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ISOLATION OF THE COVALENT BINARY COMPLEX OF 5-FLUORODEOXYURIDYLATE AND THYMIDYLATE SYNTHETASE BY TRICHLOROACETIC ACID PRECIPITATION

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SUMMARY: Strong chemical evidence for the existence of a covalent binary complex between 5-fluorodeoxyuridylate and thymidylate synthetase was provided by the isolation of the complex by trichloroacetic acid precipitation. This result together with that of a control experiment with N-ethymaleimide inactivated thymidylate synthetase demonstrated that only nucleotide covalently bound to the protein survived repeated washings of the precipitate. Under the conditions used, a maximum binding stoichiometry of about 0.9 was obtained for the covalent binary complex, K_d = 1.1 x 10^{-5} M. Also, a binding ratio of 1.7 was obtained for the methylenetetrahydrofolate-5-fluorodeoxyuridylate-thymidylate synthetase ternary complex.

Thymidylate synthetase (E.C. 2.1.1.45) catalyzes the reductive methylation of dUMP to dTMP utilizing CH_2H_4folate[1] as the source of the methyl group (1,2). This enzyme is strongly inhibited by FdUMP which is a metabolite of the cancer chemotherapeutic agent 5-fluorouracil (3). Inhibition occurs primarily through formation of the CH_2H_4folate-FdUMP-thymidylate synthetase ternary complex, Ka~10^{11} M^{-1} (2). Ample evidence has been accumulated to support the structure of this inhibitory complex (Structure E in Scheme 1) which involves a covalent bond between C-6 of the pyrimidine ring of the nucleotide and Cys-198 of the enzyme (2,4-7). This complex is believed to be analogous to the catalytically competent complex involving dUMP. Kinetic evidence (8) suggests an ordered mechanism for this reaction wherein the substrate dUMP adds initially to the enzyme to generate a binary complex which can then interact with the folate cofactor to form a ternary complex.

Abbreviation used: FdUMP,5-fluorodeoxyuridylate; CH_2H_4folate,(±)-5,10-methylenetetrahydrofolate; NEM, N-ethylmaleimide; TCA, trichloroacetic acid; DPM, disintegrations per minute.

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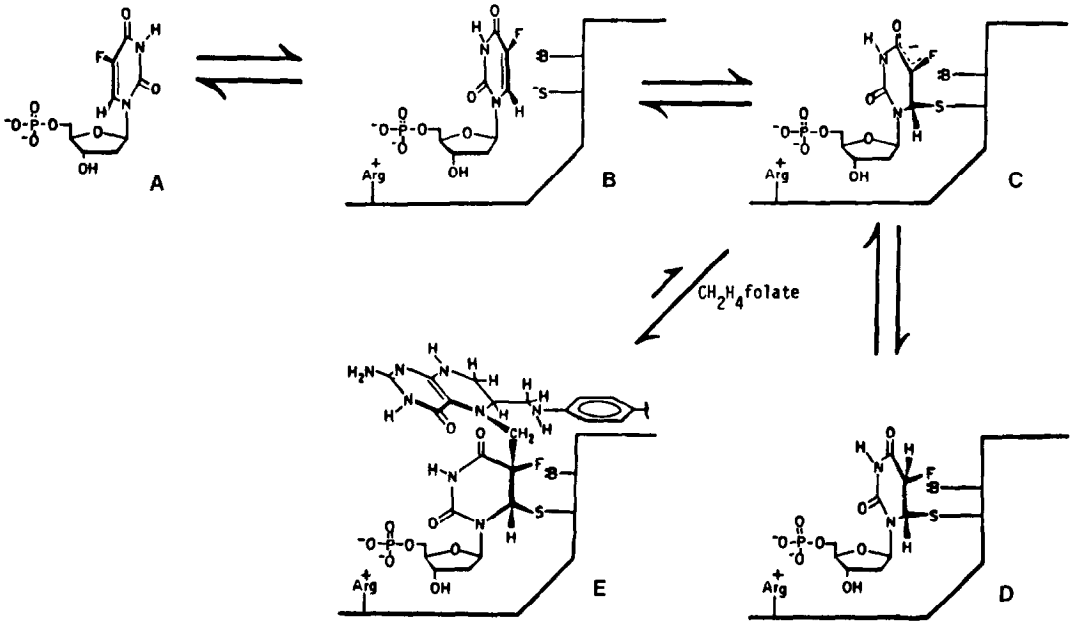


Scheme 1

The binary complex between FdUMP and thymidylate synthetase, Ka~10^4-10^6 M^{-1}, has been detected under a variety of equilibrium conditions (6). Only one of these methods, however, ^{19}F nmr spectroscopy (9), has revealed that FdUMP interacts with the enzyme to yield the expected non-covalent complex (Structure B) as well as the covalent complex (Structure D). In order to augment this physical evidence, we now report results of a chemical method that document the existence of the covalent binary complex between FdUMP and thymidylate synthetase.

EXPERIMENTAL: Thymidylate synthetase was purified from Lactobacillus casei in the presence of exogenous thiol (10 mM β-mercaptoethanol) according to the method of Lyon et al. (10). The enzyme was dialyzed against a buffer containing 100 mM Tris, 100 mM KCl, and 50 mM β-mercaptoethanol, pH 7.3 for at least 18 hr prior to use. To prepare N-ethylmaleimide modified thymidylate synthetase, the enzyme was inactivated by blocking the active site sulfhydryl groups with NEM according to the method of Plese and Dunlap (11). The unmodified enzyme showed a specific activity of about 2.5 units per mg of protein while the NEM-modified enzyme had a specific activity of less than 0.10.

[6-^{3}H]FdUMP (18 Ci/mmole) was obtained from Moravek Biochemicals (City of Industry, CA). The radiolabelled nucleotide was diluted with unlabelled FdUMP obtained from the Sigma Chemical Company. NEM was also obtained from Sigma. Trichloroacetic acid was obtained from J.T. Baker Chemical Company. (±)-Tetrahydrofolate was prepared via the catalytic hydrogenation of folic acid using the method described by Hatefi et al. (12) and was converted to (±)CH_2H_4folate by the addition of formaldehyde.

The reaction mixtures (0.5 mL) contained 2 μM thymidylate synthetase in 100 mM Tris, 100 mM KCl, pH 7.3. After incubating for 1 hr at 25° C in the dark, an equal volume of 20% TCA was added to denature the protein. The mixture was then centrifuged for 3-4 min. at 7,000 rpm. The supernatant was then decanted, the precipitate suspended in 1 mL 10% TCA, and the solution recentrifuged. This wash step was repeated, usually twice, until the supernatant contained only background radioactivity. The precipitate was then dissolved in 0.5 N NaOH, added to 10 mL of Bio-solv HP scintillation liquid (from Beckman) and counted in a Beckman LS7500 liquid scintillation counter to determine the DPM associated with the precipitate. Control experiments performed with thymidylate synthetase modified by [^{3}H]-NEM (results not shown) illustrated that the precipitation and washes employed here resulted in complete recovery of the protein. The binding ratio was calculated by dividing the amount of FdUMP bound to the precipitate by the amount of protein in the reaction mixture.

RESULTS AND DISCUSSION: Trichloroacetic acid is a well known protein precipitant, and it is generally accepted that protein-ligand complexes which survive treatment by this denaturant are covalent in nature. For example, acid precipitation has been used to isolate covalent adducts of thymidylate synthetase (13,14). In order to establish the TCA precipitation technique as a method for quantitating covalent complexes of thymidylate synthetase, we first examined the well characterized inhibitory ternary complex. As shown in Table 1, the TCA-precipitated ternary complex yields a binding ratio of 1.67 mole FdUMP per mole enzyme which is consistent with results of other methods (6). As shown in this table, free FdUMP was completely removed by washing the precipitate three times.

Table 1. Steps in the Isolation of Binary and Ternary Complexes of Thymidylate Synthetase by Trichloroacetic Acid Precipitation[a]

Step	Ternary Complex	NEM-Modified Enzyme	Binary Complex
	total disintegrations per minute		
Supernatant	1,625,223	9,033,767	1,725,742
Wash 1	76,005	62,905	42,877
Wash 2	114	482	923
Wash 3	76	83	64
Precipitate	30,194	1,000	139,967
Mole FdUMP bound/mole enzyme	1.67	0.003	0.44

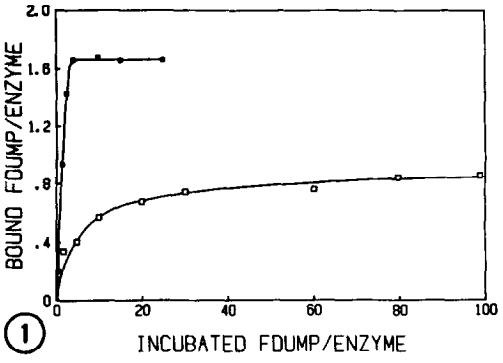
[a] A 10-fold excess of FdUMP over thymidylate synthetase was used to form the binary and ternary complexes. A 100-fold excess of CH_2H_4folate was included in the ternary complex reaction mixture. A 50-fold excess of FdUMP was used in the solution containing NEM-modified thymidylate synthetase.

Modification of the active site sulfhydryl groups of thymidylate synthetase by NEM both inactivates the enzyme and prevents its interaction with nucleotides (6,11). The possibility that nonspecific binding of the nucleotide might be occurring through adsorption or physical entrapment during precipitation was tested using NEM-modified enzyme as a control. The results in Table 1 indicate that non-specific binding of the nucleotide is insignificant and that the radioactivity retained by the precipitate from the ternary complex exclusively represents FdUMP that is covalently bound to the active site of the enzyme.

The titration of thymidylate synthetase with FdUMP in the presence of CH_2H_4folate has been studied by a variety of methods whose results indicate that a binding stoichiometry at saturation of 1.7-2.0 is achieved with as little as a 2-3 fold excess of nucleotide over enzyme (6). The results obtained using the TCA precipitation method to investigate this titration are shown in Figure 1 and are completely consistent with those from previous studies.

Having demonstrated that the TCA precipitation method can be used to quantitate covalent binding of FdUMP in the inhibitory ternary complex, we were in a position to employ this method to search for chemical evidence of the covalent binary complex between FdUMP and thymidylate synthetase. In the initial binary complex experiment, enzyme was treated with a 10-fold excess of FdUMP, in the absence of any folate, and subjected to TCA precipitation and washing as described earlier. As shown in Table 1, the results indicated that 0.44 mole of FdUMP were covalently bound per mole of thymidylate synthetase under these conditions, thus providing the first <u>chemical</u> evidence for Structure D (Scheme 1) in the mechanistic pathway for inhibition of the enzyme by FdUMP. These results stand in contrast to earlier reports which explicitly stated that the presence of CH_2H_4folate is a prerequisite to the covalent attachment of FdUMP to thymidylate synthetase (2,4,15).

It is interesting to compare the extent of covalent binding of FdUMP to the enzyme in the presence and absence of CH_2H_4folate at a 10-fold excess of



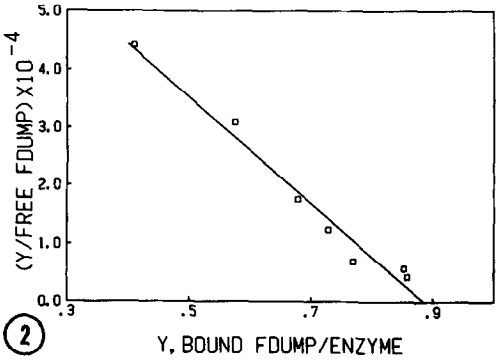


Figure 1. Plot of binding data from reaction mixtures containing different ratios of [6-^{3}H]FdUMP to thymidylate synthetase. Samples were washed until only background radioactivity (<100 DPM) was contained in a 200 µl aliquot of the wash. The precipitate was then dissolved in 0.5N NaOH and counted as described in the experimental section. The specific activity of the [6-^{3}H]FdUMP was such that 1 nmole corresponded to approximately 1 x 10^5 DPM. The open squares (□) represent samples which contained only FdUMP and enzyme while the closed squares (■), represent samples which also contained a 100-fold excess of CH_2H_4folate to enzyme.

Figure 2. Scatchard plot of the binary complex binding data from Figure 1. The line (slope =-9.1 x 10^4) was obtained by a best fit linear regression.

the nucleotide. Under these conditions, the ternary complex yields a stoichiometry of 1.7 and is well beyond its saturation point (Figure 1) while the binary complex exhibits a binding ratio of only 0.44 (Table 1). In order to further characterize the binding properties of the covalent FdUMP binary complex, the enzyme was titrated with up to a 100-fold excess of the nucleotide and analyzed using the TCA precipitation method. As shown in Figure 1, a much larger excess of FdUMP (100-fold) to the enzyme was needed to achieve saturation for the binary complex than with the ternary complex (2-3 fold). The titration data were subjected to Scatchard analysis (Figure 2) which yielded a K_d of 1.1 x 10^{-5} M for the covalent FdUMP-enzyme complex which reflects that this complex is much weaker than the ternary complex, K_d = 10^{-11}-10^{-13}M (6). However, the most striking result of the Scatchard analysis is that the binding capacity for the covalent FdUMP-thymidylate synthetase binary complex was measured to be 0.89 mole FdUMP per mole enzyme which is substantially less than the value of 1.7 for the ternary complex. In fact, even with up to a 1000-fold excess of FdUMP to enzyme (results not shown), no further increase in binding was obtained.

^{19}F nmr studies of the ternary complex (Structure E) indicate that, in the presence of CH_2H_4folate, symmetrical binding of the nucleotide occurs in the thymidylate synthetase dimer in the sense that all of the available sites are occupied by covalently bound FdUMP under saturating conditions. In contrast, ^{19}F nmr studies of the binary complex reveal, that while the same number of binding sites are available on the enzyme, asymmetric nucleotide binding exists since the FdUMP is partitioned between its noncovalent (Structure B) and covalent (Structure D) complexes with the enzyme (6). With these results in mind, we now pose the question as to why only 0.89 sites contain covalently bound FdUMP under saturating conditions in the binary complex with the remaining sites occupied by noncovalently bound nucleotide when 1.7 active sites are present on the average per enzyme molecule. In particular, it is interesting to ask what factors determine the extent of covalent nucleotide binding in the thymidylate synthetase dimer. Perhaps the mechanism which controls asymmetric binding of the nucleotide in the binary complex dictates that, at saturation, no more than one site on the dimer can be occupied by covalently bound nucleotide. Although the significance and mechanistic features associated with asymmetric nucleotide binding in the binary complex remain unknown, it is clear that the presence of CH_2H_4folate results in symmetric binding of the nucleotide in the ternary complex. We are now investigating how CH_2H_4folate triggers this response and whether other ligands (including folates) can effect the same response. We are also employing the TCA precipitation method to examine the covalent complexes of thymidylate synthetase with various nucleotides in the presence and absence of folates.

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REFERENCES:

1. Friedkin, M. (1973) Advan. Enzymol. 38, 235-292.
2. Danenberg, P. V. (1977) Biochim. Biophys. Act 473, 73-92.
3. Reyes, P. and Heidelberger, C. (1965) J. Mol. Pharmacol. 1, 14-30.

4. Pogolotti, A. L., Jr. and Santi, D. V. (1977) in Bioorganic Chemistry: Enzyme Action 1, pp 277-311, Academic Press, New York, NY.
5. Bellisario, R. L., Maley, G. F., Guarino, D. U., and Maley, F. (1979) J. Biol. Chem. 254, 1296-1300.
6. Lewis, C. A., Jr. and Dunlap, R. B. (1981) in: Topics in Molecular Pharmacology (Burgen, A. S. V. and Roberts, G. C. K., Eds.) 1, pp 169-219, Elsevier/North Holland Biomedical Press.
7. Maley, F. and Maley, G. (1981) in: Molecular Actions and Targets for Cancer Chemotherapeutic Agents (Sartorelli, A. C., Lazo, J. S., and Bertino, J. R., Eds.) pp. 265-283, Academic Press, New York, NY.
8. Daron, H. H., and Aull, J. L. (1978) J. Biol. Chem. 253, 940-945.
9. Lewis, C. A., Jr., Ellis, P. D. and Dunlap, R. B. (1980) Biochemistry 19, 116-123.
10. Lyon, J. A., Pollard, A. L., Loeble, R. B., and Dunlap, R. B. (1975) Cancer Biochem. Biophys. 1, 121-128.
11. Plese, P. C. and Dunlap, R. B. (1977) J. Biol. Chem. 252, 6139-6144.
12. Hatefi, Y., Talbert, P. J., Osborn, M. J., and Huennekens, F. M. (1960) Biochem. Prep. 7, 89-92.
13. Maley, G. F., Maley, F., and Baugh, C. M. (1982) Arch. Biochem. Biophy. 216, 551-558.
14. Bellisario, R. L., Maley, G. F., Galivan, J. H., and Maley, F. (1976) Proc. Natl. Acad. Sci. USA 73, 1848-1852.
15. Wataya, Y., Matsuda, A., and Santi, D. V. (1980) J. Biol. Chem. 255, 5538-5544.