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KINETICS OF SULFHYDRYL GROUP MODIFICATION OF THYMIDYLATE SYNTHETASE: A PROPOSAL FOR ACTIVATION OF CATALYTIC CYSTEINYL RESIDUES

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SUMMARY: The pKa of ~8.0 for the catalytic cysteine residue of thymidylate synthetase was determined from the pH dependence of inactivation by the sulfhydryl reagents methyl methanethiolsulfonate and 5,5'dithiobis(2-nitrobenzoic acid). At low pH (5.8-6.8) a rate of reaction significantly greater than can be accounted for by the concentration of thiolate anion was observed. The observed pKa and reactivity for thymidylate synthetase are comparable to those reported for papain (Little, G. L. and Brocklehurst, K. (1972) Biochem. J. 128, 475-477) (1). On the basis of these observations we propose that the cysteines of thymidylate synthetase involved in covalent catalysis may be activated through interaction with a general base in the active site of the enzyme.

Thymidylate synthetase isolated from amethopterin resistant Lactobacillus casei catalyzes the reductive methylation of dUMP to form dTMP, employing the coenzyme 5,10-methylenetetrahydrofolate. An active site cysteine has been shown to participate in a covalent ternary complex composed of the enzyme, the coenzyme and the substrate analogue 5-fluorodUMP (2,3). Recent ^{19}F nmr experiments have delineated the structure of this complex in the active site of the enzyme (4,5). This enzyme has been shown to be rapidly inactivated by various thiol specific reagents, with the extent of thiol modification parallelling both loss of activity and loss of ability to form the ternary complex (6). These results indicate that the reaction catalyzed by thymidylate synthetase is initiated by nucleophilic attack on carbon 6 of the dUMP by an essential active site cysteinyl residue. We have examined the pH dependence of the rate of inactivation of thymidylate synthetase by methyl methanethiolsulfonate (7) and 5,5'dithiobis(2-nitrobenzoic acid) (8) in order to investigate the catalytic nature of the essential cysteinyl residues.

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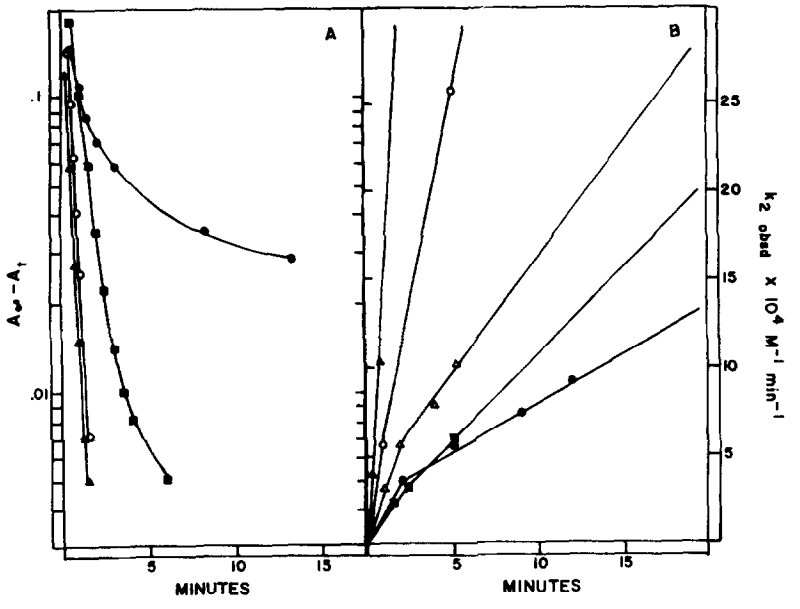

Fig. 1 Inactivation of thymidylate synthetase. Panel A, pseudo first order rate plot of modification by 59 fold excess of DTNB per catalytic thiol. Panel B, second order plot of reaction of 6 fold excess of methyl methanethiosulfonate versus pH of inactivations, 5.8 (●), 6.8 (■), 7.5 (Δ), 7.8 (+), and 8.8 (▲), in 50 mM potassium phosphate buffer, with 1 mM EDTA in DTNB reactions.

EXPERIMENTAL: Activated thymidylate synthetase was dethiolated on Sephadex G-10 columns in 50 mM potassium phosphate buffer at several pH values in the range of 5.8 to 8.8 (6). Methyl methanethiolsulfonate inactivation was performed at 0°C and halted by dilution into a standard assay containing 0.1 M potassium phosphate at pH 6.8, 2 x 10^{-4} M 5,10-methylenetetrahydrofolate, and 1 x 10^{-4} M dUMP. Activity was monitored by dihydrofolate production as indicated by increasing absorbance at 340 nm ($\Delta\varepsilon$ = 6400 $M^{-1}cm^{-1}$) at 25°C. Since DTNB[1] modification of thymidylate synthetase has previously been shown to parallel inactivation (6), this reaction was monitored by the production of 2-thio-5-nitrobenzoate as indicated by the absorbance at 412 nm (ε = 13,600 $M^{-1}cm^{-1}$) at 25°C.

RESULTS: Pseudo first order (DTNB) and second (MMTS) plots of inactivation versus time were found to be biphasic at low pH and linear at high pH for

(1) Abbreviations used: MMTS, methyl methanethiolsulfonate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TNB, 2-thio-5-nitrobenzoate.

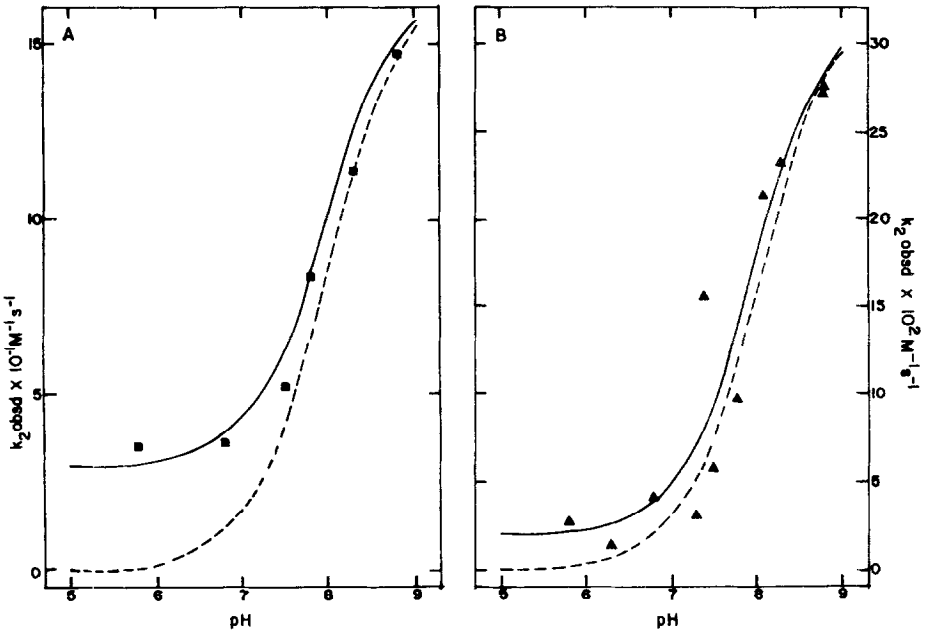

Fig. 2 pH dependence of observed total second order rate constants ($k_{2\ obsd}$) for inactivation of thymidylate synthetase by DTNB (Panel A) and MMTS (Panel B). Solid lines indicate theoretical curves calculated using equation 2, pK_a of 8.0, and k_{2S^-} = 170 $M^{-1} s^{-1}$, k_{2SH^*} = 30 $M^{-1} s^{-1}$ for the DTNB reaction and k_{2S^-} = 3260 $M^{-1} s^{-1}$, k_{2SH^*} = 200 for the MMTS reaction. Dotted lines are theoretical curves calculated using equation 1, reaction due only to thiolate anion.

both reagents (Fig. 1). Measurement of the fast reaction of MMTS was difficult due to its short duration (less than 30 seconds), severely limiting the number of data points obtainable with activity assays. For both reagents, the fast reaction was observed to be strikingly pH dependent, while the rate of the slow reaction, where observed, was independent of pH. Plots of the observed second order rate constants ($k_{2\ obsd}$) for these inactivations versus pH are shown in Figure 2.

DISCUSSION: One possible explanation for the observation of biphasic inactivation kinetics involves the state of ionization of the cysteinyl sulfur under-

going reaction with these reagents. Reaction of DTNB with protein sulfhydryl groups is normally presumed to occur through attack by the enzyme thiolate anion on the disulfide, resulting in a mixed disulfide of TNB with the enzyme (9) The reaction of MMTS is assumed to occur through a comparable mechanism. The observed rates of such reactions would be governed by the pK_a of the sulfhydryl, solution pH and the limiting rate constant for thiolate anion attack ($k_{2\ S^-}$) as expressed in Equation 1. This rate law fails to account

$$k_{2\ obsd} = \frac{k_{2\ S^-}}{1 + [H^+]/K_a} \qquad (1)$$

for the reaction rates observed at low pH, where the concentration of thiolate anion is insufficient to account for the observed rates (see dashed lines in Figure 2). In studies of the DTNB inactivation of papain, Little and Brocklehurst (1) also observed an enhanced reactivity at low pH. They attributed this rate to the enhanced nucleophilicity of the thiol form of cysteine-25, resulting from its activation through interaction with a neighboring histidyl residue. The rate law describing the reactivities of both the activated thiol ($k_{2\ SH^*}$) and thiolate ($k_{2\ S^-}$) forms is given in Equation 2.

$$k_{2\ obsd} = \frac{(k_{2\ S^-}) - (k_{2\ SH^*})}{1 + [H^+]/K_a} + (k_{2\ SH^*}) \qquad (2)$$

Just as observed with papain, the pH dependence of $k_{2\ obsd}$ in Figure 2 is more properly described by this treatment (solid lines). Table 1 presents comparative limiting rate constants for inactivation of papain and thymidylate synthetase by DTNB.

The data for inactivation of thymidylate synthetase by MMTS show much greater scatter than the DTNB data due to the experimental limitations discussed above. The best fit of the experimental data to a theoretical curve were determined using a pK_a of 8.0 and limiting rate constants given in Figure 2.

In the past x-ray crystallographic structures of serine and cysteinyl proteases have been used to infer the presence of charge relay systems, based upon the proximity of active site (10-13) residues. Subsequently, chemical

Table I

Limiting Rate Constants for Reaction of Papain and Thymidylate Synthetase with DTNB

	Papain[a]	Thymidylate Synthetase
k_{2S^-}	284 M^{-1} s^{-1}	170 M^{-1} s^{-1}
k_{2SH^*}	40 M^{-1} s^{-1}	30 M^{-1} s^{-1}
pK_a	8.0	8.0

[a]Data of Little and Brocklehurst (1).

methods have been employed to demonstrate their involvement in enzymatic catalysis. Based upon analogy with other thiol enzymes, notably papain and glyceraldehyde-3-phosphate dehydrogenase (14), we wish to propose that the covalent catalysis exhibited by thymidylate synthetase may result from activation of the catalytic cysteine residue by a general base. The enhanced nucleophilicity observed at low pH (Figure 2) may be an indication of the participation of the sulfhydryl group in a charge relay system. As a means of testing this hypothesis, we have initiated chemical studies to identify the general base and to implicate it as essential for the catalytic activity of thymidylate synthetase.

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