SUBSTRATE BINDING TO HYDROXYKETOGLUTARATE ALDOLASE

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4-Hydroxy-2-ketoglutarate (HKG), a product of hydroxyproline degradation in animal tissues (Kuratomi and Fukunaga, 1960; Goldstone and Adams, 1962; Maitra and Dekker, 1963), can also be formed reversibly from glyoxylate and pyruvate, as first reported by Kuratomi and Fukunaga (1960). HKG aldolase, which catalyzes the latter reaction, has been partly purified from rat liver by Kuratomi and Fukunaga (1963) and Maitra and Dekker (1964). An unusual feature of this reaction is its apparent nonspecificity for cleavage or formation of the HKG antipodes (Adams and Goldstone, 1963; Maitra and Dekker, 1964), although the possibility of a separate racemase has not been excluded.

In several aldolases (Horecker et al, 1961; Grazi et al, 1962; Grazi et al, 1963), azomethine-linkage with an enzyme-lysine selectively involves that condensing substrate which would form a carbanion in the condensation. Pyruvate rather than glyoxylate might therefore be expected to complex with a lysine of HKG aldolase. We report here that both glyoxylate and pyruvate can be recovered from enzyme hydrolysates as N^6-lysine derivatives after treatment of enzyme-substrate mixtures with $NaBH_4$. In addition we describe the synthesis and isolation of the reference compounds, N^6-(carboxymethyl)-lysine and N^6-(1'-carboxyethyl)-lysine.*

EXPERIMENTAL

HKG Aldolase - Enzyme was purified 600-800 fold from rat-liver acetone-

*To our knowledge, chemical synthesis of this compound has not previously been reported, although Dr. L. Hellerman has informed us privately that the compound was also recently prepared by a similar method in his laboratory.

powder extracts by a procedure summarized in Table I for a specific preparation from 600 gm of fresh rat liver. The first three steps were like those of Kuratomi and Fukunaga (1963). Succeeding steps involved first, filtration through Sephadex G-200 (the entire $(NH_4)_2SO_4$ fraction, brought up in 25 ml of .05 M Tris, pH 8.4, was passed through a 3 x 90 cm column and washed off with the same buffer), and second, adsorption of the appropriate Sephadex fractions on a 2.2 x 60 cm DEAE column and elution with 0.04 M potassium phosphate, pH 7.4

Polyacrylamide gel electrophoresis of the purified enzyme revealed several faint bands and a single major band representing most of the stainable protein. The major band corresponded both to the position from which enzyme activity was eluted, as well as radioactivity (after labeling enzyme with ^{14}C-glyoxylate in the presence of $NaBH_4$ as described below). The molecular weight of the enzyme was estimated at 117,000 both by sucrose gradient centrifugation (Martin and Ames, 1961) and Sephadex gel-filtration (Andrews, 1965).

Table I

Purification of HKG Aldolase

Fraction	Total Units*	Total Protein	Specific Activity
		mg	units/mg
Acetone Powder Extract	16,700	94,300	0.18
Supernatant after heating(67°,4')	8,700	10,600	0.82
$(NH_4)_2SO_4$, 0.30-0.45	8,200	1,140	7.2
Sephadex G-200	10,700	300	36
DEAE	7,700	64	120

*1 unit is defined by formation of 1 μmole of glyoxylate in 15 minutes; assay conditions and glyoxylate measurement were essentially those of Maitra and Dekker (1964).

Substrates - DL-HKG was made chemically by the method of Ruffo et al (1962) and isolated by a modification of the procedure described earlier (Goldstone and Adams, 1962). 1-^{14}C-Glyoxylate and 1-^{14}C-pyruvate were purchased from Nuclear-Chicago.

N^6-(carboxymethyl)-lysine - This was prepared both by alkylation of poly-L-lysine (Mann Research Laboratories) with iodoacetate (Gundlach et al, 1959), and also by reduction of the Schiff-base formed between glyoxylate and poly-

lysine. In the second procedure, polylysine (15 mg) was incubated with 0.5 mmoles of glyoxylate (100°, 15 minutes, 2 ml volume adjusted to pH 8.0 with NaOH). The reaction mixture was chilled to 0°, adjusted to pH 6.0 (acetic acid) and treated with 1 mmole of $NaBH_4$. Unreacted glyoxylate was removed by dialysis from the substituted polylysine and the latter was hydrolyzed in acid to obtain the N^6-substituted products.

Products were separated on a Dowex-1-formate column (1 x 25 cm): free lysine was washed from the column with water while presumptive N^6-(monocarboxymethyl)-lysine was eluted with a linear gradient in formic acid (0 to 6 N), appearing at about 0.5 N formic acid; a second peak, presumably N^6-(dicarboxymethyl)-lysine, appeared only after the first synthetic method and was eluted at about 1 N formic acid. The material eluted at 0.5 N formic acid was purified by Dowex-50 H^+ chromatography and behaved identically (solvents of Table III) when made by either method. Identity of the product obtained by the two independent methods served to verify each as N^6-(carboxymethyl)-lysine.

N^6-(1'-carboxyethyl)-lysine - Polylysine (60 mg) was treated with 2.5 mmoles of methyl-2-bromopropionate (pH 9, 27°, 12 hours, 4 ml volume). The reaction mixture was dialyzed against water, the dialysate was lyophilized, hydrolyzed in acid, evaporated, and adsorbed on Dowex 50 H^+ (1 x 25 cm). On elution with a linear gradient to 4 N HCl, free lysine appeared first and a second ninhydrin-positive peak appeared at about 2 N HCl. Material in the second peak was further purified by paper electrophoresis at pH 1.8 (formic-acetic acid). Its identification as N^6-(1'-carboxyethyl)-lysine was based on a comparison (Table III) with the radioactive hydrolysis product of the reduced complex of crystalline 2-keto-3-deoxy-6-phosphogluconate aldolase with ^{14}C-pyruvate (Ingram and Wood, 1965). The enzyme-derived compound had earlier been identified by conversion to authentic N^6-(1-hydroxypropyl)-lysine (Ingram and Wood, 1965).

The reference compounds were synthesized both with 1-^{14}C-glyoxylate or 1-^{14}C-pyruvate and with unlabeled precursors. Yields were low (30% or less) but in the radioactive syntheses the products described above accounted for

essentially all the incorporation of glyoxylate or pyruvate into polylysine.

Complex Formation with HKG Aldolase - The data of Table II indicate inactivation of HKG aldolase on treatment with $NaBH_4$ after either glyoxylate or pyruvate addition. After enzyme inactivation, significant radioactivity from either ^{14}C-glyoxylate or ^{14}C-pyruvate (relative to controls lacking $NaBH_4$ or enzyme) was bound to the protein, as determined both by dialysis and Sephadex gel-filtration.

Table II

Inactivation of Enzyme and Binding of Substrates after Borohydride

Purified enzyme (0.6 mg) was incubated for 5 minutes at 37° with 1.5 μmoles of 1-^{14}C-pyruvate (9 x 10^6 cpm/μmole) or 1-^{14}C-glyoxylate (1.9 x 10^6 cpm/μmole) in 2 ml of 13 mM Tris (pH 8.4). Mixtures were chilled to 0° and treated with 2.2 μmoles of $NaBH_4$ added in portions during 25 minutes, the pH being kept at about 6 with dilute acetic acid. Enzyme was assayed in aliquots before (initial units) and immediately after $NaBH_4$ addition (final units); all mixtures were then dialyzed against cold distilled water until the dialysis fluid contained negligible radioactivity, following which residual radioactivity of the dialysates was measured. In these assays 1 unit is defined by the formation of 1 μmole of pyruvate in 15 minutes at 37° and pH 8.5, by coupling HKG cleavage to DPNH oxidation catalyzed by excess lactate dehydrogenase.

Incubation Mixture	Total Enzyme Units		Total ^{14}C after dialysis
	Initial	Final	
			c.p.m.
Enzyme, 1-^{14}C-pyruvate, $NaBH_4$	65	0	51,280
minus $NaBH_4$	65	65	11,300
minus 1-^{14}C-pyruvate	65	50	-
Enzyme, 1-^{14}C-glyoxylate, $NaBH_4$	72	16	16,760
minus $NaBH_4$	62	62	4,120
minus 1-^{14}C-glyoxylate	72	60	-

Identification of Enzyme Complexes - After $NaBH_4$ treatment, aliquots of the substrate-enzyme incubation mixtures were dialyzed, lyophilized and hydrolyzed (6 N HCl, 110°, 20 hours). The hydrolysates were passed through Dowex 50 H^+ and eluted with 2 N NH_4OH; eluates containing radioactivity were concentrated and compared by paper chromatography and electrophoresis with the unlabeled synthetic reference compounds described above.

Table III shows R_f values for the ninhydrin-detected reference compounds, cochromatographed with ^{14}C-hydrolysis products derived from the enzyme-substrate

complexes. The radioactive compound isolated after hydrolysis of the reduced ^{14}C-pyruvate-HKG aldolase complex migrated identically with unlabeled chemically-synthesized N^6-(1'-carboxyethyl)-lysine in the solvents shown. Radioactivity from ^{14}C-pyruvate introduced into 2-keto-3-deoxy-6-phosphogluconate aldolase by the borohydride method also chromatographed identically with the unlabeled synthetic reference compound. In addition, the labeled compounds obtained by hydrolyzing the reduced ^{14}C-pyruvate complexes of either aldolase moved identically with each other and with the unlabeled reference compound on electrophoresis at pH 1.8 (formic-acetic acid) and pH 9.7 (bicarbonate).

Similar observations were made in comparing the radioactive compound obtained by hydrolyzing the reduced ^{14}C-glyoxylate-HKG aldolase complex with synthetic N^6-(carboxymethyl)-lysine. Radioactivity migrated with the synthetic reference compound on the solvents shown in Table III as well as on electrophoresis at pH 1.8 (formic-acetic acid), pH 5.7 (pyridine-acetic acid) and pH 9.7 (bicarbonate).

Table III

Cochromatography of Synthetic Reference Compounds with ^{14}C-Pyruvate or ^{14}C-Glyoxylate Derivatives of HKG

Solvent	N^6-(carboxymethyl)-lysine*	N^6-(1'-carboxyethyl)-lysine**
	R_f	R_f
Acetone-0.5% urea (60:40)	0.56	0.50
t-Butanol-formic acid-water (70:15:15)***	0.41	0.27
Pyridine-water (13:7)	0.20	0.48
n-Butanol-ethanol-3NNH_3 (2:1:2)	0.34	0.72
Phenol-water (80:20), NaCN	--	0.72

* Cochromatographed with radioactive hydrolysis product from 1-^{14}C-glyoxylate-HKG aldolase complex, $NaBH_4$-treated.

** Cochromatographed with radioactive hydrolysis product from 1-^{14}C-pyruvate-HKG aldolase complex and from 1-^{14}C-pyruvate complex with 2-keto-3-deoxy-6-phosphogluconate aldolase, $NaBH_4$ treated.

***Descending chromatography; all others ascending.

DISCUSSION

The foregoing observations show that both pyruvate and glyoxylate can separately form a Schiff-base complex with the 6-amino group of one or more lysine

in HKG aldolase, demonstrated by inactivation of the enzyme on treatment with $NaBH_4$ and by the subsequent isolation of the expected lysine-N^6-secondary amines. Failure to detect significant ^{14}C in other forms suggested that these were the major or exclusive bound forms of either substrate under the conditions used.

The unexpected binding of glyoxylate to an enzyme lysine raises the question if this represents an obligatory step in the enzymatic reaction. Observations made to date suggest that glyoxylate is bound by the same lysine(s) normally occupied by pyruvate, and that glyoxylate binding may therefore represent competition for a pyruvate site rather than a required step. Our preliminary findings indicate that that quantity of enzyme reductively inactivated with unlabeled pyruvate or glyoxylate is incapable of binding radioactivity from subsequently-added glyoxylate or pyruvate respectively. Further, maximal binding to enzyme of either substrate leads to incorporation of about the same molar quantity per mg of protein, although glyoxylate concentration must be 5 to 10 times that of pyruvate for saturation. Competitive glyoxylate binding to a pyruvate site in HKG aldolase would imply substrate inhibition by glyoxylate in the reaction forming HKG; such an apparent effect has been reported (Kuratomi and Fukunaga, 1963). More direct information should come from a comparison of the lysine-containing peptides which bind glyoxylate or pyruvate, to determine if the adjacent sequence suggests that these are the same or different lysines.

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