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Oxidation of Sulfhydryl Groups of Bovine Liver 2-Keto-4-hydroxyglutarate Aldolase by Tetranitromethane†

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ABSTRACT: Treatment of bovine liver 2-keto-4-hydroxyglutarate aldolase with a 42-fold molar excess of tetranitromethane at pH 8.0 and 20° rapidly and completely destroys both the aldolase and oxaloacetate β-decarboxylase activities of the enzyme. Various substrates and competitive inhibitors partially protect both enzymatic activities. Loss of aldolase and β-decarboxylase activities occurs concomitant with the modification of free sulfhydryl groups; four such groups are oxidized in the completely inactivated enzyme. Spectral and amino acid analyses demonstrate that the reaction is limited solely to cysteinyl residues. Partial recovery of enzymatic activity is obtained when the inactivated aldolase is incubated with an excess of several thiols. Cysteic acid is present in acid hydrolysates of the tetranitromethane-treated protein indicating that oxidized forms of cysteine other than disulfides are produced. The apparent Michaelis constants for substrates of the enzyme are not altered by modification of the protein with tetranitromethane. In contrast, tetranitromethane-inactivated aldolase does not bind either pyruvate or glyoxylate when aldolase is incubated with these compounds in the presence of sodium borohydride nor is glyoxylate stably bound by cyanide addition to the aldimine. Loss of both aldolase and β-decarboxylase activities, therefore, is most likely due to impaired Schiff-base binding of substrates. Since the native and oxidized enzymes show no significant differences in molecular weight or polyacrylamide gel electrophoretic mobility, the formation of more than one molecular species of the aldolase does not seem to occur.

Bovine liver 2-keto-4-hydroxyglutarate aldolase is a bifunctional enzyme catalyzing the β-decarboxylation of oxaloacetate as well as the reversible dealdolization of KHG.[1] The mechanism of aldol cleavage or condensation involves Schiff-base formation between the carbonyl group of KHG or pyruvate and the ε-amino group of a reactive lysyl residue in the protein molecule (Kobes and Dekker, 1966, 1971). The demonstration (Kobes and Dekker, 1967) that aldolase and β-decarboxylase activities are both lost by treatment of KHG-aldolase with substrates in the presence of $NaBH_4$ implicates a common functional role for the active site lysyl residue in the two catalytic activities of this enzyme. No information is presently available, however, on the nature of other aminoacyl residues in KHG-aldolase which may be essential for enzymatic activity.

Since its initial description as a mild and selective nitrating agent for tyrosine (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966), tetranitromethane has proved to be a convenient reagent to investigate the role of tyrosyl residues in protein structure–function relationships (Riordan *et al.*, 1967b; Cuatrecasas *et al.*, 1968; Atassi and Habeeb, 1969; Kassab *et al.*, 1970; Vincent *et al.*, 1970). It has been used successfully to delineate the chemical factors contributing to the unusual reactivity of phenolic side chains (Riordan *et al.*, 1967a; Cuatrecasas *et al.*, 1968), to demonstrate the occurrence of carbanion intermediates in enzymatic reaction mechanisms (Christen and Riordan, 1968; Riordan and Christen, 1969; Shlyapnikov and Karpiesky, 1969), and also to detect localized conformational changes that appear synchronous with enzymatic catalysis (Christen and Riordan, 1970). Amino acid residues other than tyrosine have been shown to react with tetranitromethane, notably sulfhydryl groups of rabbit muscle fructose diphosphate aldolase (Riordan and Christen, 1968) and a tryptophanyl residue in the extracellular nuclease of *Staphylococcus aureus* (Cuatrecasas *et al.*, 1968).

In an earlier brief report (Lane and Dekker, 1969b), we noted the rapid and concomitant loss of both aldolase and β-decarboxylase activities of KHG-aldolase when the enzyme is treated with low molar quantities of tetranitromethane under mild conditions. The data presented here indicate that this loss is associated with the preferential oxidation of four cysteinyl residues; disulfides and other oxidation products

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[1] The abbreviations used are: KHG, 2-keto-4-hydroxyglutarate; KHB, 2-keto-4-hydroxybutyrate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FDP, fructose diphosphate.

are formed. Inactivation of KHG-aldolase by tetranitromethane appears to be related to impaired formation of imine intermediates involved in the mechanisms of both enzymatic reactions.

Materials

KHG-aldolase was purified from extracts of bovine liver according to the procedure of Kobes and Dekker (1969). Final preparations of the enzyme showed one protein component when subjected to polyacrylamide gel electrophoresis (Davis, 1964). The enzyme was routinely stored at 4° at a concentration of approximately 5 mg/ml in 0.025 M Tris-HCl buffer (pH 7.4) containing 5 mM 2-mercaptoethanol and had a specific activity of 150–180 (μmoles of glyoxylate formed in 20 min at 37° per mg of protein).

DL-KHG, L-KHG, D-KHG, and 2-keto-4-hydroxybutyrate were prepared as described before (Maitra and Dekker, 1963; Lane and Dekker, 1969a). Rabbit muscle lactate dehydrogenase was purchased from Calbiochem; beef liver catalase and ovalbumin from Worthington Biochemical Corp.; and egg-white lysozyme from Sigma Chemical Co. 5,5′-Dithiobis-(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co. and was twice recrystallized from glacial acetic acid before use. Tetranitromethane was a product of Nutritional Biochemical Corp. Solutions of tetranitromethane were diluted to the desired concentration with 95% ethanol and used immediately. Sodium [1-^{14}C]glyoxylate (7.4 mCi/mmole) and sodium [2-^{14}C]pyruvate (31.0 mCi/mmole) were procured from Nuclear-Chicago Corp. All other compounds were of the highest purity commercially available.

Methods

Unless otherwise indicated, KHG-aldolase solutions were dialyzed overnight at 4° against several changes of 0.05 M Tris-HCl buffer (pH 8.0) prior to use. Protein concentrations were determined by the method of Lowry *et al.* (1951) or by a modified biuret procedure (Gornall *et al.*, 1949) with crystalline bovine serum albumin as a standard; the values obtained by these two procedures were in excellent agreement with one another. A value of 120,000 was used for the molecular weight of bovine liver KHG-aldolase (Kobes and Dekker, 1969).

KHG- and KHB-aldolase activities were determined as described earlier (Maitra and Dekker, 1964; Lane *et al.*, 1971). Oxaloacetate β-decarboxylase activity was detected by coupling pyruvate formation with excess NADH and lactate dehydrogenase. The assay mixture (1.0 ml) contained 100 μmoles of Tris-HCl buffer (pH 8.1), 0.48 μmole of NADH, 0.05 mg of lactate dehydrogenase, 3.3 μmoles of oxaloacetate, and appropriate amounts of the aldolase. Addition of oxaloacetate initiated the reaction. Initial reaction velocities were followed at 25° by measuring the rate of NADH oxidation spectrophotometrically at 340 nm; the rate of reaction was linear with time for at least 5 min. All initial rates were corrected for a low but measurable amount of NADH oxidation which occurred by nonenzymatic decarboxylation of oxaloacetate under these conditions.

Amino acid analyses were performed with a Beckman Model B amino acid analyzer equipped with long path length photometer cells. The procedure of Spackman *et al.* (1958) was used; the neutral and acidic amino acids were determined by the modification of Spackman (1967). Protein samples were hydrolyzed in sealed tubes under nitrogen with 6 N HCl at 108° for 22 hr.

The extent to which tyrosyl residues were nitrated in tetranitromethane-treated KHG-aldolase was determined either spectrophotometrically in alkaline solution due to the absorption of 3-nitrotyrosine at 428 nm or by amino acid analyses (Sokolovsky *et al.*, 1966).

Sulfhydryl groups were determined at 25° by titration with 5,5′-dithiobis(2-nitrobenzoic acid), according to the procedure of Ellman (1959). The reaction was initiated by the addition of 0.05 ml of a 10 mM solution of DTNB in 0.1 M potassium phosphate buffer (pH 7.0) to 0.95 ml of a solution of native or tetranitromethane-modified KHG-aldolase in 0.05 M Tris-HCl buffer (pH 8.0). A molar extinction coefficient of 13,600 was used for calculations. All spectral measurements were corrected by a reagent blank which contained no enzyme.

Sucrose density gradients (5–20%) were prepared in 0.05 M Tris-HCl buffer (pH 7.5) according to the method of Martin and Ames (1961). The gradients were stored for 5–6 hr at 4° prior to centrifugation. Samples (0.2 ml of a 2.5% sucrose solution containing 50–150 μg of protein) were carefully layered onto 12 ml of the sucrose gradient. Sedimentation was performed with an SW-40 rotor in a Beckman Model L2-65B ultracentrifuge at 39,000 rpm for 18 hr at 3°. After centrifugation, the gradients were analyzed continuously for absorbance at 280 nm with a Gilford Model 2000 automatic recording spectrophotometer equipped with an LKB 0.2-ml flow cell. Approximately 17 fractions (0.7 ml) were collected per gradient and assayed for KHG-aldolase activity.

Absorbancy measurements at a single wavelength were made at 25° with a Gilford spectrophotometer equipped with a thermostatted cuvet holder and a digital absorbance meter; a Cary Model 15 recording spectrophotometer was used to obtain continuous absorption spectra. Levels of radioactivity were measured with a Nuclear-Chicago thin-window gas flow counter having an efficiency for ^{14}C of approximately 32%.

Results

Reaction of KHG-Aldolase with Tetranitromethane. INACTIVATION OF ALDOLASE AND β-DECARBOXYLASE ACTIVITIES. Reaction of KHG-aldolase with increasing amounts of tetranitromethane at pH 8.0 and 20° results in a progressive and simultaneous loss of aldolase and β-decarboxylase activities (Figure 1); the extent of inactivation observed after 30 min at each concentration of tetranitromethane is virtually the same for both activities. Incubation of the enzyme with a 42-fold molar excess of tetranitromethane rapidly inactivates both catalytic activities 85–90% within 5 min; complete loss of activity results after 15–20 min under these conditions.

PROTECTION BY SUBSTRATES OR COMPETITIVE INHIBITORS. Table I shows the effect of substrates and substrate analogs on the extent of inactivation when KHG-aldolase is incubated with an excess of tetranitromethane. Loss of both catalytic activities can be appreciably diminished but not completely prevented by the presence of substrates of the aldol cleavage or condensation reaction or by oxaloacetate. Protection against inactivation is also provided by 2-ketoglutarate and 2-ketobutyrate, competitive inhibitors of aldolase activity (Lane *et al.*, 1971), and also by oxalate, a glyoxylate analog. The degree of protection observed in every case is essentially the same for both aldolase and β-decarboxylase activities.

EFFECT OF pH. Inactivation of KHG-aldolase by tetranitromethane is markedly dependent on pH (Figure 2). The kinet-

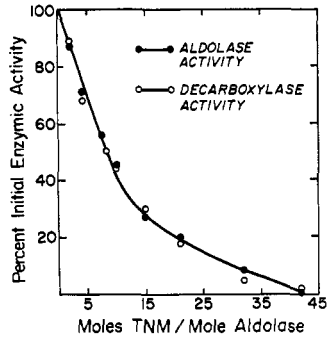


FIGURE 1: Inactivation of aldolase and β-decarboxylase activities by varying molar excesses of tetranitromethane. KHG-aldolase (0.24 mg) was incubated in 0.20 ml of 0.05 M Tris-HCl buffer (pH 8.0) for 30 min at 20° with the indicated concentrations of tetranitromethane. Aliquots were withdrawn, diluted 50-fold with the same buffer at 0°, and assayed for aldolase and β-decarboxylase activities.

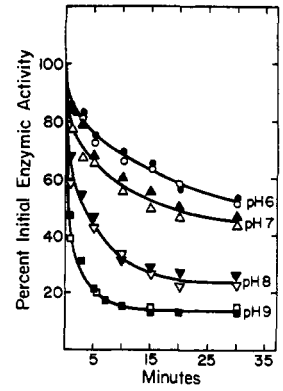


FIGURE 2: Effect of pH on the rate of inactivation by tetranitromethane. The reaction mixtures (0.20 ml) contained 0.26 mg of KHG-aldolase in 0.15 M Tris-acetate buffers at the four pH values indicated. A 15-fold molar excess of tetranitromethane was added at 20°, and at the indicated times aliquots were withdrawn, diluted, and assayed for aldolase (closed symbols) and β-decarboxylase (open symbols) activities.

ics of inactivation at the pH values studied are the same for both aldolase and β-decarboxylase activities.

NATURE AND NUMBER OF MODIFIED AMINOACYL RESIDUES. The finding that a significant loss of enzymatic activity occurs at pH 6 (Figure 2) whereas nitration of tyrosyl residues in proteins normally does not occur below pH 7 (Sokolovsky *et al.*, 1966) was the first indicator that the observed inactivation of KHG-aldolase by tetranitromethane might be due to modification of cysteinyl rather than nitration of tyrosyl residues. With this in mind, the sulfhydryl titer of aldolase samples which had been progressively inactivated with increasing concentrations of tetranitromethane was determined by titration with DTNB and correlated with the loss of both enzymatic activities. Figure 3 shows that the rate of reaction of DTNB with KHG-aldolase decreases as the enzyme is progressively inactivated by tetranitromethane. Four sulfhydryl groups in the native enzyme are titrated by DTNB within 120 min; this value does not change significantly for an additional 2 hr. The number of thiol groups modified by tetranitromethane, therefore, was calculated on the basis of the absorbancy change observed at 412 nm after a 120-min titration period with DTNB; values obtained after 180 min gave virtually identical results.

As shown in Figure 4, inactivation of both aldolase and β-decarboxylase activities by tetranitromethane is directly proportional to the number of sulfhydryl groups modified; complete inactivation of KHG-aldolase is associated with the oxidation of 4 thiol groups. All four sulfhydryl groups appear

TABLE I: Protection against Tetranitromethane Inactivation by Substrates or Competitive Inhibitors.[a]

Compound Added	Concn (M)	Initial Activity Remaining: Aldolase (%)	Initial Activity Remaining: Decarboxylase (%)
None		21	23
DL-2-Keto-4-hydroxyglutarate	1×10^{-2}	57	48
2-Keto-4-hydroxybutyrate	2×10^{-2}	56	62
Pyruvate	1×10^{-2}	55	55
Glyoxylate	1×10^{-2}	54	55
Oxaloacetate	5×10^{-2}	60	56
2-Ketoglutarate	5×10^{-2}	48	53
2-Ketobutyrate	5×10^{-2}	79	77
Oxalate	5×10^{-2}	42	45

[a] The reaction mixtures (1.0 ml) contained 0.05 M Tris-HCl buffer (pH 8.0), 0.265 mg of KHG-aldolase, 0.042 μmole of tetranitromethane (19-fold molar excess). Substrates or substrate analogs were added as indicated and preincubated with the enzyme for 5 min at 20° prior to adding tetranitromethane. After 30 min at 20°, the solutions were dialyzed overnight against 4000 volumes of 0.05 M Tris-HCl buffer (pH 8.0) at 4°. The dialyzed solutions were then assayed for aldolase and β-decarboxylase activities.

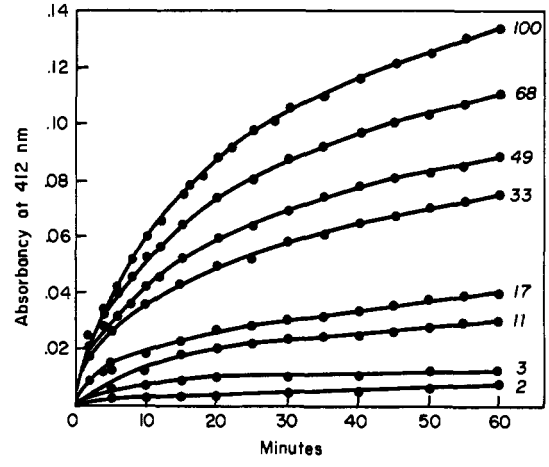


FIGURE 3: Kinetics of the reaction of DTNB with native and tetranitromethane-modified KHG-aldolase. Titrations were carried out at 25° in 1.0 ml of 0.05 M Tris-HCl buffer (pH 8.0) with 0.5 mM DTNB. The protein concentration was approximately 0.4 mg/ml. The values listed in the right-hand margin represent the percent initial KHG-aldolase activity remaining after the enzyme (1.04 mg) was incubated in 0.8 ml of 0.05 M Tris-HCl buffer (pH 8.0) for 30 min at 20° with increasing amounts of tetranitromethane (molar excess 2- to 39-fold). The protein solutions were exhaustively dialyzed against 0.05 M Tris-HCl buffer (pH 8.0) at 4° prior to titration with DTNB.

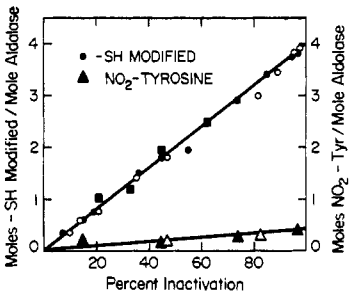


FIGURE 4: Extent of tetranitromethane inactivation as a function of the number of modified cysteinyl and tyrosyl residues. Experimental conditions for the attainment of differential levels of inactivation were the same as outlined in the legend of Figure 3. After reaction for 30 min at 20°, the protein solutions were exhaustively dialyzed against 0.05 M Tris-HCl buffer (pH 8.0) at 4°. The dialyzed solutions were used to determine protein content, aldolase activity (closed symbols), β-decarboxylase activity (open symbols), sulfhydryl content (○,●), and nitrotyrosyl content (△,▲), as described in the section entitled Methods. The difference in sulfhydryl content between native and tetranitromethane-inactivated KHG-aldolase was taken as the number of sulfhydryl groups modified. (■), number of thiol groups modified when the reaction was carried out in the presence of 10 mM DL-KHG.

to react with tetranitromethane at the same rate. Moreover, the relationship between the extent of tetranitromethane inactivation and the loss of free sulfhydryl groups is the same when the reaction is carried out in the presence of 10 mM DL-KHG. Complete inactivation of the enzyme by tetranitromethane occurs in the presence of substrate only after modification of 4 thiol groups. Incubation of the aldolase with higher concentrations (40- to 60-fold molar excess) of tetranitromethane results in no further loss of sulfhydryl groups under these conditions (Figure 5).

Evidence that reaction of KHG-aldolase with tetranitromethane does not result in significant nitration of tyrosyl residues was obtained by spectral analysis for nitrotyrosine; only 0.4 mole of nitrotyrosine per mole of enzyme is formed after 30 min at pH 8.0 whereas 4 thiol groups are oxidized (Figure 4). Furthermore, the absorption spectrum of KHG-aldolase treated with tetranitromethane for a shorter time (20 min, 40-fold molar excess, pH 8.0, 20°, 5% residual KHG-cleavage activity) was identical with that of the native enzyme between 350 and 550 nm in 0.05 M Tris-HCl buffer (pH 8.0); no peak at 428 nm was observed. In addition, no difference spectrum (tetranitromethane-oxidized minus native aldolase) in the same region of the spectrum and under identical conditions could be obtained. It appears highly improbable, therefore, that the dramatic loss of enzymatic activity which occurs when KHG-aldolase is treated with tetranitromethane can be accounted for by nitration of a tyrosyl residue (or residues).

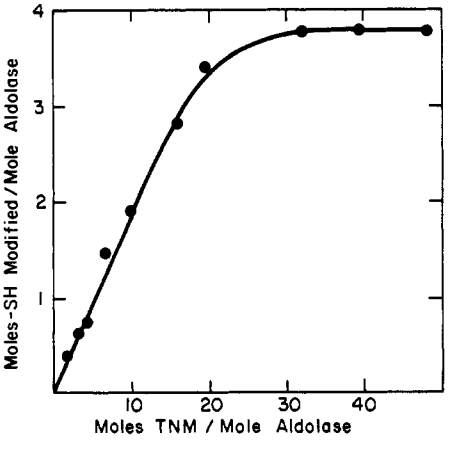


FIGURE 5: Extent of sulfhydryl group modification as a function of tetranitromethane concentration. Reaction conditions are described in the legend of Figure 3. After incubation for 30 min at 20°, the protein solutions were dialyzed exhaustively against 0.05 M Tris-HCl buffer (pH 8.0) at 4° and sulfhydryl groups were determined by DTNB titration as described in Methods.

TABLE II: Amino Acid Composition of Native and of Tetranitromethane-Modified Aldolase.[a]

Amino Acid	Molar Ratio[b]	
	Native Aldolase	Tetranitromethane-Modified Aldolase
Lysine	33.9	33.4
Histidine	16.8	16.7
Arginine	42.1	42.8
Cysteic acid	0	2.56
Aspartic acid	86.8	87.5
Threonine	73.7	74.8
Serine	83.6	84.2
Glutamic acid	122.2	121.4
Proline	76.1	78.1
Glycine	124.3	123.8
Alanine	(110)	(110)
Valine	96.8	94.6
Methionine	22.3	22.2
Isoleucine	31.9	29.7
Leucine	136.4	138.6
Tyrosine	33.7	33.0
Phenylalanine	44.3	43.8
Nitrotyrosine	0	0

[a] To prepare tetranitromethane-modified enzyme, KHG-aldolase (2.38 mg) was incubated for 20 min at 20° in 1.5 ml of 0.05 M Tris-HCl buffer (pH 8.0) with a 40-fold molar excess of tetranitromethane. Aliquots were then withdrawn and assayed for KHG-aldolase activity; the enzyme was 96% inactivated. The samples were dialyzed exhaustively against 0.01 M Tris-HCl buffer and then against water for 10 hr at 4° prior to hydrolysis in 6 N HCl for 22 hr at 108°. [b] Values are expressed as moles of amino acid per mole of aldolase and are calculated on the basis of 110 moles of alanine per mole of protein.

Further evidence that inactivation of KHG-aldolase by tetranitromethane is due to selective modification of cysteinyl residues is provided by amino acid analysis of the modified enzyme. As shown in Table II, the amino acid compositions of native and of tetranitromethane-reacted aldolase are not significantly different (other than for cysteic acid content). In particular, the histidine, lysine, and methionine contents of the native and oxidized enzymes are virtually the same. Of more importance is the fact that the amino acid analyses show no significant difference in the tyrosine content of native and modified KHG-aldolases, and nitrotyrosine cannot be detected in acid hydrolysates of the tetranitromethane-inactivated enzyme. A small but measurable amount

TABLE III: Reactivation of Tetranitromethane-Inactivated Aldolase by 2-Mercaptoethanol.[a]

Initial Enzymatic Activity Remaining					
Aldolase Activity			β-Decarboxylase Activity		
After Reaction with Tetranitromethane (%)	After Incubation with Mercapto-ethanol (%)	Reactivation[b] (%)	After Reaction with Tetranitromethane (%)	After Incubation with Mercapto-ethanol (%)	Reactivation[b] (%)
73	99	96	67	95	85
59	78	46	49	67	35
27	40	18	20	30	12
20	30	12	14	18	5
15	22	8	4	8	4

[a] The reaction mixtures (0.20 ml) contained 0.05 M Tris-HCl buffer (pH 8.0) and 0.265 mg of KHG-aldolase. Molar excesses of tetranitromethane from 4- to 38-fold were employed to achieve the different levels of inactivation. After incubating for 45 min at 20°, an aliquot was withdrawn, diluted 50-fold with the same buffer at 0°, and assayed. A second aliquot was similarly diluted in the presence of 0.1 M 2-mercaptoethanol (final concentration) and reassayed for activity after incubating for an additional 60 min at 0°. Levels of decarboxylase activity were determined on different enzyme samples than those used to measure aldolase activity. [b] Per cent reactivation is defined as (activity recovered/activity lost) × 100.

of cysteic acid was found *only* in protein hydrolysates of the tetranitromethane-modified aldolase.

REACTIVATION BY THIOLS. The data listed in Table III show that only partial reactivation can be achieved by incubating tetranitromethane-modified aldolase with 0.1 M 2-mercaptoethanol for 60 min at 0°. In addition, the extent of reactivation by added thiol decreases as the enzyme is more extensively inactivated. For example, when aldolase which had been inactivated 27% is incubated with 2-mercaptoethanol, 96% of the initial enzymatic activity is recovered. In contrast, enzyme that retained only 4% of its initial activity cannot be reactivated to any significant extent (maximum of about 4%) by added mercaptoethanol. A greater degree of reactivation cannot be achieved by increasing either the concentration of 2-mercaptoethanol (0.2 M), the time of incubation (120 min), or the temperature of incubation (37°). Similar levels of reactivation are obtained with cysteine (50 mM), reduced glutathione (10 mM), or dithiothreitol (0.1 M).

Properties of Tetranitromethane-Modified KHG-Aldolase. KINETIC CONSTANTS. Table IV compares kinetic parameters for native and tetranitromethane-modified aldolase using substrates which undergo aldolytic cleavage. It can be seen that the apparent Michaelis constants for DL-KHG, L-KHG, D-KHG, and KHB are not changed significantly using tetranitromethane-modified enzyme, but under the same conditions their respective V_{max} values are appreciably diminished. It is improbable, therefore, that decreased substrate affinity of the modified aldolase accounts for the loss of enzymatic activity that is associated with the oxidation of sulfhydryl groups in the protein molecule.

REDUCTIVE BINDING OF SUBSTRATES. Since the mechanism of action of KHG-aldolase involves the formation of imine intermediates, the ability of the tetranitromethane-inactivated enzyme to bind substrates *via* azomethine linkages was tested. The extent to which ^{14}C-labeled substrates are bound to native and tetranitromethane-oxidized KHG-aldolase in the presence of $NaBH_4$ is shown in Table V. The native enzyme is irreversibly inactivated when incubated with either ^{14}C-labeled pyruvate or ^{14}C-labeled glyoxylate plus borohydride, and 1 mole of radioactive substrate is bound per 120,000 g molecular weight of protein (experiments 4 and 5, respectively), confirming our previous observations (Kobes and Dekker, 1966). In contrast, when the aldolase is first inactivated by treatment with tetranitromethane, essentially no radioactive pyruvate or glyoxylate can subsequently be stably bound to the protein in the presence of borohydride (experiments 7 and 9, respectively). Appropriate controls established that, under the conditions specified, negligible amounts of radioactivity are stably bound to either native or modified aldolase in the absence of $NaBH_4$ (experiments 1, 2, 6, and 8). These data indicate that tetranitromethane inactivation of KHG-aldolase activity is

TABLE IV: Michaelis Constants for Substrates Using Native and Tetranitromethane-Inactivated Aldolase.[a]

	Native Aldolase		Tetranitromethane-Oxidized Aldolase	
Substrate	K_m (M)	Rel V_{max}[b] (%)	K_m (M)	Rel V_{max}[c] (%)
DL-KHG	1.0×10^{-4}	100	7.5×10^{-5}	46
			1.25×10^{-4}	23
L-KHG	7.1×10^{-5}	105	6.9×10^{-5}	23
D-KHG	1.4×10^{-4}	84	1.5×10^{-4}	24
KHB	3.1×10^{-3}	35	3.0×10^{-3}	55

[a] KHG-aldolase (0.24–0.26 mg) was incubated in 0.20 ml of 0.05 M Tris-HCl buffer (pH 8.0) with either a 10- or 21-fold molar excess of tetranitromethane. After 30 min at 20°, aliquots were withdrawn and appropriately diluted with 0.05 M potassium glycylglycine buffer (pH 8.1) at 0°. The Michaelis constants were determined in 0.1 M potassium glycylglycine buffer at pH 8.1 and 37°. [b] Values expressed relative to the V_{max} value obtained with DL-KHG as substrate. [c] Values expressed relative to the corresponding V_{max} value obtained with a given substrate in the presence of native enzyme.

TABLE V: Substrate Binding to Native and Tetranitromethane-Inactivated Aldolase in the Presence of $NaBH_4$.[a]

Expt	Additions to Enzyme	Initial Enzymatic Activity (%)	Radioactivity Bound (cpm/μmole of Protein)	Substrate Bound[b] (Moles/120,000 g of Protein)
1	[^{14}C]Pyruvate	96	0.283×10^6	0.07
2	[^{14}C]Glyoxylate	107	0.142×10^6	0.03
3	$NaBH_4$	100	0	0
4	[^{14}C]Pyruvate + $NaBH_4$	2	4.17×10^6	0.98
5	[^{14}C]Glyoxylate + $NaBH_4$	0	5.34×10^6	1.03
6	Tetranitromethane; then [^{14}C]pyruvate	10	0.260×10^6	0.06
7	Tetranitromethane; then [^{14}C]pyruvate + $NaBH_4$	10[c]	0.550×10^6	0.13
8	Tetranitromethane; then [^{14}C]glyoxylate	10	0.247×10^6	0.05
9	Tetranitromethane; then [^{14}C]glyoxylate + $NaBH_4$	10[c]	1.36×10^6	0.26

[a] The reaction mixtures (0.30 ml) contained 50 μmoles of potassium phosphate buffer (pH 6.0), 1.43 μmoles of sodium [2-^{14}C]pyruvate (4.27×10^6 cpm per μmole), 1.35 μmoles of sodium [1-^{14}C]glyoxylate (5.18×10^6 cpm per μmole), and 0.17–0.24 mg of native or tetranitromethane-inactivated aldolase. Enzyme was preincubated with substrate for 5 min at 0° prior to reduction with $NaBH_4$, as described previously (Kobes and Dekker, 1966). The reaction time for borohydride reduction was reduced to 15 min, however, since the derivatized protein precipitated after 30-min incubation at pH 6.0. Following reduction, aliquots were removed and immediately assayed for KHG-aldolase activity. The proteins were then precipitated with 0.3 ml of 12% trichloroacetic acid solution and centrifuged, and the residues dissolved in 0.3 ml of 0.5 N NaOH solution. This procedure was repeated twice. The final protein solutions were then dialyzed exhaustively against 0.025 M potassium phosphate buffer (pH 7.4) at 4°. The dialyzed solutions were used to determine protein content and levels of radioactivity. Tetranitromethane-inactivated aldolase was prepared by incubating the enzyme (0.98 mg) in 0.7 ml of 0.05 M Tris-HCl buffer (pH 8.0) with a 30-fold molar excess of tetranitromethane for 30 min at 20°. The oxidized protein derivative was then dialyzed exhaustively against 0.025 M potassium phosphate buffer (pH 7.4) at 4°. [b] Calculated on the basis of μmoles of enzyme inactivated. [c] Complete loss of enzymatic activity occurred after treatment of the tetranitromethane-modified aldolase (90% inactivated) with $NaBH_4$ in the presence of substrate.

TABLE VI: Glyoxylate Binding to Native and Tetranitromethane-Inactivated Aldolase in the Presence of NaCN.[a]

Expt	Additions to Enzyme	Initial Enzymatic Activity (%)	Radioactivity Bound (cpm/μmole of Protein)	Substrate Bound[b] (Moles/120,000 g of Protein)
1	[^{14}C]Glyoxylate	102	2.09×10^5	0.04
2	NaCN	96	0	0
3	[^{14}C]Glyoxylate + NaCN	7	5.39×10^6	1.04
4	Tetranitromethane; then [^{14}C]glyoxylate	8	7.43×10^5	0.14
5	Tetranitromethane; then [^{14}C]glyoxylate + NaCN	8[c]	1.31×10^6	0.25

[a] The reaction mixtures (0.30 ml) contained 50 μmoles of potassium phosphate buffer (pH 7.4), 1.35 μmoles of sodium [1-^{14}C]glyoxylate (5.18×10^6 cpm per μmole), and 0.11–0.24 mg of native or tetranitromethane-inactivated aldolase. Enzyme was preincubated with glyoxylate for 10 min at 20° prior to treatment with 8 μmoles of NaCN for 30 min at 20°. Aliquots were withdrawn and immediately assayed for KHG-aldolase activity. The proteins were precipitated with 0.4 ml of 12% trichloroacetic acid solution and centrifuged, and the residue was dissolved in 0.3 ml of 0.5 N NaOH solution. This procedure was repeated twice. The final protein solutions were then dialyzed exhaustively against 0.025 M potassium phosphate buffer (pH 7.4) at 4°. The dialyzed solutions were used to determine protein content and levels of radioactivity. Tetranitromethane-inactivated KHG-aldolase was prepared as described in the legend of Table V. [b] Calculated on the basis of μmoles of enzyme inactivated. [c] Complete inactivation occurred after incubation of the tetranitromethane-modified enzyme (8% residual aldolase activity) with [^{14}C]glyoxylate and NaCN.

due to impaired formation of Schiff-base intermediates in the catalytic mechanisms of both the aldolase and β-decarboxylase reactions.

AMINONITRILE FORMATION WITH GLYOXYLATE. Table VI shows the binding of ^{14}C-labeled glyoxylate to native and tetranitromethane-modified aldolase in the presence of cyanide. Cyanide readily and irreversibly inactivates the Schiff-base complex formed between ^{14}C-labeled glyoxylate and native aldolase and radioactivity is stably bound to the enzyme in the ratio of 1 mole of glyoxylate per mole of protein (experiment 3). However, negligible radioactive glyoxylate can be bound to the tetranitromethane-modified aldolase in the presence of NaCN (experiment 5). Controls again established that [^{14}C]glyoxylate is also not bound to native or modified enzyme in the absence of NaCN (experiments 1 and 4, respectively). Hence, the novel feature of cyanide stabilizing the glyoxylate–aldolase aldimine complex exhibited by native KHG-aldolase is lost as a consequence of tetranitromethane oxidation of sulfhydryl groups.

PHYSICAL PROPERTIES. The molecular weight of bovine liver KHG-aldolase was previously estimated to be 120,000 by gel filtration on a calibrated column of Sephadex G-200 (Kobes and Dekker, 1969). The molecular weight of the enzyme has now also been determined by sucrose density gradient centrifugation according to the procedure of Martin and Ames (1961). The sedimentation properties of the aldolase were de-

termined in 0.05 M Tris-HCl buffer (pH 7.5) at 3° and compared with those of catalase, lysozyme, and ovalbumin as reference standards. The apparent sedimentation coefficient of KHG-aldolase determined in this manner was 6.9 ± 0.2 S. Calculation of the molecular weight of the protein using this sedimentation coefficient gives a value of 119,000, which is in excellent agreement with that obtained by gel filtration methods.

The molecular size of KHG-aldolase is not significantly altered by treatment with tetranitromethane; both the native and the tetranitromethane-modified aldolases sediment as single symmetrical components. The apparent sedimentation coefficient of the inactive enzyme does not differ significantly from that of the native enzyme and larger molecular species of the aldolase are not detected. In addition, when samples of KHG-aldolase that have been oxidized to varying extents (19–95% inactivated) by tetranitromethane are electrophoresed on cylinders of polyacrylamide gel in 0.19 M glycine–0.02 M Tris buffer at pH 9.3 (Davis, 1964), a single protein component identical in mobility with that of the native enzyme is observed in every case.

Discussion

Bovine liver KHG-aldolase has a number of unusual properties that make it a desirable enzyme for studies relating structure to function. These novel characteristics include (1) a lack of stereospecificity toward the two optical isomers of KHG; (2) the formation of an "abortive" Schiff base with glyoxylate which can be stabilized by reduction with sodium borohydride, an interaction which is competitive with the binding of either KHG or pyruvate as azomethines; (3) the irreversible inactivation of enzymatic activity by cyanide only in the presence of glyoxylate (not with KHG or with pyruvate); and (4) a high level of β-decarboxylase activity toward oxaloacetate (relative to aldolase activity). So far, only the ε-amino group of a lysyl residue (or possibly residues) has definitively been established as a part of the catalytically active site of the enzyme.

In this report, it is shown that tetranitromethane rapidly inactivates both the aldolase and β-decarboxylase activities of KHG-aldolase concomitant with the loss of free sulfhydryl groups; four cysteinyl residues are implicated as being essential for both enzymatic activities. The linear relationship that exists between the extent of inactivation and the number of thiol groups modified indicates that there is little distinction in reactivity toward tetranitromethane among these four sulfhydryl groups, although it is still quite possible that these SH groups are functionally very distinct from one another.

Earlier studies on the reaction specificity of tetranitromethane with amino acids at pH 8 and low reagent concentrations indicated that only tyrosine and cysteine were susceptible to modification (Sokolovsky *et al.*, 1966). Recent reports (Cuatrecasas *et al.*, 1968; Sokolovsky *et al.*, 1969, 1970), however, have shown that tryptophan, histidine, and methionine also react when higher levels of tetranitromethane are employed at pH values above neutrality. Modification of KHG-aldolase by tetranitromethane appears to be limited to sulfhydryl groups; spectral measurements indicate that virtually no tyrosyl residues are nitrated when all four cysteinyl residues have been modified and nitrotyrosine cannot be detected in acid hydrolysates of the tetranitromethane-inactivated enzyme. Moreover, amino acid analyses show that no other aminoacyl residues in the KHG-aldolase molecule are destroyed by tetranitromethane, under the conditions described. Modification of tryptophan also seems unlikely since samples of the native and the tetranitromethane-treated aldolase have the same tyrosine/tryptophan ratio, as determined spectrophotometrically (Goodwin and Morton, 1946). No reaction of tetranitromethane with tryptophanyl residues of KHG-aldolase is further supported by the observation that appreciable inactivation of enzymatic activity occurs at pH 6 (Sokolovsky *et al.*, 1969).

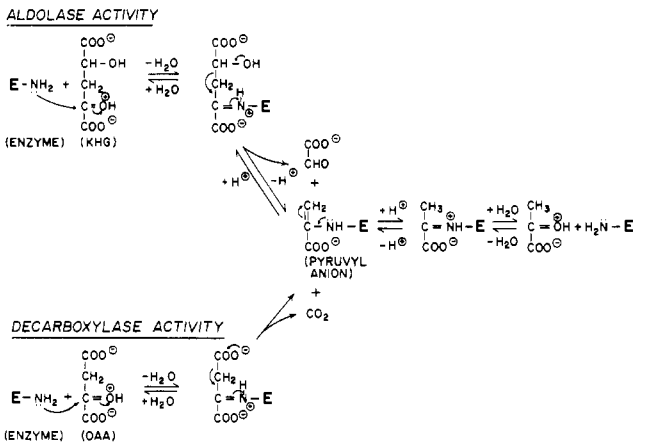
Disulfides or sulfinic acids are mainly formed when model thiol compounds are oxidized by tetranitromethane at pH 5.5 (Sokolovsky *et al.*, 1969), the amount of either product depending on the nature of the mercaptan oxidized and the molar excess of tetranitromethane used. Treatment of mercaptoethanol-reduced ribonuclease, pepsin, or lysozyme with tetranitromethane under identical conditions results largely in the formation of disulfide bonds. However, the extent of enzymatic activity recovered when tetranitromethane-inactivated KHG-aldolase is treated with thiols indicates that only limited oxidation of SH groups to disulfides occurs in this instance. The per cent reactivation by added thiols of aldolase and β-decarboxylase activities is inversely proportional to the extent of inactivation (*cf.* Table III), and tetranitromethane-treated aldolase which has lost essentially all enzymatic activity can be reactivated to only a negligible degree under a variety of conditions. In contrast, essentially all KHG-aldolase activity which is destroyed by disulfide exchange with DTNB can be recovered by treatment with excess mercaptoethanol (Lane and Dekker, manuscript in preparation). Treatment of KHG-aldolase, therefore, with a 42-fold molar excess of tetranitromethane (the amount required for complete inactivation) most likely results in the formation of oxidation products of cysteine other than disulfides; oxidation to the level of cysteinesulfinic acid and cysteic acid would seem to predominate. The presence of the latter compound in acid hydrolysates of the tetranitromethane-modified aldolase and not of the native enzyme substantiates this interpretation. Riordan and Christen (1968) demonstrated that 8–10 sulfhydryl groups of rabbit muscle FDP-aldolase are oxidized by tetranitromethane to the level of the sulfinic acid and/or cysteic acid. As more cases are studied, therefore, it appears that the chemical environment of protein SH groups will be an important factor in determining what product is formed by reaction with tetranitromethane.

Protection against tetranitromethane inactivation of both the aldolase and the β-decarboxylase activities of KHG-aldolase is afforded by several substrates and competitive inhibitors of the enzyme. These results suggest that one or more of the cysteinyl residues which are oxidized by tetranitromethane may be components of the active center of the enzyme. Alternatively, it is possible that these effects are due to substrate- or inhibitor-induced conformational changes in the protein molecule which make the SH groups less reactive toward tetranitromethane. Recent results suggest that pyruvate and glyoxylate both affect the structure of KHG-aldolase in some manner since the presence of either cosubstrate reduces the reactivity of SH groups toward DTNB in native and urea-denatured enzyme.[2]

Another possibility is that substrates and competitive inhibitors protect against tetranitromethane inactivation because they decrease the effective concentration of tetranitromethane in solution. It has been reported (Christen and Riordan, 1968; Riordan and Christen, 1969) that tetranitromethane reacts with an aldolase–substrate carbanion inter-

[2] R. S. Lane and E. E. Dekker, unpublished data.

SCHEME I



mediate in the catalytic mechanisms of rabbit muscle and yeast FDP-aldolases as well as with several model compounds having carbanion character. We have, as yet, been unable to demonstrate the existence of a tetranitromethane-sensitive intermediate in the mechanism of KHG-aldolase. We have observed, however, that tetranitromethane reacts directly with keto acid substrates or substrate analogs[2] in the absence of KHG-aldolase under the reaction conditions employed in these studies. These findings may explain the peculiar inactivation kinetics we reported earlier (Lane and Dekker, 1969b) when KHG-aldolase is reacted with tetranitromethane in the presence of α-ketoglutarate as well as the high level of protection provided by α-ketobutyrate (Table I). However, it is significant that, although tetranitromethane may undergo side reactions in certain cases, protection against tetranitromethane inactivation is observed in the presence of glyoxylate and oxalate, compounds which are incapable of forming carbanions.

Inactivation of KHG-aldolase activity by tetranitromethane is probably not due to weaker binding of substrates by an intrinsically active but modified enzyme; the apparent Michaelis constants for substrates of the dealdolization reaction are essentially the same when determined with partially active, tetranitromethane-oxidized enzymes or with the native aldolase.

A comparison of the proposed mechanisms of KHG cleavage and oxaloacetate decarboxylation as dual catalytic activities of KHG-aldolase (Scheme I) shows that enzyme–substrate imine intermediates participate in both enzymatic reactions. Both KHG and oxaloacetate form Schiff-base complexes with the ϵ-amino group of a lysyl residue in the aldolase molecule; formation of these azomethine intermediates facilitates carbon–carbon bond cleavage eliminating either glyoxylate or CO_2 and yielding the enzyme-bound pyruvyl anion. Ketonization of this species yields the ketimine form of the pyruvate–aldolase complex, which is subsequently hydrolyzed liberating pyruvate and the free enzyme. The oxidation of SH groups in KHG-aldolase by tetranitromethane results in impaired formation of azomethine complexes common to the mechanisms of aldol cleavage and β-decarboxylation since pyruvate or glyoxylate cannot be reductively bound to the modified enzyme nor is a stable aminonitrile formed with glyoxylate in the presence of NaCN. Although these results demonstrate directly that formation of the aldolase–pyruvate complex, an obligatory intermediate in both catalytic mechanisms, does not occur, it is quite probable that Schiff-base bindings of KHG and of oxaloacetate are also equally affected, thereby blocking a step early in the sequence of reactions.

Sucrose density gradient centrifugation shows no significant difference in the apparent sedimentation coefficients of native and of tetranitromethane-oxidized KHG-aldolase and the modified enzyme is homogeneous by the criterion of polyacrylamide gel electrophoresis. These results would seem to exclude tetranitromethane-induced formation of intermolecular disulfide linkages, a result which has been shown to occur by treating mercaptoethanol-reduced ribonuclease with tetranitromethane (Sokolovsky *et al.*, 1969). The data do not, however, eliminate the possibility of a localized conformational change in the enzyme molecule within the vicinity of the active center lysyl residue, thereby disrupting its functional integrity and appreciably diminishing its unusual nucleophilicity for addition to substrate carbonyl bonds. The essential nature of the tetranitromethane-susceptible cysteinyl residues for enzymatic activity may, therefore, be due to their role in maintaining the catalytic architecture of the active center whereby the reactive lysyl residue is placed in an environment optimal for efficient Schiff-base formation with substrates and for effective catalytic activity.

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Identity of the Tryptic and α-Chymotryptic Reactive Sites on Soybean Trypsin Inhibitor (Kunitz)†

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ABSTRACT: Incubation of soybean trypsin inhibitor (Kunitz) with catalytic quantities of tosyllysine chloromethyl ketone treated bovine α-chymotryspin leads to the hydrolysis of one specific peptide bond in the inhibitor molecule. Upon kinetic control dissociation at pH 2 of a complex made from α-chymotrypsin-modified inhibitor and from trypsin, the hydrolyzed bond is completely resynthesized; therefore, the bond is identified as Arg^{63}–Ile (the tryptic reactive-site peptide bond). In order to show that this peptide bond is the reactive-site peptide bond in the α-chymotrypsin–inhibitor complex, a complex was prepared by incubation of equimolar amounts of trypsin-modified inhibitor (Arg^{63}–Ile hydrolyzed) and of α-chymotrypsin for several hours at pH 7. Kinetic control dissociation of this complex was achieved by incubation with a large excess of the active-site titrant *p*-nitrophenyl *N*-acetyl-*N*-benzylcarbazate. The recovered free inhibitor was predominantly virgin (Arg^{63}–Ile intact). On these bases it is concluded that the principal reactive site for the interaction of soybean trypsin inhibitor (Kunitz) with α-chymotrypsin and trypsin is the same.

Soybean trypsin inhibitor (Kunitz) (STI)[1] is a strong inhibitor of trypsin-like enzymes. However, Kunitz (1947) showed that it also inhibits bovine α-chymotrypsin, albeit considerably more weakly than trypsin. Extensive work in this laboratory (for a review, see Laskowski and Sealock, 1971) has shown that on the surface of the soybean trypsin inhibitor molecule there is a relatively small cluster of amino acid residues, called the reactive site, which comes into intimate contact with the active site of trypsin in the stable trypsin–STI complex. Within this site there is a special peptide bond (Arg^{63}–Ile)[2] which is particularly important to the enzyme–inhibitor interaction and which is referred to as a reactive-site peptide bond. In view of the lack of detailed knowledge of the structure of the stable complex this bond is temporarily only operationally defined, although it is hoped that X-ray crystallographic studies will replace the operational definition by a more fundamental one.

Therefore, it was of interest to inquire whether the interaction of STI with α-chymotrypsin follows a mechanism similar to that of its interaction with trypsin and if it does to identify the α-chymotryptic reactive-site peptide bond. We had expected that this bond would differ from the tryptic reactive-site peptide bond since for several protein proteinase inhibitors, which inhibit both trypsin and α-chymotrypsin, it has been amply demonstrated that the inhibitions occur on separate reactive sites (*e.g.*, turkey ovomucoid (Rhodes *et al.*, 1960; Stevens and Feeney, 1963), soybean inhibitor (Bowman-Birk) (Birk *et al.*, 1967; Odani *et al.*, 1971; Seidl and Liener, 1971, 1972), lima bean inhibitor (Tan and Stevens, 1971a,b; Krahn and Stevens, 1972)).

When preliminary evidence indicated that the tryptic and α-chymotryptic reactive-site peptide bonds were the same, the intent of the work switched from a search for a new reactive site to providing as conclusive a proof as possible that the two sites are identical. The approach used in this paper is therefore closely similar to that employed by Hixson and Laskowski (1970a) in showing that cocoonase and bovine trypsin interact with the same reactive site in STI. However, there are some differences. In the work on cocoonase there was

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[1] Abbreviations used are: STI, soybean trypsin inhibitor (Kunitz) in all of its forms; S, soybean trypsin inhibitor (Kunitz) (Arg^{63}–Ile reactive-site peptide bond intact); S*, modified soybean trypsin inhibitor (Kunitz) (Arg^{63}–Ile peptide bond hydrolyzed); NPABC, *p*-nitrophenyl N_2-acetyl-N_1-benzylcarbazate; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; TPCK, tosylphenylalanine chloromethyl ketone; TLCK, tosyllysine chloromethyl ketone.

[2] This bond was originally identified as Arg^{64}–Ile by Ozawa and Laskowski (1966), and this designation was kept in many of the papers from our and other laboratories. Since the complete amino acid sequence of STI has now been determined (Koide *et al.*, 1972), the numbering system used here is based on that sequence.