Purification, Substrate Specificity and Binding, β-Decarboxylase Activity, and Other Properties of Escherichia coli 2-Keto-4-hydroxyglutarate Aldolase

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SUMMARY

2-Keto-4-hydroxyglutarate aldolase has been purified to homogeneity from extracts of Escherichia coli grown on nutrient broth. Values of 62,500 and 64,000, respectively, are obtained for the molecular weight of the enzyme by the procedures of gel filtration and sucrose density gradient centrifugation. The aldolase has a pH optimum of 8.6 and does not appear to require divalent metal ions for activity. p-Mercuriphenylsulfonate, iodoacetate, and N-ethylmaleimide partially inhibit enzymatic activity.

The aldolase from this source preferentially cleaves or forms the L isomer of 2-keto-4-hydroxyglutarate. When the enzyme is tested with a number of compounds as substrate, it catalyzes the cleavage of only 2-keto-4-hydroxybutyrate and oxalacetate besides 2-keto-4-hydroxyglutarate. A higher degree of specificity is manifested for binding analogs of pyruvate than of glyoxylate, as determined by the extent of enzyme inactivation when the aldolase is incubated with analog compounds in the presence of NaBH₄.

Extensive loss of aldolase activity occurs when the enzyme is incubated with either pyruvate or glyoxylate in the presence of NaBH₄, and 1 mole of ¹⁴C-labeled substrate is stably bound per mole of enzyme. Competition studies indicate that pyruvate, glyoxylate, and 2-keto-4-hydroxyglutarate all bind at the same active site of the aldolase. Incubation of 2-keto-4-hydroxyglutarate aldolase with glyoxylate in the presence of cyanide also causes irreversible loss of enzymatic activity, suggesting the formation of a stable aminonitrile with this substrate. Under these conditions, approximately 1 mole of [¹⁴C]glyoxylate is bound per mole of aldolase. Irreversible inactivation of aldolase activity by cyanide is not observed with pyruvate.

The pure aldolase from E. coli has a higher relative level of β-decarboxylase activity toward oxalacetate than does the enzyme obtained from extracts of bovine liver. Both aldolase and β-decarboxylase activities are lost when the E. coli aldolase is treated with glyoxylate or pyruvate in the presence of NaBH₄, suggesting that the same active site participates in both reactions.

2-Keto-4-hydroxyglutarate aldolase (2-oxo-4-hydroxyglutarate glyoxylate-lyase: 2-oxo-4-hydroxyglutarate = pyruvate + glyoxylate) has been shown to catalyze a terminal step in hydroxyproline degradation by mammals (1-9). Kuratomi and Fukunaga (1) first detected this aldolase in rat liver preparations in 1960. Since that time, many reports have appeared concerning the presence, purification, and properties of KHG-aldolase, as obtained from either rat liver (5, 9-11) or bovine liver (12, 13) extracts. A short note describing a 10-fold purification of this aldolase from a soil bacterium grown on α-ketoglutarate has also appeared (14). The samples of KHG-aldolase obtained from these three sources (rat liver, bovine liver, and a soil bacterium) are all similar in that they have no divalent metal ion requirement and function via a Schiff-base mechanism (i.e. are Class I aldolases, classification according to Rutter (15)). Furthermore, KHG-aldolase from these sources uniformly exhibits the unusual features of (a) being nonstereospecific for cleavage or formation of the v and L isomers of KHG (4, 9-14) and (b) binding glyoxylate, as well as pyruvate, via an azomethine linkage to the ε-amino group of the active-site lysyl residue (10-12, 16). Using purified preparations of bovine liver KHG-aldolase, we have uncovered a number of other novel and interesting properties of this enzyme, including the following. First, the detection of species having molecular weights in the order of 120,000 and 240,000 suggests that there may be monomeric and dimeric forms of the bovine liver enzyme (13). Secondly, this aldolase forms a stable adduct when incubated with glyoxylate in the presence of cyanide, which adduct has been isolated and characterized as an aminonitrile formed by cyanide addition to the double bond of the azomethine (17). Thirdly, and most striking, pure bovine liver KHG-aldolase is also an effective β-decarboxylase in that it catalyzes the loss of CO₂ from oxalacetate with formation of pyruvate at approximately 50% of the rate of KHG-cleavage (18, 19).

In view of these unique features of bovine liver KHG-aldolase, we have demonstrated that these aldolases, characterized as an aminonitrile formed by cyanide addition to the double bond of the azomethine (17). Thirdly, and most striking, pure bovine liver KHG-aldolase is also an effective β-decarboxylase in that it catalyzes the loss of CO₂ from oxalacetate with formation of pyruvate at approximately 50% of the rate of KHG-cleavage (18, 19).

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coupled with the report that KHG is an intermediate in bacterial metabolism (20) and also the phylogenetic concept presented by Rutter (15) that Class I aldolases may structurally resemble a previously existing enzyme such as a β-decarboxylase, we decided to attempt to isolate KHG-aldolase in homogeneous form from bacteria in order to compare its properties and role with the mammalian enzyme. In this report, we describe a method for preparing pure KHG-aldolase from extracts of *E. coli* K-12 and some of its properties. When *E. coli* KHG-aldolase is compared with the mammalian enzyme, it is strikingly different in molecular size, in substrate stereospecificity, and in its decarboxylase activity toward oxaloacetate. Brief reports of some of these findings have appeared (21, 22).

## EXPERIMENTAL PROCEDURE

### Materials—

The syntheses of three-γ-hydroxy-DL-glutamic acid and of DL-KHG have been described before (7, 8). L- and D-KHG were prepared by the nonenzymatic transamination of three-γ-hydroxy-L-glutamate and erythro-γ-hydroxy-L-glutamate, respectively (7); the convention is followed that L-malic acid is formed from L-KHG and D-KHG yields α-malic acid by oxidative decarboxylation (4, 8). The three- and erythro isomers of γ-hydroxy-L-glutamate were prepared enzymatically by reductive amination of DL-KHG with NADH and NH₄⁺ in the presence of glutamate dehydrogenase and subsequent resolution of the two diastereoisomers of the hydroxymino acid by use of a column of Dowex 1 (acetate) resin (23). 2-Keto-4-hydroxybutyrate was prepared from homoserine (24). The method of Shannon and Marcus (25) for the acid-catalyzed aldol condensation of pyruvic acid was followed to synthesize 2-keto-4-hydroxy-4-methylglutaric acid. Acetoacetate was obtained by alkaline hydrolysis of ethyl acetoacetate (26).

Sodium [1-14C]pyruvate and sodium [1-14C]glyoxylate were purchased from Nuclear Chicago Corporation. The following compounds were gift samples: 2-ketoglutarate, 5-ketogluconate, and 5-keto-4-deoxygluconate from Dr. W. A. Wood (Michigan State University); 2-keto-3-deoxyglactonate from Dr. W. Shuster (Case Western Reserve University); 2-keto-4,5-dihydroxyvalerate from Dr. R. Abeles (Brandeis University). The barium salt of 2-deoxyribose 5-phosphate (Calbiochem) was converted to the potassium salt prior to use by passing the solution over a column of Dowex 50 (K⁺) resin and washing the resin with water.

Sephadex G-100, G-200, and blue dextran were purchased from Pharmacia. DEAE cellulose (0.06 meq per g), ovalbumin, glyceroldehyde 3-phosphate dehydrogenase, and alcohol dehydrogenase were purchased from Sigma Chemical Co. Special enzyme grade ammonium sulfate, crystalline bovine serum albumin, and chymotrypsin were obtained from Mann Research Labs. Lactate and glutamate dehydrogenases were products of Calbiochem whereas catalase was from Worthington Biochemical Corp. All other compounds and reagents were of the best quality commercially available.

*E. coli* K-12 was grown at 37° in a medium of 2.5% nutrient broth (Difco Labs.); cells were harvested in late log phase and kept frozen until used. Frozen cell pastes obtained commercially (late log phase cells grown on nutrient broth; Grain Processing Corporation, Muscatine, Iowa) were also used. Extracts prepared from cells obtained in either manner had similar protein content and KHG-aldolase activity.

### Methods—

Protein concentrations were routinely measured by the method of Lowry et al. (27) with crystalline bovine serum albumin as a standard; in some cases, particularly in the assay of column eluates, it was estimated by absorbance at 280 nm and 260 nm (28). Glyoxylic acid was determined as previously described (7). The method of Friedemann and Haugen (29) was used as a general procedure to measure the concentrations of keto acids. Quantitative ninhydrin determinations were performed according to the method of Rosen (30).

Polyacrylamide gel electrophoresis was routinely carried out with 7.5% gels in glycine-Tris buffer, pH 9.3, for 1 hour according to the procedure of Davis (31). Electrophoresis at pH 7.5 was performed with 7.5% gels in Veronal buffer for approximately 2.7 hours, with slight modifications of the method described by Williams and Reisfeld (32). An adaptation of the procedure of Reisfeld et al. (33) was used to carry out electrophoresis for 2.5 hours at pH 4.3 in alanine-acetate buffer with 15% gels. In each case, 5 μl of current were applied per gel.

Sucrose density gradient centrifugation was performed according to a modification of the technique of Martin and Ames (34). Samples (0.2 ml) were layered on linear gradients (12.0 ml) prepared with 5 and 20% (w/v) sucrose solutions in 0.05 M Tris-HCl buffer, pH 7.4, containing 5 mm 2-mercaptoethanol. After centrifuging for 18 hours at 39,000 rpm at 3° in a Beckman model L2 65B centrifuge (SW 40 swinging bucket rotor), the centrifuge tubes were perforated at the bottom with a needle and the sucrose solutions passed through a flow cell in a Gilford spectrophotometer which continuously recorded absorbance at 280 nm; fractions (0.7 ml) were subsequently collected for enzyme assays. The enzymatic activities of catalase and lactate dehydrogenase were measured by following the decrease in absorbance at 240 nm (35) and at 340 nm (36), respectively.

Radioactivity measurements were made by placing aliquots of solutions on planchets, drying the samples either with a heat lamp or by placing them in vacuo over calcium chloride for 3 hours, 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.

### Enzyme Assay—

KHG-aldolase activity was determined by colorimetric estimation of glyoxylate (7). One unit of enzyme activity is defined as the amount of protein that liberates 1.0 μmole of glyoxylate in 20 min at 37°. Specific activity is expressed as units per mg of protein.

For substrate specificity studies, enzymatic assays were performed as follows. KHG-aldolase activity was determined at pH 8.1 and 25° with 10 mM DL-KHG or 5 mM L-KHG as substrate. The reaction mixtures for this purpose contained 100 μmoles of Tris-HCl buffer, pH 8.1, 5 μmoles of GSH, and 70 pg of lactate dehydrogenase and NADH oxidation; the decrease in absorbance at 340 nm was followed: 2-ketoglutarate, 2-keto-4-hydroxy-4-methylglutarate, 2-ketovalerate, 2-keto-4,5-dihydroxyvalerate, 2-keto-2,3-deoxyglactonate, 5-keto-4-deoxygluconate, and oxaloacetate. The reaction mixtures (1.0 ml) contained the following components (in micromoles): Tris-HCl buffer, pH 8.1, 100; NADH, 0.49; substrate analog, 10; 70 μg of lactate dehydrogenase; and 21 μg of purified aldolase.

The release of formaldehyde, determined by the chronometric acid method (37), was used to measure the cleavage of 2-keto-4-hydroxybutyrate. Purified aldolase (10 to 20 μg) was incubated...
with 100 μmoles of glycylglycine buffer, pH 8.1, 5 μmoles of GSH, and 10 μmoles of 2-keto-4-hydroxybutyrate in a final volume of 1.0 ml for 20 or 30 min at 25°. The reaction was terminated by adding 0.5 ml of 12% metaphosphoric acid, and aliquots of the resulting mixture were taken for analysis. The decarboxylation of acetoacetate was measured by a modification of the method of Fridovich and Westheimer (38). The reaction mixtures (1.0 ml) contained 100 μmoles of potassium phosphate buffer, pH 6.0, 15 μmoles of acetoacetate, and 21 μg of purified aldolase. The decrease in absorbance at 270 nm was followed.

The cleavage of isocitrate was determined by a modified assay for isocitrate lyase (39). KHG-aldolase, 21 or 42 μg, was incubated at 25° for 20 min with 100 μmoles of Tris-HCl buffer, pH 7.4, 5 μmoles of GSH, 3 μmoles of MgCl₂, and 4 μmoles of isocitrate in a final volume of 1.0 ml. The reaction was stopped by adding 0.4 ml of 12% metaphosphoric acid; the resulting solution was assayed for glyoxylate (7).

The formation of glyceraldehyde 3-phosphate from fructose 1,6-diphosphate was determined by incubating 21 μg of purified aldolase, 66 pmoles of L-threonine in a volume of 1.0 ml. The reaction was started by adding 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.4, 15 pmoles of acetoacetate, and 5 pmoles of NADH, 60 μg of alcohol dehydrogenase, and 10 pmoles of either 2-deoxyribose or 2-deoxyribose 5-phosphate in a final volume of 1.0 ml. The amount of acetaldehyde produced was measured at 340 nm after the mixture was incubated at 25°. Likewise, the liberation of acetaldehyde from threonine was coupled with alcohol dehydrogenase (41). The incubation mixtures contained 100 μmoles of potassium phosphate buffer, pH 7.4, 0.5 μmoles of NADH, 60 μg of alcohol dehydrogenase, and 10 μmoles of either 2-deoxyribose or 2-deoxyribose 5-phosphate in a final volume of 1.0 ml. The amount of acetaldehyde produced was observed by following the decrease in absorbance at 340 nm at 25°. Likewise, the liberation of acetaldehyde from threonine was also coupled with alcohol dehydrogenase (41). The incubation mixtures contained 100 μmoles of potassium phosphate buffer, pH 7.4, 0.01 μmol of pyridoxal phosphate, 5 μmoles of NADH, 0.15 mg of alcohol dehydrogenase, 91 μg of KHG-aldolase, and 66 μg of L-threonine in a volume of 1.0 ml. In all of these experiments, routine controls included an incubation mixture lacking aldolase and another lacking substrate.

**RESULTS**

**Purification of Enzyme from E. coli K-12**

KHG-aldolase was purified from extracts of E. coli K-12 by the following procedure; all operations were carried out at 4° except Step 3.

**Step 1—**Frozen cells (423 g, wet weight) of E. coli K-12 were thawed overnight in the cold room and then suspended in 1,000 ml of 0.05 M potassium phosphate buffer, pH 7.4, containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA. All of the buffers used in the following steps contained the same components. The cell suspension was divided into two equal portions, and each portion was subjected to sonication. Sonication was carried out for a total of 10 min at a power setting of 5 using a Branson Sonifier, model MS2 (Branson Instruments, Danbury, Connecticut). The temperature of the cell suspension was kept below 10° during this process. The two portions of crude extract were then combined and an additional 690 ml of buffer-mercaptoethanol-EDTA mixture added. The extract was stirred overnight, then centrifuged at 90,000 × g for 30 min, and the precipitate discarded.

**Step 2—**Crystalline ammonium sulfate was added slowly with stirring to the supernatant fluid until the salt concentration was 50% saturation (303 g per liter); the pH of the solution was adjusted as necessary to 7.4 with 5 N NH₄OH. The mixture was stirred for an additional 2 hours and centrifuged at 20,000 × g for 30 min; the precipitate was discarded. Crystalline ammonium sulfate was then added with stirring to the supernatant fluid until the salt concentration was 80% saturation (230 g additional per liter of original volume). This solution was stirred for 2 hours, after the pH had been adjusted to 7.4, and subsequently centrifuged at 20,000 × g for 30 min. The precipitate obtained was dissolved in about 150 ml of buffer mixture, and the resulting solution was dialyzed against three changes (4 liters each) of the same buffer for a period of 24 hours. Any precipitate which formed during dialysis was removed by centrifuging at 20,000 × g for 30 min.

**Step 3—**The dialyzed enzyme solution was divided into 15-ml portions in test tubes. Each portion was heated in a 90° water bath, the fluid contents being stirred continuously with a thermometer until the temperature reached 80°. Thereafter, the tube was immediately plunged into an ice-water bath and stirring was continued until the temperature dropped below 30°. The precipitate of denatured protein was removed by centrifugation and discarded.

**Step 4—**Ninety-two milliliters (0.041 ml per mg of protein) of a 1% solution of neutralized protamine sulfate were added dropwise with stirring to the supernatant fluid obtained in Step 3. The turbid solution was stirred for an additional 20 min and then centrifuged at 20,000 × g for 30 min.

**Step 5—**Crystalline ammonium sulfate was added slowly with stirring to the resulting supernatant fluid until the salt concentration was 52% saturation (317 g per liter); if necessary, the pH was adjusted to 7.4 with 5 N NH₄OH. The mixture was stirred for an additional 2 hours and centrifuged at 20,000 × g for 30 min. The precipitate was discarded. Crystalline ammonium sulfate was again added slowly to the supernatant fluid until the salt concentration was 80% saturation (230 g per liter of original volume). After the pH of the solution was adjusted to 7.4 with 5 N NH₄OH, it was stirred for an additional 2 hours and then centrifuged at 20,000 × g for 30 min. The precipitate obtained was dissolved in a minimal volume of the phosphate buffer mixture, and the resulting solution dialyzed against several changes (4 liters each) of the same buffer for a total period of 24 hours. This solution was then concentrated to approximately 15 ml by ultrafiltration and dialyzed overnight against 2 liters of the same phosphate buffer mixture. If any precipitates occurred during dialysis or ultrafiltration, they were removed by centrifuging at 20,000 × g for 30 min and were discarded.

**Step 6—**The dialyzed enzyme solution was then applied to a column (3.5 × 65 cm) of Sephadex G-100 which had been equilibrated with the 0.05 M potassium phosphate buffer mixture. The column was washed with the same buffer solution at a flow rate of approximately 25 ml per hour; 10-ml fractions were collected. Those fractions (tubes 25 to 30) containing enzyme solution having the highest specific activity were pooled and concentrated by ultrafiltration for 24 hours. The resulting solution was dialyzed overnight against 2 liters of 0.005 M potassium phosphate buffer, pH 7.4. Any precipitates that formed either during
ultrafiltration or dialysis were removed by centrifugation as before.

Step 7—A DEAE-cellulose column (2.3 x 27 cm) was packed under 5 pounds of pressure and equilibrated with 0.005 M potassium phosphate buffer, pH 7.4. The enzyme solution from the preceding step was applied to the column; the column was subsequently washed first with 400 ml of 0.005 M and then with 400 ml of 0.017 M potassium phosphate buffer, pH 7.4. Finally, the enzyme was eluted with 0.030 M potassium phosphate buffer, pH 7.4. The flow rate was maintained at approximately 36 ml per hour and 5-ml fractions were collected. Those fractions (tubes 95 to 110) with highest specific activity were pooled and immediately subjected to concentration by ultrafiltration. The concentrated enzyme solution (about 1.0 ml) was then dialyzed overnight against 2 liters of 0.05 M Tris-HCl buffer, pH 7.4. This procedure provides KHG-aldolase which is over 2000-fold purified; the results obtained in a typical run are summarized in Table 1.

We have found this procedure to be highly reproducible in more than 10 different preparations. It can be successfully scaled up to process 900 g (wet weight) of frozen cells by changing the size of the Sephadex column (Step 6) to 3.5 x 92 cm. The final enzyme preparation, which is water-clear, loses 40 to 50% of its original activity by storage at 4°C for 1 month. The enzyme was eluted with 0.030 M potassium phosphate buffer, pH 7.4. The flow rate was maintained at approximately 36 ml per hour and 5-ml fractions were collected. Those fractions (tubes 95 to 110) with highest specific activity were pooled and immediately subjected to concentration by ultrafiltration. The concentrated enzyme solution (about 1.0 ml) was then dialyzed overnight against 2 liters of 0.05 M Tris-HCl buffer, pH 7.4.

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pH 7.4, containing no 2-mercaptoethanol; this process of dialysis caused about a 10% decrease in specific activity of the enzyme (Table II). No significant requirement for added thiol compounds could be demonstrated. Some recovery (specific activity = 75 to 79) of the original activity was obtained by subsequent incubation of the dialyzed enzyme with low concentrations (1 \times 10^{-3} M to 1 \times 10^{-2} M) of either glutathione, 2-mercaptoethanol, or dithiothreitol; thiomalate and thioglycolate at these concentrations had essentially no effect. As shown in Table II, the most effective sulphydryl-reacting compound was p-mercuriphenylsulfonate which, at 1 \times 10^{-3} M concentration, inhibited enzymatic activity 56%. A high concentration (0.05 M) of iodoacetate caused almost complete inhibition, whereas N-ethylmaleimide (2.5 \times 10^{-3} M) had little effect on E. coli KHG-aldolase activity. The inhibition caused by 1 \times 10^{-4} M p-mercuriphenylsulfonate was only partially prevented by a prior incubation of the enzyme with either 5 \times 10^{-4} M 2-mercaptoethanol or 5 \times 10^{-4} M reduced glutathione. Likewise, the inhibitory effect is only partially reversed by the addition of these sulhydryl compounds (5 \times 10^{-3} M) after the enzyme is first treated with 1 \times 10^{-4} M p-mercuriphenylsulfonate. Only suggestive evidence, therefore, can be gathered indicating the requirement of a free sulphydryl group or groups for the bacterial enzyme to be catalytically active.

Tests for the effect of metal ions and chelating agents on KHG-aldolase activity were carried out with enzyme (1.6 \mu g) that was first exhaustively dialyzed against 0.05 M Tris-HCl buffer, pH 7.4, containing 5 mM 2-mercaptoethanol and 1 mM EDTA, and then exhaustively dialyzed against the same buffer solution containing no EDTA. The bacterial aldolase apparently does not require any metal ion for its catalytic activity; preliminary incubation of the dialyzed enzyme with the following cations (1 \times 10^{-4} M or 5 \times 10^{-5} M, final concentration, all tested as their chloride salts) has no effect on aldolase activity: Mg++, Mn+++, Zn++, Cu++, Ba++, Co++, Cu++, Ni++, Fe++, Hg++, K+ or Fe++. Furthermore, a prior incubation of the enzyme with either EDTA, 1,10-phenanthroline, 8-hydroxyquinoline, or \alpha,\alpha'-dipyridyl (at concentrations of 10^{-4} M, 10^{-3} M, and 3 \times 10^{-3} M) has no effect on its catalytic activity.

**Substrate Specificity and Binding Studies**

**Substrate Stereospecificity**—KHG-aldolase from E. coli utilizes L-KHG as substrate much more effectively than it does the D isomer. The rates of reaction as a function of substrate concentration are shown in Fig. 3. Results of the type shown were obtained reproducibly with several different enzyme preparations and also with many different substrate solutions. The apparent Michaelis constants, calculated from Lineweaver-Burk double reciprocal plots (43) of these data, are 2.3 \times 10^{-3} M, 2.5 \times 10^{-2} M, and 4.2 \times 10^{-3} M for L-KHG, D-KHG, and DL-KHG, respectively.

![Fig. 3](https://example.com/fig3.png)

**Table II**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final concentration</th>
<th>Specific activity (units/mg)</th>
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<tbody>
<tr>
<td>None</td>
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<tr>
<td>p-Mercuriphenylsulfonate</td>
<td>0.001, 0.05, 1.00</td>
<td>72, 43, 32</td>
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<tr>
<td>N-Ethylmaleimide</td>
<td>0.01, 0.10, 1.00</td>
<td>80, 81, 71</td>
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<tr>
<td>Iodoacetate</td>
<td>1.00, 5.00, 10.00</td>
<td>68, 39, 17</td>
</tr>
</tbody>
</table>

* Enzymatic activity prior to exhaustive dialysis was 82 units per mg.

![Fig. 3](https://example.com/fig3.png)
tively, whereas the corresponding \(V_{\text{max}}\) values are 7.90, 1.53, and 6.65 (pmoles of glyoxylate formed per min per mg of protein). Since this preferential cleavage of one of the optical isomers of KHG by the \(E.\ coli\) enzyme is in sharp contrast with all other KHG-aldolase preparations so far examined, a two-step experiment was performed to determine how much of each of the two isomers is formed by the aldolase-catalyzed condensation of \(\text{[14C]}\)glyoxylate with pyruvate. For this purpose, the KHG formed initially is subsequently converted enzymatically to the \(\text{erythro}\) and \(\text{threo}\)-isosomers of \(\gamma\)-hydroxy-L-glutamate which diastereoisomers can be resolved by ion exchange column chromatography (44); slight modifications of the procedure described before (9) were used. Calculation of the ratio of \(L\)-KHG to \(D\)-KHG on the basis of the amount of radioactivity present in each of the two amino acid peaks (i.e. \(L\)-KHG and \(D\)-KHG being formed from \(\text{threo}\) and \(\text{erythro}\)-\(\gamma\)-hydroxy-L-glutamate, respectively) gives a value of about 11.4 to 1. This ratio is in good agreement with the rates of utilization observed for the two isomers of KHG in the direction of cleavage.

The finding that the stereospecificity of the bacterial aldolase is not absolute but strongly favors the \(L\) isomer of KHG raised two possible complications—i.e. the apparent slight utilization of \(D\)-KHG could possibly be due to either a small contamination of the \(L\) isomer in the preparation of \(D\)-KHG used or the presence of racemase activity in the aldolase preparations. The formation of a significant amount of \(D\)-KHG by the aldolase-catalyzed condensation of pyruvate with glyoxylate eliminates the first possibility. The second possibility was explored by incubating 100 \(\mu\)moles of \(L\)-KHG with a large amount (56 \(\mu\)g) of pure aldolase for 20 min; thereafter, the remaining KHG was converted to the corresponding \(\alpha\)-l-amino acid by the action of L-glutamate dehydrogenase, and the \(\text{erythro}\) and \(\text{threo}\)-isosomers of \(\gamma\)-hydroxy-L-glutamate were then resolved by ion exchange column chromatography (44). No trace of the \(\text{erythro}\) isomer could be detected by quantitative ninhydrin determination, indicating no significant conversion of \(L\)-KHG to \(D\)-KHG by possible racemase activity.

Borohydride Reduction of Substrate-Aldolase Complexes; Substrate Binding Ratio—Like the bovine liver enzyme (12, 16), KHG-aldolase from \(E.\ coli\) is markedly inactivated when treated with NaBH\(_4\) in the presence of one of its substrates. Incubation of the enzyme with either \([\text{14C}]\)pyruvate or \([\text{14C}]\)glyoxylate, followed by borohydride reduction as described before (12, 16), results in 85 to 97\% loss of aldolase activity and stable binding of either radioactive substrate to the enzyme. Such results are 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 amount inactivated, the specific activity of the respective radioactive substrate, and taking 63,000 as the molecular weight of the bacterial aldolase, we calculated that essentially 1 mole (0.94 and 1.17, respectively) of either pyruvate or glyoxylate is bound per mole of pure enzyme. Competition studies indicate that all three substrates (KHG, pyruvate, or glyoxylate) are apparently bound at the same site on the enzyme. For example, the presence of one substrate unlabelled and the other labeled with carbon 14 significantly dilutes the total amount of radioactivity bound to the enzyme. Likewise, a prior incubation of the aldolase with either unlabeled pyruvate, glyoxylate or KHG, in the presence of borohydride severely blocks the subsequent binding of a radioactive cosubstrate.

Reaction of Cyanide with the Glyoxylate-Aldolase Imine—A number of enzymes known to function via Schiff base mechanisms are inactivated when treated with cyanide in the presence of a substrate capable of forming a carbanion (45–47). Kobes and Dekker (16), using bovine liver KHG-aldolase, unexpectedly noted that no inhibition of KHG-aldolase occurs when the enzyme is treated with cyanide in the presence of either pyruvate or KHG. Peculiarly, however, bovine liver KHG-aldolase is almost completely inactivated when incubated with cyanide plus its other substrate, namely glyoxylate; cyanide addition to the glyoxylate-aldolase imine yielding an inactive, stable amino-nitrite was postulated. KHG-aldolase of \(E.\ coli\) behaves similarly in this respect. Table III shows that no aldolase activity is lost when the enzyme is incubated with cyanide alone or with cyanide in the presence of \([\text{14C}]\)pyruvate and no radioactivity is stably bound to the protein (Experiments 3 and 4). However, extensive enzymatic activity is lost when the aldolase is treated with cyanide plus \([\text{14C}]\)glyoxylate and radioactivity is bound to the enzyme in the ratio of about 1 mole of glyoxylate to 63,000 \(\mu\)g of inactive protein (Experiment 5).

**Table III**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions to enzyme</th>
<th>Initial enzymatic activity</th>
<th>Radioactivity bound</th>
<th>Substrate bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>([\text{14C}])Pyruvate</td>
<td>106</td>
<td>0.19 (\times 10^6)</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>([\text{14C}])Glyoxylate</td>
<td>98</td>
<td>0.17 (\times 10^6)</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>NaCN</td>
<td>102</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>([\text{14C}])Pyruvate + NaCN</td>
<td>87</td>
<td>0.31 (\times 10^6)</td>
<td>0.02</td>
</tr>
<tr>
<td>5</td>
<td>([\text{14C}])Glyoxylate + NaCN</td>
<td>13</td>
<td>4.69 (\times 10^6)</td>
<td>0.91</td>
</tr>
</tbody>
</table>
TABLE IV

Azomethine formation with analogs of pyruvate or glyoxylate

The reaction mixtures (0.20 ml) contained 100 μmoles of potassium phosphate buffer, pH 6.0, 1 μmole of EDTA, 4 μmoles of each substrate or substrate analog, and 82 μg of KHG-aldolase (specific activity, 71). Each reaction mixture, except the controls, was treated at 60°C with four 0.010-ml portions of NaBH₄ (0.1 M in 20 mM NaOH) added alternately with four 0.005-ml portions of 0.1 M acetic acid over a period of 30 min. All of the reaction mixtures were subsequently subjected to dialysis overnight against 0.025 M potassium phosphate buffer, pH 7.4, containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA. The resulting enzyme solutions were tested for KHG-aldolase activity and protein content.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions to enzyme</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate analogs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2-Ketobutyrate</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Glyoxal</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Glycolaldehyde</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

The enzyme alone with each analog listed.

* Only 0 to 5% inactivation was observed with the following analogs: hydroxyacetone, acetone, monohydroxyacetone, dihydroxyacetone, α, β-diglyceroldehyde.

Table IV shows that the enzyme activity is not lost when the aldolase is treated with NaBH₄ alone but only when the enzyme is incubated with NaBH₄ in the presence of either pyruvate or glyoxylate. These results indi-

β-Decarboxylase Activity toward Oxalacetate

In view of the strikingly high ratio (approximately 0.50) of oxalacetate β-decarboxylase to aldolase activity found for bovine liver KHG-aldolase (19), we also tested the ability of E. coli KHG-aldolase to catalyze pyruvate formation with oxalacetate as substrate. Fig. 4 shows that the pure bacterial aldolase catalyzes the decarboxylation of oxalacetate at a rate which is proportional to the amount of enzyme added, heat-denatured enzyme does not catalyze the reaction.

Because of the unstable nature of oxalacetate under typical assay conditions (spontaneously liberating pyruvate), we attempted to measure the $V_{\text{max}}$ values for both KHG-aldolase and oxalacetate-decarboxylase activities; data so obtained were used to calculate the ratio of the two activities. Three different enzyme preparations were used in these studies; one (Preparation XI) was used 2 weeks after being purified and the other two preparations were tested immediately after being obtained in homogeneous form. Unfortunately, as shown in Table V, consistent $V_{\text{max}}$ values were not obtained with these three preparations for either oxalacetate-decarboxylase or KHG-aldolase activity (using L-KHG or D-L-KHG as substrate). The relative ratio of the two activities, however, is consistently higher in every case than that found for bovine liver KHG-aldolase. At present, it is not known whether the variability of these kinetic values is due to the nature of the E. coli cells used for purification of the enzyme (large-scale, commercially grown cells versus cells grown locally on a shaker in small batches) or to other factors; likewise, no obvious explanation is available to explain the high
TABLE VI
Oxalacetate decarboxylase activity after treatment with sodium borohydride

Reduction with sodium borohydride was carried out as described in the legend of Table IV. β-Decarboxylase activity was measured as described in the legend of Fig. 4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial aldo activity</th>
<th>Decarboxylase activity*</th>
<th>Initial decarboxylase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>A.O.D./min/μg protein</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>0.0103</td>
<td>100</td>
</tr>
<tr>
<td>NaBH₄</td>
<td>95</td>
<td>0.0103</td>
<td>100</td>
</tr>
<tr>
<td>Pyruvate + NaBH₄</td>
<td>20</td>
<td>0.0015</td>
<td>15</td>
</tr>
<tr>
<td>Glyoxylate + NaBH₄</td>
<td>2</td>
<td>0.0009</td>
<td>9</td>
</tr>
</tbody>
</table>

* Corrected by subtracting the value obtained with boiled enzyme (0.0008 per min per μg of protein). Boiled enzyme was prepared by heating at 100° for 10 min.


dicate that the two enzymatic processes, decarboxylation and aldol cleavage or condensation, are catalyzed by the same active site.

DISCUSSION

To date, essentially all work concerning the metabolic role and enzymology of KHG-aldolase has been done with rat and bovine liver preparations (4-13, 16-19). A single short paper by Aronson et al. (14) describes a 10-fold purification of KHG-aldolase from extracts of a soil bacterium grown on α-ketoglutarate as sole carbon source; the properties of this enzyme, to the extent examined, are the same as those of the liver aldolase. One objective of the present work, therefore, was to obtain KHG-aldolase in pure form from a bacterium and most desirably from a bacterium which could be used for subsequent genetic studies. This paper describes a highly reproducible procedure which yields pure KHG-aldolase from extracts of E. coli K-12. Limited studies indicate that changes in the growth medium have little effect on the level of this enzymatic activity; it would appear, therefore, that KHG-aldolase of E. coli is quite likely a constitutive enzyme.

The data presented in this paper show several similarities but also many sharp differences for pure KHG-aldolase obtained from either liver or E. coli extracts. The enzyme from either source (a) has no divalent metal ion requirement; (b) functions by a Schiff base mechanism; (c) undergoes azomethine formation with pyruvate (as anticipated mechanistically) but also forms a mechanistically "abortive" Schiff base with glyoxylate; (d) is irreversibly inactivated by cyanide only in the presence of glyoxylate (not in the presence of pyruvate or KHG); (e) binds 1 mole of any of its substrates per mole of enzyme (as determined by borohydride reduction or by cyanide addition to Schiff base intermediates); and (f) in both cases all three substrates (KHG, pyruvate, or glyoxylate) appear to be bound at the same catalytically active site. In contrast, (a) the molecular weights are 63,000 and 120,000, respectively, for KHG-aldolase from E. coli and liver; (b) the liver aldolase is nonstereospecific, catalyzing the cleavage or the formation of both optical antipodes of KHG at essentially the same rate and to the same extent, whereas the E. coli enzyme utilizes L-KHG at a rate 10 times greater than the D isomer; (c) E. coli KHG-aldolase catalyzes the aldol cleavage of fewer analogs of KHG, and (d), in terms of β-decarboxylase activity toward oxalacetate, the E. coli aldolase is considerably more effective than the enzyme from bovine liver.

It should be recalled that fructose 1,6-diphosphate aldolases appear to be of two different types (15). Those of Class I have no divalent metal ion requirement and function via a mechanism involving azomethine (Schiff base) formation; this type is found most commonly in plants and higher animals. Class II aldolases, in contrast, are metalloproteins with divalent metal catalysis and are usually found in bacteria. It is apparent, therefore, that KHG-aldolase from either a mammalian source or E. coli is related mechanistically to the Class I fructose 1,6-diphosphate aldolases.

Furthermore, this is the first report of a KHG-aldolase preparation which is highly stereospecific toward one of the two optical isomers of KHG; KHG-aldolase from all other sources tested (i.e., rat liver (9, 11), bovine liver (19), and a soil bacterium (14)) utilize both isomers equally well. The finding that KHG-aldolase from E. coli has a molecular weight approximately half that of the liver enzyme and binds 1 mole of substrate per 63,000 molecular weight again suggests that the molar ratio of substrate binding for the liver enzyme (1:1, using a mol wt of 120,000) should not be regarded as an absolute number.

The one feature of this aldolase which is especially intriguing is its high relative efficiency as a β-decarboxylase toward oxalacetate. Although the precise magnitude of the β-decarboxylase to aldolase ratio could not be determined for E. coli KHG-aldolase, it seems quite clear that it is higher than for the aldolase from bovine liver. This finding appears to be the first instance of experimental results which support the phylogenetic concept of Rutter (15) that Class I aldolases may structurally resemble a previously existing enzyme such as a β-decarboxylase. It seems apparent that having KHG-aldolase in pure form from these two sources (liver and E. coli) provides a highly interesting system for studies which correlate enzymatic properties, molecular structure, and function. Further studies are in progress.

At present and until definitive experiments are done, one can only speculate as to the function of KHG aldolase in E. coli cells. Although it is well established that this aldolase catalyzes a terminal step in the catabolism of L-hydroxyproline by mammals this enzyme quite possibly does not have the same role in bacteria. Whether or not the same results apply to E. coli is not known, but Adams and his associates (48, 49) have shown that an induced metabolic pathway for hydroxyproline degradation by Pseudomonas putida is quite different from that for mammalian hydroxyproline breakdown. On the basis of what is presently known about KHG metabolism in general, however, two other metabolic involvements of KHG can be considered. One is that KHG is a possible regulator of the tricarboxylic acid cycle; KHG has been found to inhibit enzymes of the cycle such as aconitase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (50). The other interesting possibility is that KHG functions as an intermediate in the cyclic oxidation of glyoxylate (51). As envisaged in this process, pyruvate condenses with glyoxylate yielding KHG. Dehydrogenase activity then catalyzes the oxidative decarboxylation of KHG forming malate which, in turn, is converted to oxalacetate and finally pyruvate. Since E. coli K12 KIH-aldolase has a very high level of oxalacetate β-decarboxylase activity, the one enzyme could possibly be catalyzing two necessary reactions in this cyclic process.

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Purification, Substrate Specificity and Binding, β-Decarboxylase Activity, and Other Properties of *Escherichia coli* 2-Keto-4-hydroxyglutarate Aldolase

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