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THE EFFECT OF THIOLS ON THE SULFHYDRYL CONTENT AND NUCLEOTIDE BINDING OF THYMIDYLATE SYNTHETASE

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SUMMARY: Variability in the endpoints for catalytic sulfhydryl group modification, nucleotide binding, and ternary complex formation has been observed in thymidylate synthetase from amethopterin-resistant Lactobacillus casei. Electrophoretic analysis of ternary complexes of various enzyme preparations revealed the presence of as many as three enzyme forms containing one, two, or no nucleotide binding sites or catalytic sulfhydryl groups per enzyme dimer. The relative quantities of multiple forms were dependent on exogenous thiols in the purification scheme, thus resulting in the variability in active site content obtained for thymidylate synthetase.

Although thymidylate synthetase consists of apparently identical subunits, (1-3) recent investigations of nucleotide binding and sulfhydryl group modification have resulted in values ranging from 0.9-1.7 for the number of active sites present in the enzyme (1,2,4-8). The results of circular dichroism and equilibrium dialysis studies of substrate (dUMP) binding (4,5,7) and of sulfhydryl group modification experiments with certain preparations (1,2,6,7) were interpreted to indicate the presence of a single active site per enzyme dimer. A clear distinction between the binding of dUMP and the potent inhibitor FdUMP[1] was noted; although dUMP was bound at a single site, FdUMP was found to interact at one or two sites depending on the buffer and the presence or absence of folates (4). In contrast, recent work from this laboratory revealed the presence of ∿1.6 essential cysteine residues per enzyme dimer and an equivalent number of FdUMP binding sites present in the ternary complexes involving FdUMP, 5,10-CH_2H_4folate and the enzyme (8). In addition, an equal number of binding sites for FdUMP and dUMP was suggested by the parallel effect of sulfhydryl modification on enzymatic activity and

1. The abbreviations used are: FdUMP, 5-fluoro-2'-deoxyuridylate; 5,10-CH_2H_4folate, 5,10-methylenetetrahydrofolate.

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the ability of the enzyme to form ternary complexes (8).

As a possible means of explaining the discrepancies which were noted above in the extent of substrate and inhibitor binding and the stoichiometry of essential sulfhydryl group modification, differences with regard to the presence of exogenous thiols in the purification schemes for amethopterin-resistant L. casei thymidylate synthetase in various laboratories were investigated. In this communication thymidylate synthetase isolated in the presence of exogenous thiols is compared to enzyme isolated in the absence of thiols (thiol-free enzyme) and to thiol-free enzyme activated by dialysis against thiols. The existence of multiple forms of the enzyme and the effect of thiols on the relative quantities of each form in the various preparations were revealed by the determination of active site sulfhydryl content and the ability of the enzyme preparations to form ternary complexes with FdUMP and 5,10-CH_2H_4folate or binary complexes with either FdUMP or dUMP.

EXPERIMENTAL

Thymidylate synthetase was isolated by the method of Lyon et al. (9) in either the presence or absence of 10 mM 2-mercaptoethanol. Circular dichroic spectra were recorded at room temperature on a Jasco J-40C Recording Spectropolarimeter. The nucleotide titrations were performed as described by Leary et al. (7) using enzyme that had been activated and dethiolated as described previously (8). Circular dichroic studies of FdUMP binding were monitored at 269 nm which was the wavelength of maximum ellipticity for the nucleotide alone. Values for the decrease in θ_{290} were obtained by subtracting the ellipticity at 290 nm for the nucleotide-enzyme binary mixture from the ellipticity of the enzyme alone at 290 nm. Sulfhydryl modification studies and the formation of the ternary complexes were performed as described (8). The number of FdUMP sites present per enzyme molecule in each enzyme preparation was determined by analysis of the polyacrylamide gels of the ternary complexes (10) using the following equation:

$$\text{FdUMP sites per enzyme molecule} = \frac{(\%\ \text{From II})(1) + (\%\ \text{Form III})(2)}{100}$$

RESULTS AND DISCUSSION

As reported by Leary et al. (7), the interaction of dUMP with thymidylate synthetase resulted in an increase in negative ellipticity at 267 nm which was attributed to a change in the conformation of the nucleotide on binding to the protein. In contrast to the single site binding obtained by Leary et al., titration of the enzyme purified in the presence of thiol and activated by dialysis against thiol resulted in an end point of 1.5 for dUMP

binding (Figure 1). In further contrast to the studies of Leary et al., we have found that the binding of dUMP to the enzyme elicited a decrease in positive ellipticity at 290 nm which is attributed directly to the protein. Monitoring the titration at 290 nm also resulted in an end point of 1.5 for dUMP binding, indicating that changes in both the enzyme and the nucleotide regions of the CD spectra yield equivalent end points. Further, the titration experiments were repeated with the inhibitor, FdUMP, and an end point of 1.6 FdUMP binding sites per enzyme dimer was obtained (Figure 2). Thus, these data illustrate the fact that native thymidylate synthetase (Form I) isolated from amethopterin-resistant L. casei in the presence of thiols contains an average of 1.5-1.6 substrate or inhibitor binding sites as well as catalytically essential sulfhydryl groups per enzyme dimer (8).[2]

Gel electrophoretic analysis of the ternary complexes derived from the latter enzyme preparation (Figure 3, gel 3) reveals that 30% of the enzyme can form only a 1:1:1 ternary complex (FdUMP to 5,10-CH_2H_4folate to enzyme) (Form II) while 70% of the enzyme forms a 2:2:1 complex (Form III) (10,11). Thus, the maximum nucleotide binding ratio of $\sim$1.7 sites per enzyme is obtained, a value which correlates with the stoichiometry of both essential sulfhydryl group modification and nucleotide binary complex formation. These data provide strong evidence for the existence of a heterogeneous population of thymidylate synthetase molecules; multiple forms of the enzyme are indicated --- one form with a single nucleotide binding site (30%) and another form with two nucleotide binding sites (70%).

We have found that enzyme isolated in the absence of exogenous thiols, designated as thiol-free enzyme, appears to possess a single active site by

2. Initial studies reported in 1971 of the interaction of thymidylate synthetase with p-chloromercuribenzoate suggested that 1.1 to 1.2 catalytic sulfhydryl groups were present per enzyme dimer (1). Although this enzyme was isolated in the presence of 5-10 mM 2-mercaptoethanol, no activation step with high concentrations of thiol (25 to 100 mM) was performed. Our studies indicate that thymidylate synthetase isolated in the presence of thiols must be activated by dialysis against thiols to achieve maximum ternary complex formation and thus sulfhydryl reagent incorporation.

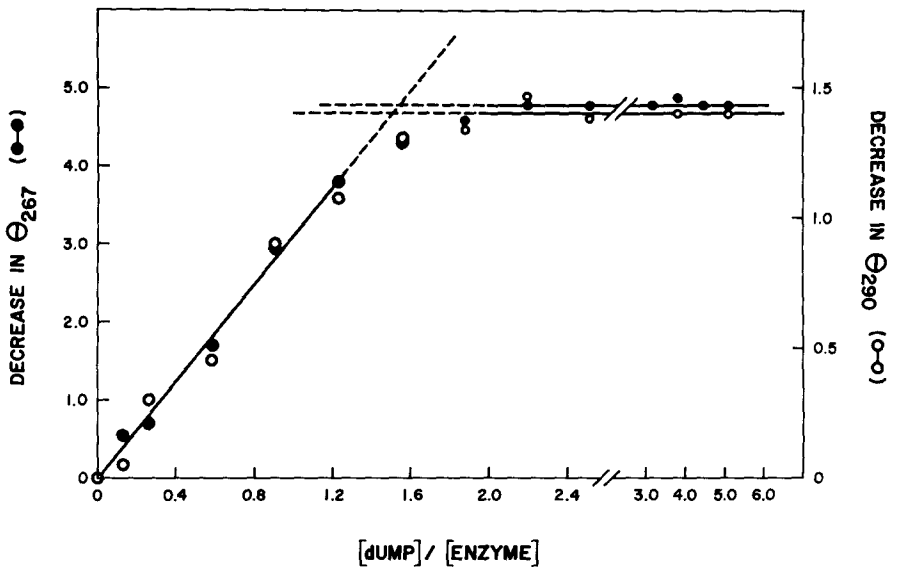

Figure 1: Circular dichroic titration of thymidylate synthetase with dUMP monitored at 267 nm (● - ●) and 290 nm (○ - ○). Enzyme concentration: 5.83 x 10^{-6}M. Buffer: 50 mM Tris, pH 7.4, containing 1 mM EDTA.

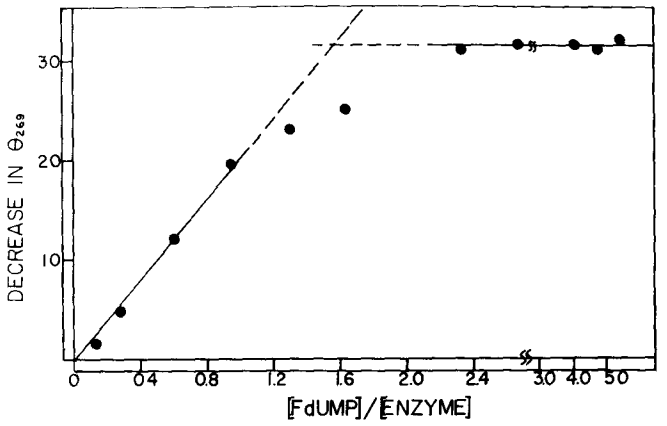

Figure 2: Circular dichroic titration of thymidylate synthetase with FdUMP monitored at 269 nm. Enzyme concentration: 5.92 x 10^{-6}M. Buffer: 50 mM Tris, pH 7.4, containing 1 mM EDTA.

sulfhydryl modification: 0.9 and 0.8 active site sulfhydryl groups per enzyme dimer in Tris and phosphate buffer, respectively, as detected by iodoacetamide modification. The results are in agreement with Leary et al.

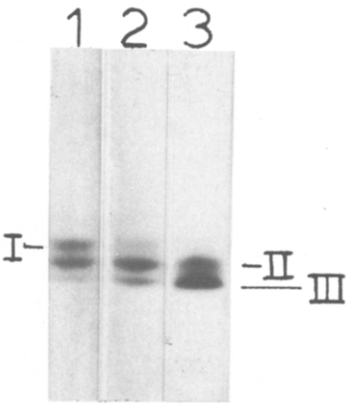

Figure 3: Electrophoretic patterns of the ternary complexes of thymidylate synthetase formed with thiol-free enzyme (gel 1); activated, thiol-free enzyme (gel 2); and enzyme isolated completely in the presence of exogenous thiols (gel 3). Identification of the protein bands was accomplished as described by Aull et al. (10).

(7) and Galivan et al. (6) using enzyme isolated similarly. The fact that the modification of catalytic sulfhydryl groups in thymidylate synthetase correlates directly with both the loss of enzymic activity and the loss in the ability of the enzyme to form ternary complexes suggested a means of testing the single active site hypothesis. If each thymidylate synthetase dimer exhibited a single active site, then treatment of the enzyme preparation with FdUMP and 5,10-CH_2H_4folate would be expected to convert all of the enzyme molecules into 1:1:1 ternary complexes (Form II). However, polyacrylamide gel electrophoresis of the ternary complexes (10,11) formed with thiol-free enzyme actually revealed the presence of three species which corresponded to the native enzyme (Form I, 33%) and its two ternary complexes (Forms II, 52% and III, 15%) (Figure 3, gel 1). These results indicated an average of 0.8 FdUMP sites per enzyme molecule which correlates with the sulfhydryl group modification data (see above and refs 6 and 7) and the extent of dUMP binding reported by Leary et al. (7).

Although the presence of a single active site in the thiol-free enzyme[3] is suggested by the overall stoichiometry of ternary complex formation and sulfhydryl modification, the results of the preceding gel electrophoresis experiment strongly suggest that the value obtained for the active site content is a product of the isolation procedure and actually represents an average value of three forms of the protein present in solution which have two, one, or no sites available for nucleotide binding or modification by sulfhydryl group reagents.

Attempts were made to activate the thiol-free enzyme to yield values of sulfhydryl incorporation and ternary complex formation found in protein isolated in the presence of thiol. The thiol-free enzyme was dialyzed for 24 hours in buffer containing 0.1 M 2-mercaptoethanol. The level of incorporation of iodoacetamide required to inactivate this enzyme preparation was increased from 0.8 to 1.2 per enzyme dimer in phosphate buffer. The number of FdUMP binding sites as determined in the presence of 5,10-CH_2H_4folate by gel electrophoresis was also increased from 0.8 to 1.2 per enzyme molecule (Figure 3, gel 2, 14%I:55%II:31%III). However, the levels of iodoacetamide incorporation and ternary complex formation with the activated, thiol-free enzyme were lower than those found for enzyme isolated with thiols (Figure 3, gels 2 and 3). These results indicate that the addition of thiols to the thiol-free enzyme mediates a change in the relative quantities of multiple enzyme forms such that the active site content is increased.

The analysis of catalytic sulfhydryl group modification, nucleotide binary complex formation, and ternary complex formation for each of the three enzyme preparations studied herein yielded internally consistent stoichiometry values. Electrophoresis of the ternary complexes revealed the heterogeneity of the various enzyme preparations and indicated the presence of as many as three multiple enzyme forms. The relative quantities of

3. The possibility of nucleotide binding to a second site on the thiol-free enzyme was suggested by equilibrium gel filtration studies reported by Beaudette et al. (12).

enzyme forms were found to be dependent on the presence of thiols in the purification procedure, thus providing a basis for explaining the variable active site content obtained for thymidylate synthetase.

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