EFFECT OF TETRANITROMETHANE ON THE ALDOLASE AND β-DECARBOXYLASE ACTIVITIES OF BOVINE LIVER 2-KETO-4-HYDROXYGLUTARATE ALDOLASE*

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Summary: Treatment of bovine liver 2-keto-4-hydroxyglutarate aldolase with tetranitromethane at pH 8.0 and room temperature rapidly and irreversibly destroys two known catalytic properties of this enzyme, namely, the reversible aldolytic cleavage of 2-keto-4-hydroxyglutarate and also the β-decarboxylation of oxaloacetate. Loss of both enzymatic activities proceeds at the same rate and to the same extent with low molar quantities of tetranitromethane. 2-Ketoglutarate, a competitive inhibitor, protects the enzyme against inactivation by this reagent. The rate of inactivation increases with increasing pH and corresponds well with the pH dependency of aldolase activity. Identical inactivation kinetics are obtained regardless of whether the D- or the L-isomer of 2-keto-4-hydroxyglutarate is used as substrate. The results are consistent with the proposal that tetranitromethane modifies an active site (or sites) involved in both the aldolase and β-decarboxylase activities of the enzyme.

2-Keto-4-hydroxyglutarate aldolase catalyzes the reversible cleavage of KHG[1] yielding pyruvate and glyoxylate. This reaction represents a terminal step in the normal catabolism of L-hydroxyproline by mammals. KHG-aldolase, recently obtained in homogeneous form from bovine liver extracts in our laboratories (1,3) is a remarkably versatile enzyme. In addition to being involved in the pathway for hydroxyproline degradation, this aldolase seems to have a functional role in the mammalian metabolism of L-homoserine; we recently showed (4) that it catalyzes the formation of formaldehyde plus pyruvate by aldolytic cleavage of 2-keto-4-hydroxybutyrate, the α-keto analog of homoserine. Furthermore, pure KHG-aldolase from bovine liver is a very effective β-decarboxylase toward OAA (2). The enzyme also has the novel ability to catalyze the cleavage of both optical antipodes of KHG; i.e. it is nonstereospecific (1).

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[1]Abbreviations used: KHG, 2-keto-4-hydroxyglutarate; TNM, tetranitromethane; KG, 2-ketoglutarate; OAA, oxaloacetate.

The mechanism of action of liver KHG-aldolase (10,1,2) and certain β-decarboxylases (13) has been shown to involve the formation of an azomethine linkage between the carbonyl group of a substrate and the ε-amino group of a lysyl residue in the protein molecule. We observed earlier that both the aldolase (1) and the decarboxylase (2) activities of KHG-aldolase are destroyed by incubating the enzyme with pyruvate plus $NaBH_4$. These results, which indicated that the same active site lysyl residue is involved in the two catalytic processes, have prompted us to examine the effect selective chemical modification of the protein molecule has on the diverse catalytic properties of this enzyme.

Tetranitromethane has been described as a mild and specific reagent for the nitration of tyrosyl residues (8,12) and the oxidation of sulfhydryl groups (9) in proteins. We have studied the effect that TNM treatment of bovine liver KHG-aldolase has on some of its enzymatic properties. We report here our initial results which support the view that the same active site of KHG-aldolase participates in the many reactions it catalyzes.

EXPERIMENTAL AND RESULTS

Materials and Methods - TNM was obtained from Nutritional Biochemicals Corp. and appropriately diluted with 95% ethanol; 2-ketoglutarate was a product of Aldrich Chemical Co. KHG-aldolase was purified from bovine liver extracts by the procedure of Kobes and Dekker (3); final preparations of the enzyme (sp. act. ≅ 180) were determined to be homogeneous by polyacrylamide gel electrophoresis. The molecular weight of the aldolase was taken to be 120,000 (3). Aldolase and β-decarboxylase activities were measured as described before (7,2). Protein was estimated by the method of Lowry, et al. (5).

TNM-Inactivation of Aldolase and β-Decarboxylase Activities - Treatment of KHG-aldolase with TNM at pH 8.0 and 20° results in a dramatic loss of both aldolase and β-decarboxylase activities (Figure 1); under the conditions specified, both activities are completely destroyed within 20 minutes when the enzyme is treated with a 42-fold molar excess of reagent. Enzyme inactivated in this manner is also unable to catalyze the cleavage of 2-keto-4-hydroxybutyrate.

The presence of KG, a competitive inhibitor of aldolase activity (4), in the incubation mixture substantially reduces the rate of inactivation. Under the conditions indicated (Figure 1), the rate of inactivation is very slow after 10 minutes and the extent of reaction approaches a constant value. These results indicate protection of the active center of the enzyme; however, these data also suggest a utilization of TNM by a reaction or reactions other than by modification of amino acid residues in the protein molecule. Studies are in progress to examine such possibilities. It is clear, nevertheless, that the loss of both enzymatic activities proceeds at the same rate in the presence or absence of inhibitor when the aldolase is treated with either a 19- or 42-fold molar excess of TNM.

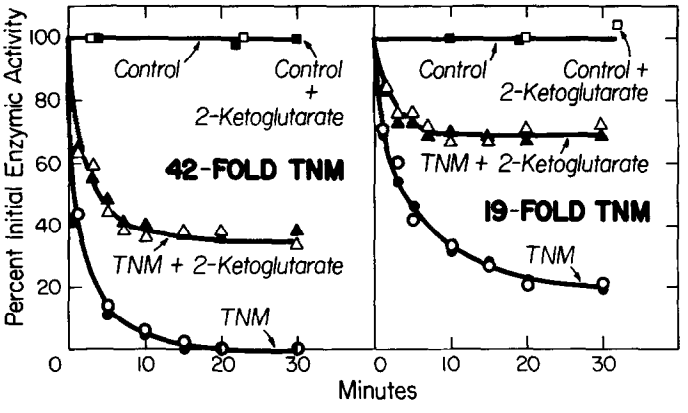

Fig. 1. TNM-Inactivation of Aldolase (closed symbols) and β-Decarboxylase (open symbols) Activities of KHG-Aldolase in the Presence (Δ,▲) and Absence (o,●) of KG. Aldolase (0.24 mg) was incubated in 0.05 M Tris-HCl buffer (0.20 ml, pH 8.0) at 20° containing either 0.038 μmole (19-fold excess) or 0.084 μmole (42-fold excess) of TNM. Aliquots (20 μl) were removed at the times indicated, diluted 50-fold with 0.05 M Tris-HCl buffer (pH 8.0) and assayed immediately for aldolase and β-decarboxylase activities. Control samples (□,■) contained no TNM. Enzyme was initially incubated alone with KG (50 mM, final concentration) for 10 min. at 20° prior to adding TNM.

Table I shows that the aldolase and β-decarboxylase activities of KHG-aldolase are both progressively abolished by increasing concentrations of TNM. The degree of inactivation observed in the presence of a given excess of reagent after 30 minutes is virtually the same for both catalytic activities and does not change even after incubating the mixture for 3 hours at 20°; corresponding-

TABLE I

Inactivation of Aldolase and β-Decarboxylase Activities by Varying Molar Excesses of TNM

Molar Excess TNM	Initial Enzymic Activity		Aldolase to Decarboxylase Ratio
	Aldolase	Decarboxylase	
	%	%	
0	100	100	1.00
2	90	89	1.01
4	71	68	1.04
8	58	50	1.16
10	45	45	1.00
21	20	18	1.11
42	0	0	——

Reaction conditions were the same as those described in the legend of Figure 1. After incubating the enzyme for 30 min. with a given concentration of TNM, an aliquot of the treated enzyme was removed, diluted and assayed for the two activities. In each case, the activity of the untreated enzyme was taken to be 100% and the ratio of the two rates set at 1.00.

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ly, the aldolase to β-decarboxylase activity ratio remains constant and essentially unity throughout the inactivation process.

pH Dependency of TNM-Inactivation - Figure 2 shows that inactivation of aldolase activity by TNM is characteristically dependent on pH; the rate of inactivation increases with increasing pH. When the enzyme is incubated with a 42-fold excess of TNM (the amount required for complete inactivation), a high level of inactivation is observed within 5 minutes even at pH 6 where aldolase activity is low. The observed pH effects are more pronounced when lower concentrations of reagent are employed. Incubation of KHG-aldolase with a 15-fold excess of TNM for 15 minutes results in 90% loss of aldolase activity at pH 9 whereas only 35% inactivation occurs at pH 6. The rates of aldolase activity and TNM-inactivation as a function of pH are strikingly similar; both plots are sigmoidal showing an inflection at pH 7.4 to 7.5.

Substrate Stereospecificity of TNM-Modified Aldolase - An unusual feature

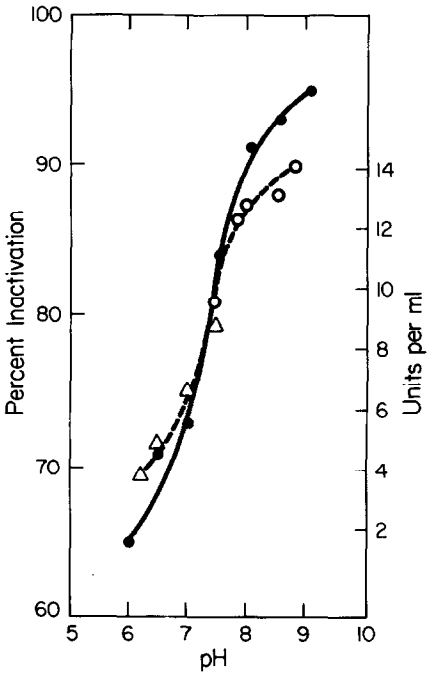

Fig. 2. Effect of pH on Aldolase Activity and Rate of Inactivation by TNM. The reaction mixtures (0.20 ml) contained 0.24 mg of enzyme and 0.084 μmole (42-fold excess) of TNM in 0.15 M Tris-acetate buffers (●). After incubating for 5 min. at 20°, aliquots (1 μl) were removed and assayed for aldolase activity. The pH-aldolase activity profile was determined with 0.48 μg of enzyme under normal assay conditions except 0.25 M potassium phosphate (Δ) or glycylglycine (o) buffers were used.

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of KHG-aldolase from bovine liver is its ability to cleave or form equally well either of the two optical antipodes of KHG (1). As shown in Figure 3, the kinetics of TNM-inactivation are essentially identical regardless of whether the D- or the L-isomer of KHG is used as substrate. Aldolase that is completely inactive toward L-KHG is also devoid of activity with D-KHG as substrate. It would appear, therefore, that those features of the KHG-aldolase molecule which enable it to be nonstereospecific towards the isomers of KHG cannot be selectively altered or differentiated by reaction of the protein with TNM.

DISCUSSION

Reaction of KHG-aldolase from bovine liver with TNM rapidly and completely inactivates both aldolase and β-decarboxylase activities of the enzyme. KG, a competitive inhibitor, protects the enzyme against this inactivation. Similar

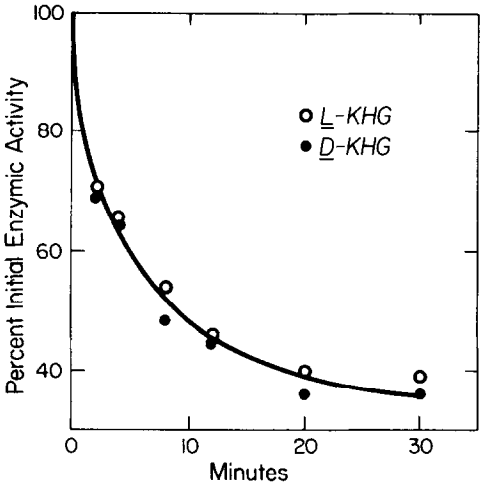

Fig. 3. Activity of TNM-Modified Aldolase with the Optical Antipodes of KHG. The experimental conditions were the same as those outlined in the legend of Figure 1, except KHG-aldolase was treated with a 12-fold excess of TNM. The activity of the enzyme in the absence of TNM was set at 100% for a given isomer of KHG. L-KHG and D-KHG were prepared by nonenzymatic transamination of threo-γ-hydroxy-L-glutamate and erythro-γ-hydroxy-L-glutamate, respectively, according to the procedure of Maitra and Dekker (6).

protection is afforded by other substrates and substrate analogs (Lane and Dekker, unpublished data). Such observations suggest that reaction of TNM with the aldolase involves functional groups which may be components of the active center. The conclusion that TNM-inactivation is due to an alteration of the same active site which catalyzes both enzymatic reactions (aldolase and β-decarboxylase) is supported by these observations: (1) the kinetics of inactivation are identical for both catalytic activities when the aldolase is treated with either a 19- or a 42-fold molar excess of TNM; (2) the level of inactivation obtained with varying concentrations of TNM is essentially the same for both activities; (3) the protective effects of KG against TNM-inactivation are the same for aldolase and decarboxylase activities.

The very similar effect that variation of pH has on KHG-aldolase activity and on the rate of TNM-inactivation provides additional indirect evidence that TNM modifies an active site of the enzyme. Analogous pH effects on the inactivation of rabbit muscle fructose diphosphate aldolase by TNM have been observed

by Riordan and Christen (9). They demonstrated that the loss of fructose diphosphate aldolase activity was associated with the oxidation of sulfhydryl groups in the enzyme molecule. Since bovine liver KHG-aldolase is known to require free sulfhydryl groups for catalytic activity (3), it seems quite possible that in this instance also TNM might be reacting with protein sulfhydryl rather than with tyrosyl residues. Our finding that KHG-aldolase activity is abolished to a very significant extent at pH 6 (Figure 2), whereas nitration of tyrosyl residues in proteins does not occur below pH 7 (12), reinforces that possibility. We have, indeed, found more recently that the deleterious effect of TNM on the catalytic activities of KHG-aldolase reported here is due to the oxidation of four sulfhydryl groups in the enzyme molecule. The details concerning such experiments will be reported elsewhere.

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