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ESSENTIAL TYROSYL RESIDUES IN LACTOBACILLUS CASEI THYMIDYLATE SYNTHETASE

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SUMMARY: Sulfhydryl-blocked thymidylate synthetase (EC 2.1.1.4.5) is rapidly inactivated by low concentrations of tetranitromethane. This reagent first nitrates two non-essential tyrosines per dimeric enzyme molecule followed by two essential tyrosines with no oxidation of sulfhydryl groups. dUMP affords significant protection against inactivation. These results suggest that essential tyrosyl residues are present in the active sites of the enzyme.

Thymidylate synthetase catalyses the conversion of dUMP to dTMP in a reaction in which the substrate (+)CH_2H_4folate serves first as the donor of the one carbon fragment and later as the source of reducing equivalents. One study has suggested that arginyl residues participate in the functional binding of dUMP to enzyme (1). Numerous other studies have indicated a catalytic role for cysteine as the nucleophile which attacks carbon 6 of the uracil ring in the initial step of the reaction sequence (2). When FdUMP is used in the reaction in place of dUMP, a stable covalent ternary complex of FdUMP:enzyme:CH_2H_4folate occurs which is thought to be analogous to the catalytically competent complex involving dUMP (3,4). In this article we present evidence for essential tyrosyl residues in the active sites of thymidylate synthetase.

MATERIALS AND METHODS

Thymidylate synthetase was purified from an amethopterin strain of Lactobacillus casei according to the procedure of Lyon et al. (5). Enzyme assays were performed spectrophotometrically at 25°C by following the increase in absorbance at 340 nm using a Beckman Acta III ultraviolet visible spectrophotometer. The assay mixture contained 0.1 M potassium

Abbreviations used are: (+)CH_2FH_4, + 5,10-methylenetetrahydrofolate; FdUMP, 5-fluoro-2'-deoxyuridylate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); MMTS, methyl methanethiolsulfonate; DTE, dithioerythreitol; pHMB, para-hydroxymercuribenzoate; SDS, sodium dodecyl sulfate.

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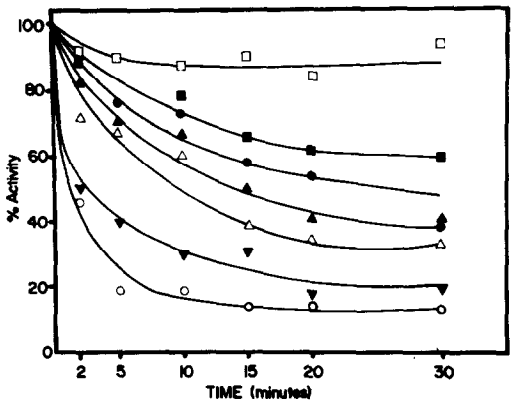


Figure 1. Effect of varying concentrations of tetranitromethane on thymidylate synthetase activity as a function of time. Enzyme (0.68 μM) was incubated with 25 μM (□), 50 μM (■), 100 μM (●), 250 μM (▲), 1000 μM (△), 2000 μM (▼) and native enzyme alone (○) in 50 mM Tris Cl buffer. Aliquots were removed at various time intervals and assayed as described in Materials and Methods.

phosphate buffer, pH 6.8, 1 mM dUMP, 2 mM (+) CH_2H_4folate, 25 mM 2-mercaptoethanol, 10 mM DTE and enzyme (6). Tetrahydrofolate was prepared by catalytic hydrogenation of folic acid (Calbiochem) (7).

Nitration was carried out according to the procedure of Sokolovsky et al. (8). Enzyme that had been activated overnight by dialysis against 50 mM Tris-Cl, pH 8.0, 25 mM 2-mercaptoethanol was freshly dethiolated using a Sephadex G-25 column. With the exception of the nucleotide protection studies, the sulfhydryl groups of the enzyme were protected by modification with a 20 fold excess of the sulfhydryl reagent pHMB which resulted in complete inactivation in 20 min. The enzyme-pHMB complex was then incubated with the desired concentration of tetranitromethane at 25°C, 50 mM Tris HCl, pH 8. Dilutions were made from a freshly prepared stock solution of 0.84 M tetranitromethane in methanol. The nitration reaction was quenched and the sulfhydryl blocking group removed simultaneously by incubation of enzyme with 100 mM DTE at 37°C for 5 min. Enzyme assays were then performed to quantitate inactivation.

Sulfhydryl groups were quantitated by titration with DTNB according to the procedure of Ellman (9), and with ^{14}C MMTS as described previously (10). After gel filtration to remove nitroformate, the nitration of the enzyme was quantitated by measuring the absorbance at 381 nm using $\varepsilon_{NO_2\text{-tyr}} = 2200\ M^{-1}cm^{-1}$ (8).

RESULTS

Thymidylate synthetase was treated with increasing concentrations of tetranitromethane. Low enzyme concentrations were employed in order to prevent dimer formation. As can be seen in Fig. 1, initially enzyme activity decreased rapidly, and then leveled off.

Table I. Effect of inactivation of thymidylate synthetase on the number of titratable sulfhydryl groups of the enzyme.

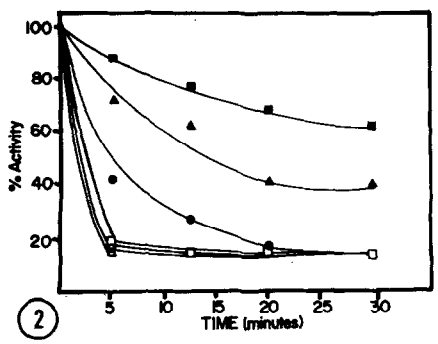
Protecting group of enzyme	% control activity remaining	Sulfhydryl groups titratable under non-denaturing conditions	Sulfhydryl groups titratable under denaturing conditions (1% SDS)
pHMB	100%	1.65	3.2
	72%	2.4	3.3
	65%	2.65	3.3
	34%	2.65	3.3
	30%	2.5	3.2
	28%	3.0	3.3
	24%	2.6	3.2
	21%	3.6	3.8
	15%	3.6	3.9
	11%	3.5	4.0
	100%	1.7*	3.4*
	70%	3.0*	4.0*
	58%	2.4*	3.8*
	37%	2.8*	3.8*
	30%	3.2*	4.0*
dUMP	100%	1.6	3.2
	85%	0.6	1.9
	82%	0.6	1.9
	72%	0.2	1.6
	27%	0.1	0.8

Portions of thymidylate synthetase (ca. 2 mg) protected with pHMB or dUMP were inactivated to varying extents by incubation with 500 μM tetranitromethane. The reaction was quenched and the blocking group removed as described in Materials and Methods. The enzyme was dethiolated by passage through a G-10 column. The enzymic sulfhydryl groups were then measured as described in Materials and Methods.

*Values determined using ^{14}C MMTS as a titrating reagent.

Since tetranitromethane is known to react with sulfhydryl groups and thymidylate synthetase possesses an essential cysteine (2), it was necessary to verify that no loss of sulfhydryl groups occurred as a result of the sequence of blocking, inactivation and deblocking steps performed in the experiment. DTNB titrations of modified enzyme were performed on enzyme samples inactivated to various extents. As seen in Table 1, the number of titratable sulfhydryl groups before and after denaturation actually increased with increasing extent of inactivation, perhaps due to conformational changes in the protein. This phenomenon was confirmed using ^{14}C MMTS to quantitate sulfhydryl groups.

The effect of the substrate on this inactivation is seen in Figure 2.



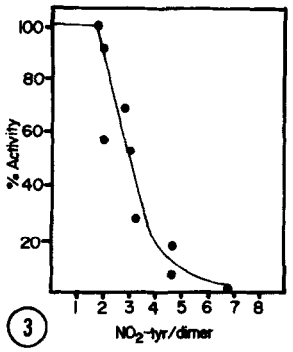


Figure 2. Effect of nucleotides on inactivation of thymidylate synthetase. Enzyme (0.68 μM) was incubated with 100 μM tetranitromethane and 10 mM dUMP (■), 10 mM dTMP (●), 10 mM dCMP (□), 10 mM dAMP (○), enzyme blocked with pHMB (▲), and native enzyme (△).

Figure 3. Enzyme (6.8 μM) was inactivated to varying extents and dethiolated as described in the legend to Table I. The number of nitrotyrosines were quantitated from the absorbance of the protein at 381 nm. Protein concentration was estimated from the absorbance at 280 nm after it was determined that the extinction coefficient was not detectably changed.

dUMP at high concentrations provided marked protection of the enzyme against inactivation in spite of the fact that the catalytically essential sulfhydryl group was not blocked with a reversible sulfhydryl reagent. Loss of activity in this reaction mixture was due in part, if not totally, to the oxidation of sulfhydryl groups as seen in Table 1. However, since inactivation in the presence of dUMP still occurred more slowly than with the sulfhydryl group blocked, we conclude that the nucleotide was also providing protection of the essential tyrosyl residues of the enzyme. Because of sulfhydryl group oxidation, we were unable to show protection of tyrosyl residues with dTMP in the reaction mixture, which showed an inactivation rate intermediate between unprotected enzyme and pHMB protected enzyme. The presence of other nucleotides in the reaction mixture at the same concentrations provided no protection.

The extent of nitration of thymidylate synthetase (6.8 μM) was followed as a function of inactivation using the absorption of the protein at 381 nm. Gel electrophoresis of nitrated enzyme showed less than 5% dimer formation under these conditions. As seen in Fig. 3, apparently two

non-essential tyrosines were rapidly nitrated. Modification of the next two tyrosyl residues occurred more slowly but resulted in the loss of 80% of the initial activity. Complete inactivation occurs on further nitration, possibly by means of a change in the native conformation of the protein.

DISCUSSION

The data obtained by nitration of the enzyme suggested the presence of a tyrosyl residues at the active sites. Evidence for this conclusion is derived from the extensive protection of the enzyme from inactivation by its substrate dUMP. We were unable to evaluate the protection by the other substrate $(\pm)CH_2FH_4$ due to its oxidation by tetranitromethane. Tetranitromethane possesses a high reactivity and a high specificity toward tyrosyl residues. However, it also readily oxidizes cysteine, and for this reason it was necessary to protect the essential sulfhydryl group before nitration. pHMB has been used before as a protecting agent for nitration studies (11,12). The data in Fig. 3 show that in our case as well pHMB served as a reversible blocking group which afforded complete protection from oxidation by titranitromethane.

Two other amino acid residues, cysteine (2) and arginine (1), have been previously shown to participate in mechanism of action of thymidylate synthetase. This report provides evidence which implicates tyrosine as the third known essential amino acid in the active site.

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