary complex using a large sample tube probe.[21] The line width of this resonance was observed to be concentration dependent, having a value of 30 Hz for an enzyme concentration of 0.25 mM and a value of 22 Hz at an enzyme concentration of 0.09 mM. This observation was confirmed in a ^{19}F NMR study of the ternary complex: at an enzyme concentration of 0.3 mM the ^{19}F line width was 270 Hz while at 0.1 mM concentration the resonance line width was only 96 Hz;[22] further dilution did not appreciably affect the line width. This situation clearly reflects the fact that the highest possible concentration of a protein is not necessarily the best concentration at which to run the NMR experiment. Hence, all ternary complex experiments were performed using an enzyme concentration of 0.1 mM.

The possibility existed that an additional factor contributing

Table I. Fluorine Chemical Shifts of the Free 5-Fluorodeoxyuridylates and Their Ternary Complexes, and the Substituted 5-Fluorouracil Model Compounds

compd	chemical shift, ppm[a,b]
FdUMP	0.0
FdUMP ternary complex	12.4
F_2dUMP	0.5 and 38.5
F_2dUMP ternary complex	12.4 and 40.9
5-fluoro-6-methoxy-5-methyl-5,6-dihydro-uracil (III)	9.4
5-fluoro-6-methoxy-5,6-dihydrouracil (II)	40.3[c]

[a] The solutions were buffered with 0.1 M Tris-SO_4, pH 7.3. [b] Positive chemical shifts are shielded with respect to FdUMP. The latter resonance is 89.7 ppm shielded with respect to trifluoroacetic acid (external). [c] Reference 13.

to line broadening could be an exchange phenomenon involving a reversible binding of the proposed sulfhydryl at the pyrimidine ring. To investigate this point, the ^{19}F NMR spectrum of ternary complex formed with F_2dUMP, instead of FdUMP, was obtained. Since the line width of the 2'-fluorine resonance, which should be relatively insensitive to such an exchange reaction, was of the same order of magnitude as that of the 5-fluorine resonance, this effect was discounted. Such an experiment does not discount, however, exchange of FdUMP on and off the enzyme. However, independent biochemical experiments have shown that such an exchange is slow on the NMR time scale.

It was pointed out above that our initial results for the native ternary complex were in substantial disagreement with those reported for the peptide isolated by Santi and co-workers.[11] In order to fully resolve the basis of these differences and to completely characterize the ternary complex, it was pertinent to this investigation to study the ^{19}F NMR of the denatured ternary complex. Despite lengthy signal averaging, no resonance was observed for the trifluoroacetonylated enzyme following denaturation in either urea or guanidine hydrochloride. However, when the trifluoroacetonylated thymidylate synthetase was denatured in 1% sodium dodecyl sulfate, a resonance was observed quite easily and exhibited a line width of 15 Hz for an enzyme concentration of 0.08 mM. These results are interpreted as reflecting aggregation of the enzyme in urea and guanidine hydrochloride which greatly increases the effective correlation time and renders the resonance too broad to be observed. On the other hand, sodium dodecyl sulfate appears to effectively denature the enzyme into nonaggregating subunits, as indicated by the narrowness of the observed resonance, and thus provides an effective system for studying the denatured ternary complex. These conclusions correlate with results of gel filtration and ultracentrifugal studies performed on native and denatured thymidylate synthetase.[23]

^{19}F NMR of the Ternary Complex. 1. Chemical Shifts of the Native Ternary Complex.
In Table I the ^{19}F chemical shifts of the model compounds 5-fluoro-6-methoxy-5,6-dihydrouracil (II), 5-fluoro-5-methyl-6-methoxy-5,6-dihydrouracil (III), F_2dUMP, and the native ternary complexes formed with either FdUMP or F_2dUMP are presented. The assignments of the spectra for the two ternary complexes are based primarily on the comparison with the spectra of the free ligands. In both of the ternary complexes, the resonance for the 5-fluorine appears ≈11–12 ppm to higher shielding of that in the free ligands. On the other hand, the 2'-fluorine resonance in F_2dUMP shifts to higher shielding by only 2.4 ppm upon formation of the ternary complex; this small shift is analogous to those observed for numerous CF_3-type ligands upon binding to proteins and is indicative of microenvironment changes, not bonding rearrangements. In contrast, the fluorine at the 5 position of the

pyrimidine ring should reflect the change from an alkenyl to an alkyl system (structure 1b). Indeed, the approximately 11–12 ppm upfield shift of this fluorine upon complex formation clearly indicates a change in its bonding environment relative to the free ligands.

Insight into the bonding of the ternary structure may be obtained through a consideration of the fluorine spectra of the model compounds II and III. As may be seen from Table I, the

II   III
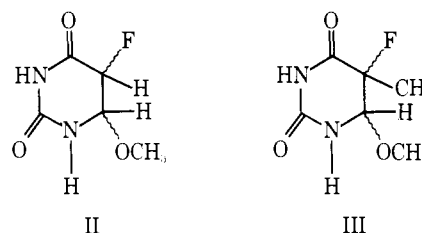
resonances for both these compounds are shielded with respect to that for FdUMP, but that of II differs considerably from the resonance of the ternary complex. This is due to the lack of an additional substituent at the 5 position. The model compound III has both a fluorine and a methyl group at the 5 position, as well as a group 6 substituent at the 6 position, and is more closely analogous to the proposed ternary complex structure. The fluorine resonance of III is shielded 9.5 ppm with respect to FdUMP and is within 2.9 ppm of the resonance observed for the ternary complex. The effect of the methyl substituent, which creates a tertiary center rather than a secondary center at the 5 position, thus contributes significantly to the chemical shift difference between II and III and these results suggest that *the native ternary complex also possesses an alkyl substituent* (*presumably the methylene group of 5,10-CH_2H_4-folate*) *at the 5 position.*
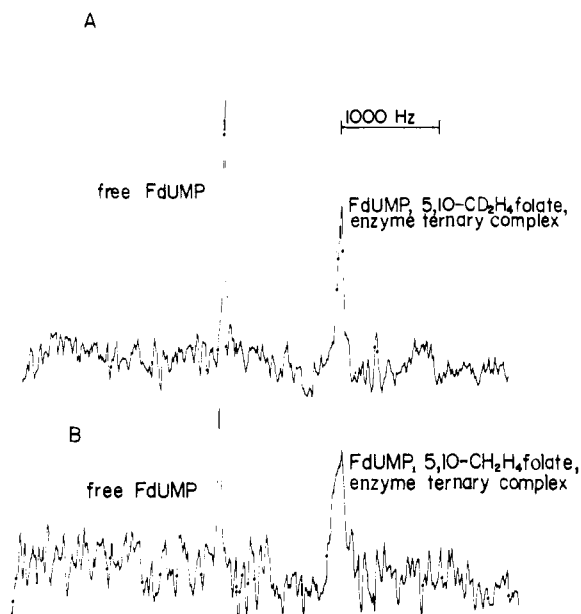
2. Heteronuclear Spin–Spin Coupling in the Native Complex.
Further characterization of the covalent nature of the ternary complex would be obtained by observing a heteronuclear spin–spin coupling from the methylene hydrogens of 5,10-CH_2H_4folate to the fluorine at carbon 5, i.e., J_{HF}. Selective ^{1}H decoupling techniques in principle could be employed to deduce the value of the coupling constant. However, there are technical problems associated with this approach; in particular, this would require locating the methylene resonance in the ^{1}H spectrum. Furthermore, it has been shown that either broadband or selective ^{1}H decoupling in a macromolecular system can lead to a negative nuclear Overhauser effect if the fluorine is relaxed predominantly by dipolar interactions with protons.[21b,24] As a result, the net intensity may approach zero and no resonance can be observed under these conditions. This phenomenon was experimentally observed for the FdUMP ternary complex under conditions of broad-band ^{1}H decoupling, indicating that the relaxation of the fluorine is due principally to ^{1}H–^{19}F dipolar interactions.

An equally attractive method of obtaining spin–spin coupling information utilizes isotopic substitution. This procedure involves a comparison of the ^{19}F line width obtained from ternary complex formed with 5,10-CH_2H_4folate and that generated from 5,10-CD_2H_4folate. The resulting differential line width will be proportional to the value of the J_{HF}. For example, if we consider that the two methylene hydrogens are magnetically equivalent, then the ^{19}F half-height line width, $\nu_{1/2}$, may be given by the following expression:

$$\nu_{1/2}(H) = 2(J_{HF}) + \text{other mechanisms} \qquad (1)$$

In this expression the term "other mechanisms" refers to other heteronuclear couplings and relaxation processes. If we now replace both of the magnetically equivalent protons by deuterons, the spin–spin coupling constant will be reduced by a

A

|1000 Hz|

free FdUMP

FdUMP, 5,10-CD₂H₄folate, enzyme ternary complex

B

free FdUMP

FdUMP, 5,10-CH₂H₄folate, enzyme ternary complex

Figure 2. The 94.1-MHz ^{19}F NMR spectra of native ternary complexes formed with FdUMP in 0.1 M phosphate buffer, pH 6.8, containing 25 mM 2-mercaptoethanol and 1 mM EDTA. The enzyme concentrations were 0.1 mM and the spectra were obtained using 60° rf pulses, a spectral width of 5000 hz, a recycle time of 0.6 μs, and 10.6-Hz broadening due to exponential multiplication. The complex used in the upper spectrum (A) was formed with 5,10-CD₂H₄folate, while that in (B) was formed with 5,10-CH₂H₄folate. The sharp resonance to the left in both spectra is that of excess, free FdUMP, added as an internal reference.

factor of 6.51 according to the expression[25]

$$J_{DF} = \frac{\gamma_D}{\gamma_H} J_{HF} \qquad (2)$$

where γ_H and γ_D refer to the magnetogyric ratios of hydrogen and deuterium, respectively. The resulting line width would be expressed as

$$\nu_{1/2}(D) \simeq 3(J_{DF}) + \text{other mechanisms} \qquad (3)$$

The factor of 3 arises due to the splitting pattern for two equivalent spin-1 nuclei coupling to a spin-$\frac{1}{2}$ nucleus which generates a quintet with intensities 1:2:3:2:1. By subtracting eq 1 and 3, and substituting in eq 2, an expression is obtained for the reduction in line width due to isotopic substitution:

$$\Delta\nu_{1/2} = 2\left[1 - \left(\frac{3}{2}\right)\frac{\gamma_D}{\gamma_H}\right] J_{HF} \qquad (4)$$

The fundamental assumption of this analysis is that the "other mechanisms" which contribute to the line width are constant in the two experiments. This assumption is not completely rigorous; however, it may be shown that any deviations from this assumption fall within the experimental error of our line width determinations (see Appendix within the supplementary material). Similarly, if only one hydrogen is involved in the coupling, such as the hydrogen at C-6 of the pyrimidine ring, then deuteration at this position will result in a differential line width that is given by

$$\Delta\nu_{1/2} = \left[1 - 2\frac{\gamma_D}{\gamma_H}\right] J_{HF} \qquad (5)$$

The preceding analysis assumes that all of the couplings are present but unresolved owing to the large natural line width of the ^{19}F resonance in comparison to expected values of J_{HF} and J_{DF} (e.g., ≤35 Hz). However, in certain instances, there will be no residual J_{DF} coupling contributing to the line width of the ^{19}F resonance. This arises because, at correlation times

exhibited by proteins as large as thymidylate synthetase (e.g., 50-100 ns),[20] rapid relaxation of the deuterium nucleus via the quadrupolar mechanism effectively decouples the residual deuterium spin-spin coupling to the fluorine. This is not the case, however, for the proton-fluorine spin-spin coupling. Consequently, in these cases the reduction in line width is directly a measure of the J_{HF} contribution, such that eq 4 and 5 become simply

$$\Delta(\nu_{1/2}) = 2J_{HF} \qquad (6)$$

and

$$\Delta(\nu_{1/2}) = J_{HF} \qquad (7)$$

respectively.

The ^{19}F NMR spectrum obtained from ternary complex containing 5,10-CD₂H₄folate is shown in Figure 2A. Comparison of this spectrum with that of the isotopically normal species shown in Figure 2B clearly indicates a reduced line width, demonstrating that the alkyl substituent at the 5 position is, in fact, the methylene group of 5,10-CH₂H₄folate. The measured differential line width is 48 ± 4 Hz and is presented in Table II, along with other coupling constant data to be discussed below. If the methylene protons are magnetically equivalent, then the differential line width will represent $2J_{HF}$, as described above (see eq 6), and will yield a value of $J_{HF} = 24 \pm 4$ Hz. In order for the coupling to be equivalent to both protons, the dihedral angles between the C5–F and C–H bonds must be equal. This can only occur in two geometries: when the dihedral angle is either 60° (IVa) or 120° (IVb). However, calculations[26] and experimental results for J_{HF} indicate a value in the range of 8-12 Hz for such geometries. Consequently,

IVa

IVb

IVc

IVd
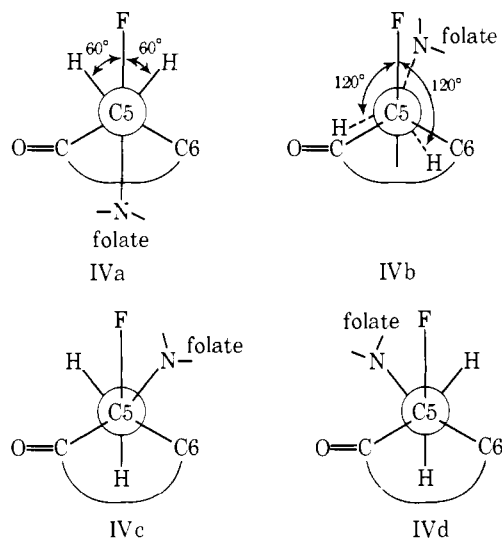
this case does not accurately explain the observed differential line width. Structures, such as IVc and IVd, provide the only viable explanation of these data. In these structures one proton is truly trans and one proton is gauche to the fluorine, and motional interconversion of these protons is slow on the NMR time scale. Calculations show (see below) that the ratio $J_{HF}^{trans}/J_{HF}^{gauche}$ is on the order of 3-5 such that the expression for the line width becomes

$$\Delta\nu_{1/2} = J_{HF}^{trans} + J_{HF}^{gauche} \simeq \frac{4}{3}J_{HF}^{trans} \qquad (8)$$

From the experimentally observed differential line width, this leads to values of $J_{HF}^{trans} \simeq 36 \pm 4$ and $J_{HF}^{gauche} \simeq 12 \pm 4$ Hz. No particular significance is attached to these actual values except that they are of the appropriate magnitudes for the proposed geometry and indicate that the methylene group in the native ternary complex is motionally restricted. Such a

Table II. Chemical Shifts, Line Widths, and Coupling Constants Derived from Isotopic Derivatives of the Ternary Complex

| composition of the complex[a]	chemical shift,[b] ppm	conformational state[c]	temp °C	line width,[d] Hz	coupling constants,[e] Hz		
					J_{HF}[f]	J_{H_6F}	J_{CF}
FdUMP:CH$_2$FH$_4$:TS	12.4	N	20	96			
FdUMP:CH$_2$FH$_4$:TS	1.9	D	20	65			
FdUMP:CD$_2$FH$_4$:TS	12.4	N	20	48	24[g]		
FdUMP:CD$_2$FH$_4$:TS	1.9	D	20	45	14		
			32	29			
FdUMP:^{13}CD$_2$FH$_4$:TS	12.4	N	20	62			14
FdUMP:^{13}CD$_2$FH$_4$:TS	1.9	D	20	58			13
			32	43			14
6-^{2}H-FdUMP:CD$_2$FH$_4$:TS	12.4	N	20	46		0[h]	
6-^{2}H-FdUMP:CD$_2$FH$_4$:TS	1.9	D	20	31		22[i]	

[a] Composition is represented according to the abbreviations used in the text, except for TS = thymidylate synthetase. [b] Chemical shifts are reported relative to FdUMP; positive values indicate higher shielding. [c] The conformational state is designated as either native, N, or denatured, D. [d] Half-height line widths are reported in hertz and corrected for broadening due to exponential weighting; experimental error is ±4 Hz. [e] Experimental error is assumed to be ±3–4 Hz. [f] J_{HF} represents the coupling constant between the methylene protons and the fluorine. [g] This value assumes the motionally restricted conformer leading to a doublet of doublets (see text). [h] Within experimental error, this value is zero. [i] This value is based on the triplet structure of residual J_{DF} coupling (see text).

result is not unexpected for the ternary complex, which can be viewed as a transition state for the enzyme-catalyzed reaction. The rigidity of the methylene group in the ternary complex is simply a manifestation of the very specific relative orientation in which the reacting groups are being held at the active site of the enzyme.

Based upon the chemical shift and J_{HF} data for the native ternary complex, the bonding in the proposed structure has been verified. However, our data for the native ternary complex clearly disagree with the results reported by Santi and co-workers for the isolated FdUMP peptide.[11] We chose to use the denatured ternary complex which is more representative of the latter peptide in order to resolve these discrepancies and fully characterize the relative stereochemistry of the groups involved in the ternary complex.

The ^{19}F NMR spectrum obtained from ternary complex containing FdUMP, 5,10-CH$_2$FH$_4$folate, and thymidylate synthetase following denaturation by the presence of 1% SDS is shown in Figure 3. The denatured ternary complex exhibits a single resonance with a chemical shift of 1.9 ppm shielded with respect to FdUMP, a result which agrees very closely with the ^{19}F chemical shift of the FdUMP peptide reported by Santi et al.[11] However, this shift is also remarkably similar to that of free FdUMP, and it was necessary to confirm that the methylene group was still attached at the 5 position of the pyrimidine ring. A method which would establish the covalent attachment of the methylene moiety in the native and denatured ternary complex that is independent of any conformational effects involves labeling the methylene group with ^{13}C and observing a carbon–fluorine spin–spin coupling constant, geminal J_{CF}. This parameter is not dependent upon *any* structural feature other than the existence of a covalent bond between the methylene carbon and the 5 position of the pyrimidine ring; consequently, it should be almost the same for both the native and denatured states of the ternary complex. The difference in line width obtained by comparing the ^{19}F NMR spectrum of ternary complex containing 5,10-^{13}CD$_2$H$_4$folate with that of ternary complex containing 5,10-CD$_2$H$_4$folate provides a direct measure of J_{CF}.

The methylene carbon was labeled with ^{13}C by preparing the cofactor as described in the Experimental Section, except that dideuterioformaldehyde-^{13}C was used in place of formaldehyde, yielding 5,10-^{13}CD$_2$H$_4$folate. The dideuterio species was used because the narrower resonance affords better signal to noise ratio and a more accurate measure of J_{CF}. The measured line widths of the ^{19}F resonances observed for the native and denatured forms of the ternary complex are presented in Table II. Comparison of these values indicates a coupling of
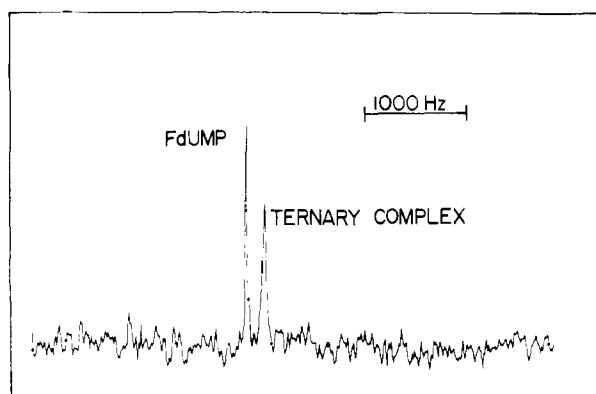
Figure 3. The 94.1-MHz ^{19}F NMR spectrum of ternary complex formed with FdUMP and 5,10-CD$_2$H$_4$folate and denatured in the presence of 1.2% SDS. The enzyme concentration is 0.1 mM in 0.1 M phosphate buffer; pH 6.8, containing 50 mM 2-mercaptoethanol and 1 mM EDTA. This spectrum represents 140 000 accumulations following 60° rf pulses, a spectral width of 5000 Hz, a recycle time of 0.5 s, and 10.6-Hz broadening due to exponential multiplication.

14 ± 3 Hz for both the native and denatured states. These data confirm that the methylene carbon is still covalently bound to the 5 position of the pyrimidine ring in the denatured state. Furthermore, these results imply that there must be some mechanism other than bond cleavage responsible for the observed deshielding of the ^{19}F resonance by 10.5 ppm upon denaturation.

Having demonstrated the covalent nature of the denatured ternary complex, the differential line width of 20 ± 4 Hz observed in the denatured state of the ternary complex containing 5,10-CD$_2$H$_4$folate compared to the denatured ternary complex containing 5,10-CH$_2$H$_4$folate may now be used to calculate the value of J_{HF} in the denatured complex. A priori there is no reason to expect any motional restriction of the methylene group in the denatured state. Hence, rapid motional averaging of the H–F couplings for the two protons would render them equal yielding a triplet structure in which the contribution to the line width is $2J_{HF}$. This leads to a value of 10 ± 4 Hz assuming that there is no residual D–F coupling; however, the effective correlation time for the deuterons in the denatured state may be short enough such that there is residual coupling contributing to the line width (see Appendix within the supplementary material). In this case, the differential line width will be as expressed in eq 5 and will lead to a value of J_{HF} = 14 ± 4 Hz. This result compares favorably with the value of 19.5 Hz reported for the peptide.[11]
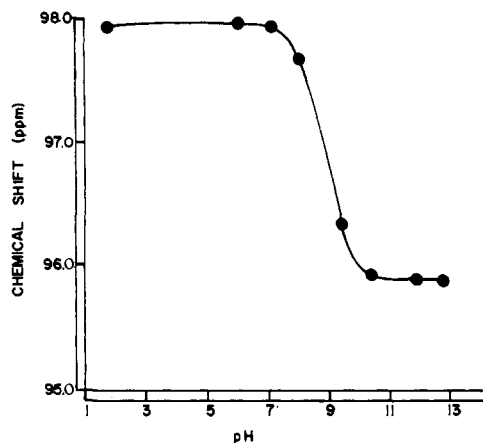
Figure 4. The effect of pH on the ^{19}F chemical shift of 5-fluoro-6-methoxy-5-methyl-5,6-dihydrouracil (III). The chemical shifts are in parts per million, shielded with respect to external trifluoroacetic acid.

R= 2'-deoxyribosyl 5'-phosphate

Figure 5. Structures of the ternary complex proposed for the native (A) and denatured (B) states of the enzyme.

The above data point to similarities in the structures of the native and denatured forms of the ternary complex, i.e., denaturation in SDS is not accompanied by *bond* cleavage and the FdUMP remains covalently attached to both thymidylate synthetase and 5,10-CH$_2$H$_4$folate. Furthermore, the denatured ternary complex appears to exhibit the same characteristics as the peptide; nevertheless, the origin of the significant (10.5 ppm) chemical shift must be ascertained to prevent false conclusions from being drawn from data observed for either the peptide or the denatured ternary complex. One possible explanation of the observed chemical shift upon denaturation is ionization of the N(3) ring hydrogen due to a different solvent environment in the denatured state relative to the native state. To investigate this we studied the effect of pH upon the ^{19}F chemical shift of the model compound III. The results of this experiment are shown in Figure 4. Although the chemical shift dependence on pH does describe a titration curve and reflect an ionization of the pyrimidine ring ($pK_a \sim 8.5$), it is clear that this ionization cannot account for the observed 10.5-ppm shift, since the total change in chemical shift due to pH is only 2.4 ppm.

Another possible mechanism which could account for the observed shift is a conformational change of the pyrimidine ring. It has been established that the carbonyl groups of 5,6-dihydrouracil derivatives possess a trigonal geometry which imparts a half-chair conformation to this portion of the ring such that the substituents at the 5 and 6 positions are staggered relative to one another, as found in substituted cyclohexane.[27] A structure of this geometry could be envisioned to undergo a simple ring inversion which corresponds to a rotation about the 5,6 carbon–carbon bond and interchanges the relative axial and equatorial position of the substituents. The two isomers of the pyrimidine ring in the ternary complex which would correspond to such a conformational change are shown in Figure 5. The methylene group and the sulfhydryl group of the enzyme are shown trans to one another; however, evidence has not yet been presented to substantiate this feature, and the opposite relative configuration could be envisioned. These structures are not intended to represent any absolute stereochemistry with respect to which side of the ring is attacked by the sulfhydryl group—rather they merely represent one possible orientation.

An interesting feature of these two structures is the different relative orientation of the fluorine atom with respect to the carbonyl group at the 4 position of the pyrimidine ring. The question arises how this different relative orientation might affect the chemical shift of the ^{19}F resonance. The chemical shift reflects a composite of local magnetic fields and the external magnetic field which determines the actual resonance
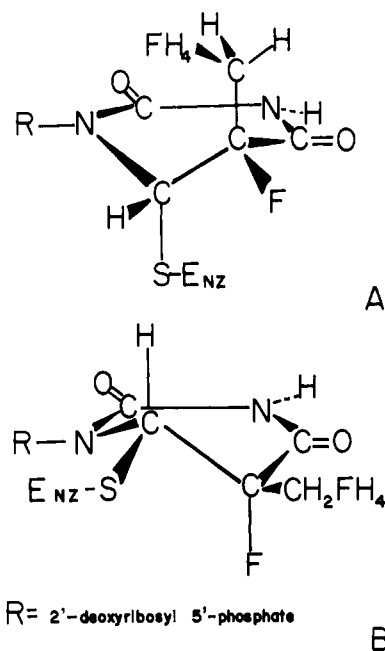
frequency. Since the local magnetic field associated with the carbonyl group is inherently anisotropic, the effective field experienced by the fluorine nucleus will certainly be different for different orientations of the fluorine atom with respect to the carbonyl group. Consequently, the two conformations shown in Figure 5 should exhibit different ^{19}F chemical shifts. Evidence of this type of effect has been reported in terms of the observed ^{19}F chemical shifts for the two conformers of 4-*tert*-butyl-2-fluorocyclohexanone.[28] This molecule provides a model for the equatorial and axial positions of a fluorine atom adjacent to a carbonyl as exists in the proposed conformers of the ternary complex (Figure 5). The ^{19}F chemical shift of the equatorial isomer of 4-*tert*-butyl-2-fluorocyclohexanone is reported to be 7.5 ppm shielded with respect to the axial isomer. The magnitude of this chemical shift indicates that a conformational change of this nature could, in fact, account for the observed shift difference between the native and denatured states of the ternary complex. Also, these data implicate the equatorial conformer shown in Figure 5 as the structure of the native ternary complex.

The rationalization of the change in chemical shift observed for the ternary complex upon denaturation as a conformational change is attractive, but it could not be used to conclusively assign the structure of the ternary complex. It did, however, suggest another experiment which could test this proposition and confirm its validity. Assuming that the conformers depicted in Figures 5A and 5B accurately represent the native and denatured states of the ternary complex, the relative orientation of the 6 proton to the fluorine changes from a pseudo-trans-diequatorial relationship to a trans-diaxial relationship. The pseudo-trans-diequatorial structure (Figure 5A) positions the 6 proton and fluorine in a gauche configuration, whereas the trans-diaxial structure of Figure 5B represents a purely trans configuration. Gutowsky et al.[29] have reported a value of $J_{HF}{}^{gauche} = 2.8$ and $J_{HF}{}^{trans} = 38.0$ Hz for CHCl$_2$–CHFCl. Consequently, if ring inversion does occur, a measure of the spin–spin coupling constant between the 6 proton and the fluorine, J_{H_6F}, could verify this hypothesis. Furthermore, it was stated previously that the trans relationship of the sulfhydryl vs. 5,10-CH$_2$H$_4$folate was arbitrary, and it is clear that this relative orientation could also be cis without altering the proposed chemical shift change associated with

the ring inversion. However, if this geometry did occur, the 6 proton and the fluorine would exist in a gauche equatorial-axial relationship for both conformers, In light of this possibility, it is pertinent to ask whether a measure of J_{H_6F} could distinguish between these two interpretations.

The sensitivity of J_{H_6F} to different geometrical conformers was evaluated by calculating J_{H_6F} as a function of the dihedral angle between the C6-H6 bond and the C5-F bond.[26] The calculation was based upon structural parameters obtained from the crystal structure of 1-methyl-5-fluoro-6-methoxy-5,6-dihydrouracil[27] and the results are shown in Figure 6 as a plot of J_{H_6F} vs. the dihedral angle. Examination of this plot indicates that if the sulfhydryl group of the enzyme and the methylene group of the cofactor are oriented trans to one another with respect to the 5,6 carbon–carbon bond, *and* the native state of the ternary complex represents the diequatorial conformation of the fluorine atom and H6, then J_{H_6F} will be very small (ca. 4 Hz) in the native state and large (ca. 38 Hz) in the denatured state. However, if the enzyme sulfhydryl group and the methylene group are oriented cis to one another, then H6 and the fluorine atom will be in a gauche relationship for both states, where the dihedral angle, ϕ, will be approximately 60°. In this case, the value of J_{H_6F} in both states constitutes a sensitive evaluation of the relative stereochemistry of the sulfhydryl group and methylene group *and* information regarding the validity of the conformational change proposed to explain the chemical shift data.

The same method used to measure the coupling constant between the methylene protons and the fluorine was employed to measure J_{H_6F}. The existence of a coupling will result in a reduction of the ^{19}F line width equal to J_{H_6F} when 6-D-FdUMP as opposed to FdUMP is employed to form the ternary complex. This experiment was performed for both the native and denatured states. These data are presented in Table II and indicate that J_{H_6F} is very small and not measurable within experimental error for the native state. This value for J_{H_6F} corresponds to a dihedral angle of ~60° as is found in the conformer shown in Figure 5A. For the denatured state J_{H_6F} was determined to be in the range of 15-22 Hz. This range allows for the existence of residual deuterium–fluorine coupling. The lower limit corresponds to an absence of residual coupling, whereas the upper limit takes into account the maximum residual coupling. These limits correspond to a range of dihedral angles of 130-150° (Figure 6) which indicates that the 6 proton and the fluorine are essentially trans as in Figure 5B. In summary, these data correlate very well with the trans attachment of the enzyme sulfhydryl relative to 5,10-CH2H4folate across the 5,6 double bond yielding a structure for the native ternary complex as shown in Figure 5A. Denaturation of the ternary complex then translates structurally into a conformational change to yield the 6 proton and the fluorine in a trans-diaxial relationship according to the large value of J_{H_6F} and a conformation as depicted in Figure 5B. This interpretation is consistent with the previous chemical shift analysis.

It is pertinent to indicate that this stereochemical structure is a relative one, and the true structure could easily exist as the mirror image of this structure with respect to the plane of the pyrimidine ring. Nevertheless, these composite results lead to the assignment of the detailed geometrical relationship of the substituents directly attached to the pyrimidine ring within the *native* ternary complex as that depicted in Figure 5A.

Discussion

In assessing the implications that this structural elucidation has upon our understanding of the reaction mechanism of thymidylate synthetase, the question arises as to whether or not there is a difference in the covalent complexes formed at the two active sites in the enzyme. It has been well established
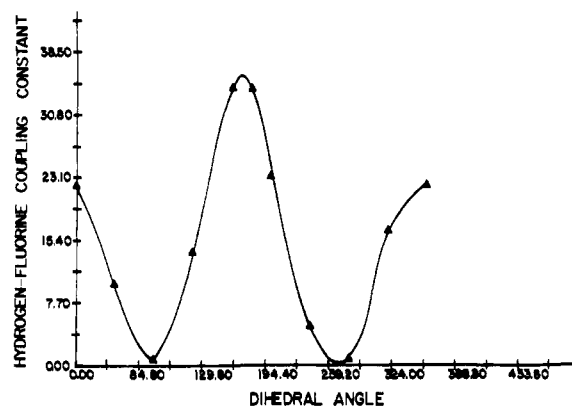

Figure 6. The dependence of J_{H_6F} upon the dihedral angle, ϕ, between the C6-H6 and C5-F bonds in 5-fluoro-5,6,-dihydrouracils.

that, for an otherwise homogeneous preparation of the enzyme, approximately 70% of the enzyme forms ternary complexes with a stoichiometry of 2:2:1 (referring to FdUMP:5,10-CH2H4folate:enzyme) and 30% forms a 1:1:1 complex.[7f,8] Both of these stoichiometric components were, in fact, present in all of our experiments. If we assume initially that the covalent complexes formed at the two sites are distinct, then based on the observed data structural differences can be readily eliminated. Since only a single broad resonance was observed, the chemical shifts must be very similar. The data presented for the model compounds indicate that, under this restriction, both forms of the complex must possess an alkyl substituent at C5 of the pyrimidine ring, and the fluorine must reside in an equatorial position relative to the C4 carbonyl. The only plausible bonding variation which could meet these criteria would be for the second site to represent a cis attachment of the sulfhydryl and 5,10-CH2H4folate. The formation of ternary complex is stereospecific for the *l*-L isomer of 5,10-CH2H4folate,[7] and the reduction of the methylene carbon to the methyl group of thymidylate has been shown to be very stereospecific.[4c] It seems highly unlikely that this sort of stereospecificity could accommodate both cis and trans reaction mechanisms. Furthermore, such an arrangement is inconsistent with the observed changes in J_{H_6F}. Consequently, there is only *one* structural geometry which fits all of the observed data.

The structure of the native ternary complex determined in this study substantiates that the initial steps in the catalytic mechanism involve a nucleophilic attack by the enzyme at the 6 position of the pyrimidine ring to generate a carbanionic center at the 5 position which in turn participates in nucleophilic attack on the methylene carbon of 5,10-CH2H4folate. That the enzyme nucleophile is the sulfhydryl group of a cysteine residue cannot be deduced from the NMR data but may be inferred from other work. On the other hand, the attachment of the methylene carbon of 5,10-CH2H4folate to the pyrimidine ring and the fact that it adds stereospecifically trans relative to the sulfhydryl group are unambiguously confirmed by the NMR data. Furthermore, the diaxial relationship of the sulfhydryl and methylene groups in the native ternary complex indicates that these groups were positioned in the active site such that they approached the substrate approximately perpendicular to the plane of the carbon–carbon double bond. In the presence of FdUMP the reaction stops at this point (with the fluorine in an equatorial position) because of the inability of the enzyme to remove the fluorine. When dUMP is the substrate, abstraction of the 5 hydrogen must lead to a cis elimination of the sulfhydryl group.

It is pertinent in this discussion to compare the results and interpretations presented by Santi and co-workers[11] based on ^{19}F NMR data of a peptide fragment derived from the ternary complex to those reported herein, and the inferences these data

bear on the thymidylate synthetase mechanism. Both studies confirm that the initial steps proceed to a trans relationship of the sulfhydryl group and methylene carbon. However, in the peptide the conformation of these two groups was found to be transdiequatorial, an orientation which they recognized to be at odds with an initial attack perpendicular to the plane of the pyrimidine ring.[11] On the assumption that the peptide is representative of the native ternary complex, Santi and co-workers rationalized that a ring inversion occurs in the formation of the ternary complex. This consideration then led them to propose that a ring inversion is required as part of the catalytic mechanism. The results presented in this study clearly demonstrate that a trans-diaxial relationship of the sulfhydryl and methylene groups is retained in the native ternary complex. The abstraction of the proton from the 5 position (by an, as yet, unidentified base in the active site) of ternary complex formed with dUMP (a transient species in this case) would not necessarily require that this proton be axial; the ensuing cis elimination of the sulfhydryl group would occur perpendicular to the plane of the ring if this group remains axial. The NMR data do not provide any information beyond formation of the ternary complex, however. The pyrimidine ring is observed to undergo a conformation change upon denaturation to give the trans-diequatorial orientation of sulfhydryl and methylene groups found in the peptide but this does not appear to have any bearing on the catalytic mechanism; rather, this simply represents a conversion to an energetically more stable species when released from the stringent steric requirements imposed by the active site of the native enzyme. As a consequence, we consider the complicated sequence of ring inversions postulated by Santi and co-workers to be entirely speculative.

Since covalently bonded hydrogen is nearly isosteric with covalently bonded fluorine, inhibition of thymidylate synthetase by FdUMP is probably a result of the inability to abstract F^+ from the 5 position of the pyrimidine ring. This argues that the proximal base is unable to abstract F^+, or, perhaps, the next step involves a concerted mechanism which cannot be triggered owing to the chemical nature of fluorine. Nevertheless, the results of this study do not conclusively eliminate the possibility that a conformational change is a subsequent part of the reaction mechanism.

Experiments are currently in progress in this laboratory to further evaluate subsequent mechanistic steps and elucidate other amino acid residues present in the active site. Of particular interest is the identification of the base involved in the proton abstraction and the proximal location of this residue relative to the pyrimidine ring. Other important features under investigation are the identification of which nitrogen in the folate moiety is linked to the methylene carbon, and the relative orientation of the hydrogen at carbon 6 of the pterin ring with respect to the methylene carbon. The success achieved in the present study with regard to measuring the existence and structural significance of coupling constants suggests that this approach may be taken with regard to the folate moiety.

Supplementary Material Available: An Appendix describing line width determinations (4 pages). Ordering information is given on any current masthead page.

References and Notes

(1) (a) Taken in part from the thesis of R.A.B. submitted in partial fulfillment of the requirements for the Ph.D. degree. (b) Alfred P. Sloan Foundation Fellow, 1977–1979. (c) Recipient of a Faculty Research Award (FRA-144) from the American Cancer Society.

(2) Abbreviations used: 5,10-CH$_2$H$_4$folate, 5,10-methylenetetrahydrofolate; FdUMP, 5-fluoro-2'-deoxyuridylate; dUMP, 2'-deoxyuridylate; SDS, sodium dodecyl sulfate.

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