*Biochimica et Biophysica Acta*, 438 (1976) 324—331


BBA 67834

# ON THE STRUCTURE OF THE COFACTOR IN THE COMPLEX FORMED WITH THYMIDYLATE SYNTHETASE, 5,10-METHYLENETETRAHYDROFOLATE AND 5-FLUORO-2'-DEOXYURIDYLATE

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(Received February 9th, 1976)

## Summary

A study was undertaken to ascertain whether dihydrofolic acid is produced in the complex formed with 5,10-methylenetetrahydrofolate, 5-fluorodeoxyuridylate and thymidylate synthetase, as suggested by ultraviolet difference spectral studies. The complex was formed using the cofactor specifically labeled with tritium at the 6-position. After dissociation by equilibration with unlabeled cofactor, it was demonstrated that the tritium remained exclusively at the 6-position. Had oxidation of the cofactor occurred within the complex to give a methylated enzyme form, tritium should have been transferred to the one-carbon unit of the cofactor. It was also found that the difference spectrum of the ternary complex which resembles that of dihydrofolic acid can also be produced by substituting an analog of the cofactor which is not susceptible to oxidation. The results described here demonstrate that oxidation of the cofactor does not occur in the ternary complex and suggest that the unusual ultraviolet spectrum results from perturbations of a chromophore of the bound cofactor.

## Introduction

Thymidylate synthetase catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to thymidylate (dTMP) with the concomitant conversion of 5,10-methylenetetrahydrofolic acid ($CH_2$-$H_4$folate) to 7,8-dihydrofolic acid ($FAH_2$). 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 [1,2].

dUMP + $CH_2$-$H_4$folate → dTMP + $H_2$folate

This hypothesis was strengthened by the finding that 5-fluoro-2′-deoxy-uridylate (FdUMP) behaves as a quasi-substrate for thymidylate synthetase [3—6]; that is, in the presence of $CH_2$-$H_4$folate, a covalent bond is formed between an amino acid residue of the enzyme and the 6-position of FdUMP to provide a stable complex which is believed to be analogous to a steady-state intermediate of the enzymic reaction. For this reason, a number of laboratories have been interested in delineating the exact nature of the interaction of FdUMP with the enzyme and identifying the stable complex which is formed. One feature of the native enzyme · $CH_2$-$H_4$folate · FdUMP complex which remains puzzling is the ultraviolet spectrum which is obtained upon subtraction of the absorbance of the enzyme · $CH_2H_4$folate complex [4,5,7—9]; the resultant difference spectrum is very similar to that obtained when $CH_2$-$H_4$-folate is subtracted from $H_2$folate [10], the product of the normal enzymic reaction. If $CH_2$-$H_4$folate is converted to $H_2$folate within the enzyme · cofactor · FdUMP complex, as these studies might imply, a dramatic revision of the currently accepted mechanism of this reaction would be warranted. In this report, we describe experiments which demonstrate that this conversion does not occur and that the spectral changes observed are probably the result of perturbations of a chromophore of the bound cofactor.

## Materials and Methods

Thymidylate synthetase was a homogeneous preparation obtained from an amethopterin resistant strain of *Lactobacillus casei* as previously described [4]. [$^3$H]$NaBH_4$ (6.5 Ci/mmol) and [$^{14}$C]$H_2CO$ (59 Ci/mol) were obtained from New England Nuclear. Other materials have been previously described [4] or were the purest commercial grade available. Buffer A refers to 50 mM *N*-methylmorpholine/HCl, 30 mM $MgCl_2$, 1.0 mM EDTA and 90 mM 2-mercaptoethanol. Ultraviolet spectra were obtained with a Cary 118 recording spectrophotometer.

Nitrocellulose filter assays of enzyme · $CH_2$-$H_4$folate · FdUMP complexes were performed by reported methods [11]. Isotopes were counted to an accuracy of ±0.5% in a Nuclear Chicago Isocap 300 liquid scintillation counter and dpm calculations were aided by a PDP-10 tape-fed computer. dUMP was separated from TMP by high-pressure chromatography (300 lbs/inch$^2$) on an Aminex A27 column (0.6 × 13 cm) using 0.12 M $NH_4HCO_3$ containing 8% (v/v) *n*-propanol. The procedure will be described in detail in a forthcoming publication.

(±)-L-[6-$^3$H]$H_4$folate was prepared by [$^3$H]$NaBH_4$ (3 mg; 450 mCi) reduction of 7,8-$H_2$folate (30 mg), purified by DEAE-cellulose chromatography [12] and stored in 200 mM 2-mercaptoethanol under argon at −20° C. The product had $1.12 \times 10^8$ dpm/$A_{298nm}$. Assuming that no ultraviolet-absorbing impurities were present, and using $\epsilon_{298} = 29\,100$, the specific activity of the (±)-isomer was calculated to be 6800 dpm/pmol. The purity of the cofactor and position of tritium incorporation were established by the following criteria: using excess dUMP, the thymidylate-synthetase catalyzed conversion of (−)-L-$CH_2$-$H_4$folate

to $H_2$folate was monitored spectrophotometrically [10]. From $\epsilon_{340} = 6400$, it could be calculated that 43% of the total ultraviolet-absorbing material of the preparation was (—)-L-$H_4$folate. Assuming that equal amounts of both the (+)-L- and (+)-L-diasteriomers were formed upon reduction, this indicates the preparation to be 86% in (±)-L-$H_4$folate. The TMP was isolated by high-pressure chromatography and shown to contain over 42% of the total tritium used; this is in good agreement with the spectrophotometric assay, and verifies that at least 82% of the tritium on $H_4$folate is at the 6-position.

## Results

*Preparation of enzyme · FdUMP · $CH_2$-$H_4$folate complexes*

A solution (300 μl) containing 4.2 μM (1.26 nmol) thymidylate synthetase 1.4 mM [$^{14}$C]$H_2CO$, 20 μM [6-$^3$H]$H_4$folate, and 0.3 mM FdUMP in buffer A was allowed to stand at room temperature for 1 h. The solution was filtered through Sephadex G-25 (1.0 × 22 cm) using buffer A as eluent to separate the bound from the free ligands. The collected excluded volume (3.0 ml) contained 760 pmol (0.12 μM) of complex having $6.41 \times 10^4$ dpm $^{14}$C and $2.41 \times 10^6$ dpm $^3$H ($^3$H/$^{14}$C = 37.6). Filtration of an aliquot through nitrocellulose gave quantitative retention of radioactivity and $^3$H/$^{14}$C = 36.2. FdUMP was added to the solution to give a final concentration of $1.6 \times 10^{-5}$ M and the solution was stored under argon at 0° C. A similar procedure was used to prepare the enzyme · $CH_2$ [6-$^3$H]-$H_4$folate complex except that unlabeled formaldehyde was used.

*Identification and position of isotopic substitution of the cofactor form released upon dissociation of the enzyme · FdUMP · $CH_2$[6-$^3$H]$H_4$ folate complex*

The enzyme · FdUMP · $CH_2$-[6-$^3$H]$H_4$folate complex was prepared and isolated by gel filtration as described above; the solution contained $1.68 \times 10^6$ dpm (530 pmol complex). Unlabeled (±)-L-$CH_2$-$H_4$folate and $H_2CO$ were added to give final concentrations of 0.65 mM and 7 mM, respectively (total volume, 3.6 ml) and equilibration was allowed to proceed at 37° C under argon until over 95% of the nitrocellulose filterable counts were lost (approx. 6 h). Two procedures were then used to ascertain the identity of the radioactive cofactor released from the complex and the position of isotopic substitution: (i) a mixture (1.7 ml) was made containing 0.56 ml ($9.4 \times 10^5$ dpm) of the dissociated complex, 0.02 mM thymidylate synthetase and 0.13 mM FdUMP. Nitrocellulose filtration of an aliquot demonstrated that 76% of the dissociated [$^3$H]$CH_2$-$H_4$folate could be rebound to the enzyme; it should be noted that this is a minimal value since corrections were not made for filtration efficiency [4]; (ii) a mixture (1.68 ml) containing 0.56 ml of the dissociated complex ($9.4 \times 10^5$ dpm), 1.0 ml of buffer A containing 1.0 mM dUMP and 628 pmol of thymidylate synthetase was incubated at 3° C for 20 min under argon. The TMP formed was isolated and shown to contain 95% ($8.9 \times 10^5$ dpm) of the total radioactivity in the effluent.

*Reaction of dissociated [$^3$H,$^{14}$C] cofactor with dimedone*

To 1.0 ml of the double-labeled complex (264 pmol; $2.2 \times 10^4$ dpm $^{14}$C,

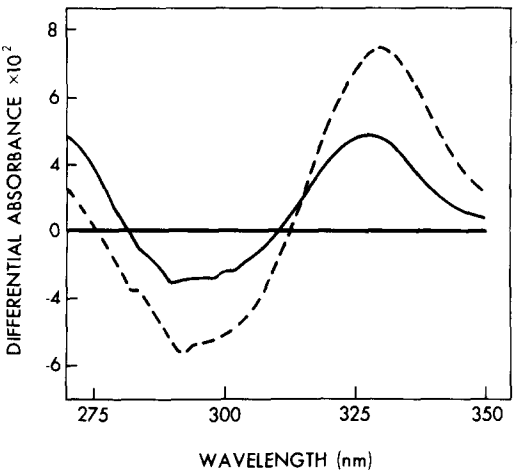


**Fig. 1. Ultraviolet difference spectra of FdUMP, $CH_2$-$H_4$folate and thymidylate synthetase versus $CH_2$-$H_4$-folate and thymidylate synthetase (- - - - -); and FdUMP, 5,8-deazafolate and thymidylate synthetase versus enzyme and 5,8-deazafolate (———).**

$8.36 \times 10^5$ dpm $^3H$) was added 35 μl of 7.5 mM (±)-L-$CH_2$-$H_4$folate and 5 μl of 1.5 M $H_2CO$ to give final concentrations of 0.26 and 7.5 mM, respectively. The solution was kept at 37°C for 6 h under argon to "chase" the bound labeled cofactor into the solution. To this solution was added 0.1 ml of 1.5 M $H_2CO$ and 10 ml of 0.5% dimedone in 100 mM potassium phosphate (pH 6.2) [13]. The dimedone complex was extracted with 10 ml $CH_2Cl_2$ and washed with 10-ml portions of water until the washings contained no tritium. After evaporation of the solvent, and repeated recrystallization from MeOH, the dimedone complex retained $^{14}C$ = 18 535 dpm (84% yield) and 734 dpm $^3H$ ($^3H/^{14}C$ = 0.040); m.p. 191—192.5°C (literature 189°C [13]). This result demonstrates that there is no transfer of tritium from the 6-position of $CH_2$-$H_4$folate to the methylene group within the ternary complex.

*Ultraviolet difference spectra*

The spectra shown in Fig. 1 were obtained by adding 20 μl of a 0.24 mM solution of FdUMP or an equivalent amount of water to two previously balanced cells containing in a volume of 0.50 ml: 5.7 μM thymidylate synthetase, 6.5 mM dithiothreitol, 25 mM $MgCl_2$, 1 mM EDTA, 50 mM *N*-methylmorpholine/HCl (pH 7.4) and either 44 μM (±)-L-$CH_2$-$H_4$folate or 54 μM 5,8-deazafolate. Titrating with FdUMP (not shown), $\Delta\epsilon_{330nm}$ for complexes formed with $CH_2$-$H_4$folate and 5,8-deazafolate may be calculated to be 17 700 and 10 600, respectively; these calculations are based on $\Delta A_{330nm}$/mol of nucleotide in the concentration range where titrant is limiting.

## Discussion

A number of workers [4,5,7—9] have recently observed that when the ultraviolet spectrum of $CH_2$-$H_4$folate and thymidylate synthetase is subtracted from that of the complex formed with enzyme, $CH_2$-$H_4$folate and FdUMP, the resultant difference spectrum in the 280—350 nm range is strikingly similar to

that obtained for 7,8-$H_2$folate minus $CH_2$-$H_4$folate [9]. The Laser-Raman spectrum of the thymidylate-synthetase · FdUMP · $CH_2$-$H_4$folate also shows a C=N stretching band for which $H_2$folate has been suggested as a possible source. We and others have been proceeding with studies of the mechanism of this enzyme with the assumption that the redox reaction occurs after transfer of the one-carbon unit to dUMP. If $H_2$folate does exist in the enzyme · cofactor · FdUMP complex, dramatic revision of current concepts of the mechanism of this enzyme would be necessary. Although the isolation of a peptide covalently bound to FdUMP and cofactor [6] argues against this possibility, it is conceivable that this peptide may be an artifact resulting from covalent bond changes during denaturation or digestion of the complex. The pertinent question is the structure of the native complex; the experiments described here seek to establish whether $H_2$folate is formed upon interaction of thymidylate synthetase with FdUMP and $CH_2$-$H_4$folate.

In the conversion of dUMP to TMP, $CH_2$-$H_4$folate acts as both the donor of the one-carbon unit and reductant; it is well established that the hydrogen at C-6 of $H_4$folate is transferred to the methyl group of TMP [15—17]. We can envision two mechanisms in accord with this fact which might accommodate the formation of $H_2$folate in the enzyme · FdUMP · $CH_2$-$H_4$folate complex: the first, shown in eqn. 1, involves reversible transfer of the 6-H of $H_4$folate to the one-carbon unit of the cofactor to produce a methylated form of the enzyme, as previously suggested for the normal enzymic reaction [18]. Alternatively, an oxidized form of the enzyme could be reversibly reduced by the 6-H, leaving the one-carbon unit at the formaldehyde level of oxidation (eqn. 2).

$$\text{FdUMP} + \text{E} \cdot \text{CH}_2\text{-H}_4\text{folate} \rightleftharpoons \text{FdUMP} \cdot \text{E-CH}_3 \cdot \text{H}_2\text{folate} \quad (1)$$

$$\text{FdUMP} + \text{E}^{ox} \cdot \text{CH}_2\text{-H}_4\text{folate} \rightleftharpoons \text{FdUMP} \cdot \text{EH}^{red}\text{-CH}_2\text{OH} \cdot \text{H}_2\text{folate} \quad (2)$$

To ascertain whether a methylated form of the enzyme is produced in the ternary complex we used the following approach: as depicted in Fig. 2, if $H_2$-folate is formed by transfer of the 6-H to the methylene group of the cofactor, the tritium of [6-$^3$H]$H_4$folate would be quantitatively transferred to the methylene group, reducing it to the methanol oxidation level. Since methyl groups rotate approx. $10^7$/s in a short period of time its hydrogens would become equivalent. Upon dissociation by equilibration with excess unlabeled $CH_2$-$H_4$folate, hydrogen rather than tritium should preferentially be transferred to the 6-position of $CH_2$-$H_4$folate. From probability alone, one would predict that the specific activity of [6-$^3$H]$H_4$folate released would be 1/3 of that bound to the enzyme and that 2/3 of the tritium should be found in the formaldehyde unit; considering the sizable discrimination of $^1$H vs. $^3$H, the effect should be even more dramatic. Thus, if $H_2$folate and a methylated form of the enzyme were produced, (i) tritium would be lost from the 6-position of the cofactor prior to dissociation from the complex, and (ii) tritium would be found in the methylene group of the dissociated $CH_2$-$H_4$folate.

We first prepared the complex containing enzyme, FdUMP and $CH_2$-[6-$^3$H]-$H_4$folate, and separated it from unbound ligands by gel filtration. The isolated complex was equilibrated with excess $CH_2$-$H_4$folate until all radioactivity was

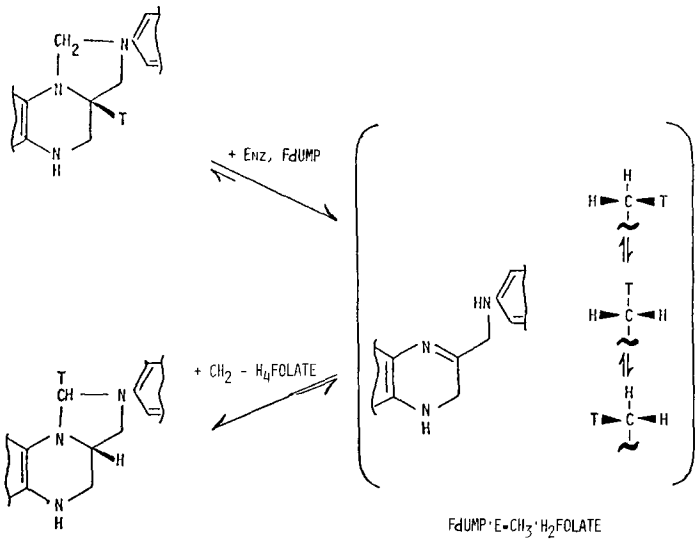


**Fig. 2. Transfer of tritium from the 6-position of $CH_2$-$H_4$folate to the methylene group upon formation and dissociation of the hypothetical FdUMP · E-$CH_3$ · $H_2$folate complex; equilibration of the tritiated methyl group within the complex is depicted.**

released from the protein. The dissociated radioactive ligand was shown to be (—)-L-$CH_2$-$H_4$folate by the following experiments: upon addition of excess FdUMP and thymidylate synthetase, at least 76% of the radioactivity could be rebound to the enzyme as a tight complex, retained on nitrocellulose filters. This was indicative that the radioactivity was present as $CH_2$-$H_4$folate or a related form of the cofactor which could stimulate the formation of a tight ternary complex. Upon addition of excess enzyme and dUMP, at least 95% of the tritium of the dissociated cofactor could be transferred to TMP. This result indicates that in the presence of excess (±)-L-$CH_2$-$H_4$folate, the tight complexes formed with FdUMP and thymidylate synthetase preferentially bind the (—)-L-diastereoisomer of the cofactor. Most important, it provides unequivocal evidence that the cofactor form released from the enzyme · FdUMP · $CH_2$-$H_4$-folate complex is (—)-L-$CH_2$-$H_4$folate and demonstrates that all tritium is covalently bound to the dissociated cofactor. To ascertain whether the tritium on the dissociated cofactor resides at the 6-position or on the formaldehyde unit, we prepared the complex containing enzyme, FdUMP and [$^{14}$C]$CH_2$-[6-$^3$H]$H_4$-folate ligands; the complex formed had $^3H/^{14}C = 36$. After dissociation by equilibration with excess $CH_2$-$H_4$folate the formaldehyde was isolated as its dimedone complex. The derivatized [$^{14}$C]formaldehyde was recovered with a yield greater than 80% and shown to possess less than 0.1% tritium ($^3H/^{14}C$ = 0.04). These experiments unequivocally establish that the 6-hydrogen of $H_4$-folate is not transferred to the methylene group in the enzyme · FdUMP · $CH_2$-$H_4$folate complex, nor is it lost from the cofactor.

The remaining mechanism which could accommodate both formation of $H_2$-folate and the results discussed above is one in which an oxidized form of the enzyme receives the 6-hydrogen of the cofactor and quantitatively transfers it back to the 6-position upon dissociation (eqn. 2); in the normal enzymic reac-

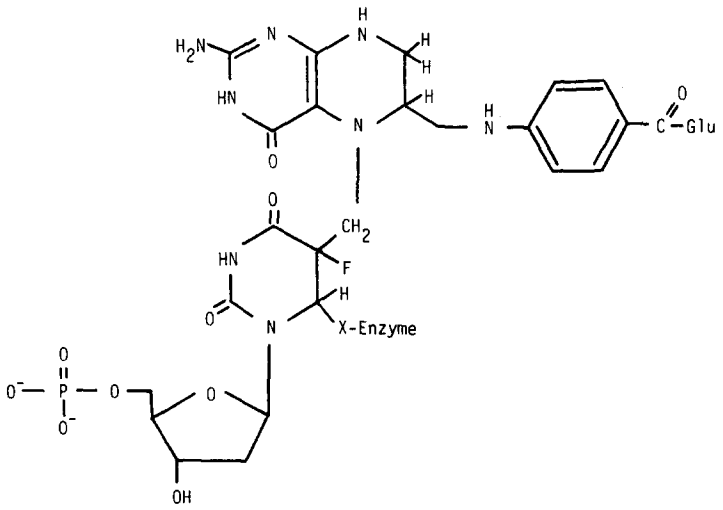


Fig. 3. Proposed structure of thymidylate-synthetase · $CH_2$-$H_4$folate · FdUMP complex where X is a nucleophilic group of the enzyme.

tion, the reduced form of the enzyme would donate hydride to the incipient methyl group of TMP and represent a heretofore unrecognized intermediate. We consider this possibility unlikely. There is no precedent for an amino acid residue of an enzyme acting as a direct hydride carrier. In addition, in its activated form, the native protein has been reported to possess no disulfide bonds [19], the functional group of a protein which is most susceptible to reversible reduction.

It is our belief that the unusual ultraviolet spectrum of $CH_2$-$H_4$folate in the ternary complex results from perturbation of a chromophore of the cofactor and not from the formation of $H_2$folate; this could result from interaction with tryptophan residues of the enzyme, as suggested by fluorescence studies of the ternary complex [20]. In support of this contention, the enzyme · FdUMP · $CH_2$-$H_4$folate complex has $\Delta\epsilon_{330nm}$ = 17 700, a value which is almost three-fold higher than that of $H_2$folate vs. $CH_2$-$H_4$folate ($\Delta\epsilon_{340nm}$ = 6400).

Moreover, this difference spectrum is not unique to $H_2$folate: 5,8-deazafolate, a cofactor analog which is not susceptible to oxidation or covalent bond modification under the conditions used, yields a similar difference spectrum as observed with $CH_2$-$H_4$folate. Thus, from all available evidence, it appears that the structure of the thymidylate-synthetase · FdUMP · $CH_2$-$H_4$folate complex is best depicted as shown in Fig. 3.

## Acknowledgement

This work was supported by USPHS Grant No. CA-14394 from the National Cancer Institute. DVS is a recipient of a N.I.H. Career Development Award.

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