BEEF LIVER 2-KETO-4-HYDROXYGLUTARATE ALDOLASE: SUBSTRATE STEREOSPECIFICITY AND SCHIFF BASE FORMATION WITH PYRUVATE AND GLYOXYLATE*

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Aldolases are of vital importance in carbohydrate and amino acid metabolism. Several of these enzymes have been highly purified and studied in considerable detail, such as fructose 1,6-diphosphate aldolase (Horecker, 1959; Grazi et al., 1962; Horecker et al., 1963), 2-keto-3-deoxy-6-phosphogluconate aldolase (Grazi et al., 1963; Ingram and Wood, 1965) and 2-deoxyribose-5-phosphate aldolase (Hoffee et al., 1965). Results obtained with such aldolases indicate that they have, as group characteristics, strict optical isomer specificities, no metal ion requirements, and a common mechanism involving the formation of an azomethine linkage between one substrate (capable of forming a carbanion) and an ϵ-amino group of a lysyl residue in the protein molecule.

2-Keto-4-hydroxyglutarate (KHG) aldolase catalyzes the reversible cleavage of KHG, an intermediate in hydroxyproline breakdown by animals, into pyruvate and glyoxylate. In previous studies, this enzyme was partially purified from rat liver extracts by Kuratomi and Fukunaga (1963), by Maitra and Dekker (1964), and more extensively by Rosso and Adams (1966). The latter investigators reported that in the presence of $NaBH_4$ the rat liver enzyme forms N^6-lysine derivatives with both substrates, glyoxylate and pyruvate.

We have recently purified KHG-aldolase from beef liver extracts to near homogeneity. Among other properties, we report here the novel ability of this purified enzyme to cleave both optical isomers of KHG, its ability to bind (via Schiff base intermediates) 1 mole of either glyoxylate or of pyruvate per mole of enzyme, and the absence of a requirement for divalent metal ions.

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EXPERIMENTAL AND RESULTS

Materials and Methods - 1-^{14}C-Pyruvate and 1-^{14}C-glyoxylate were obtained from Nuclear Chicago Corp. Protein was estimated by the method of Lowry et al. (1951); aldolase activity was determined by measuring the formation of glyoxylate from DL-KHG (Maitra and Dekker, 1964). One unit of enzyme activity is defined as the amount that liberates 1.0 μmole of glyoxylate in 20 minutes.

KHG-Aldolase - The enzyme was purified 1300-fold from beef liver extracts by the procedures outlined in Table I (starting with 1 kg of frozen liver). Electrophoresis of Fraction VI on cellulose polyacetate (Sepraphore III) showed a single major and single minor stainable band; enzymic activity was associated only with the major band. When this same fraction was applied to and eluted from Sephadex columns (G-100 and G-200), a single symmetrical protein peak which coincided with aldolase activity was obtained. Fraction VII, prepared with an increase in specific activity, showed two protein bands when subjected to electrophoresis on either cellulose polyacetate (Sepraphore III) or disc polyacrylamide gel. These two bands were of essentially equal concentration and of very similar mobility (just resolvable) under the conditions employed. Ultracentrifugation of Fraction VII at 42,040 rpm for 174 min revealed a single protein peak with an s-value of 4.0 Svedbergs. The molecular weight of the enzyme was estimated to be 120,000 by Sephadex gel-filtration (Andrews, 1965).

TABLE I

Purification of KHG-aldolase

	Fraction	Protein	Specific Activity	Total Units	Recovery
		mg/ml	units/mg		%
I.	Crude extract	64.0	0.11	16,870	100
II.	Heated to 70° C	6.9	1.2	13,760	82
III.	$(NH_4)_2SO_4$, 20-38%	17.0	12	7,600	45
IV.	DEAE cellulose eluate	0.16	71	4,750	28
V.	Calcium phosphate-cellulose eluate	2.3	77	4,650	27
VI.	$(NH_4)_2SO_4$, 0-40%	8.5	100	3,160	19
VII.	DEAE-cellulose (eluted with 0.1 M pyruvate)	4.5	145	650	4

Optical Isomer Specificity and Metal Ion Requirement - As shown in Table II, highly purified beef liver aldolase shows a unique nonstereospecific character in that it cleaves both optical isomers of KHG. A slight preference for the L-isomer would seem to be indicated, but a very significant degree of reactivity is observed in both cases.

TABLE II

Specificity of Aldolase for Isomers of KHG

Compound Tested	Initial Concentration	Glyoxylate Formed
	μmoles/ml	μmoles/mg protein
DL-KHG	2.5	135
DL-KHG	5.0	135
L-KHG	2.5	140
L-KHG	5.0	130
D-KHG	2.5	117
D-KHG	5.0	112

DL-KHG, L-KHG, and D-KHG were prepared by the nonenzymatic transamination of threo-Y-hydroxy-DL-glutamate (Y-HG), threo-L-Y-HG, and erythro-L-Y-HG, respectively, according to the procedure of Maitra and Dekker (1963). The convention is being continued here (cf. Goldstone and Adams, 1962; Maitra and Dekker, 1964) that D-KHG→D-malic acid and L-KHG→L-malic acid by oxidative decarboxylation. Fraction VII (0.5 μg protein) was used as enzyme in these experiments.

The purified enzyme (Fraction VII) has no apparent divalent metal ion requirement; preincubation of dialyzed enzyme (Fraction VII) with Mg^{++}, Ni^{++}, Co^{++}, Zn^{++}, Cu^{++} or Ca^{++} (0.005 M, final concentration) followed by usual assay of the enzyme resulted in no stimulation of activity. In addition, a prior incubation of the purified aldolase with either ethylene diaminetetraacetate, 8-hydroxyquinoline, or 1,10-phenanthroline (0.003 M final concentration) had no effect on its catalytic activity.

Substrate-Aldolase Binding - For KHG-aldolase, one would expect that pyruvate but not glyoxylate would be bound to the enzyme via an azomethine linkage analogous to that formed by dihydroxyacetone phosphate with fructose 1,6-diphosphate aldolase, by pyruvate with 2-keto-3-deoxy-6-phosphogluconate aldolase, and by acetaldehyde in the case of 2-deoxyribose-5-phosphate aldolase. When tested in this regard, KHG-aldolase

showed no loss of activity when incubated with either pyruvate or glyoxylate alone (Exp. 1,2) or when treated with $NaBH_4$ alone (Exp. 3) (Table III). In the absence of borohydride reduction, negligible ^{14}C-pyruvate or ^{14}C-glyoxylate was bound to the precipitated enzyme (Exp. 1,2). KHG-aldolase was completely inactivated, however, when treated with $NaBH_4$ in the presence of either ^{14}C-pyruvate or ^{14}C-glyoxylate and radioactivity was bound to the enzyme in the ratio of 1 mole of substrate to 120,000 gm of protein (Exp. 4,5). A tentative estimate of the number of binding sites, assuming homogeneity of the enzyme, would be one (for pyruvate or for glyoxylate) per mole of enzyme. Exp. 6 shows that adding both ^{14}C-substrates concomitantly to the enzyme in the presence of $NaBH_4$ does not result in the binding of an additive amount of radioactivity by the aldolase. The presence of one substrate unlabeled and the other labeled with carbon-14 dilutes the total amount of radioactivity incorporated (Exp. 7,8). Exps. 9 and 10 involve two steps. The purified enzyme is first incubated for 30 minutes with either unlabeled glyoxylate or pyruvate plus $NaBH_4$. The data show that when this "enzyme" is subsequently incubated with ^{14}C-pyruvate or ^{14}C-glyoxylate, respectively, in the presence of $NaBH_4$, essentially no radioactivity is bound to the aldolase.

DISCUSSION

These findings with purified beef liver KHG-aldolase support the general concept for the mechanism of action of aldolases as involving the formation of Schiff base complexes between the ϵ-amino group of lysyl residues in the enzyme and the carbonyl group of a substrate. Results reported in this regard for the beef liver enzyme are also in complete accord with those reported recently by Rosso and Adams (1966) for rat liver KHG-aldolase. Both rat liver and beef liver KHG-aldolase, however, are uniquely distinct in that each enzyme binds <u>either</u> one of its two substrates (glyoxylate or pyruvate) via azomethine linkages which are stabilized by reduction with $NaBH_4$. The simplest explanation for the results of Experiments 6-10 (Table III) is that pyruvate and glyoxylate are bound at the same site (lysyl residue) in the enzyme molecule. Most likely, the binding of pyruvate is mechanistically necessary for the enzyme-catalyzed reaction; glyoxylate binding, on the other hand, is possibly competitive in nature by virtue of being an analog of pyruvate (analog binding is also seen with 2-keto-3-deoxy-6-phosphogluconate aldolase; Ingram and Wood, 1966).

The finding that highly purified KHG-aldolase shows no optical isomer specificity is without precedence in the study of aldolases. This observation suggests that in the enzyme-catalyzed cleavage of KHG,

TABLE III

Enzymic Activity and Substrate Binding after $NaBH_4$ Treatment

Exp.	Additions to Enzyme	Per cent of Initial Enzymic Activity	Radioactivity Bound	Substrate Bound
			cpm/120,000 μg protein	moles/120,000 gm protein
1.	^{14}C-Pyruvate	102	0.02×10^6	<0.01
2.	^{14}C-Glyoxylate	107	0.20×10^6	0.04
3.	$NaBH_4$	96	0	0
4.	^{14}C-Pyruvate + $NaBH_4$	3	6.30×10^6	0.97
5.	^{14}C-Glyoxylate + $NaBH_4$	2	4.74×10^6	1.01
6.	^{14}C-Pyruvate + ^{14}C-Glyoxylate + $NaBH_4$	2	6.85×10^6	----
7.	^{14}C-Pyruvate + Glyoxylate + $NaBH_4$	2	1.15×10^6	0.18
8.	Pyruvate + ^{14}C-Glyoxylate $NaBH_4$	2	2.87×10^6	0.61
9.	Glyoxylate + $NaBH_4$; then ^{14}C-Pyruvate + $NaBH_4$	8	0.36×10^6	0.05
10.	Pyruvate + $NaBH_4$; then ^{14}C-Glyoxylate + $NaBH_4$	15	0.47×10^6	0.10

The reaction mixtures (0.33 ml) contained the following components (in μmoles), as indicated: 50, potassium phosphate buffer, pH 6.3; 0.629, sodium 1-^{14}C-pyruvate (6.51×10^6 cpm/μmole); 0.885, sodium 1-^{14}C-glyoxylate (4.69×10^6 cpm/μmole); 2.15, sodium pyruvate; 2.98, sodium glyoxylate. Purified enzyme (Fraction VII, 0.58 mg) was used uniformly. Each reaction mixture, except 1 and 2, was treated at 4° C with four 0.01 ml portions of 1 M $NaBH_4$ added alternately with four 0.005 ml portions of 2 M CH_3COOH over a period of 30 minutes. The enzyme was then precipitated with 185 mg of $(NH_4)_2SO_4$, centrifuged, and washed 2 times with 1 ml of 80% saturated $(NH_4)_2SO_4$ solution. The washed precipitates were dissolved in 0.4 ml of 0.05 M Tris-HCl buffer, pH 7.4, plus 0.005 M 2-mercaptoethanol, and the resulting solutions tested for aldolase activity. The enzyme was then precipitated 2 more times with 0.4 ml of 10% TCA at 4° C and each time redissolved in 0.4 ml of 0.75 N NaOH solution. The protein content of this final solution was then determined and aliquots were plated for radioactivity measurements. Levels of radioactivity were determined with a Nuclear Chicago thin-window gas flow counter.

two stereochemically different carbanions of the enzyme-pyruvate complex are formed.

The properties of beef liver KHG-aldolase, here reported, indicate that this enzyme is a Class I and not a Class II aldolase (classification according to Rutter, 1964). The former group of enzymes is characterized by no divalent metal ion requirement and the formation of Schiff base intermediates that are inactivated by $NaBH_4$ treatment. Class II enzymes, in contrast, are metalloproteins whose catalytic activity is severely inhibited by chelating agents; the catalytic mechanism of these aldolases may not involve Schiff base formation but rather utilize the divalent metal ion, instead of lysine, as an electrophile.

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