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Substrates of Hydroxyketoglutarate Aldolase

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The substrate specificity of the condensation reaction catalyzed by rat liver 4-hydroxy-2-ketoglutarate aldolase has been investigated. It was found that an enzyme-mediated condensation between glyoxylate and several "activated" carbonyl compounds could be performed. Two classes of these "activated" carbonyls were tested—the first of which are pyruvate analogs differing by substitution at C-3, whereas the second include some C-1 analogs of pyruvate as well as other simple carbonyl compounds. The possible synthetic uses of such a system are discussed as well as possible insights into the structure of the active site of this enzyme.

INTRODUCTION

The enzyme, 4-hydroxy-2-ketoglutarate aldolase (EC 4.1.3.16), catalyzes the reversible cleavage of 4HKG,[1] an intermediate in mammalian hydroxyproline catabolism, to form pyruvate and glyoxylate. The enzyme was first observed in extracts of rat liver acetone powder by Kuratomi and Fukunaga (*1*), and its role in hydroxyproline metabolism was elucidated by Goldstone and Adams (*2*), and Maitra and Dekker (*3*). The enzyme has been partially purified from several sources including *Escherichia coli* (*2*), bovine liner (*4, 5*), and rat liver (*6–8*). We have also reported (*6*) the kinetics of both the 4HKG cleavage reaction and the 4HKG synthesis reaction catalyzed by the rat liver form of 4HKG aldolase. This other investigation also explored several inhibitors of both reactions as well as the effects of ions and buffers on the isolated enzyme system. This present work reports on the substrate specificity of the condensation reaction catalyzed by this enzyme.

MATERIALS AND METHODS

[1-^{14}C]Glyoxylic acid, as the sodium salt, was obtained from Amersham (Arlington Heights, Ill.). All other scintillation supplies were purchased from Research Products International (Elk Grove, Ill.). Mercaptopyruvate was obtained from Research Organics, Inc. (Cleveland, Ohio), and acetaldehyde was

[1] Abbreviations used: 4HKG, 4-hydroxy-2-ketoglutarate; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; DTT, dithiothreitol.

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purchased from Aldrich Chemical Company (Milwaukee, Wisc.). All other chemicals and chromatography supplies were purchased, in the highest grade possible, from Sigma Chemical Company (St. Louis, Mo.).

4HKG was synthesized by the general method of Ruffo *et al.* (*9*) via a base-catalyzed aldol condensation between oxaloacetate and glyoxylate, followed by an acidic decarboxylation yielding the desired 4HKG (*6*).

Animals. Male Long–Evans rats were provided by the School of Biological Sciences, University of Nebraska-Lincoln. They were kept at 22–25°C on a 12-hr light schedule, fed Lab-Blox animal diet (Allied Mills, Inc., Chicago, Ill.), and watered *ad libitum*.

Enzyme assays. 4HKG aldolase activity was measured in the cleavage direction by a modification of the assay described by Maitra and Dekker (*10*). This assay was used in the purification of the enzyme and also in the determination of the specific activity of each enzyme fraction used in the condensation studies. In all cases, the final concentration of 4HKG used was 0.88 mM. This was the highest concentration that could be utilized without observing substrate inhibition (*6*).

Protein was determined by the method of Bradford (*11*) using Bio-Rad protein assay dye reagent concentrate and lyophilized bovine γ-globulin (Bio-Rad Laboratories, Richmond, Calif.) as the standard.

The condensation assay employed is modeled after that described by Rosso and Adams (*12*), and utilizes the fact that, when [1-^{14}C]glyoxylate condenses with pyruvate (or other compounds of interest), the glyoxylate carboxyl group becomes a peroxide-resistant carboxyl adjacent to the aldol-formed hydroxyl. Subsequent decarboxylation with H_2O_2 in an acidic solution causes the loss of $^{14}CO_2$ from unreacted glyoxylate, and leaves the only significant label incorporated into the newly formed adduct.

The assay mixture consisted of 100 mM Epps, pH 8.8, 10 mM [1-^{14}C]glyoxylate, pyruvate (or alternate substrate), enzyme extract, and water to a volume of 1 ml. After 30–90 min of incubation at 37°C, the reaction was stopped by the addition of 0.2 ml 5 N HCl, followed by 0.2 ml 20% H_2O_2. The mixture was kept at 37°C for 45 min to assure complete decarboxylation. To remove the dissolved $^{14}CO_2$ in the mixture, 0.2 ml 5 N HCl was added along with a small piece of dry ice, and the mixture was shaken intermittently for 30 min at 37°C. Finally 0.2 ml 1 M sodium carbonate was added to neutralize the mixture, and 0.9 ml of each sample was counted in 10 ml 3a70B scintillation fluid in a Beckman LS 8000 liquid scintillation counter. Several duplicates were performed in each case, as well as blanks (omitting enzyme from reaction mixture) and zero time points. Whereas the amount of blank and zero time counts varied somewhat with the concentrations of glyoxylate and pyruvate (or other substrate), the residual counts never exceeded 2–4% of the total counts added (possibly attributed to H_2O_2-resistant impurities or dimers present in the glyoxylate).

Enzyme purification. 4HKG aldolase was purified from rat liver by the following procedure, with all steps carried out at 0–4°C unless otherwise noted. Rats were sacrificed by cervical dislocation, and their livers were removed and placed in ice-cold 0.5 M potassium chloride. The livers were drained, weighed, and then placed in 1 vol 0.5 M potassium chloride, minced, and filtered through miracloth. The

minced livers were placed in 1 vol 0.5 M potassium chloride, 1 mM EDTA, and blended for 4 min on low and 1 min on high speed in a Waring Blender. This solution was next stirred with 1 vol 0.5 M potassium chloride, 50 mM Tris–Cl, pH 7.8, 5 mM DTT for 20 min. This homogenate was centrifuged for 50 min at 8300*g* in a GS-3 rotor in a Sorvall RCB-5 centrifuge. The supernatant was saved and heated while stirring in a water bath to 70°C. The solution was then cooled to 10°C in an ice bath and 1 M Tris-Cl, pH 7.8 (180 ml/kg liver), was added slowly. Heat-denatured protein was removed by centrifugation under the same conditions as described above. The supernatant was saved and brought to 40% saturation with solid ammonium sulfate. After stirring for 20 min, the precipitate was collected by centrifugation at 27,000*g* for 30 min in a Sorvall SS-34 rotor. The pellet was resuspended in a minimal amount volume of 50 mM Tris–Cl, 2.5 mM DTT, pH 7.8 (Buffer A), and frozen for at least 48 hr until a green flocculent precipitate formed. The extract was thawed and the precipitate was removed by centrifugation at low speed in a table-top centrifuge. An aliquot of this extract was dialyzed against several liters of Buffer A for 18–24 hr and then applied to a 5 × 15-cm column packed with DEAE A-50 equilibrated with Buffer A. Protein was eluted off the column with 100 ml Buffer A, followed by 200 ml 1.0 M potassium chloride in Buffer A. The eluate containing 4HKG aldolase was collected from 180 to 220 ml. This fraction contained 4HKG aldolase with specific activities in the range of 0.6 to 0.8 μmol product min^{-1} mg protein measured as described above. This purification scheme, which starts with 70 g rat liver, affords a 17% yield of 4HKG aldolase with a 2400-fold purification over the crude liver homogenate. A typical purification scheme is shown in Table 1.

RESULTS AND DISCUSSION

This investigation of the enzymatic condensation between glyoxylic acid and a host of various "activated" carbonyl compounds using 4HKG aldolase can be

TABLE 1

PURIFICATION OF RAT LIVER 4HKG ALDOLASE

Fraction[a]	Protein (mg/ml)	Specific activity (μmol min^{-1} mg)	Fold purification
Crude liver extract	86	0.00034	—
Supernatant from 8300*g*	67	0.00074	2.2
Supernatant from heat/cold	13	0.0010	26
0–40% $(NH_4)_2SO_4$	8.4	0.035	100
$(NH_4)_2SO_4$ frozen 48 hr	3.4	0.077	220
Eluate from DEAE	0.053	0.80	2400

[a] See text for an explanation of purification.

divided into two classes based on the type of carbonyl compound investigated. The first class are those analogs of pyruvic acid differing only by substitutions at C-3. The second class would then include the remaining compounds, some of which are C-1 pyruvate analogs.

The kinetic parameters K_m and V_{max} were determined for each of the compounds by measuring the amount of product formed at a constant (10 mM) concentration of glyoxylic acid with substrate concentrations from 0.5 to 20 mM. Double-reciprocal plots were used, and all data were fit with a linear weighted least-squares program. The V_{max} values measured were then adjusted for comparison purposes by dividing each by the specific activity of the enzyme preparation used in the experiment. Table 2 lists the K_m, V_{max}, and V_{max}/K_m values for the C-3 pyruvate analogs, and Table 3 lists the same information for the remaining compounds. It should be noted that some of these data are based on fitting nonlinear double-reciprocal data to the best weighted least-squares line. This was done in all the cases in lieu of a detailed kinetic analysis into the cause of the noncompliance with the Michaelis–Menten model. This detailed analysis could be further investigated, yet for the purposes of this communication our approach is justifiable.

The ramifications of such results yield insight into the possible uses for 4HKG aldolase as a synthetic tool in carbon–carbon bond formation as well as a possible structural inference regarding the active site of the enzyme. Based on the size and nature of the substituent R group, one could infer that the active site of this enzyme is not very specific or hindered by space considerations. It is known (*8*, *12*) that the enzyme forms a Schiff's base intermediate with pyruvate, and Meloche and Mehler (*13*) have postulated that this step is partially rate limiting. Meloche and Mehler (*13*) also have shown that the configuration at C-3 of pyru-

TABLE 2

KINETIC PARAMETERS FOR THE CONDENSATION OF GLYOXYLATE WITH 3-SUBSTITUTED PYRUVATE ANALOGS USING 4HKG ALDOLASE

Substrate	V_{max}[a]	K_m (mM)	V_{max}/K_m
Oxaloacetate	56	30	1.9
3-(4-Hydroxyphenyl)pyruvate[b]	8.3	7.7	1.1
3-(4-Imidazole)pyruvate[b]	5.0	7.4	0.68
3-Phenylpyruvate	7.6	24	0.32
3-Mercaptopyruvate	1.1	3.6	0.30
3-Hydroxypyruvate	1.7	2.9	0.26
3-Bromopyruvate	3.1	13	0.24
2-Ketoglutarate	1.2	7.2	0.17
2-Ketobutyrate	4.5	78	0.06
Pyruvate	1.7	25	0.07

[a] V_{max} values reported are obtained by dividing the observed V_{max} values for the condensation reactions by the specific activity of the enzyme preparation utilized.

[b] Does not adhere to Michaelis–Menten model. Data are fit to best least-squares line through the curve. (See text for explanation.)

TABLE 3

KINETIC PARAMETERS FOR THE CONDENSATION OF GLYOXYLATE WITH LISTED COMPOUNDS USING 4HKG ALDOLASE

Substrate	V_{max}[a]	K_m (mM)	V_{max}/K_m
Pyruvaldehyde	4.9	5.1	0.97
Pyruvic acid: methyl ester[b]	2.4	3.2	0.75
Pyruvic acid: ethyl ester[b]	2.1	2.8	0.75
Acetaldehyde	1.6	10	0.16
Pyruvic acid	1.7	25	0.07

[a] V_{max} values reported are obtained by dividing the observed V_{max} values for the condensation reactions by the specific activity of the enzyme preparation utilized.

[b] Does not adhere to Michaelis–Menten model. Data are fit to best least-squares line through the curve. (See text for explanation.)

vate is retained during C–C synthesis using the bovine 4HKG aldolase, whereas the condensation products formed give a racemic mixture at C-4. They postulate a mechanism in which the pyruvyl-enolate is formed and then attacked by a randomly oriented glyoxylate. One explanation that they give, that can be supported by our findings, is that the active site is very near the surface of the molecule and the pyruvyl-enolate can be attacked by either face of an aldehyde in solution. This model is supported by the apparent nonspecificity this enzyme exhibits for the aldehyde receptor (*5, 12, 14, 15*), but is unattractive in the sense that the stereochemistry of C–C bond formation is random. A second possibility—presently under investigation in this laboratory—is that larger, substituted aldehydes will not exhibit the random addition to the pyruvyl-enolate that the small, unsubstituted glyoxylate shows. Regardless of the mechanistic basis for the condensation reaction, these results indicate that 4HKG aldolase could be used to catalyze a wide variety of aldol condensations. The nature of the reacting carbonyl compounds (see Tables 2 and 3) indicate that this enzyme could be used to form C–C bonds with many different functional groups adjacent to the newly formed bond. This makes this enzyme an attractive tool for future organic synthesis.

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