A SULFHYDRYL GROUP IS NOT THE COVALENT CATALYST IN THE THYMIDYLATE SYNTHETASE REACTION

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SUMMARY: The total sulfhydryl content of the covalent complex formed between thymidylate synthetase and 5-fluoro-2'-deoxyuridylate was found to be identical to that of the free enzyme. This result demonstrates that cysteine is not the amino acid residue which links the nucleotide to the enzyme. Since covalent binding of 5-fluoro-2'-deoxyuridylate to thymidylate synthetase proceeds by a mechanism similar to the initial stages of the normal enzymic reaction, it is concluded that a sulfhydryl group is *not* the nucleophile which adds to the 6-position of 2'-deoxyuridylate. This conclusion refutes proposals of other workers.

Thymidylate synthetase (EC 2.1.1.6) catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to thymidylate with the concomitant conversion of 5,10-methylene tetrahydrofolic acid (CH_2FAH_4) to 7,8-dihydrofolic acid. Model studies (1-5) of the thymidylate synthetase reaction have led to the proposal that a nucleophile in the active site of the enzyme catalyzes the reaction by attacking the 6-position of dUMP to form a reactive, covalently-bound intermediate. In support of this hypothesis it has been demonstrated that a stable covalent bond is formed between a nucleophile of the enzyme and the 6-position of 5-fluoro-2'-deoxyuridylate (FdUMP)(Figure 1), a close structural analog of the nucleotide substrate, dUMP (6-9).

A number of workers have strongly supported the proposal that the aforementioned nucleophile is a sulfhydryl group of a cysteine residue of the enzyme (4,10-14). Although this proposal is not based upon firm evidence, it has appeared in review articles (15,16) and is gaining increasing acceptance among workers interested in this area. In this report we describe evidence which demonstrates that sulfhydryl is *not* the nucleophile which covalently links FdUMP to thymidylate synthetase.

Materials and Methods

Thymidylate synthetase was obtained from an amethopterin-resistant strain of *Lactobacillus casei* (17) and purified to homogeneity by published procedures (8,18). 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Aldrich. N-Ethylmaleimide (NEM) was purchased from General Biochemicals and was recrystallized twice from ethanol-water; [^{14}C]NEM (24.4 dpm/pmol) was purchased from New England Nuclear. The concentration and specific activity of all [^{14}C]NEM solutions was determined by

spectrophotometric measurement using ϵ_{305} = 620 (19); purity was determined by silica gel tlc (pet ether-ethyl acetate, 8:1). Other materials and methods have been described (7,8).

Thymidylate synthetase-FdUMP-CH_2FAH_4 complexes were formed and nitrocellulose filtration assays were performed by published procedures (7). Radioactivity determinations were performed as described (8). Sephadex G-25 chromatography was performed at 4° on a 50 cm x 9 mm column using 80 m*M* sodium phosphate (pH 8.0)-0.5 mg/ml EDTA as the eluant. Protein determinations were performed by the method of Lowry (20) using homogeneous thiol-free thymidylate synthetase as a standard.

DTNB Thiol Determination. An enzyme-FdUMP-CH_2FAH_4 complex (1.3 ml) was formed from thymidylate synthetase (59 nmol), 153 nmol [^{14}C]FdUMP (5.6 dpm/pmol) and 1.2 µmol *dl*,L-CH_2FAH_4. Low molecular weight thiols were removed by gel filtration, the eluant was continuously purged with argon and fractions (*ca.* 1.4 ml) were collected in cuvettes under argon and immediately stoppered. Determination of the radioactivity and protein in the macromolecular peak indicated that 80% of the sites were bound to FdUMP, a value identical to that obtained with this preparation prior to the experiment. A sulfhydryl determination was performed by standard procedures (21) in 2% sodium dodecyl sulfate (SDS) using ϵ_{312} = 11,400 (22). Gel filtration of the protein after the reaction indicated that all FdUMP was still bound. A free enzyme sample was treated as the enzyme-FdUMP-CH_2FAH_4 complex described above.

[^{14}C]*NEM Thiol Determination.* Enzyme-FdUMP-CH_2FAH_4 complexes were formed by adding 50 µl of a solution containing 32.2 nmol [^{3}H]FdUMP (8 dpm/pmol) to two 250 µl solutions containing 26.8 µ*M* thymidylate synthetase (1.72 [^{14}C]FdUMP binding sites/mol enzyme), 53 m*M* potassium phosphate (pH 6.8), 22 m*M* dithiothreitol, 0.80 m*M* *dl*,L-CH_2FAH_4, 10.6 m*M* HCHO, 8.5 m*M* N-methylmorpholine, 7.9 m*M* $MgCl_2$, and 0.35 m*M* EDTA. After 4 hr, 20 µl was filtered to ensure complex formation was complete. FdUMP was deleted from the two controls. To one solution of the enzyme-FdUMP-CH_2FAH_4 complex and one control was added 25 µl of 20% SDS to denature the enzyme; these solutions were incubated for 1 hr at 37°. Then 125 µl of a [^{14}C]N-ethylmaleimide solution (32 µmol, 1.5 dpm/pmol) was added to all solutions and incubated for 1 hr at 37°. The protein was precipitated with two volumes of ethanol and centrifuged. The pellet was vortexed vigorously in 2 ml ethanol and recentrifuged. This precipitated protein was dissolved in 1 ml 2% Na_2CO_3-0.1 *N* NaOH. Aliquots were removed from this solution and assayed for protein (80 to 98% recovery) and radioactivity to determine [^{14}C]NEM and [^{3}H]FdUMP.

Results and Discussion

It appears to be generally accepted (15,16) that a primary step of the thymidylate synthetase reaction is the addition of a nucleophile of the enzyme to the 6-position of dUMP. Numerous reports have recently appeared which propose that this nucleophile is the sulfhydryl group of a cysteine residue (4,10-14). The basis of this proposal

are as follows: *(i)* Sulfhydryl compounds catalyze chemical reactions of uracil heterocycles which are analogous to those catalyzed by thymidylate synthetase (4,10). *(ii)* The catalytic activity of thymidylate synthetase is stabilized and stimulated by thiols (17,23). When cysteines of the enzyme from *L. casei* are modified, catalytic activity is completely lost (10,23). *(iii)* Showdomycin (3-β-D-ribofuranosylmaleimide) and its 5'-phosphate, analogs of dUR and dUMP which have similar chemical reactivity to NEM, irreversibly inactivate thymidylate synthetase (12). The enzyme is protected against these reagents by hydroxyethyldisulfide and the substrate, dUMP. Clearly, one or more of the four sulfhydryl groups (10,23) of thymidylate synthetase is necessary for catalytic activity. Together with the high nucleophilicity of sulfhydryl groups, the aforementioned evidence makes cysteine a prime candidate for the amino acid residue which adds to the 6-position of dUMP in the catalytic sequence. However, the evidence forwarded in support of this proposal is indirect and does not constitute proof. Firstly, numerous nucleophiles other than sulfhydryl groups act as catalysts in model reactions of thymidylate synthetase (1-3,5). Secondly, the loss of catalytic activity concomitant with sulfhydryl modification could be due to a secondary effect and does not provide convincing evidence that cysteine is the nucleophilic catalyst. Thirdly, inactivation of the enzyme by showdomycin and its 5'-phosphate is explicable as a simple bimolecular reaction with sulfhydryl group(s) of the enzyme. With the exception of protection by dUMP, there is no evidence that either of these analogs bind reversibly to the active site of the enzyme prior to irreversible reaction. As we will describe later, binding of a nucleotide to the native protein affords protection against NEM, a sulfhydryl titrant which certainly reacts bimolecularly.

To ascertain whether the amino acid residue in question is cysteine, we compared the free sulfhydryl content of thymidylate synthetase which was covalently bound to FdUMP to that of the free enzyme. Thymidylate synthetase possesses four cysteines (10,23), two binding sites for FdUMP (8), and two apparently identical subunits (24). A nucleophilic group of the enzyme attacks the 6-position of FdUMP (Figure 1) to form a covalent bond which is stable towards denaturants (8); all lines of evidence indicate that this nucleophile is the same as the one involved in catalysis. Thus, if a sulfhydryl group is the nucleophile which covalently links FdUMP to the enzyme, two of the four cysteines of thymidylate synthetase should not be reactive towards thiol reagents when FdUMP is covalently bound. On the other hand, if a sulfhydryl group is not the nucleophile, the enzyme-FdUMP-CH_2FAH_4 complex should possess the same number of sulfhydryl groups as does the unbound enzyme. Since the covalent bond is stable toward protein denaturants, we could perform titrations under conditions where all free sulfhydryls should be exposed to the titrant. The results of our experiments are given in Table I.

In the first experiment, the FdUMP-enzyme complex and the unbound enzyme were

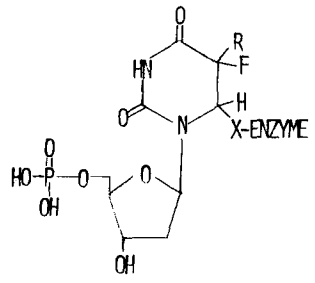

Figure 1. Covalent thymidylate synthetase-FdUMP-CH_2FAH_4 complex; R = -H or -CH_2FAH_4, X = unknown nucleophilic group in thymidylate synthetase active site.

Table I. DTNB and [^{14}C]NEM Sulfhydryl Determinations[a]

Enzyme Form	Reagent Used	Cysteine/Enz	FdUMP/Enz
Enz-FdUMP	DTNB-SDS	3.2	1.6
Enz	DTNB-SDS	3.5	-
Enz-FdUMP	NEM-SDS	4.1	1.8
Enz	NEM-SDS	4.0	-
Enz-FdUMP	NEM	1.9	1.8
Enz	NEM	3.4	-

[a]Determinations were performed as described in Materials and Methods.

freed of low molecular weight thiols, denatured with SDS, and treated with excess DTNB. Although the total sulfhydryl content was somewhat below theoretical, the bound complex contained over 90% of the thiols found in the control. There was no detectable loss of covalently bound FdUMP over the course of this experiment. In the second experiment, we desired to determine sulfhydryl content in the presence of all components of the reaction mixture (*i.e.* excess thiol, CH_2FAH_4). We formed the complex in the usual manner and omitted [^{3}H]FdUMP from the control. Both were denatured with SDS and treated with [^{14}C]NEM. After isolation and Lowry estimation of protein, radioactivity measurements showed that four sulfhydryl groups per enzyme

molecule had reacted with [^{14}C]NEM in both the denatured FdUMP complex and the control In addition, the NEM-modified protein contained 1.8 mol [^{3}H]FdUMP/mol enzyme. Interestingly, when a similar experiment was performed in the absence of denaturant, FdUMP protected approximately two sulfhydryl groups from reaction with NEM.

These results conclusively demonstrate that the nucleophile which covalently links FdUMP to thymidylate synthetase is *not* a sulfhydryl group. From analogies established for the reaction of FdUMP with thymidylate synthetase and the catalytic mechanism, we conclude that the nucleophilic catalyst of thymidylate synthetase is likewise not a sulfhydryl group. The fact remains that the protein does possess sulfhydryl groups which are necessary for catalysis and which are protected by FdUMP, providing the enzyme is not denatured. Whether such groups have a catalytic function is an important question which is not yet answered.

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