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SULFHYDRYL GROUPS IN RELATION TO THE STRUCTURE AND CATALYTIC ACTIVITY OF 2-OXO-4-HYDROXYGLUTARATE ALDOLASE FROM BOVINE LIVER

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Summary

Bovine liver 2-oxo-4-hydroxyglutarate aldolase (suggested name: 2-oxo-4-hydroxyglutarate glyoxylate-lyase catalyzing the reaction: 2-oxo-4-hydroxyglutarate \rightleftharpoons pyruvate + glyoxylate) contains eight to ten sulfhydryl groups as determined by titration of the enzyme with either 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs₂) or *p*-mercuribenzoate in the presence of 1% sodium dodecyl sulfate. In the absence of a denaturant, all of the cysteinyl residues react with *p*-mercuribenzoate whereas only four are accessible to titration with Nbs₂. No differences in -SH group reactivity can be detected during titration of the aldolase with *p*-mercuribenzoate. In contrast, two classes of sulfhydryls can be differentiated in the disulfide exchange reaction with Nbs₂ in the absence of a denaturant; one -SH group (Class I) reacts rapidly whereas three additional thiols (Class II) titrate at approx. 0.1 the rate of the Class I -SH residue. Both pyruvate and glyoxylate protect one of the three -SH residues in Class II from reaction with Nbs₂. Either substrate also prevents titration of one to two thiol groups by *p*-mercuribenzoate and decreases the rate of reaction of aldolase -SH groups with Nbs₂ in 8 M urea. These ligand-induced changes in -SH reactivity provide a sensitive indication that the enzyme exists in an altered conformational state in the presence of either of its cosubstrates.

Titration of the enzyme with either Nbs₂ or *p*-mercuribenzoate results in a progressive loss of aldolase activity which is not proportional to the number of -SH groups modified. The enzyme retains 50% of the activity of the native enzyme when Class I and Class II thiols (i.e. four -SH groups total) are modified

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*** To whom correspondence should be addressed. Abbreviation: Nbs₂, 5,5'-dithiobis(2-nitrobenzoic acid).

with Nbs_2 ; 15% residual activity is still observed following titration of all of the cysteinyl residues with *p*-mercuribenzoate. Pyruvate and glyoxylate provide partial protection against inactivation. It is concluded that inactivation of 2-oxo-4-hydroxyglutarate aldolase by Nbs_2 or *p*-mercuribenzoate is a consequence of alterations in protein structure which accompany modification of -SH groups. The data argue against the direct participation of an active-site thiol group in the catalytic mechanism of 2-oxo-4-hydroxyglutarate aldolase, be that aldol cleavage and condensation or β -decarboxylation.

Introduction

2-Oxo-4-hydroxyglutarate aldolase from bovine liver, an important enzyme in L-hydroxyproline catabolism, catalyzes the reversible dealdolization of 2-oxo-4-hydroxyglutarate yielding pyruvate and glyoxylate. The enzyme also exhibits appreciable β -decarboxylase activity toward oxalacetate, catalyzing the formation of pyruvate and carbon dioxide at about one-half the rate of 2-oxo-4-hydroxyglutarate cleavage [1,2]. Both reactions proceed by Schiff-base mechanisms involving the ϵ -amino group of an active-site lysyl residue; this has been shown by (a) demonstrating the irreversible loss of catalytic activity and stable incorporation of isotope following reduction of the enzyme with NaBH_4 in the presence of ^{14}C -labeled substrates and/or products [3,4] and (b) isolating the predicted secondary amine from acid hydrolysates of the borohydride-reduced pyruvate · aldolase ketimine complex [4]. Similar experiments using either BH_4^- or CN^- as a trapping agent [3–5] indicated that glyoxylate also forms an azomethine with the active-site region of 2-oxo-4-hydroxyglutarate aldolase, an interaction which is mutually exclusive with imine formation with either pyruvate or 2-oxo-4-hydroxyglutarate.

Earlier work [6] showed that 2-oxo-4-hydroxyglutarate aldolase activity is sensitive to inhibitors normally assumed to react with protein sulfhydryl groups, including *p*-mercuribenzoate, *N*-ethylmaleimide, and iodoacetate. More recently, it was demonstrated that both the aldolase and β -decarboxylase activities of the enzyme are abolished when four thiol residues are oxidized with tetranitromethane [7]. The studies reported here were undertaken to determine the number of sulfhydryl groups in bovine liver 2-oxo-4-hydroxyglutarate aldolase, to examine the reactivity of these groups in the absence and presence of substrates, and to investigate more fully the relationship of sulfhydryl groups to the catalytic activities of this aldolase.

Materials and Methods

Materials

2-Oxo-4-hydroxyglutarate aldolase was purified from extracts of bovine liver [6]; final preparations of the enzyme were determined to be homogeneous by polyacrylamide gel electrophoresis at pH 9.3. The aldolase used in these studies had a specific activity of 150–180 (μmol glyoxylate formed/20 min per mg protein at 37°C) and, before being used, was dialyzed overnight at 4°C against the appropriate buffer.

DL-2-Oxo-4-hydroxyglutarate was prepared by non-enzymic transamination of *threo*- γ -hydroxy-DL-glutamate [8]. Nbs₂ was purchased from Aldrich (Milwaukee, U.S.A.) and recrystallized twice from glacial acetic acid. *p*-Mercuribenzoate was obtained from Sigma (St. Louis, U.S.A.). Solutions of *p*-mercuribenzoate were prepared by dissolving 10 mg of the sodium salt in 4 ml of 0.01 M NaOH followed by dilution to a volume of 25 ml with 10 mM potassium phosphate buffer (pH 7.0). Such solutions were stable for at least 2 weeks when stored in the dark at room temperature. The concentration of each solution was determined spectrophotometrically at 232 nm using a molar extinction coefficient of $1.69 \cdot 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [9] and verified by titrating a primary reference solution of reduced glutathione. Sodium pyruvate, sodium glyoxylate and oxalacetate were obtained from Nutritional Biochemicals (Cleveland, U.S.A.), and "ultra pure" sodium dodecyl sulfate from Sigma (St. Louis, U.S.A.). Solutions of urea were freshly prepared from material which had been recrystallized twice from 70% ethanol, washed with cold ether, and then dried for 24 h at 60°C.

Methods

The concentration of purified 2-oxo-4-hydroxyglutarate aldolase was determined by the method of Lowry et al. [10] or by a modified biuret procedure [11] with crystalline bovine serum albumin as a standard. Molar concentrations of the enzyme were calculated on the basis of a molecular weight of 120 000 [6].

2-Oxo-4-hydroxyglutarate aldolase activity was determined by the colorimetric assay of Maitra and Dekker [12]. Oxalacetate β -decarboxylase activity was monitored spectrophotometrically at 340 nm by coupling pyruvate formation with excess NADH and lactate dehydrogenase [7].

Titration of 2-oxo-4-hydroxyglutarate aldolase with Nbs₂ was performed at 25°C in 50 mM Tris · HCl buffer (pH 8.0) in a final reaction volume of 1.0 ml. Potassium glycyglycine buffer (pH 8.0) was substituted for Tris in certain instances as specified. The reaction was initiated by the addition of 0.05 ml of a 10 mM solution of Nbs₂ in 0.1 M potassium phosphate buffer (pH 7.0) to 0.95 ml of a solution of the aldolase (0.3–0.9 mg/ml); the rate of reaction was monitored by following the change in absorbance at 412 nm with time using a Gilford spectrophotometer equipped with a thermostatted cell compartment and a digital readout meter. A molar extinction coefficient of $13\,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used to calculate the number of sulfhydryl groups titrated [13].

The kinetics of Nbs₂ titrations were analyzed by pseudo first-order plots of $\log(a-x)$ versus time where a is the -SH titer after completion of the reaction and x is the number of thiols titrated at a given time, t . With biphasic plots (see Fig. 1A), values for the apparent first-order rate constant (k_{obs}) of sulfhydryl residues with different reactivities and the number of residues in each category were determined as described by Frost and Pearson [14].

2-Oxo-4-hydroxyglutarate aldolase was titrated spectrophotometrically with *p*-mercuribenzoate as described by Boyer [9] and further elaborated by Benesch and Benesch [15]. Titration of the enzyme (0.45–0.50 mg/ml) was carried out in 10 mM Tris · HCl buffer (pH 7.4) at 26°C by sequential addition of 5 μ l aliquots of *p*-mercuribenzoate (approx. 1 mM). The increase in absor-

bance at 250 nm was measured after each addition and plotted against the amount of *p*-mercuribenzoate added [9]. Aliquots of *p*-mercuribenzoate were usually added 15–20 μ l past the equivalence point.

Results

Number and reactivity of thiol groups of bovine liver 2-oxo-4-hydroxyglutarate aldolase

The number of sulfhydryl groups present in 2-oxo-4-hydroxyglutarate aldolase was determined under various conditions; the results are shown in Table I. In the native enzyme, 8–10 -SH residues per 120 000 daltons of protein are titrated with *p*-mercuribenzoate. The reaction is complete within a few minutes after each addition of *p*-mercuribenzoate and no differences in the rate of reaction of these thiol groups can be detected during sequential addition of the reagent. The total number of residues titrated with *p*-mercuribenzoate is not increased significantly when the reaction is carried out in 1% sodium dodecyl sulfate. It appears, therefore, that even in the absence of a denaturant all of the -SH groups in the molecule are readily accessible to *p*-mercuribenzoate.

Reaction of 2-oxo-4-hydroxyglutarate aldolase with Nbs₂ in 1% sodium dodecyl sulfate solution also reveals a total of 8–10 sulfhydryl groups in the protein molecule. Under these conditions, the titration is complete in 20–30 min. If 6 M guanidine · HCl is used in place of sodium dodecyl sulfate, Nbs₂ reacts instantaneously with all of the thiol groups. In the absence of a denaturant, however, only four cysteinyl residues are titrated with Nbs₂. In this case, the reaction is complete in 120–180 min and no significant increase in the sulfhydryl titer is observed beyond this time; prolonged incubation (6 h) of the enzyme with Nbs₂ results in the development of turbidity in the reaction mixture. A pseudo first-order plot of the rate of reaction with excess Nbs₂ is biphasic (Fig. 1A) indicating the presence of two distinct classes of -SH groups

TABLE I

TITRATION OF THE SULFHYDRYL GROUPS OF BOVINE LIVER 2-OXO-4-HYDROXYGLUTARATE ALDOLASE

Titration of the enzyme with Nbs₂ and *p*-mercuribenzoate were carried out as described in the section entitled Methods. Each number listed represents the average value of several determinations with a number of different preparations of the aldolase.

Method of titration	Number of sulfhydryl groups titrated * (equivalents/120 000 daltons protein)
Nbs ₂	4.07 ± 0.15 **
Nbs ₂ in 1% sodium dodecyl sulfate	8.20 ± 0.05
<i>p</i> -Mercuribenzoate	10.15 ± 0.05
<i>p</i> -Mercuribenzoate in 1% sodium dodecyl sulfate	10.00 ± 0.20

* For reasons not yet established, different preparations of the aldolase show a total of either eight or ten -SH groups. Hence the variation in the values listed. If, however, a given sample showed eight -SH groups by titration with Nbs₂ in 1% sodium dodecyl sulfate, a total of eight likewise reacted with *p*-mercuribenzoate (with or without denaturant present). The same was routinely true for preparations having ten titratable -SH groups.

** Average deviation of the mean.

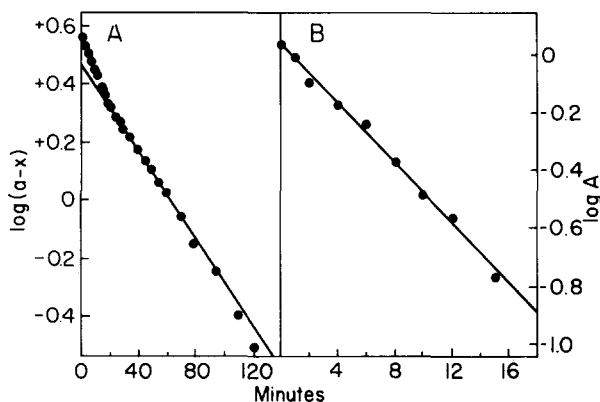


Fig. 1. (A) Pseudo first-order plot for the titration of 2-oxo-4-hydroxyglutarate aldolase -SH groups with Nbs_2 in the absence of a denaturant. Aldolase (0.38 mg/ml) was incubated at 25°C in 50 mM Tris \cdot HCl buffer (pH 8.0) containing 0.5 mM Nbs_2 . The labeling of the ordinate is defined in Methods. (B) Replot of the data obtained in the fast phase of the reaction shown in A. The ordinate represents the logarithm of the concentration of the more reactive component corrected for the contribution of the slower reacting -SH groups [14]. The change in scales from A should be noted.

with different reactivities toward Nbs_2 . The apparent first-order rate constant (k_{obs}) for the slow phase of the titration is computed to be $1.7 \cdot 10^{-2} \text{ min}^{-1}$; this class (Class II) was found to consist of three -SH groups. If one corrects the experimental results obtained in the early phase of the reaction for the contribution of the slower reacting -SH groups (Fig. 1B), the data indicate that Nbs_2 reacts with a single more reactive thiol (Class I, rate constant = $12 \cdot 10^{-2} \text{ min}^{-1}$).

Reactivity of thiol groups in the presence of substrates

Pyruvate and glyoxylate, cosubstrates of the 2-oxo-4-hydroxyglutarate aldolase reaction, markedly influence the titration of enzyme -SH groups by Nbs_2 in the absence of a denaturing agent. As shown in Table II, one -SH group of Class II is protected from reaction in the presence of either 10 mM pyruvate or 10 mM glyoxylate. Moreover, either one of these cosubstrates diminishes the rate of titration of the Class I thiol as well as of the two remaining Class II

TABLE II

REACTIVITY OF ALDOLASE SULFHYDRYL GROUPS WITH Nbs_2 OR *p*-MERCURIBENZOATE IN THE PRESENCE OF SUBSTRATES

Titration of the enzyme (0.45 mg/ml) with *p*-mercuribenzoate was carried out in 10 mM Tris \cdot HCl buffer (pH 7.4) as outlined in Methods. Aldolase (0.33 mg/ml) was titrated with 0.5 mM Nbs_2 at 25°C in 50 mM potassium glycyglycine buffer (pH 8.0). Substrates were added as indicated.

Additions	Number of reactive sulfhydryl groups		Titration rate constant with Nbs_2 (k_{obs}) ($\text{min}^{-1} \times 10^2$)		
	<i>p</i> -Mercuribenzoate	Nbs_2 (mol/mol protein)		Class I	Class II
		Class I	Class II		
None	10.3	1.1	2.9	19	1.6
10 mM pyruvate	8.5	1.2	1.7	10	1.0
10 mM glyoxylate	9.3	1.0	1.8	10	1.0

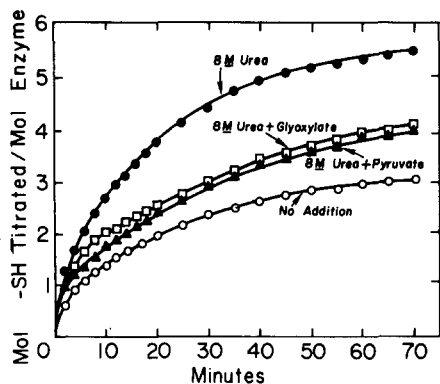


Fig. 2. Effect of substrates on the rate of reaction of thiol groups of 2-oxo-4-hydroxyglutarate aldolase with Nbs_2 in the presence of urea. The protein concentration was 0.33 mg/ml; other experimental conditions were the same as in Fig. 1A. Where indicated, 8 M urea (\bullet), 8 M urea plus 10 mM pyruvate (\blacktriangle), or 8 M urea plus 10 mM glyoxylate (\square) was added.

cysteinyI residues approx. 2-fold. The effects of pyruvate and glyoxylate are not additive; no additional protection or alteration in titration behavior is observed when both compounds, each at a concentration of 10 mM, are present in the same reaction mixture. Added substrates also change the reactivity of 2-oxo-4-hydroxyglutarate aldolase -SH groups with Nbs_2 in the presence of a denaturant; the rate of reaction in 8 M urea is diminished considerably in the presence of 10 mM pyruvate or 10 mM glyoxylate (Fig. 2). Again, no additive effects are observed if both ligands (10 mM each) are present simultaneously during the titration.

A similar protective influence of substrates against sulfhydryl group modification occurs when the native enzyme is titrated with *p*-mercuribenzoate. Although the rates of reaction of the thiol groups with *p*-mercuribenzoate are essentially the same in the presence or absence of either cosubstrate, glyoxylate in this case also protects one -SH group and pyruvate about 1–2 from reacting (Table II). Experiments with numerous preparations of the aldolase showed that either substrate protects 1–2 sulfhydryl groups from being titrated by *p*-mercuribenzoate.

*Inhibition of 2-oxo-4-hydroxyglutarate aldolase activity by Nbs_2 or *p*-mercuribenzoate and reactivation by 2-mercaptoethanol*

Fig. 3 illustrates the loss of 2-oxo-4-hydroxyglutarate aldolase activity which accompanies titration of aldolase -SH groups by Nbs_2 or *p*-mercuribenzoate. One -SH group may be titrated with Nbs_2 and at least two with *p*-mercuribenzoate before the enzyme undergoes any significant loss of catalytic activity. Although further modification with either reagent results in a progressive decline in activity, in both cases the observed loss of enzymic activity does not correlate with the number of thiol groups modified. In addition, only 50% inactivation of enzyme activity is observed when the Class I and Class II cysteinyI residues are titrated with Nbs_2 , and 15% residual activity still persists after *p*-mercuribenzoate reacts with all -SH groups in the protein molecule (a total of eight sulfhydryls were titratable in this experiment). Incubation of the

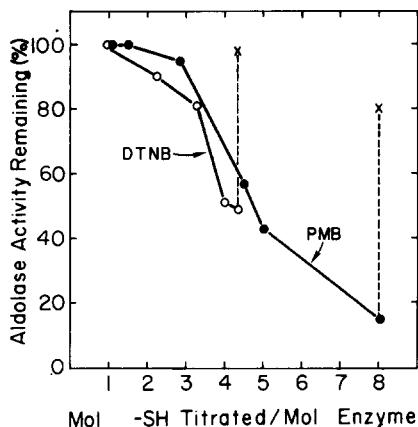


Fig. 3. Extent of inactivation of 2-oxo-4-hydroxyglutarate aldolase by Nbs_2 or *p*-mercuribenzoate as a function of sulphydryl group modification; reactivation by 2-mercaptoethanol. Aldolase (0.33 mg/ml) was titrated with Nbs_2 under the conditions specified in the legend of Fig. 1A. Aliquots were removed at set time intervals and assayed for aldolase activity. The number of sulphydryl groups modified was determined from the absorbance change at 412 nm. Aldolase (0.94 mg/ml) in 0.1 M Tris · HCl buffer (pH 8.0) was titrated with *p*-mercuribenzoate at 26°C by addition of 5- μ l aliquots of *p*-mercuribenzoate solution (0.544 mM) in a stepwise manner. The sulphydryl titer was determined spectrophotometrically at 250 nm. After each addition, aliquots were removed and assayed for aldolase activity. (○), activity of the enzyme titrated with Nbs_2 ; (●), activity of the enzyme treated with *p*-mercuribenzoate; X, maximum reactivation of the enzyme obtained after incubation with 0.1 M 2-mercaptoethanol.

p-mercuribenzoate-treated enzyme with excess 2-mercaptoethanol (0.1 M) restores 80% of the initial enzymic activity; virtually complete reactivation of Nbs_2 -enzyme occurs under the same conditions (Fig. 3).

Protection against Nbs_2 inactivation by substrates

2-Oxo-4-hydroxyglutarate aldolase activity is decreased 2-fold when the enzyme has four of its -SH groups (Classes I and II) titrated with Nbs_2 . With three -SH groups modified in the presence of pyruvate or glyoxylate (Table II), only 10% of the activity of the native enzyme is lost. This protection against Nbs_2 inactivation by substrates can also be demonstrated when the aldolase is reacted with 0.5 mM Nbs_2 at 37°C (Table III). At this higher temperature, about 85% of the initial enzymic activity is destroyed within 90 min in the absence of substrates, whereas over the same interval of time only about 50% inactivation occurs in the presence of either 5 mM pyruvate or 5 mM glyoxylate. No further protection occurs in the presence of both substrates (5 mM each)*. Extended incubation (3 h) of the native enzyme with Nbs_2 at 37°C results in only a small additional decrease (3–5%) in activity.

* When 2-oxo-4-hydroxyglutarate aldolase is treated with Nbs_2 (0.5 mM) at 37°C, 85% inactivation is associated with the titration of six -SH groups; the protective influence of pyruvate (5 mM), glyoxylate (5 mM), pyruvate plus glyoxylate (5 mM each), 2-oxo-4-hydroxyglutarate (5 mM), or even the competitive inhibitor, 2-oxoglutarate (5 mM), against inactivation is accompanied by a decrease in one titratable -SH group.

TABLE III

INACTIVATION OF 2-OXO-4-HYDROXYGLUTARATE ALDOLASE BY Nbs_2 IN THE PRESENCE OF SUBSTRATES

Reaction of the enzyme (0.33 mg/ml) with Nbs_2 was carried out under the conditions specified in the legend of Fig. 1A, except that the temperature was 37°C. Substrates were added as indicated. After a 90 min incubation, the solutions were assayed for aldolase activity after being dialyzed against 10 mM Tris · HCl buffer (pH 7.4) at 4°C. 2-Oxoglutarate is a competitive inhibitor of aldolase activity.

Additions	Aldolase activity remaining (%)
None	17
5 mM pyruvate	51
5 mM glyoxylate	54
5 mM pyruvate + 5 mM glyoxylate	50
5 mM 2-oxo-4-hydroxyglutarate	55
5 mM 2-oxoglutarate	51

Effect of Nbs_2 on the β -decarboxylase activity of 2-oxo-4-hydroxyglutarate aldolase

Results obtained previously [7] indicated that inactivation of 2-oxo-4-hydroxyglutarate aldolase activity by tetranitromethane occurs with the concomitant loss of oxalacetate β -decarboxylase activity. These two catalytic activities of the enzyme are also affected equally by treatment of the aldolase with Nbs_2 (Table IV). In addition, the protective effect of pyruvate on the rate of Nbs_2 inactivation is the same for both aldolase and β -decarboxylase activities.

Discussion

8–10 sulfhydryl groups of bovine liver 2-oxo-4-hydroxyglutarate aldolase react with Nbs_2 in the presence of sodium dodecyl sulfate or guanidine · HCl. All of these -SH residues can be titrated with *p*-mercuribenzoate in the absence of a denaturing agent, but the rate of interaction with *p*-mercuribenzoate does

TABLE IV

EFFECT OF Nbs_2 ON THE ALDOLASE AND β -DECARBOXYLASE ACTIVITIES OF 2-OXO-4-HYDROXYGLUTARATE ALDOLASE

Aldolase (0.33 mg/ml) was incubated for 90 min at 37°C with Nbs_2 or Nbs_2 plus pyruvate, as indicated, in 50 mM Tris · HCl buffer (pH 8.0). Aliquots were withdrawn, diluted and assayed for aldolase and β -decarboxylase activities.

Additions	Aldolase activity remaining (%)	β -Decarboxylase activity remaining (%)
None	100	100
0.02 mM Nbs_2	62	60
0.50 mM Nbs_2	18	18
0.50 mM Nbs_2 + 5 mM pyruvate	51	47

not show any differences among the thiol groups in the enzyme. With Nbs_2 , however, three distinct types of sulfhydryls in 2-oxo-4-hydroxyglutarate aldolase can be differentiated. The first class consists of one -SH group which is highly reactive in the disulfide-exchange reaction. Class II is composed of three cysteinyl residues which titrate with Nbs_2 at approx. 0.1 the rate of the single Class I -SH group. 4–6 additional thiols in the protein become available for reaction with Nbs_2 only in the presence of a denaturant. This difference in titration behavior between *p*-mercuribenzoate and Nbs_2 may be related to their molecular sizes; whereas the relatively small *p*-mercuribenzoate molecule apparently reacts readily with less accessible cysteinyl residues, the larger size of Nbs_2 may hinder reaction with buried thiol groups.

Pyruvate and glyoxylate alter the reactivity of -SH groups in 2-oxo-4-hydroxyglutarate aldolase. Both ligands abolish the reactivity of one thiol group in Class II towards Nbs_2 and also substantially protect the enzyme against inactivation by this reagent. It is not likely that these protective effects are due to masking of this residue because of direct binding of substrate since a significant reduction in reactivity of the other three Class I and II -SH groups is also observed; steric hindrance considerations alone would not seem adequate to explain such results. Rather, these results suggest that 2-oxo-4-hydroxyglutarate aldolase exists in an altered conformational state in the presence of its cosubstrates. The possibility of ligand-induced conformational changes is further strengthened by the protective influence provided by substrates against denaturation of 2-oxo-4-hydroxyglutarate aldolase by 8 M urea. Moreover, although previous binding studies have shown that 1 mol of pyruvate or glyoxylate is bound per mol of enzyme in azomethine linkage [3–5] up to two thiols in the native enzyme are protected from reaction with *p*-mercuribenzoate. Of particular interest is the observation that both ligands elicit parallel effects on the reactivity of thiol residues towards Nbs_2 or *p*-mercuribenzoate as well as in protecting the aldolase from urea denaturation and Nbs_2 inactivation. Thus, a similar conformational change may result from the binding of pyruvate and glyoxylate at a common site on the protein molecule. Competitive binding studies performed previously indicate that both ligands more than likely undergo imine formation with the same active-site lysyl residue [3–5]. Experiments are in progress to further define the nature and the magnitude of the structural changes observed by -SH titration.

Although 2-oxo-4-hydroxyglutarate aldolase is extensively inactivated by reaction with Nbs_2 or *p*-mercuribenzoate, and substrate protection against inactivation is associated with a decrease in 1–2 titratable thiol groups, it is apparent that these -SH residues cannot play a major role in substrate binding or catalysis since residual enzymic activity is consistently observed following complete titration of the enzyme with either reagent. We conclude, therefore, that the thiol groups of 2-oxo-4-hydroxyglutarate aldolase are essential in maintaining the active conformation of the enzyme required for maximum expression of both aldolase and β -decarboxylase activities (Table IV), and interpret the results with Nbs_2 and *p*-mercuribenzoate as being due to a progressive and general loss of this conformation which accompanies titration of sulfhydryl groups. A concomitant loss of aldolase and β -decarboxylase activities also occurs upon chemical modification of the enzyme with tetranitromethane [7].

In this instance, however, oxidation of four -SH groups results in complete inactivation of the enzyme. The difference between the effects of tetranitromethane and the reagents used in these studies are apparently related to the nature of the product formed and/or the change in protein structure accompanying modification. Inactivation of the aldolase by Nbs₂ or *p*-mercuribenzoate is almost completely reversible by the addition of excess 2-mercaptoethanol (Fig. 3) while thiols are ineffective in reactivating the tetranitromethane-derivatized enzyme. Oxidation of -SH groups in 2-oxo-4-hydroxyglutarate aldolase by tetranitromethane produces cysteic acid and other unidentified products [7].

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