5-Fluoro-2'-Deoxyuridylate: Covalent Complex with Thymidylate Synthetase

(affinity labeling/enzyme mechanism/inhibition/fluorinated pyrimidines)

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ABSTRACT   5-Fluoro-2'-deoxyuridylate causes a rapid inactivation of thymidylate synthetase that is dependent upon prior complexation of the cofactor 5,10-methylene-tetrahydrofolate. The enzyme–5-fluoro-2'-deoxyuridylate complex may be isolated on nitrocellulose membranes and is not disrupted by 6 M urea. Upon reaction of 5-fluoro-2'-deoxyuridylate with the enzyme in the presence of 5,10-methylenetetrahydrofolate a rapid loss of absorbance is observed at 269 nm, the absorption maximum for the pyrimidine chromophore. It is concluded that a covalent bond is formed between the 6-position of 5-fluoro-2'-deoxyuridylate and a nucleophilic group of the enzyme that is involved in catalysis.

Thymidylate synthetase catalyzes the reductive methylation of dUMP to TMP with the concomitant conversion of 5,10-methylenetetrahydrofolic acid (5,10-CH_2FAH_4) to 7,8-dihydrofolic acid (7,8-FAH_2). Unlike other folate cofactor enzymes, 5,10-CH_2FAH_4 serves the dual function of both a one-carbon carrier and reductant, and the regeneration of cofactor is required for continued TMP synthesis. Since this enzyme represents the only pathway for *de novo* TMP synthesis, and behaves as an apparent "rate limiting" step in DNA synthesis (1), considerable efforts have been expended in studies of its properties and control (2). We have been primarily interested in establishing the underlying features of the catalytic mechanism of this enzyme.

Our previous studies (3–5) on model systems of the thymidylate synthetase reaction have led to the proposal that a primary event in catalysis is the addition of a nucleophilic group of the enzyme to the 6-position of dUMP to form transient, covalently bound intermediates. As one approach to obtaining direct experimental evidence for this, we sought substrate analogs that might react by a similar mechanism to form a more stable covalent bond with the suspected nucleophilic catalyst. In this report, we describe preliminary experiments that demonstrate that the mechanism of inhibition of thymidylate synthetase by the much studied 5-fluoro-dUMP (FdUMP) involves covalent bond formation. Chemical considerations lead us to believe that the inhibitor behaves as a quasi-substrate and reacts with the same nucleophile involved in catalysis. Much of the reported controversy regarding the nature of inhibition by FdUMP may be resolved by this finding.

MATERIALS AND METHODS

Crystalline thymidylate synthetase (about 50% pure) was obtained from dichloromethotrexate-resistant *Lactobacillus casei* (6) by the method of Leary and Kisliuk (7). Tetrahydrofolic acid (FAH_4) was purchased from General Bio-

Abbreviations: FAH_4, tetrahydrofolic acid; 5,10-CH_2FAH_4, 5,10-methylenetetrahydrofolic acid; 7,8-FAH_2, 7,8-dihydrofolic acid; FdUMP, 5-fluoro-2'-deoxyuridine-5'-phosphate.

chemicals and purified by DEAE chromatography before use; concentrations were determined spectrophotometrically and by thymidylate synthetase-catalyzed conversion to 7,8-FAH_2 in the presence of excess dUMP. FdUMP was obtained from the Drug Research and Development division of the National Cancer Institute. [6-^{3}H]FdUMP (2.84 Ci/mmol) was prepared from the nucleoside (Schwarz–Mann) with carrot phosphotransferase (8). Nitrocellulose filters (2.4 cm) were obtained from Schliecher and Schuell and were soaked before use in a solution containing 50 mM phosphate buffer (pH 7.4) and 50 mM $MgCl_2$.

Assays and Incubations. Standard initial velocity assays were performed at 30° in a total volume of 1.1 ml as described (9). Reactions were initiated by the addition of a limiting amount of enzyme (about 0.075 units) and monitored spectrophotometrically (10). A unit is defined as that amount of enzyme that will produce 1 μmol of TMP per hr. Incubations in the presence of FdUMP were conducted at 30° in Siliclad (Clay-Adams, Inc.)-treated containers under an argon atmosphere. The mixtures contained formaldehyde (6.5 mM), $MgCl_2$ (25 mM), EDTA (1 mM), 2-mercaptoethanol (75 mM), *N*-methylmorpholine·HCl buffer (pH 7.4) (50 mM), the specified concentrations of FAH_4 and FdUMP, and about 1.5 units of enzyme. Under these conditions, FAH_4 is present almost exclusively as 5,10-CH_2FAH_4 (11). At appropriate time intervals 50-μl aliquots were removed and assayed for enzyme activity.

Nitrocellulose Filtration. Isolation of [6-^{3}H]FdUMP–thymidylate synthetase complexes were performed by a modification of the nitrocellulose-membrane-filter technique reported by Yarus and Berg (12). Immediately before assay, 10 μg of bovine serum albumin was applied to the filter and 100 μl of the incubation solution was filtered at a rate of 2 ml/min. The filter was then washed with 1 ml of a solution containing 50 mM potassium phosphate (pH 7.4) and 50 mM $MgCl_2$ to remove uncomplexed [6-^{3}H]FdUMP. Filters were dissolved in Bray's solution (13) containing 2% H_2O, and counted in a liquid scintillation spectrometer. Complete details of this method shall appear elsewhere.

RESULTS

Inactivation by FdUMP. In the presence of saturating amounts of 5,10-CH_2FAH_4, the inactivation of thymidylate synthetase by even very low concentrations of FdUMP occurs so rapidly that the rate cannot be measured by manual sampling techniques (Fig. 1). The level of inactivation is dependent upon the amount of FdUMP added, and calculations of the nominal stoichiometry of the reaction indicate that 2 mol of FdUMP are required per mol of enzyme. Inactivation of the enzyme in the absence of FdUMP
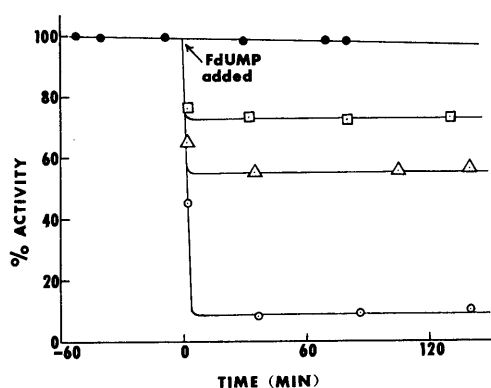
FIG. 1. Inactivation of thymidylate synthetase with various concentrations of FdUMP. [5,10-CH₂FAH₄] = 0.5 mM; ●, [FdUMP] = 0; □, [Enz] = 74 nM, [FdUMP] = 28 nM; △, [Enz] = 95 nM, [FdUMP] = 85 nM; ○, [Enz] = 95 nM, [FdUMP] = 170 nM.

proceeds with $t_{1/2} \simeq 20$ hr at 25° and is insignificant over the time scale of the experiments.

Dependence of FdUMP Inactivation on 5,10-CH₂FAH₄.
Fig. 2 shows experiments in which the concentration of 5,10-CH₂FAH₄ was varied at a concentration of FdUMP found to be sufficient for complete inactivation of the enzyme in the previous experiments. In the absence of 5,10-CH₂FAH₄, the inactivation by 0.15 μM FdUMP is slow ($t_{1/2} \simeq 6$ hr). With increasing concentrations of the cofactor, both the rate and extent of inactivation are increased. Conclusions regarding the exact stoichiometry of the requirement for 5,10-CH₂FAH₄ are not drawn from these experiments since at the low concentrations of FAH₄ used, oxidative reactions probably deplete the system of cofactor before inactivation is complete.

The deletion of formaldehyde from an experiment such as that described in Fig. 1 results in a 10-fold decrease in the rate of inactivation by FdUMP. It is to be noted that even the low level of inactivation observed may be due to the presence of 5,10-CH₂FAH₄, resulting from formaldehyde contaminants. Indeed, it has been observed (14) that FAH₄ preparations may contain as much as 5% formaldehyde, presumably arising from degradative reactions; the inactivation we observe could be accounted for by as little as 0.1 μM l,L-5,10-CH₂FAH₄, or a 0.04% formaldehyde contaminant in the FAH₄ preparation used.
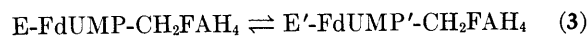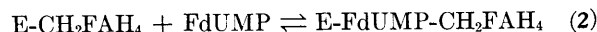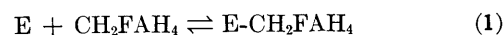
Isolation of Complexes. In the experiments shown in Table 1, 50 nM [6-³H]FdUMP and 25 nM thymidylate synthetase were incubated under varying conditions, and the complexes were isolated by filtration through nitrocellulose membranes. Under the conditions used, free FdUMP is completely washed from the membrane and enzyme-bound FdUMP is retained. The corresponding nucleoside, 5-fluoro-2′-deoxyuridine, does not form a filterable complex. With the complete preparation the amount of FdUMP retained shows little variation over periods of up to 4 hr of incubation. In the absence of 5,10-CH₂FAH₄, FdUMP is not retained on the filter. Treatment of the enzyme with 6 M urea before the addition of FdUMP prevents the formation of filterable complexes. However, when the preformed complex is treated with urea, no loss in the retained counts is observed. Simi-

larly, the ternary complex is not disrupted upon treatment with 6 M guanidine hydrochloride.

Loss of FdUMP Chromophore Upon Complexation. FdUMP (3.4 μM) was added to one of two previously balanced cuvettes containing thymidylate synthetase (1.9 μM), dithiothreitol (6.5 mM), formaldehyde (7.0 mM), MgCl₂ (25 mM), EDTA (1 mM), and N-methylmorpholine·HCl buffer (pH 7.4) (50 mM). The differential absorbance of 0.029 absorbance units at 269 nm, the maximum for FdUMP, corresponded closely to the value calculated with $\epsilon_{max} = 8.3 \times 10^3$. No changes occurred during a 5-min period, but the addition of 5,10-CH₂FAH₄ (7.2 μM) to each cuvette resulted in a rapid loss of the differential absorbance at 269 nm.

DISCUSSION

There have been numerous conflicting reports regarding the nature of the inhibition of thymidylate synthetase by FdUMP. Various investigators have claimed the inhibition to be competitive, noncompetitive, stoichiometric, and competitive changing to noncompetitive (2). The results described herein allow insight into the nature of FdUMP inhibition and bear relevance to the mechanism of catalysis. A scheme that is consistent with the observations reported is as follows:

$$E + CH_2FAH_4 \rightleftharpoons E\text{-}CH_2FAH_4 \qquad (1)$$

$$E\text{-}CH_2FAH_4 + FdUMP \rightleftharpoons E\text{-}FdUMP\text{-}CH_2FAH_4 \quad (2)$$

$$E\text{-}FdUMP\text{-}CH_2FAH_4 \rightleftharpoons E'\text{-}FdUMP'\text{-}CH_2FAH_4 \quad (3)$$

The initial event must be binding of 5,10-CH₂FAH₄ since the rate and extent of inactivation by FdUMP are dependent upon the concentration of the cofactor. In the absence of cofactor, inactivation is not observed and the formation of an isolable FdUMP–enzyme complex does not occur. Furthermore, since the extent of inactivation is also dependent upon the concentration of 5,10-CH₂FAH₄, it may be concluded that it is not readily dissociated once FdUMP is bound. It is also interesting that FAH₄ by itself is not sufficient to induce whatever changes in the protein are required for optimal FdUMP inactivation. Lomax and Greenberg (15) have observed that the 5-H exchange of dUMP catalyzed by thymidylate synthetase is greatly depressed in the absence of
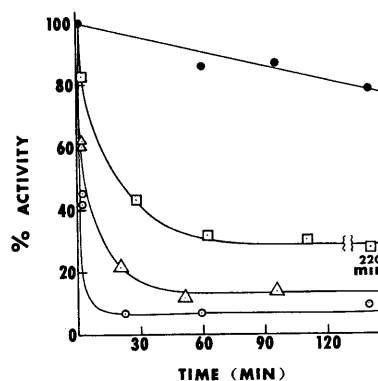

FIG. 2. Inactivation of thymidylate synthetase with various concentrations of 5,10-CH₂FAH₄. [FdUMP] = 153 nM; [Enz] = 72 nM; ●, [1-FAH₄] = 0; □, [1-FAH₄] = 0.38 μM; △, [1-FAH₄] = 3.8 μM; ○, [1-FAH₄] = 38 μM.

formaldehyde. Reasoning from the aforementioned results, it is probable that this effect is due to the necessity of 5,10-CH$_2$FAH$_4$ for binding of dUMP. This is consistent with the obligatory order of binding implicated from kinetic studies (16).

The inability of the nucleoside 5-fluoro-2'-deoxyuridine to inactivate or bind to the enzyme parallels the inefficacy of deoxyuridine as a substrate and, together with the cofactor requirement and low concentrations of inhibitor used, strongly indicates that FdUMP interacts at the same site(s) as dUMP. In all of the experiments described it appears that a nominal value of two mole equivalents of FdUMP are required to inactivate the enzyme. Since the enzyme is composed of two subunits of identical molecular weight, it is tempting to speculate that the inhibitor reacts with two identical sites of the enzyme. This postulate is undergoing experimental test at this time.

A most pertinent question concerns the nature of the complex formed: does it involve a tight reversible complex with a low rate of dissociation, or have covalent bonds been formed between FdUMP and thymidylate synthetase? The data discussed thus far are accommodated by either of these possibilities, and in order to answer this question the enzyme was denatured with 6 M urea before and after treatment with FdUMP and 5,10-CH$_2$FAH$_4$, and the resultant complexes isolated by the nitrocellulose membrane technique (Table 1). Whereas denaturation prevents the formation of an isolable FdUMP–enzyme complex, once formed the complex is not reversed by treatment with 6 M urea for as long as 4 hr. It is difficult to rationalize this result in any way other than covalent bond formation between FdUMP and thymidylate synthetase.

It has been well established that the 6-position of the 5-fluorouracil heterocycle is the most susceptible site to nucleophilic reagents (17–19). Furthermore, model studies have led us to propose that catalysis involves the addition of nucleophile of the enzyme to the 6-position of dUMP (3–5); from these considerations, it is most reasonable to suggest
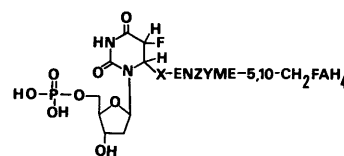

FIG. 3. Covalent thymidylate synthetase–FdUMP complex.

that a nucleophilic group of the enzyme, probably that one involved in catalysis, adds to the 6-position of FdUMP as depicted in Fig. 3. Consistent with this proposal is the observed loss of absorbance at 269 nm (the maximum of the FdUMP chromophore), which occurs upon formation of the enzyme–CH$_2$FAH$_4$–FdUMP complex, suggesting saturation of the 5,6-double bond of the pyrimidine. The reversal of this reaction would generate native enzyme and unchanged FdUMP, and would accommodate the reported observations that even the apparent noncompetitive inhibition of thymidylate synthetase by FdUMP can be reversed by prolonged dialysis (2).

The results represented in this paper are preliminary; complete results will be published elsewhere.

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TABLE 1. *Nitrocellulose filtration*

Components	Reaction conditions	[6-³H] FdUMP cpm retained
Complete*	1 hr incubation	5100
	4 hr incubation	5300
Minus 5,10-CH$_2$FAH$_4$	1 hr incubation	60
Complete with 6 M urea denatured enzyme	1 hr incubation	70
Complete	1 hr incubation, then	
	6 M urea for 1 hr	5400
	6 M urea for 4 hr	5300

* Complete preparation contains 50 nM [6-³H]FdUMP, 25 nM thymidylate synthetase, 60 µM 5,10-CH$_2$FAH$_4$, 25 mM MgCl$_2$, 1 mM EDTA, 75 mM 2-mercaptoethanol, and 50 mM *N*-methylmorpholine·HCl buffer (pH 7.4). Each 100-µl aliquot used in the filtration contained 9400 cpm.