2-Keto-4-hydroxyglutarate Aldolase of Bovine Liver. Schiff-Base Formation with 2-Keto-4-hydroxyglutarate, Pyruvate, and Glyoxylate*

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ABSTRACT: 2-Keto-4-hydroxyglutarate aldolase, isolated in pure form (1300-fold purified) from bovine liver, binds any one of its substrates (2-keto-4-hydroxyglutarate, pyruvate, or glyoxylate) *via* an azomethine linkage. Using a molecular weight of 120,000 for the aldolase, the determined molar ratio of each substrate bound by the enzyme is 1:1; this value may possibly not represent the absolute but rather the minimal number of binding sites. Azomethine formation between the enzyme and [^{14}C]glyoxylate or [^{14}C]pyruvate, involving the ϵ-amino group of a lysyl residue or residues in the protein molecule, was established by reduction with borohydride; acid hydrolysis of the stabilized adducts yields the expected radioactive N^6-lysine derivatives. Competitive binding of 2-keto-4-hydroxyglutarate, pyruvate, and glyoxylate by the enzyme in the presence of borohydride suggests that all substrates are bound at the same site (lysyl residue) or sites; confirming evidence is provided by release of identical radioactive peptides after tryptic digestion of the borohydride-reduced complexes formed by either [^{14}C]glyoxylate or [^{14}C]pyruvate with the aldolase. When 2-keto-4-hydroxyglutarate aldolase is incubated with glyoxylate in the presence of cyanide, essentially all enzymatic activity is lost and 1 mole of glyoxylate is bound per mole (120,000 g) of enzyme; in this instance, the formation of an inactive enzyme–aminonitrile is postulated.

Since no aldolase activity is lost and no substrate stably bound when enzyme is incubated with either 2-keto-4-hydroxyglutarate or pyruvate in the presence of cyanide, a distinctive imine appears to be formed between 2-keto-4-hydroxyglutarate aldolase and glyoxylate. Substrate-dependent inactivation of 2-keto-4-hydroxyglutarate aldolase in the presence of either borohydride or cyanide is proof for the participation of a Schiff-base mechanism in this enzyme-catalyzed reaction.

In recent years, considerable progress has been made in the elucidation of the mechanism of action of aldolases. Three aldolases which have been studied most extensively are fructose 1,6-diphosphate (FDP)[1] aldolase (Horecker, 1959; Grazi *et al.*, 1962a,b; Horecker *et al.*, 1963; Morse *et al.*, 1965; Lai *et al.*, 1965; Kobashi *et al.*, 1966), 2-keto-3-deoxy-6-phosphogluconate aldolase (Meloche and Wood, 1964a,b; Grazi *et al.*, 1963; Ingram and Wood, 1965, 1966), and 2-deoxyribose 5-phosphate aldolase (Grazi *et al.*, 1963; Hoffee *et al.*, 1965; Rosen *et al.*, 1964). These enzymes contain a lysyl residue (or residues) at the binding site (or sites) which activates the appropriate substrate (the one capable of forming a carbanion) by Schiff-base formation with the ϵ-amino group. In these instances, Schiff-base intermediates have most commonly been detected by observing the loss of enzymatic activity that occurs when aldolase is incubated with substrate in the presence of borohydride. This technique was first reported by Fischer *et al.* (1958) and enabled them to demonstrate that pyridoxal phosphate is bound as a Schiff base to the ϵ-amino group of a lysyl residue in phosphorylase. Borohydride reduction of Schiff-base imines, using radioactive substrates, has also been used to selectively tag aldolases for identification and quantitative determination of the number of active sites.

Another technique, used less frequently for the detection of Schiff-base complexes, is to measure the loss of activity when enzyme is incubated with substrate in the presence of cyanide. This approach has been used successfully by Westheimer (1963) with acetoacetate decarboxylase, by Cash and Wilson (1966) with FDP-aldolase, and by Brand and Horecker (1968) with transaldolase.

We recently developed a procedure for isolating pure KHG-aldolase from bovine liver extracts (Kobes and Dekker, 1969) and briefly reported before (Kobes and Dekker, 1966) that glyoxylate, as well as pyruvate, appeared to be bound *via* an azomethine linkage to the enzyme. The experiments described in this paper consider in detail the nature and extent of binding of all substrates involved in the reversible reaction, the identification of the substrate binding site on the enzyme, as well as data on radioactive peptides released after tryptic digestion of the borohydride reduced enzyme–substrate adducts. The results indicate that pure bovine liver KHG-aldolase has the novel ability to bind all three (KHG, pyruvate, and glyoxylate) of its substrates *via* Schiff-base intermediates. Binding occurs with the ϵ-amino group of a lysyl residue or residues in the protein molecule; the respective N^6-lysine derivatives for pyruvate and glyoxylate have been isolated and identified. One mole of each substrate is bound per mole of enzyme, assuming a molecular weight of 120,000, and all three substrates appear to compete for either the same site or for binding sites that are mutually exclusive when one is occupied. The finding that cyanide inactivates only the glyoxylate–enzyme Schiff-base complex and not the Schiff-base intermediates formed with KHG or pyruvate suggests

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[1] Abbreviations used are: FDP, fructose 1,6-diphosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; KHG, 2-keto-4-hydroxyglutarate.

the formation of a unique glyoxylate–enzyme imine. In the presence of cyanide, 1 mole of glyoxylate is bound per mole (120,000 g) of aldolase. These results are considered relative to the mechanism of the enzyme-catalyzed reaction.

Materials

DL-KHG and bovine liver KHG-aldolase (1300-fold purified) were prepared and assayed as described before (Kobes and Dekker, 1969). [2-^{14}C]KHG was obtained from [2-^{14}C]γ-hydroxyglutamate by the method of nonenzymatic transamination (Maitra and Dekker, 1963). Sodium borohydride was purchased from Metal Hydrides, Inc.; trypsin, twice recrystallized, from Worthington Biochemical Corp.; and [1-^{14}C]pyruvate and [1-^{14}C]glyoxylate were products of Nuclear-Chicago Corp. The specific activities of radioactive KHG, pyruvate, and glyoxylate were 0.103×10^6, 6.51×10^6, and 4.69×10^6 cpm per μmole, respectively. Synthetically prepared N^6-(carboxymethyl)lysine and N^6-(1′-carboxyethyl)-lysine, used as reference compounds in paper chromatography, were kindly provided by Dr. E. Adams of the University of Maryland School of Medicine, Baltimore, Md., and were also prepared chemically by reaction of iodoacetic acid or 2-bromopropionic acid with polylysine (Gundlach *et al.*, 1959).

Methods

Protein concentrations were determined either by the procedure of Lowry *et al.* (1951) or by the spectrophotometric method of Murphy and Kies (1960). Radioactivity measurements were made by plating aliquots of solutions on planchets, drying the sample *in vacuo* over calcium chloride for 3 hr, and determining the amount of radioactivity with a Nuclear-Chicago thin-window gas-flow counter. The efficiency of the counter was approximately 32% for carbon-14. Reference lysine derivatives were observed on paper chromatograms by dipping the sheets in a 0.2% solution of ninhydrin in acetone. Radioactive compounds on chromatograms were detected by use of a Vanguard Autoscanner 880 strip scanner and confirmed by radioautography with Kodak No-Screen X-Ray film.

Peptide mapping was carried out as follows. Solutions of peptides were applied to 16 × 19 in. sheets of Whatman No. 3MM filter paper. Descending chromatography in 1-butanol–pyridine–glacial acetic acid–water (15:10:3:12, v/v) was performed at 24° for 16 hr after equilibrating the paper sheets for 1–3 hr in an atmosphere of the same solvent. Following chromatography, the papers were dried overnight at room temperature. The dried chromatograms were moistened with pyridine–glacial acetic acid–water (500:20:4500, v/v), pH 6.5, in a way such that the peptide streak was washed into a sharp line by buffer fronts advancing from both sides. Electrophoresis was carried out in pyridine–glacial acetic acid–water (500:20:4500, v/v) for 3 hr at 1000 V (120 mA) at approximately 10° in a Savant tank with xylene as coolant. The paper sheets were subsequently dried for 48 hr at room temperature.

Results

Borohydride Reduction of Substrate–Aldolase Complexes. By analogy with the results obtained with FDP-aldolase, KDPG-aldolase, and 2-deoxyribose 5-phosphate aldolase using dihydroxyacetone phosphate (Grazi *et al.*, 1962a,b; Horecker *et al.*, 1963; Morse *et al.*, 1965; Lai *et al.*, 1965), pyruvate (Grazi *et al.*, 1963), and acetaldehyde (Grazi *et al.*, 1963), respectively, one would expect that pyruvate, but not glyoxylate, would cause loss of KHG-aldolase activity and be stably bound when incubated with the enzyme in the presence of sodium borohydride. As already reported briefly before (Kobes and Dekker, 1966), we were surprised to find that incubation of pure bovine liver KHG-aldolase with [^{14}C]-pyruvate *or* [^{14}C]glyoxylate, followed by borohydride reduction, resulted in virtually complete loss of aldolase activity and stable binding of *either* radioactive substrate to the enzyme. These effects were completely dependent upon the concomitant presence of one of the substrates, the aldolase, and borohydride. Knowing the amount of enzyme added to the incubation mixture, the specific activity of the respective radioactive substrate, and assuming a molecular weight of 120,000 for the aldolase, we calculated that 0.97 and 1.01 moles of pyruvate and of glyoxylate, respectively, are bound per mole of pure aldolase. In addition, we found that adding these two ^{14}C substrates concomitantly to the enzyme in the presence of borohydride did not result in the incorporation of an additive amount of radioactivity, and the presence of one substrate unlabeled and the other labeled with carbon-14 significantly diluted the total amount of radioactivity bound to the enzyme. Also, a prior incubation of the aldolase with either unlabeled glyoxylate or pyruvate in the presence of borohydride completely prevented the subsequent binding of the radioactive cosubstrate. Subsequent to our preliminary report (Kobes and Dekker, 1966) of these findings, we have repeated experiments of this kind several times with different preparations of pure bovine liver KHG-aldolase and find the results consistently reproducible.

We have now done similar studies with the third substrate, namely, KHG; the data are shown in Table I. As can be seen, treatment of pure KHG-aldolase from bovine liver with borohydride (expt 1) or [^{14}C]KHG (expt 2) alone causes no loss in aldolase activity, and in the absence of borohydride little radioactivity is bound to the enzyme. KHG-aldolase, however, is completely inactivated when treated with [^{14}C]-KHG in the presence of borohydride and radioactivity is stably bound to the enzyme in the approximate ratio of 1 mole of KHG to 120,000 g of protein (expt 3). In expt 4 and 5, aldolase was first incubated for 30 min with unlabeled KHG plus borohydride. Subsequently, this "enzyme protein" was treated with [^{14}C]pyruvate or [^{14}C]glyoxylate in the presence of borohydride. In these two experiments, essentially no radioactivity was bound to the aldolase. Identical results were obtained in the reverse situation wherein aldolase was first incubated with unlabeled pyruvate or glyoxylate plus borohydride followed by treatment with [^{14}C]KHG and borohydride. These findings suggest that each of the three substrates is bound to the same site (or sites) in the protein molecule.

In those experiments where the ketimine formed between the aldolase and KHG was reduced with borohydride, it was considered quite unlikely that products (pyruvate and/or glyoxylate) derived by aldolytic cleavage of KHG rather than KHG *per se* were bound. Experimental conditions ruling against this possibility included a low pH (6.0–6.3) and temperature (0–4°) as well as the known extensive (80% or greater) and instantaneous reduction of Schiff-base complexes by the first addition of borohydride. This possibility was effectively eliminated by extensive controls using either unlabeled KHG (where any glyoxylate formed could be detected colorimetrically in amounts less than 0.01 of 1 μmole) or [2-^{14}C]KHG (where the liberation of ^{14}C-labeled pyruvate could be monitored).

Identification of Borohydride-Reduced Substrate–Aldolase

TABLE I: Inhibition of Enzymatic Activity and Substrate Binding after Borohydride Treatment.[a]

Experiment	1	2	3	4	5
Additions to aldolase	$NaBH_4$	[^{14}C]KHG	[^{14}C]KHG + $NaBH_4$	KHG + $NaBH_4$; then [^{14}C]pyruvate + $NaBH_4$	KHG + $NaBH_4$; then [^{14}C]glyoxylate + $NaBH_4$
Enzymatic activity lost (%)	3	4	97	99	99
Radioactivity bound (cpm/120,000 μg of protein)	0	0.03×10^6	0.13×10^6	0.72×10^6	0.53×10^6
Substrate bound[b] (μmoles/120,000 μg of protein)	0	0.29	1.20	0.11	0.11

[a] The reaction mixtures (0.33 ml) contained the following components (in micromoles), as indicated: 50, potassium phosphate buffer (pH 6.3); 2.52, [2-^{14}C]KHG; 0.629, sodium [1-^{14}C]pyruvate; 0.885, sodium [1-^{14}C]glyoxylate; and 5.00, KHG. Purified enzyme (0.22 mg; specific activity 150) was used. Each reaction mixture, except in expt 2, was treated at 4° over a period of 30 min with four 10-μl portions of 1 M sodium borohydride added alternately with four 5-μl portions of 2 M acetic acid. The enzyme was then precipitated with 186 mg of ammonium sulfate, removed by centrifugation, and washed twice with 1 ml of 80% saturated ammonium sulfate solution. The washed precipitates were dissolved in 0.4 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.005 M 2-mercaptoethanol, and the resulting solutions were tested for aldolase activity. The enzyme was then precipitated two more times with 0.8 ml of 10% trichloroacetic solution at 4° and each time redissolved in 0.4 ml of 0.75 M NaOH solution. The protein content of the final solution was determined by the method of Lowry *et al.* (1951) and aliquots were plated for radioactivity measurements. [b] The values shown were calculated on the basis of micromoles of enzyme inactivated.

TABLE II: Chromatographic Identification of the Borohydride-Reduced Substrate–Aldolase Adducts Released after Acid Hydrolysis.

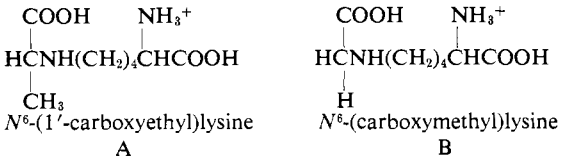
Solvent	N^6-(1′-Carboxyethyl)lysine (R_F)	Hydrolysis Product from [^{14}C]Pyruvate–Aldolase Complex (R_F)	N^6-(Carboxymethyl)lysine (R_F)	Hydrolysis Product from [^{14}C]Glyoxylate–Aldolase Complex (R_F)
Acetone–aqueous 0.5% urea (60:40, v/v)	0.48	0.48	0.40	0.40
2-Butanol–formic acid–water[a] (70:15:15, v/v)	0.36	0.37	0.22	0.23
Pyridine–H_2O (13:7, v/v)	0.35	0.37	0.30	0.29
1-Butanol–ethanol–3 N NH_4OH (2:1:2, v/v)	0.38	0.39	0.31	0.31
Phenol–H_2O (80:20, w/v)	0.63	0.66	0.53	0.54

[a] Descending chromatography on Whatman No. 3MM filter paper; all other solvents were used with ascending solvent flow and Whatman No. 1 filter paper.

Adducts. In order to determine how glyoxylate and pyruvate are bound to bovine liver KHG-aldolase, samples (0.22 mg) of the pure enzyme were treated with [^{14}C]pyruvate or [^{14}C]-glyoxylate in the presence of borohydride, as described in Table I. Instead of precipitating the enzyme with ammonium sulfate, the reaction mixtures were dialyzed against 0.01 M Tris-HCl buffer (pH 7.4) for 24 hr with two changes of buffer. Aliquots of these dialyzed solutions were removed for protein and radioactivity determinations; again, in each case we found about one mole of substrate was bound per 120,000 g of protein. The remaining portions of the solutions were separately hydrolyzed in sealed tubes with 6 N HCl at 110° for 24 hr. After applying the hydrolysates to columns (0.5 × 1 cm) of Dowex 50 (H^+) resin, the resins were washed with ten column volumes of water; no radioactivity was eluted. The resin columns were then eluted with 2 N NH_4OH; quantitative recovery of radioactivity was obtained. The radioactive eluates were evaporated *in vacuo* to dryness four times and the final residues dissolved in 0.2 ml of water. These concentrated radioactive hydrolysates were compared by paper chromatography to unlabeled synthetic N^6-(1′-carboxyethyl)lysine and N^6-(carboxymethyl)lysine; labeled hydrolysis products were also cochromatographed with reference compounds. Table II shows that, in the five solvent systems listed, the radioactive compound isolated after hydrolysis of the borohydride-reduced [^{14}C]pyruvate-aldolase

adduct migrates identically with known N^6-(1′-carboxyethyl)-lysine. Likewise, reference N^6-(carboxymethyl)lysine has the same R_F values as the radioactive product isolated after hydrolysis of the reduced Schiff-base formed between [^{14}C]-glyoxylate and KHG-aldolase. When cochromatographed, radioactivity and ninhydrin responses were superimposable in every case.

These results indicate that, before reduction with sodium borohydride, both pyruvate and glyoxylate form Schiff bases with the ϵ-amino group of a lysyl residue in the KHG-aldolase molecule; after reduction and acid hydrolysis, the isolated adducts have these structures (A, from pyruvate; B, from glyoxylate).



Tryptic Digestion of Borohydride-Reduced Substrate–Aldolase Adducts. Two individual portions of bovine liver KHG-aldolase (0.21 mg each; specific activity 145) were separately treated with 2.15 μmoles of sodium [1-^{14}C]pyruvate or 3.75 μmoles of sodium [1-^{14}C]glyoxylate and reduced with borohydride as described in the legend of Table I. The enzyme was then precipitated with 168 mg of ammonium sulfate, removed by centrifugation, and washed twice with 0.3 ml of 80% saturated ammonium sulfate solution. The washed precipitate was dissolved in 0.3 ml of 0.2 M ammonium bicarbonate buffer (pH 8.5) and dialyzed against the same buffer for 15 hr.

The general techniques of Chernoff and Liu (1961) were followed for carrying out tryptic digestion of the aldolase. The samples of radioactive aldolase were denatured by heating at 100° for 5–6 min. The denatured protein was cooled to room temperature, collected by centrifugation, washed twice by suspending in 0.3 ml of 0.2 M ammonium bicarbonate buffer (pH 8.5), and finally suspended in 0.3 ml of the same buffer. An aqueous solution of trypsin (2 mg/ml) was added to the suspensions of denatured aldolase in an amount equal to 5% of the aldolase protein. These solutions were incubated for 8 hr at 24° with constant stirring; a second portion of trypsin, equal to the first, was added after 4 hr. Other digestions were carried out in an identical manner but with 50% trypsin solutions, and the digests were incubated either for 4 or 24 hr. In all cases, the final opaque solutions were centrifuged at the end of the incubation to remove a small amount of insoluble material. The resulting supernatant fluids were concentrated to about 60 μl and immediately subjected to chromatography. Peptide mapping was carried out as described under Methods; ^{14}C-labeled peptides were detected by radioautography.

In all these studies, the radioautograms invariably showed two distinct radioactive spots (Figure 1). The radioactive peptides released by digestion of the [^{14}C]pyruvate–enzyme complex (reduced with borohydride) cochromatographed with the peptides obtained by digestion of the corresponding [^{14}C]glyoxylate–enzyme adduct. All digestion conditions tried showed the same two radioactive spots, but the 8-hr digestion and lower concentration of trypsin resulted in the best resolution of the radioactive peptides.

Reaction of Cyanide with the Glyoxylate–Aldolase Imine. Since Schiff-base intermediates have more recently been

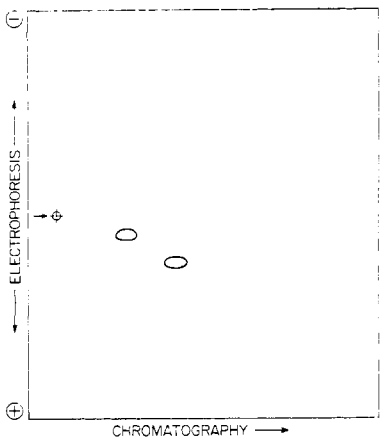


FIGURE 1: Paper chromatographic–electrophoretic pattern of peptides obtained by tryptic digestion of KHG-aldolase labeled with either [^{14}C]pyruvate or [^{14}C]glyoxylate. The arrow indicates the point of application; the shaded areas represent the observed radioactive spots.

detected by reaction with cyanide (Westheimer, 1963; Cash and Wilson, 1966; Brand and Horecker, 1968), we decided to test KHG-aldolase in this manner. We expected that cyanide treatment of the Schiff base formed by pyruvate with KHG-aldolase would cause loss of enzymatic activity, analogous to the inactivation of FDP-aldolase caused by cyanide in the presence of dihydroxyacetone phosphate (Cash and Wilson, 1966). As can be seen in Table III, there is no inhibition of KHG-aldolase activity when the enzyme is treated with cyanide alone or with cyanide in the presence of either [^{14}C]KHG or [^{14}C]pyruvate and no radioactivity is stably bound to the protein (expt 1, 2, and 3). The same results are obtained with these two substrates when the time of incubation with cyanide is decreased to 2 min, extended to 120 min, or when the concentration of pyruvate is increased to 200 μmoles/0.2 ml. However, essentially all enzymatic activity is lost when the aldolase is incubated with cyanide plus [^{14}C]glyoxylate and radioactivity is bound to the enzyme in the ratio of one mole of glyoxylate to 120,000 g of protein (expt 4). This inactivation of KHG-aldolase activity and binding of [^{14}C]glyoxylate cannot be reversed by dilution or exhaustive dialysis. Experiments 5 and 6 show that when KHG-aldolase is initially treated with unlabeled glyoxylate or pyruvate in the presence of borohydride and subsequently incubated with cyanide plus [^{14}C]glyoxylate, no radioactivity is bound to the enzyme. Likewise, no radioactivity is bound to the enzyme if the aldolase is first incubated with cyanide in the presence of unlabeled glyoxylate and then treated with either ^{14}C-labeled pyruvate or glyoxylate plus borohydride (expt 7 and 8).

Discussion

Other investigators have shown that the active sites of FDP-aldolase (Horecker, 1959; Grazi *et al.*, 1962a,b; Horecker *et al.*, 1963), KDPG-aldolase (Grazi *et al.*, 1963; Ingram and Wood, 1965), and 2-deoxyribose 5-phosphate aldolase (Grazi *et al.*, 1963; Hoffee *et al.*, 1965; Rosen *et al.*, 1964) contain lysyl residues; in the condensation reaction, the one substrate capable of forming a carbanion (dihydroxyacetone phosphate, pyruvate, and acetaldehyde, respectively) is activated by Schiff-base formation with the ϵ-amino group of a lysyl residue in the enzyme molecule. Pure KHG-aldolase

TABLE III: Inhibition of Aldolase Activity and Substrate Binding after Cyanide Treatment.[a]

Experiment	1	2	3	4	5	6	7	8
Additions to aldolase	NaCN	[^{14}C]KHG + NaCN	[^{14}C]Pyruvate + NaCN	[^{14}C]Glyoxylate + NaCN	Pyruvate + $NaBH_4$; then [^{14}C]glyoxylate + NaCN	Glyoxylate + $NaBH_4$; then [^{14}C]glyoxylate + NaCN	Glyoxylate + NaCN; then [^{14}C]pyruvate + $NaBH_4$	Glyoxylate + NaCN; then [^{14}C]glyoxylate + $NaBH_4$
Enzymatic activity lost (%)	0	0	9	95	100[b]	100[b]	94[c]	92[c]
Radioactivity bound[c]	0	0	0.05×10^6	4.60×10^6	0.13×10^6	0.13×10^6	0.26×10^6	0.66×10^6
Substrate bound[d,f]	0	0	<0.01	0.98	0.03	0.03	0.04	0.14

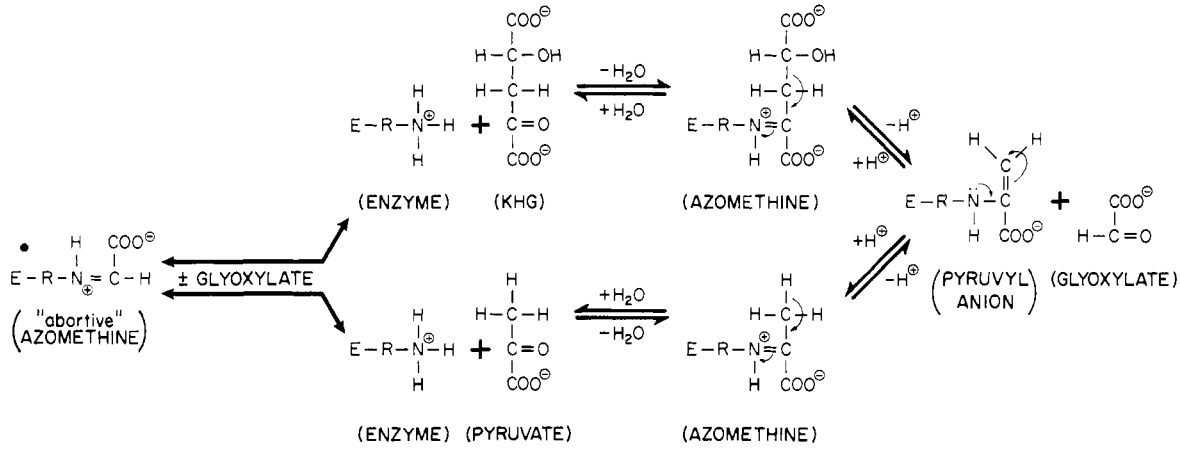
[a] The reaction mixtures (0.20 ml) contained the following components (in micromoles), as indicated: 50, potassium phosphate buffer, pH 7.4; 1.26, [2-^{14}C]KHG; 0.725, sodium [1-^{14}C]pyruvate; 1.14, sodium [1-^{14}C]glyoxylate; 9, sodium pyruvate; and 9, sodium glyoxylate. Purified aldolase (0.22 mg; specific activity 150) was used uniformly. Borohydride reductions were carried out as described in Table I. Each reaction mixture was treated with 0.1 ml (8 μmoles) of NaCN solution for 15 min at 25°. The enzyme was then precipitated, washed, and dissolved in buffer as outlined in Table I. The resulting solutions were dialyzed against 0.05 M Tris-HCl buffer (pH 7.4) containing 0.005 M 2-mercaptoethanol for 24 hr. Aliquots of these dialyzed solutions were used to determine aldolase activity, protein content (Murphy and Kies, 1960; Lowry *et al.*, 1951), and levels of radioactivity. Under the conditions of this experiment, no radioactivity was stably bound and no enzymatic activity lost when KHG-aldolase was incubated alone with either [^{14}C]-KHG, [^{14}C]pyruvate, or [^{14}C]glyoxylate. [b] KHG-aldolase activity determined after $NaBH_4$ treatment in the presence of unlabeled substrate. [c] KHG-aldolase activity determined after NaCN treatment in the presence of unlabeled glyoxylate. [d] The values shown were calculated on the basis of micromoles of enzyme inactivated. [e] cpm/120,000 μg of protein. [f] μmoles/120,000 μg of protein.

of bovine liver also forms an azomethine linkage with its substrate (KHG, pyruvate, or glyoxylate), as demonstrated by loss of enzymatic activity after reduction of the enzyme–substrates imines with borohydride and concomitant binding of radioactive substrate to the protein. Furthermore, actual isolation of the anticipated secondary amine N^6-lysine derivatives of pyruvate and glyoxylate, after reduction of the corresponding Schiff-base intermediates, indicates that the binding of substrates by this enzyme also involves the ϵ-amino group of a lysyl residue (or residues) in the KHG-aldolase molecule. In these respects, therefore, KHG-aldolase shares properties common with the other aldolases mentioned.

The feature which is unique to bovine liver KHG-aldolase, and which has also been found to be true for KHG-aldolase from rat liver (Rosso and Adams, 1966, 1967), is that it not only binds as a Schiff base that substrate which is able to form a carbanion, namely, pyruvate, but also glyoxylate. Proposal of a mechanism for the reaction catalyzed by KHG-aldolase analogous to those for FDP-, KDPG-, and 2-deoxyribose 5-phosphate aldolase (Horecker, 1965; Meloche and Wood, 1964a) calls for a sequence of events as outlined in Scheme I. Enzymatic attack of KHG (or pyruvate) involves the ϵ-amino group of a lysyl residue in the protein molecule yielding an azomethine intermediate. In the cleavage reaction, the KHG-lysyl(Enz)azomethine rearranges with elimination of glyoxylate and formation of an enzyme-bound pyruvyl anion. Similarly, in the condensation reaction, the pyruvyl-lysyl(Enz)azomethine yields the enzyme-bound pyruvyl anion by loss of a proton. The pyruvyl anion can then either accept a proton yielding pyruvate (cleavage reaction) or react with glyoxylate yielding KHG (condensation reaction). Binding of glyoxylate by an ϵ-amino group of a lysyl residue in the protein molecule is not required by such a mechanism; yet, this is what we observe experimentally. A complete scheme of the enzymatic events that occur in the KHG-aldolase-catalyzed process, therefore, must include a reversible equilibrium between the aldolase, glyoxylate, and the corresponding azomethine.

What, then, is the significance of glyoxylate binding by this aldolase? No definitive answer can be given on the basis of currently available data. Rosso and Adams (1967) presented some of the alternatives that are relevant to this question and we comment briefly in this regard only for completeness. Certainly, as noted above, there is no mechanistic requirement for the binding of glyoxylate as an azomethine to a lysyl residue in the enzyme in order to bring about a condensation of pyruvate with glyoxylate. Secondly, there appears to be little basis for the concept that glyoxylate controls KHG-aldolase activity. This seems to be particulary true for KHG-aldolases from liver. High concentrations (above 6 mM (Rosso and Adams, 1967)) of glyoxylate have been observed to inhibit the condensation reaction with pyruvate. We have, however, never detected any significant quantity of free glyoxylate in a large number of homogenates prepared from livers of a variety of species. The analytical methods used would have responded positively to levels of glyoxylate as low as 0.02–0.03 mM. Since the equilibrium constant for KHG-aldolase from rat liver and bovine liver is 75–95% in favor of KHG cleavage (Maitra and Dekker, 1964; Rosso and Adams, 1967; Kobes and Dekker, 1969), it would also appear more desirable to examine the effects of varying concentrations of glyoxylate on KHG cleavage rather than on the condensation of glyoxylate with pyruvate; apparently, this has not been done. Possibly, a concept of glyoxylate controlling KHG-aldolase activity is more relevant to plants

SCHEME I: Proposed Mechanism of Action for KHG-Aldolase



where a very measurable level of free glyoxylate in crude homogenates is frequently encountered.[2] In this same context, there is also some indication that high glyoxylate concentrations (8 or 12 mM) may have an indirect effect on the binding of pyruvate by rat liver KHG-aldolase (Rosso and Adams, 1967), but here, too, there is no apparent reason why glyoxylate should be uniquely bound as an azomethine in order to act as a modifier.

A third possibility is that KHG-aldolase not only catalyzes the reversible cleavage of KHG but also effects an internal oxidation–reduction of KHG whereby carbon-2, originally having the carbonyl function, is reduced to a secondary alcohol, and carbon-4 originally having the hydroxyl functional group, is oxidized to the carbonyl level. Enzyme binding sites to hold both ends (or both pieces) of the KHG molecule could then be utilized. This possibility appears to be eliminated by studies already published (Maitra and Dekker, 1964), in which we used partially purified rat liver KHG-aldolase. Were this oxidation–reduction process to occur, some portion (or all) of carbons-1 and -2 of KHG would form glyoxylate and also some of carbons-3, -4, and -5 would be released as pyruvate. We found, however, that incubation of the aldolase with either [5-^{14}C]- or [2-^{14}C]KHG yielded only [^{14}C]glyoxylate or [^{14}C]pyruvate, respectively.

We are left with the remaining idea, which we briefly indicated we favored earlier (Kobes and Dekker, 1966), that glyoxylate is bound nonspecifically by virtue of being an analog of pyruvate and its binding by the enzyme may not be physiologically significant. This proposal appears valid in view of our finding that a large number of aldehydes and keto acids (other than glyoxylate) appear to be nonspecifically bound *via* azomethine linkages to KHG-aldolase[3] and that the binding of all substrates seems to occur at the same site or sites.

There can be little doubt that pyruvate and glyoxylate are *both* bound by bovine liver KHG-aldolase to the ϵ-amino group of one or more lysyl residues in the protein molecule. This is shown by isolation, after acid hydrolysis, of the respective radioactive adducts formed after treatment of ^{14}C substrate–aldolase mixtures with borohydride. In all solvent systems tried, the isolated radioactive compound obtained from aldolase treated with [^{14}C]pyruvate or [^{14}C]-glyoxylate has the same paper chromatographic mobility as chemically synthesized N^6-(1′-carboxyethyl)lysine or N^6-(carboxymethyl)lysine, respectively.

There also appears to be fairly strong evidence that both glyoxylate and pyruvate (as well as KHG) are bound at the same site (lysyl residue) or sites in the enzyme molecule. Competitive binding of substrates in the presence of borohydride may be cited as first evidence. Data presented previously (Kobes and Dekker, 1966) and also outlined in Table I show that, in the presence of borohydride, the addition of one unlabeled substrate (pyruvate, glyoxylate, or KHG) to the aldolase at the same time or before adding a different radioactive substrate greatly dilutes or completely prevents the binding of radioactivity to the enzyme. Furthermore, competitive binding data of essentially the same kind are obtained when Schiff-base intermediates are not inactivated by borohydride reduction but rather by cyanide addition (Table III). The second form of supporting evidence comes from analysis of radioactive peptides released after tryptic digestion of the ^{14}C substrate–aldolase complexes. The observation that, under the conditions employed, two radioactive peptides of identical chromatographic and electrophoretic behavior are liberated when either the [^{14}C]pyruvate–enzyme adduct or the [^{14}C]glyoxylate–enzyme adduct is digested would also appear to indicate that both substrates are bound at the same site or sites on the enzyme. Normally, if these two substrates were bound at different sites, a nonidentical peptide pattern would be expected. Results obtained by peptide mapping of this sort, however, must be viewed with some caution since, by this criterion alone, it is still possible that glyoxylate and pyruvate are bound at two different sites which are very similar in amino acid environment. In such a case, the peptides released by tryptic digestion would be very difficult to distinguish by the method employed and whether or not there is a single unique site for the substrates of KHG-aldolase must await the sequencing of the individual fingerprint peptides. Nevertheless, the release of apparently identical radioactive peptides after tryptic digestion of the individual enzyme–substrate adducts is consistent with the binding of the substrates at the same site or sites.

Another reasonably defined feature of KHG-aldolase, from bovine as well as from rat liver (Rosso and Adams, 1967), is that the ratio of substrate bound per 120,000 g of protein is the same for KHG, pyruvate, and glyoxylate (see Tables I and III of this paper and Table III of Kobes and

[2] E. E. Dekker, unpublished data.

[3] R. D. Kobes and E. E. Dekker, details to be published.

Dekker, 1966). All data obtained to date in our laboratories, wherein substrate is stably bound to the enzyme by borohydride reduction of the azomethine, have been consistent with 1:1 stoichiometry (moles of substrate bound per mole (120,000 g) of enzyme) for any one of the three substrates. When a different method is used to stabilize the Schiff-base intermediate, *i.e.*, cyanide addition, the same binding ratio is obtained for glyoxylate. A ratio of 1:1 is also obtained when the quantitative binding of formaldehyde by bovine liver KHG-aldolase in the presence of borohydride is determined (Lane and Dekker, 1968). We hasten to add, however, that this may not necessarily represent the absolute number of binding sites in the molecule but merely a minimum value. The changing picture over the years regarding the actual number of binding sites per mole of FDP-aldolase (Grazi *et al.*, 1962a; Horecker *et al.*, 1963; Kobashi *et al.*, 1966) naturally engenders caution in this regard; final conclusions on this point for KHG-aldolase must await further determinations of the binding ratio under a variety of experimental conditions and with as many different substrates as possible.

If the assumption is made that there is one binding site per mole (120,000 g) of enzyme, why are two radioactive peptides released after tryptic digestion of either the reduced [^{14}C]pyruvate- or [^{14}C]glyoxylate–enzyme complex? Obviously, two radioactive peptides are more compatible with two binding sites, a point relevant to the discussion in the preceding paragraph. Other possibilities, however, should also be considered. Two different radioactive peptides could be formed by incomplete digestion by trypsin of the reduced enzyme–substrate complexes. This complication does not seem likely here in view of the low (5%) and high (50%) trypsin concentrations used and times of incubation with trypsin varying from 4 to 24 hr; certainly, this is not pertinent to the fact that apparently *identical* peptides are formed when the separate enzyme–substrate complexes (for glyoxylate and pyruvate), reduced with borohydride, are digested under the same experimental conditions.

Another alternative, considering together the binding competition studies, the magnitude of the binding ratio (1 or higher), and the two radioactive peptides, is that there are two binding sites which are juxtaposition or mutually exclusive with respect to each other. In this instance, both sites will accept either glyoxylate or pyruvate, but as soon as one site is filled by any of the substrates, the other site is completely masked and unavailable. Substrate binding competition and a 1:1 binding ratio would then be experimentally observed. If random binding of a given substrate by either of these two sites occurs (such that some glyoxylate is present on site 1 *and* site 2, for example) and each of these sites has a different surrounding primary structure, two radioactive peptides could conceivably be released when the individual ^{14}C substrate–aldolase complexes are digested by trypsin.

Another feature unique to bovine liver KHG-aldolase, in addition to its binding glyoxylate as well as pyruvate and KHG in the presence of borohydride, is that enzymatic activity is lost and radioactivity is stably bound *only* when the aldolase is treated with cyanide and glyoxylate (Table III). Other investigators (Cash and Wilson, 1966; Fridovich and Westheimer, 1962; Westheimer, 1963; Brand and Horecker, 1968) previously observed enzyme inactivation in the presence of cyanide with FDP-aldolase, acetoacetate decarboxylase, and transaldolase. In every instance, this inactivation is believed to be due to the addition of cyanide to the double bond of the imine formed between the substrate and the ϵ-amino group of a lysyl residue in the protein molecule yielding an inactive aminonitrile. The chemical addition of cyanide to imines is well known (Stewart and Li, 1938). In the case of the three enzymes just mentioned, however, cyanide inactivation occurs only in the presence of substrates capable of forming a carbanion.

In conclusion, the demonstration that KHG-aldolase functions *via* a Schiff-base mechanism further supports its designation as a class I, rather than a class II, aldolase (classification according to Rutter (1964, 1965)). This is in agreement with other class I aldolase properties noted before (Kobes and Dekker, 1969) for KHG-aldolase. Also, the results obtained with bovine liver KHG-aldolase and with rat liver KHG-aldolase (Rosso and Adams, 1967) provide additional support for the general concept that all class I aldolases form Schiff-base intermediates involving the ϵ-amino group of lysyl residues.

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On the Nature of the Lactic Dehydrogenase–Oxidized Coenzyme–Pyruvate Complex*

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ABSTRACT: Complexes of the type chicken heart lactic dehydrogenase–oxidized coenzyme–pyruvate have been obtained with DPN and with a number of DPN analogs. These complexes were stabilized by reduction with $NaBH_4$ and isolated on a Sephadex column. The complexes showed a marked decrease in enzymatic activity which was, at least in part, restored upon dilution or treatment with DPNH. *p*-Hydroxymercuric benzoate partially prevented the formation of the complexes and reacted with them more slowly than with the free enzyme. After treatment with $NaBH_4$, the coenzyme–substrate portion of the complexes could be dissociated from the enzyme by precipitating the latter with heat or acids. The enzyme–coenzyme–substrate complex could also be dissociated by treating it with snake venom phosphodiesterase. The behavior of the coenzyme–substrate complex on column and thin-layer chromatography indicates the existence of a covalent bond between the coenzyme and the substrate. The coenzyme is in 1,4-dihydro form, as shown by its behavior with phenazine methosulfate. Also the substrate is in reduced form. The coenzyme–substrate complex obtained after precipitation of the enzyme has a structure different from that of the adducts of DPN and pyruvate obtained in the absence of enzyme. A tentative structure of the coenzyme–substrate complex, consistent with the available evidence, is given.

The formation of inactive, "abortive" ternary complexes of the type LDH[1]–DPN–pyruvate has been studied by Fromm (1961). In this and in other papers (Fromm, 1963; Zewe and Fromm, 1965), it was postulated that the inhibition of the enzymatic activity of LDH's by high concentrations of pyruvate was due to the formation of these complexes.

The formation of "abortive" complexes has been further investigated by Vestling and Künsch (1968) and by Gutfreund *et al.* (1968). Kaplan *et al.* (1968) and Everse *et al.* (1970) have discussed their physiological significance. Griffin and Criddle (1970) have shown that the formation of the ternary complex LDH–DPN–pyruvate involves the monomeric unit of the enzyme.

The isolation of complexes of the type CHLDH–DPN–pyruvate and CHLDH–DPN–pyruvate ($NaBH_4$) has been described by Di Sabato (1968b). In the present paper these observations have been extended and some details of the structure of the coenzyme–substrate portion of these complexes are presented.

Materials and Methods

The chemicals used in this work were obtained from the following sources; Boehringer, Mannhein Co. (DPN, DPNH, TPN), PL Biochemicals Co. (APDPN, DeAPDPN, PADPN, DePADPN, THDPN), Sigma Chemical Co. (NMN, PHMB, PMS, 2,4-dinitrophenylhydrazine), Nutritional Biochemical Co. (sodium pyruvate and sodium borohydride). [2-^{14}C]Pyruvate, [^{14}C]DPN (DPN labeled on the carboxamide group), [^{3}H]DPNAdH (DPN labeled on the adenine ring) and [$^{3}H_4$]-$NaBH_4$ were obtained from New England Nuclear. [4-^{3}H]DPN (DPN labeled in position 4 of the nicotinamide ring) was obtained from Amersham/Searle.

CHLDH was a gift of Dr. N. O. Kaplan. It was prepared according to Pesce *et al.* (1964). Rabbit muscle LDH, DPNase from *Neurospora crassa*, and phosphodiesterase from snake venom were purchased from Worthington Biochemcial Co.

In a typical experiment, suitable amounts of enzyme, coenzyme, and substrate (Di Sabato, 1968b) were incubated in 0.1 M sodium phosphate buffer, pH 6.90, for about 1 hr at room temperature. In the early stages of this work, the incubation time of the sample was about 12–14 hr. These

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[1] The following abbreviations were used in this paper: LDH, lactic dehydrogenase; CHLDH, chicken heart lactic dehydrogenase; APDPN, 3-acetylpyridine analog of DPN; DeAPDPN, desamino-3-acetylpyridine-DPN; PADPN, 3-pyridinecarbaldehyde analog of DPN; DePADPN, desamino-3-pyridinecarbaldehyde-DPN; THDPN, thionicotinamide-DPN; PHMB, *p*-hydroxymercuric benzoate; PMS, phenazine methosulfate. The composition of the complex followed by ($NaBH_4$) indicates that the complex has been treated with $NaBH_4$.