THYMIDYLATE SYNTHETASE: FLUORINE-19 nmr CHARACTERIZATION OF THE ACTIVE SITE PEPTIDE COVALENTLY BOUND TO 5-FLUORO-2'-DEOXYURIDYLATE AND 5,10-METHYLENETETRAHYDROFOLATE

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SUMMARY: The fluorine-19 nmr spectra of an active-site peptide of thymidylate synthetase covalently bound to FdUMP and CH_2-H_4folate has been obtained. The splitting pattern, a doublet of triplets, provides strong confirmatory evidence for the previously proposed structure in which the 6-position of the nucleotide is covalently bound to the peptide, and the 5-position is attached to the one-carbon unit of the cofactor. From the magnitude of coupling constants we have ascertained that the nucleophile of the enzyme and the cofactor have added across the 5,6-double bond of FdUMP in a *trans* fashion and, in the isolated FdUMP-CH_2-H_4folate peptide, exist in pseudoequatorial positions. Consequently, we are able to propose a stereochemical pathway involving transient intermediates in the normal catalytic reaction.

Thymidylate synthetase catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to thymidylate (dTMP) with the concomitant conversion of 5,10-methylenetetrahydrofolic acid (CH_2-H_4folate) to 7,8-dihydrofolic acid

$$dUMP + CH_2\text{-}H_4folate \longrightarrow dTMP + H_2folate$$

(H_2folate). Model studies have led to the suggestion that a primary event in the catalytic sequence involves the addition of a nucleophilic group of the enzyme to the 6-position of the substrate, dUMP (1-3). This hypothesis was strengthened by the finding that 5-fluoro-2'-deoxyuridylate (FdUMP) behaves as a quasi-substrate for thymidylate synthetase (4-6); that is, in the presence of CH_2-H_4folate, a covalent bond is formed between an amino acid residue of the enzyme and the 6-position of the nucleotide to provide a stable complex which is believed to be analogous to a steady state intermediate of the enzymic reaction. Proteolytic digestion of this complex provided a peptide which was attached to both FdUMP and CH_2-H_4folate (7,8). From spectral and chemical properties it was proposed that the 6-position of FdUMP was covalently bound to an amino acid residue of the peptide, and the formaldehyde unit of the cofactor linked the 5-position of the nucleotide to the 5-nitrogen of H_4folate.

We report herein the ^{19}F-nmr spectrum of the FdUMP-CH_2-H_4folate-peptide.

The results provide strong supportive evidence for the previously proposed structure, and permit assignment of the stereochemistry of the addition product. By analogy with the proposed catalytic mechanism of the enzyme, these data permit assignment of the stereochemical pathway of the enzymic reaction, allow deduction of the stereochemistry of a number of transient intermediates, and provide further insight into the transformations required for catalysis.

MATERIALS AND METHODS: Thymidylate synthetase was obtained from an amethopterin resistant strain of *Lactobacillus casei* (9) and purified by the procedure of Galivan *et al.*, (10). [6-^{3}H]H_4folate (1.13 Ci/mmol) was prepared as previously described (11) and [2,6-^{14}C]FdUMP (106 mCi/mol) was prepared by phosphorylation of [2,6-^{14}C] 5-fluoro-2'-deoxyuridine (Nuclear Dynamics) with thymidine kinase from *Escherichia coli* (12). Deuterium oxide (100 atom %D) was obtained from Diaprep Inc. and nuclease free Pronase from Calbiochem. All other reagents have been previously described (5). The double labeled thymidylate synthetase-FdUMP-CH_2-H_4folate complex was prepared, digested with Pronase and purified by TEAE-cellulose chromatography as previously described (7). Calculating from ε_{max}^{290} = 30,000 (7), the effluent contained 1.1 μmol of the FdUMP-CH_2-H_4folate peptide. The solvent and volatile buffer ($NH_4 \cdot HCO_3$) were removed by repeated lyophilization; the residue was lyophilized three times from D_2O and dissolved in 1 ml D_2O to give a 0.9 mM solution of peptide which had 4.3 x 10^6dpm ^{3}H and 4.3 x 10^3 dpm ^{14}C.

The ^{19}F nmr spectrum was obtained at 94.077 MHz on a Varian XL-100 spectrometer equipped with a Nicolet Fourier transform accessory at 25°. The theoretical spectrum was calculated using the ITRCAL computer program supplied by Nicolet Transform Corp.

RESULTS: As shown in Figure 1, the 94 MHz ^{19}F spectrum of the FdUMP-CH_2-H_4folate-peptide in D_2O shows an apparent quintet of intensity ratio 1:2:2:2:1 which is located 87.2 ppm upfield of the signal from the external reference trifluoroacetic acid. This is interpreted to result from overlapping of the two inner lines of a doublet of triplets. The doublet is due to splitting of the ^{19}F resonance by the ring proton H_A (Figure 2) and exhibits a coupling constant J_{AF} of 34.0 Hz. Each component of the doublet is further split into a triplet (intensity ratio 1:2:1) due to coupling of the fluorine with the methylene protons H_B, the value for the coupling constant J_{BF} being 19.5 Hz. Also shown in Figure 1 is the theoretical spectrum calculated using the above coupling constants and a line width of 10.0 Hz.

The magnitude of the coupling between the fluorine and the vicinal protons, in particular J_{AF}, shows the fluorine and proton H_A both to be in pseudoaxial positions as depicted in Figure 2. This was not evident *a priori*; unlike the situation with substituted cyclohexanes, the peptide moiety or the methylenetetrahydrofolate moiety in a pseudoaxial position would not lead to appreciable nonbonded interactions (13). The assigned conformation of the FdUMP-CH_2-H_4folate-peptide is based on the following arguments.

It has been established that the trigonal geometry of the carbonyl

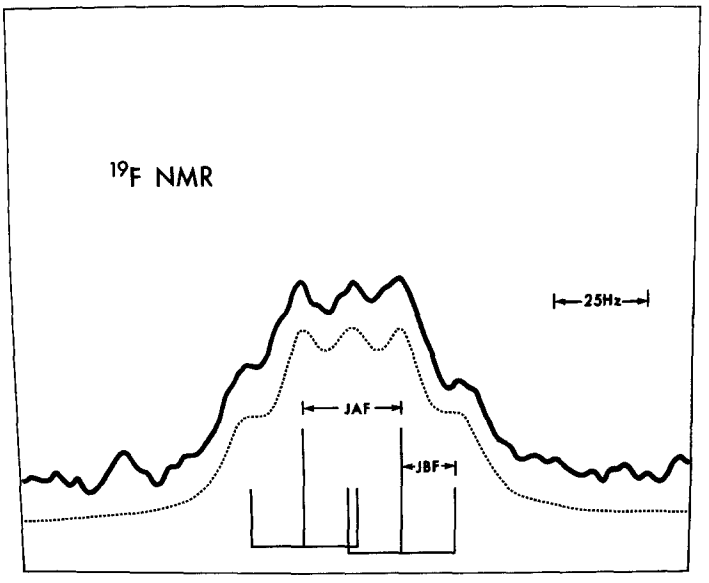


Figure 1. 94 MHz Fluorine-19 nmr spectrum of the FdUMP-CH_2-H_4folate-peptide (———); theoretical spectrum calculated by ITRCAL (.....).

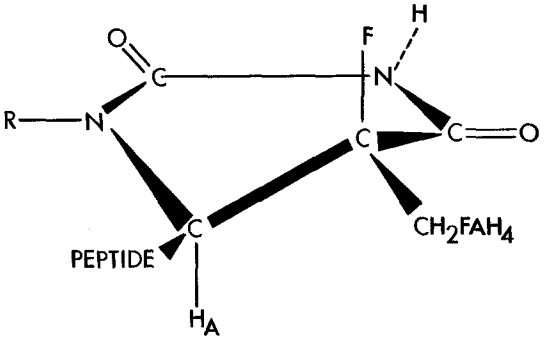
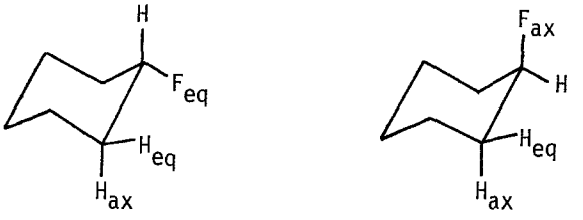


Figure 2. Stereochemical projection of the FdUMP-CH_2-H_4folate-peptide as determined by its ^{19}F nmr spectrum; R = 1-(2'-deoxyribosyl)-5'-phosphate.

atoms in uracil derivatives saturated across the 5,6-double bond gives rise to the half-chair conformation with substituents of carbon atoms 5 and 6 staggered (14-16) as normally found in cyclohexane. The conformational assignment of the fluorine atom and H_A in pseudoaxial positions is based largely on the work of Bovey (17) with fluorocyclohexanes. It was found for the fluorocyclohexanes that the vicinal fluorine-proton coupling constants

$J_{F_{eq}H_{eq}}$, $J_{F_{eq}H_{ax}}$ and $J_{F_{ax}H_{eq}}$ were all less than 3-4 Hz, whereas $J_{F_{ax}H_{ax}}$ was 43.4 Hz (17).



By analogy, the observed coupling constant J_{AF} for the FdUMP-CH_2-H_4folate-peptide, (34.0 Hz) indicates that both fluorine and H_A are in pseudoaxial positions. Two additional observations which support this assignment are that the *trans* fluorine-proton vicinal coupling constant for $CHCl_2CHFCl$ is 38.0 Hz, and a *gauche* fluorine-proton coupling constant is 2.8 Hz (18). One cannot however, rule out the existence of a small amount (e.g., 10-20%) of a minor conformer, with both the fluorine and H_A in pseudoequatorial positions, existing in rapid equilibrium with the major conformer. Such an equilibrium

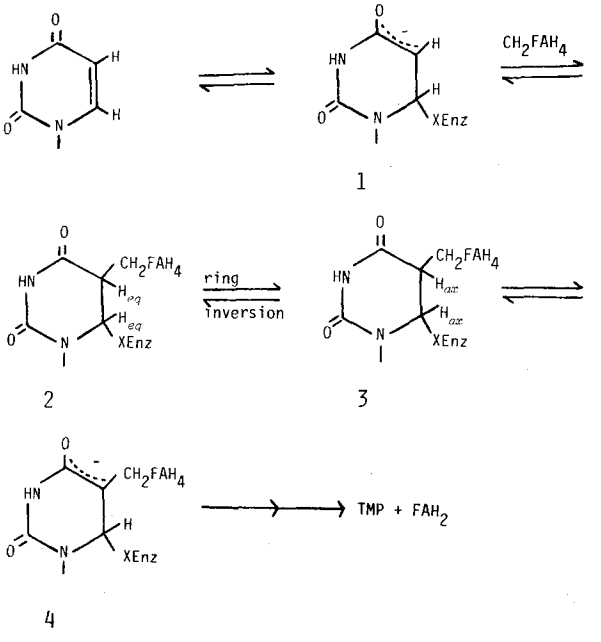


Figure 3. Proposed partial mechanism for the thymidylate synthetase reaction showing stereochemistry of addition-elimination reactions.

may explain the somewhat smaller value of J_{AF} = 34.0 Hz compared with fluorocyclohexane, although the smaller value may be due to the substituent groups.

DISCUSSION: The ^{19}F splitting pattern of the FdUMP-CH_2-H_4folate-peptide has two possible interpretations: (1) As argued here, it is a doublet of triplets arising from a single fluorine species, or (2) it may result from two overlapping triplets arising from chemically shifted fluorine species. The latter could occur if two distinct fluorine species were formed in identical amounts, such as a diastereomeric mixture. All evidence thus far available indicates that FdUMP interacts with the enzyme-cofactor complex in a manner directly analogous to the enzymic reaction, and a non-stereospecific interaction would *a priori* appear highly unlikely. Further, it has recently been established (11) that over 95% of the cofactor in the FdUMP-CH_2-H_4folate-thymidylate synthetase complex is the natural *l*,L-isomer, regardless of whether the *d*,L-isomer is present during preparation of the complex. Nevertheless, unequivocal assignment awaits decoupling experiments which are in progress. With the reasonable assumption that the FdUMP-CH_2-H_4folate-peptide possesses a single fluorine species, a number of interesting conclusions may be derived.

The ^{19}F splitting pattern, together with previously reported spectral data (11) of the FdUMP-CH_2-H_4folate-peptide, provides definitive evidence for its structure. The doublet of triplets indicates that the carbon bearing the fluorine is flanked by CH and CH_2 groups (i.e. $CHCFCH_2$). The CH is certainly the 6-carbon of the nucleotide which is attached to the nucleophile of the enzyme, and the most logical assignment for the CH_2 group is the carbon bridging the nucleotide and cofactor (Figure 2).

By comparison of the coupling constants with extensively studied models, we are able to deduce the stereochemistry of the substituents which have added across the 5,6-double bond of FdUMP to give the ternary complex. As depicted in Figure 2, the 5-fluoro and 6-hydrogen of the FdUMP-CH_2-H_4folate-peptide are situated in a *trans* pseudoaxial conformation; consequently, the nucleophile and cofactor are *trans* pseudoequatorial.

A primary event in the thymidylate synthetase catalyzed reaction is the addition of a nucleophile of the enzyme to the 6-position of dUMP (Figure 3). The resultant carbanion 1 reacts with CH_2-H_4folate to form an intermediate (2,3) analogous in structure to the ternary complex formed with thymidylate synthetase, FdUMP, and the cofactor. Abstraction of the 5-hydrogen followed by a series of steps involving reduction of the one carbon unit and elimination of the nucleophile results in the observed products of the reaction. These steps have been described in detail in a previous report (3). With the reasonable assumption that the normal enzymic reaction proceeds in a manner similar

to the formation of the FdUMP-CH_2-H_4folate-thymidylate synthetase complex, a number of conclusions may be reached concerning the mechanism of the enzyme-catalyzed reaction.

First, the *overall* stereochemical pathway of the enzymic reaction may be deduced. The addition of the nucleophile and cofactor across the 5,6-double bond must occur in a *trans* fashion. Subsequent loss of the 5-hydrogen and nucleophile would therefore occur as a *cis*-elimination.

Second, details of the stereochemistry of certain transient intermediates depicted in Figure 3 may be inferred. The following rationalizations are derived from the principle that a group reacting with the π-system of the uracil heterocycle will approach approximately perpendicular to the plane of the ring; by microscopic reversibility, a similar orientation is required when a group departs to reform the π-system. Thus, the initial approach of the nucleophile of the enzyme to the electrophilic 6-carbon of dUMP should be perpendicular to the plane of the heterocycle. The resultant carbanion 1 will be delocalized throughout the carbonyl groups and be high in sp^2 character. The approach of CH_2-H_4folate to the 5-position sould be perpendicular to the plane of the ring and, based on data presented here, *trans* to the nucleophile attached to the 6-position. Consequently, in 2, the cofactor would exist in a pseudoaxial position and the 5-hydrogen would be pseudo-equatorial. For the subsequent elimination reaction, the proton from the 5-position would be in the pseudoaxial position 3 prior to its abstraction to form the carbanion 4. This rationalization requires a previously unrecognized conformational change to take place after addition of the cofactor but before abstraction of the 5-proton, resulting in inversion of the 5-and 6-positions of the nucleotide intermediates (i.e. 2→3).

The results presented in this paper are preliminary; a detailed nmr study of the interaction of FdUMP with thymidylate synthetase will be published elsewhere.

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