BBA 63289

Mechanism of the inactivation of threonine dehydratase during the dehydration of serine

The ability of L-serine to serve as substrate for L-threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) has been well established for preparations obtained from microorganisms and mammals[1–5]. Equally well known is the inactivation of this enzyme as a result of action on serine, while the mechanistically similar dehydration of threonine produces no observable loss in activity. WOOD AND GUNSALUS[6] were the first to show that this inactivation resulted in an enzyme which could no longer carry out the dehydration of either serine or threonine. Several explanations of the nature of this phenomenon have been proposed. NISHIMURA AND GREENBERG[5] presented evidence that the enzyme became resolved from its coenzyme, pyridoxal phosphate, during serine dehydration and that readdition of pyridoxal phosphate or adjustment to a more alkaline pH restored activity. An oxazolidine ring conjugate between serine and pyridoxal phosphate was postulated as the intermediate which prevented further dehydrative activity. PHILLIPS AND WOOD[7], however, proposed that the highly reactive α-aminoacrylate formed as a result of enzymatic dehydration of serine became bound to some nucleophilic group which was essential for the catalytic process. Preliminary evidence was obtained that radioactivity was covalently bound to threonine dehydratase which had been inactivated by [^{14}C]serine dehydration, but the nature of the adduct was not unequivocally established. The present communication presents further evidence supporting the idea that alkylation of an essential group by aminoacrylate is responsible for the inactivation produced by serine dehydration.

Threonine dehydratase from *Clostridium tetanomorphum*, ATCC 3606, was purified to homogeneity and assayed according to the procedures developed in this laboratory. Details of the purification and crystallization are to be published elsewhere. One unit of enzyme activity is defined as that quantity of enzyme which produces the oxidation of 0.032 μmole of NADH per min in a coupled lactate dehydrogenase assay at 25°. Each assay routinely contained 1 μmole ADP, 1 μmole dithiothreitol, 0.08 μmole NADH, 10 μg crystalline lactate dehydrogenase, 20 μmoles potassium phosphate buffer, pH 8.2, and 10 μmoles L-threonine in a final reaction volume of 0.20 ml. Absorbance changes were recorded at 340 mμ with a Gilford Model 2000 spectrophotometer.

N-Ethylmaleimide (Sigma Chem. Co.) solutions were prepared fresh in 0.1 M phosphate buffer, pH 7.2. Alkylation was achieved by adding 50 μl of *N*-ethylmaleimide to 0.45 ml of enzyme (900 units) in the same buffer at 0°. Each assay cuvette contained sufficient dithiothreitol to destroy the residual *N*-ethylmaleimide transferred into the assay.

Inactivation by serine dehydration was achieved either under assay conditions with 20 μmoles of L-serine as the substrate or outside of the cuvette in a mixture containing 2 μmoles dithiothreitol, 2 μmoles ADP, 100 μmoles phosphate buffer, pH 8.2, 200 μmoles L-serine and 15 units of dehydratase in a final volume of 1.0 ml. Inactivation was carried out at 22° and the process was followed by removing aliquots to an assay mixture containing L-threonine as substrate.

Alkylation by *N*-ethylmaleimide of the threonine dehydratase from *C. tetanomorphum* resulted in a very rapid loss of catalytic activity. Within 15 min at 0°, 50 mM *N*-ethylmaleimide produced a catalytically "crippled" enzyme having only 10% of the maximum velocity toward threonine as that possessed by the native enzyme. The Michaelis constant remained unaffected by this treatment. Because the modification of an essential amino acid residue had been postulated as the means for inactivation by serine, the similarity of the inactivations produced by *N*-ethylmaleimide and serine was suspected.

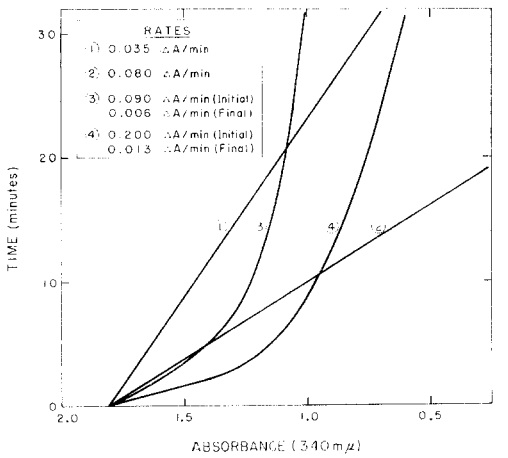


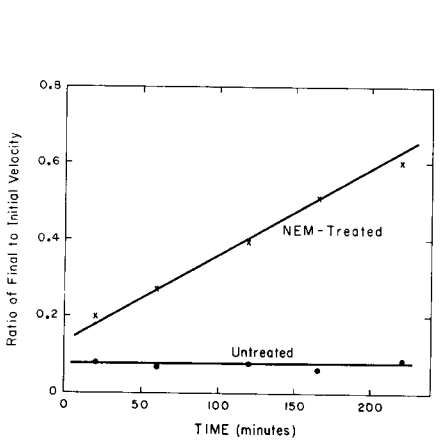
Fig. 1. Reaction rates for *N*-ethylmaleimide-treated and native dehydratase acting on serine. Reactions 1 and 2 contained 10 μl and 20 μl, respectively, of *N*-ethylmaleimide-treated enzyme prepared as described in the text. Reactions 3 and 4 contained 5 μl and 10 μl, respectively, of a 1:100 dilution of native enzyme. Concentration of L-serine was 0.1 M. *N*-Ethylmaleimide concentration for alkylation was 0.05 M.

Fig. 1 illustrates typical assays observed with native enzyme and *N*-ethylmaleimide-treated enzyme when serine served as substrate. It is readily seen that enzyme which had been altered by *N*-ethylmaleimide was not subject to inactivation by serine. Comparison of the initial and final rates of reaction shows that the inactivation of native enzyme by serine dehydration had proceeded so that only 7% of the original activity remained after 20 min, but further inactivation did not occur. This residual activity has apparently been overlooked in previous investigations. On the other hand, *N*-ethylmaleimide-treated enzyme retained its activity at a constant level throughout the entire reaction period, indicating its insensitivity to inactivaton by serine dehydration.

This observation permitted the use of initial and final reaction rates as an index of the sensitivity of the enzyme toward inactivation by serine dehydration. The ratio of final to initial velocities was unity for the completely desensitized enzyme, while sensitive native enzyme had a final to initial velocity ratio of approximately 0.07. In Fig. 2, the changes in velocity ratio are plotted as a function of the time of exposure of the enzyme to *N*-ethylmaleimide. The treated enzyme showed a progressive loss in sensitivity toward inactivation. This result is consistent with the suggestion that *N*-ethylmaleimide alkylation prohibits inhibition by serine dehydration.

The identity of the two processes was also demonstrated by experiments wherein changes in kinetic parameters were followed as serine dehydration proceeded. When inactivation by serine was conducted on a 1.0 ml scale and samples taken for determination of v_{max} and K_m for threonine, marked changes in v_{max} but not K_m were noted (Fig. 3). Under the same conditions, incubation with or without threonine resulted in less than 5% decrease in v_{max}.

The conclusion drawn from these observations, namely that alkylation of some group by either aminoacrylate or *N*-ethylmaleimide results in the observed loss in enzymatic activity, implicates the participation of a cysteinyl residue in the dehydra-



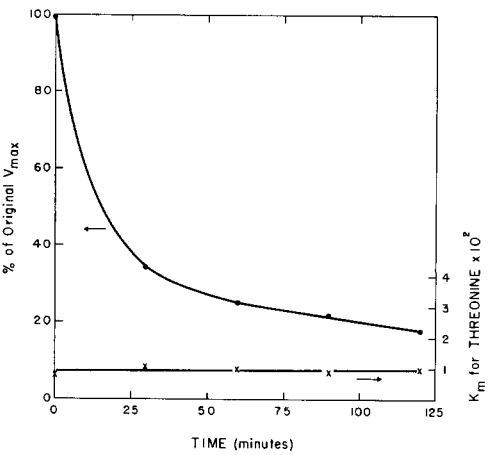


Fig. 2. Time course of *N*-ethylmaleimide (NEM) modification on the susceptibility toward inactivation by serine. These results were obtained in experiments similar to those shown in Fig. 1 except *N*-ethylmaleimide concentration was 0.002 M and samples were withdrawn for assay with serine at the stated times. Initial velocities were determined from the first 2 min, while final velocities were determined after 30 min.

Fig. 3. Changes in v_{max} and K_m during inactivation by serine dehydration. Conditions for incubation were as described in the text. Samples were withdrawn, diluted when necessary, and subjected to assay with varying concentrations of L-threonine (0.01–0.1 M) to determine K_m and v_{max}.

tion process. Although BREWER AND RIEHM[8] have recently shown that *N*-ethylmaleimide can slowly attack the ε-NH_2 group of lysyl residues when sulfhydryl is not present and *N*-ethylmaleimide concentration is high, the rapidity of the reaction between *N*-ethylmaleimide and enzyme under the rather mild conditions employed here would strongly suggest that only sulfhydryl is being modified by *N*-ethylmaleimide. A similar reaction specificity is possible for α-aminoacrylate but the exact reactive properties and mode of addition for this enamine are uncertain. An additional possibility would be that different groups are substituted by *N*-ethylmaleimide and aminoacrylate, but that, for steric reasons, binding of *N*-ethylmaleimide prevents reaction of aminoacrylate with its specific site.

It is clear that the presence of the group modified by these treatments is not strictly essential for catalysis, since alkylation merely decreases v_{max} for the catalyzed reaction. This decrease in velocity could be due to increased steric hindrance during the elimination reaction or to a conformational change induced by alteration of the

sensitive residue. No change is noted in K_m because, for this enzyme, K_m nearly approximates the dissociation constant for the enzyme–substrate complex and inactivation by *N*-ethylmaleimide or serine apparently does not affect the binding of substrate.

This work was supported in part by a research grant from the National Institutes of Health (AM 11619).

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BBA 63291

Homogenous crystalline phosphoglycerate phosphomutase of high activity. A simple method for lysis of yeast

It was shown in 1957[1] that the crystalline yeast phosphoglycerate phosphomutase (D-phosphoglycerate 2,3-phosphomutase, EC 5.4.2.1), by most criteria pure, showed 5 distinct peaks on electrophoresis and these peaks were considered to represent different amounts of bound phosphate[2]; others[3] suggested this effect to be due to different lysine contents resulting from proteolysis. By varying the length of fermentation, all the electrophoretically different forms of the mutase were crystallized and showed to have specific activities ranging from 200 to 4100, the latter being nearly twice the value first described by us[1]. The procedure of CHIBA, SUGIMOTO AND KITO[3] yields small amounts of the mutase and we have not been able to repeat it. During the last 10 years, we have found that while our method is very reproducible the specific activity of the crystals changes, presumably reflecting the activity of the contaminant proteolytic enzyme(s). Thus, a method which would consistently yield preparations of the highest specific activity would be welcome, particularly in view of the new interest being taken in this enzyme[4]. The present paper presents a simplified procedure, together with a very simple technique for lysing yeast.

3-*P*-glycerate, $(NH_4)_2SO_4$ (enzyme grade) and Lab-trol were obtained from Schwarz, Mann and Dade, respectively. Crystalline phosphoglycerate phosphomutase and phosphopyruvate hydratase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11), free of mutase, were prepared as previously described[1,5]. The enolase–mutase coupled assay[1] was used. Specific activity is defined as the enzyme units/mg of protein[6] with the use of Lab-trol as a standard. Disc electrophoresis was performed as previously