PURIFICATION AND AMINO ACID ANALYSIS OF AN ACTIVE SITE PEPTIDE FROM THYMIDYLATE SYNTHETASE CONTAINING COVALENTLY BOUND 5-FLUORO-2'-DEOXYURIDYLATE AND METHYLENETETRAHYDROFOLATE

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SUMMARY: Thymidylate synthetase forms a complex with 5-fluoro-2'-deoxyuridylate and 5,10-methylenetetrahydrofolate in which a nucleophile of the enzyme is covalently attached to the 6-position of the nucleotide. Treatment of the complex with Pronase provides a small peptide to which both the nucleotide and cofactor are covalently attached. From amino acid analysis, it may be deduced that the amino acid which is covalently attached to 5-fluoro-2'-deoxyuridylate is threonine or histidine. Implications with regard to catalysis are discussed.

Chemical model studies of the thymidylate synthetase reaction have led to the proposal 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, 2'-deoxyuridylate (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), and is, in effect an affinity labeling agent for the enzyme; that is, in the presence of 5,10-methylenetetrahydrofolate (CH_2FAH_4), a covalent bond is formed between an amino acid residue of the enzyme and the 6-position of the nucleotide. Two problems of paramount importance with regard to the inhibition of thymidylate synthetase by FdUMP, as well as the catalytic mechanism of the enzyme are: *(i)* identification of the amino acid residue attached to FdUMP, and *(ii)* ascertaining the role of the cofactor, CH_2FAH_4, in the high affinity binding of FdUMP. In this report, we describe the isolation and purification of a peptide fragment to which both FdUMP and CH_2FAH_4 are covalently linked. Furthermore, amino acid analysis permits us to deduce that either threonine or histidine is the amino acid residue of the protein to which FdUMP is covalently attached.

MATERIALS AND METHODS

Thymidylate synthetase was obtained from an Amethopterin resistant strain of *Lactobacillus casei* (7) and purified to homogeneity (>95%) by previously described methods (5). [6-^{3}H]FdUR (1.31×10^4 dpm/pmol) and [2-^{14}C]FdUR (34.4 dpm/pmol) were obtained from New England Nuclear and Schwarz/Mann respectively; un-

labeled FdUR was obtained from the Drug Research and Development division of the National Cancer Institute. The corresponding nucleotides were prepared with carrot phosphotransferase (8) which was provided by Dr. Alex Nussbaum of Hoffmann LaRoche. Pronase was purchased from Calbiochem. Folate and 5-CH_3FAH_4 were obtained from Sigma Chemical Co. Ultraviolet and fluorescence spectra were recorded on Cary 15 and Hitachi MPF 2A recording spectrophotometers, respectively. High voltage (2.5 kv, 180mA) electrophoresis was performed with Whatman 3 MM paper using 0.5% pyridine - 5% acetic acid (pH 3.5). Radioactivity measurements were made on a Nuclear Chicago Isocap 300 liquid scintillation counter using a fluid containing 0.3% Omnifluor in xylene-Triton X-114 (3:1).

Formation and Pronase Digestion of the Enzyme-[^3H]FdUMP-CH_2FAH_4 Complex. A solution (2.7 ml) containing 0.15 µmol (10.5 mg) thymidylate synthetase, 0.36 µmol [^{3}H]FdUMP, 1 µmol CH_2FAH_4, 15.5 µmol formaldehyde, 25 m*M* $MgCl_2$, 50 m*M* N-methylmorpholine (pH 7.4), 1 m*M* EDTA, and 75 m*M* 2-mercaptoethanol was allowed to stand at 22-24° for 6 hours. The solution was cooled in ice and the complex precipitated by treatment with 6 ml of cold TCA for 15 minutes. The precipitate was centrifuged and washed with 5 ml of cold TCA. The denatured protein was suspended in 2.0 ml of deaerated (argon) 0.1 *M* potassium phosphate (pH 7.4) and the pH adjusted to 7.5 with $NH_4 \cdot HCO_3$. Pronase (1 mg) and 4.5 µmol (100 µl of 45 m*M* solution) unlabeled FdUMP were added and the suspension was agitated at 37° under argon. Additional 0.5 mg portions of Pronase were added after 3 and 6 hours of the first addition. After 10 hour total incubation time the solution was diluted to 10.0 ml with 10 m*M* 2-mercaptoethanol. This solution contained 1.1 x 10^6 dpm which corresponds to 0.23 µmol (75%) of the theoretical FdUMP binding sites (5) of the native enzyme originally present.

Purification of the FdUMP-peptide was achieved by chromatography on TEAE-cellulose followed by Sephadex G-25 as described in the legend to Figure 3. High voltage electrophoresis (pH 3.7) gave a single radioactive spot which moved toward the cathode (R_f = 2.7 with respect to a serine standard).

Hydrolysis and Amino Acid Analysis of the FdUMP-peptide. The purified FdUMP-peptide (50 nmol; 2.45 x 10^5 dpm) along with 25.0 nmol of a norleucine marker was dissolved in 2.8 ml of 6 N HCl and heated at 110° *in vacuo* for 22 hr. The solvent was removed by lyophilization and the residue dissolved in 2.6 ml of 0.2 N citrate buffer pH 2.2. Radioactivity measurements showed that this solution contained 2.40 x 10^5 dpm (98% recovery of ^{3}H) at least 80% of which co-migrated on silica gel tlc (MeOH/CHl_3-1:9) with unlabeled markers of 5-FU (R_f 0.46) and 5-FdUR (R_f 0.33); not more than 10% of the radioactivity was associated with the amino acids which remained at the origin. Amino acid analysis was performed with a Beckman 121 amino acid analyzer.

RESULTS AND DISCUSSION

The interaction of FdUMP with thymidylate synthetase involves a CH_2FAH_4 dependent formation of a covalent bond between a nucleophile of the enzyme and the 6-position of FdUMP (4-6). In addition to our interest in understanding the mechanism of action of this inhibitor, this finding is pertinent because of its close relationship to the mechanism of action of the enzyme (2,5,9). It appears that the analog behaves as a quasi-substrate which undergoes conversions up to the stage of one carbon transfer from the cofactor, CH_2FAH_4, and in effect results in the build-up of an analog of a steady state intermediate of the normal enzymic reaction. The experiments described here aim to establish the structure of the covalent complex, to ascertain the role of the cofactor in this reaction, and to identify the amino acid residue which covalently links the protein to FdUMP.

To investigate these matters we have purified a small peptide from Pronase digestion of a complex formed from [^{3}H]FdUMP, CH_2FAH_4 and thymidylate synthetase. Figure 1 shows elution profiles of the [^{3}H]FdUMP-peptide[1] after TEAE-cellulose and Sephadex G-25 chromatography. That the FdUMP-peptide was sufficiently pure for the experiments described here is indicated by (*1*) identical specific activities (1.51×10^5 dpm/A_{280}) in the tubes pooled from gel-filtration, (*2*) homogeneity on high voltage electrophoresis, and (*3*) stoichiometry of the components of the peptide *(vida infra)*.

With this purified peptide, we could approach the question as to what amino acid residue is attached to the 6-position of FdUMP. The rationalization used was that hydrolysis of the purified [^{3}H]FdUMP-peptide should yield a nucleophilic amino acid in amounts stoichiometric with FdUMP. That the nucleotide was released from the covalently attached amino acid upon acid hydrolysis was demonstrated by tlc of the hydrolysate; almost all radioactivity could be accounted for by FU and FdUR, and only background levels with the amino acids. The amino acids found in the purified FdUMP-peptide and their molar ratios with respect to FdUMP are: Glu (1.1), Pro (1.7), Ala (0.94), Leu (0.92), Thr (0.98), His (1.3). As will be shown later, the glutamate residue results from a covalently attached derivative of FAH_4 and not the peptide which contains six amino acids. There is no indication of the presence of cysteine or cysteic acid in the FdUMP-peptide. This result, together with experiments involving sulfhydryl titration of the enzyme-FdUMP-CH_2FAH_4 complex (10) demonstrate that, contrary to other proposals (3,11,12), cysteine is *not* the nculeophilic catalyst in the thymidylate synthetase reaction. Since the

[1] Significant phosphatase activity was present in the Pronase preparation used. We have not established whether the "FdUMP-peptide" indeed possesses a phosphate but this point has no bearing on the conclusions reached in this report.

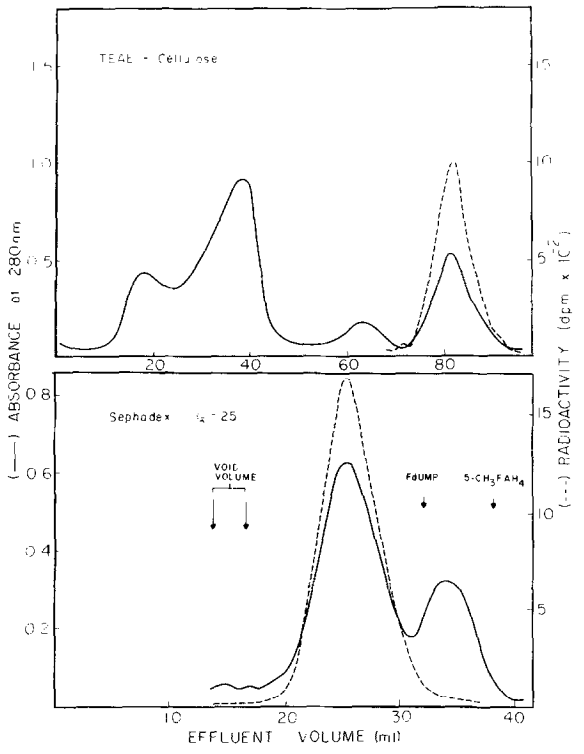

Figure 1: Purification of the FdUMP-peptide. *TEAE-cellulose Chromatography.* The Pronase digest was applied to a TEAE-cellulose column (1.3 x 4 cm) equilibrated with 10 m*M* $NH_4 \cdot HCO_3$ (pH 7.7) - 10 m*M* 2-mercaptoethanol. The column was washed with 10 ml of equilibration buffer. A linear gradient composed of 50 ml of the equilibration buffer and 50 ml of 0.3 *M* $NH_4 \cdot HCO_3$ (pH 7.7) - 10 m*M* 2-mercaptoethanol was applied and 1 ml fractions collected. Most of the radioactivity (8.4×10^5 dpm; 77% of radioactivity applied) eluted between 0.23-0.27 *M* buffer (fractions 74-91). Peak fractions (77-87) were combined and the tubes rinsed with water to give 15.2 ml of a solution containing 7.3×10^5 dpm (150 nmols) of FdUMP-peptide (1.33×10^5 dpm/A_{280}). The solution was lyophilized to dryness. *Sephadex G-25 Chromatography.* The residue was dissolved in 0.75 ml of 25 m*M* NH_4OAc (pH 7.0)-10 m*M* 2-mercaptoethanol containing 8×10^4 dpm [^{14}C]FdUMP as a marker and applied to a Sephadex G-25 column (1.1 x 40 cm) equilibrated with the same buffer. The column was eluted with the application buffer at a flow rate of 8 ml/hr, collecting 0.9 ml fractions. Tubes 24-38 contained 75% of the [^{3}H]-dpm applied and was separated from the [^{14}C]FdUMP marker. Fractions 28-32 (3.9×10^5 dpm; 79 nmol) were combined, lyophilized twice from water and redissolved in 1.0 ml of 25 m*M* NH_4OAc-10 m*M* 2-mercaptoethanol. This solution contained 1.51×10^5 dpm/A_{280} (79 nmol) of the purified FdUMP-peptide and was used for all experiments described.

N-terminus of thymidylate synthetase is methionine (13), and this amino acid is not found in the FdUMP peptide, we need not consider the possibility that the covalent attachment is *via* the N-terminal amino acid. The only amino acids *in the FdUMP-peptide which* possess nucleophiles are threonine and histidine; we conclude from this that the nucleophile of the enzyme which is covalently bound

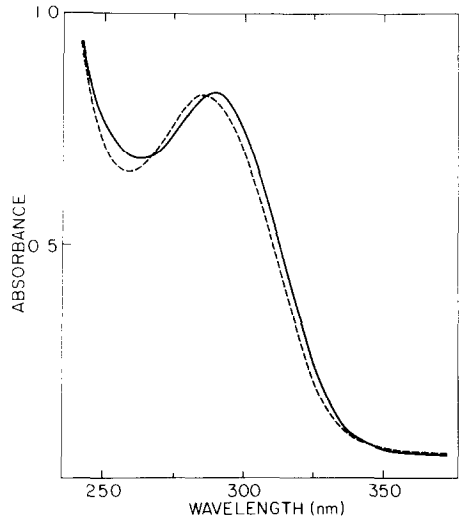

Figure 2: Ultraviolet spectra of 24 μM purified FdUMP-peptide and 24 μM 5-CH_3FAH_4 in 25 mM NH_4OAc– 10 mM β-mercaptoethanol (pH 7.0).

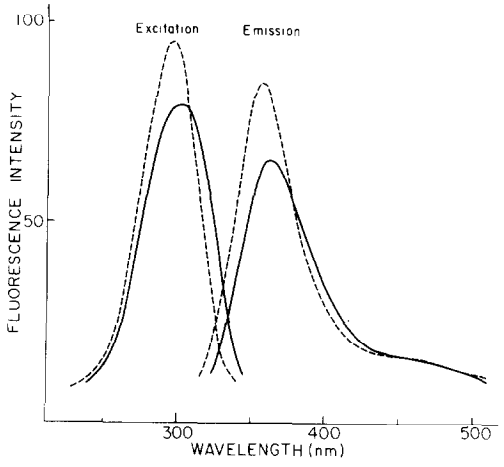

Figure 3: Fluorescence spectra of 4 μM purified FdUMP-peptide (——) and 4 μM 5-CH_3FAH_4 (– – –) in 25 mM NH_4OAc-10 mM β-mercaptoethanol (pH 7.0). For excitation spectra, emission wave length was 360 nm; for emission spectra, excitation was at 300 nm for the FdUMP-peptide and 298 nm for 5-CH_3FAH_4. Band width was 10 nm and fluorescence intensity is reported in arbitrary units.

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to FdUMP and, in all likelihood, the one involved in catalysis of the normal enzymic reaction is the hydroxyl group of threonine or the imidazole of histidine. It is most intriguing to speculate that, if threonine is the nucleophilic amino acid, the high nucleophilicity required might best be provided by hydrogen bonding

to a histidine, in analogy to the serine proteases. Although this speculation is somewhat premature, it is sufficiently attractive to warrant investigation.

Figures 2 and 3 show the ultraviolet and fluorescence spectra of the FdUMP-peptide to be almost identical to 5-CH_3FAH_4 and strongly suggest that a derivative of FAH_4 is covalently linked to the FdUMP-peptide. That this derivative is a 5-substituted FAH_4 may be deduced by its stability over the six day period separating Pronase digestion and spectral analysis; an analog of FAH_4 which is unsubstituted at N-5 would have certainly undergone oxidative degradation over this period (14). Using an extinction value of ϵ_{max} = 32,000 (15) which is characteristic of 5-alkyl derivatives of FAH_4, and the specific activity of the [^{3}H]FdUMP used, we calculate that 1.04 mol of the uv absorbing species are present in the FdUMP-peptide per mol of nucleotide. Taken in concert, these data are in best accord with the structure shown in Figure 4. The 6-position of FdUMP is linked to the hydroxyl

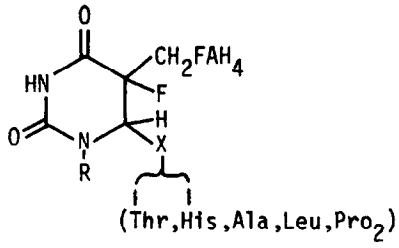

Figure 4: Structure of FdUMP-peptide where R = 5-phospho-2'-deoxyribosyl and X = imidazole nitrogen of histidine or hydroxyl oxygen of threonine. Attachment is at the 5-nitrogen of FAH_4.

group of a threonine or the imidazole of a histidine, and the 5-nitrogen of FAH_4 is coupled to the 5-position of the nucleotide *via* a methylene bridge. The latter point has been considered a possibility in previous reports (5,6). Most important, we note that the general structure shown in Figure 3 is analogous to a steady state intermediate we have proposed to exist in the normal enzymic reaction (2,9) except that fluorine is replaced by hydrogen. Because of the stability of the C-F bond, the covalent complex is stabilized and permitted to accumulate. Thus, the mechanism of formation of the covalent enzyme-FdUMP-CH_2FAH_4 complex is directly analogous to a portion of the enzymic reaction and its delineation should resolve many questions concerning the catalytic mechanism of thymidylate synthetase. For example, the results described here argue against general mechanisms which require a redox reaction to occur before condensation of the formaldehyde unit with the nucleotide; thus, we would exclude methylated enzyme, methylated derivatives of FAH_2 (16), and FAH_2 (5,17) from consideration

as intermediates. Structure assignment of the FdUMP-peptide also provides experimental support for the suggestion (18) that the iminium cation of CH_2FAH_4 which is formed on the enzyme is located at the 5-rather than 10-nitrogen. Further studies on the mechanism of FdUMP interaction with thymidylate synthetase are in progress.

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References

1. Santi, D. V., and Brewer, C. F., *J. Amer. Chem. Soc. 90*, 6236 (1968).
2. Santi, D. V., and Brewer, C. F., *Biochemistry 12*, 2416 (1973).
3. Kalman, T. I., *Biochemistry 10*, 2567 (1971).
4. Santi, D. V., and McHenry, C. S., *Proc. Nat. Acad. Sci. USA 69*, 1855 (1972).
5. Santi, D. V., McHenry, C. S., and Sommer, H., *Biochemistry 13*, 471 (1974).
6. Langenbach, R. J., Danenberg, P. V., and Heidelberger, C., *Biochem. Biophys. Res. Commun., 48*, 1565 (1972).
7. Crusberg, T. C., Kisliuk, R. L., *J. Biol. Chem., 245*, 5292 (1970).
8. Harvey, C. L., Clericuzio, E. M., and Nussbaum, A. L., *Anal. Biochem., 36*, 413 (1970).
9. Pogolotti, A. L., Jr., and Santi, D. V., *Biochemistry 13*, 456 (1974).
10. McHenry, C. S., and Santi, D. V., *Biochem. Biophys. Res. Commun.*, in press.
11. Dunlap, R. B., Harding, N. G. L., and Huennekens, F. M., *Ann. N.Y. Acad. Sci. 186*, 153 (1971).
12. Kalman, T. I., *Biochem. Biophys. Res. Commun. 49*, 1007 (1972).
13. Loeble, R. B., and Dunlap, R. B., *Biochem. Biophys. Res. Commun., 49*, 1671 (1972).
14. Blakley, R. L., "The Biochemistry of Folic Acid and Related Pteridines", American Elsevier Publishing Company, Inc., New York, 1969 p 76.
15. Blair, J. A., and Sanders, K. J., *Anal. Biochem., 41*, 332 (1971).
16. Wahba, A. J., and Friedkin, M., *J. Biol. Chem. 237*, 3794 (1962).
17. Sharma, R. K., and Kisliuk, R. L., *Fed. Proc. Fed. Amer. Soc. Exp. Biol. 31*, No. 2, 591 (1973).
18. Kallen, R. G., and Jencks, W. P., *J. Biol. Chem. 241*, 5851 (1966).