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VARIANT PROPERTIES OF BOVINE LIVER 2-KETO-4-HYDROXY-GLUTARATE ALDOLASE; ITS β-DECARBOXYLASE ACTIVITY, LACK OF SUBSTRATE STEREOSPECIFICITY, AND STRUCTURAL REQUIREMENTS FOR BINDING SUBSTRATE ANALOGS

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SUMMARY

1. 2-Keto-4-hydroxyglutarate aldolase (2-oxo-4-hydroxyglutarate glyoxylate-lyase; reaction: 2-oxo-4-hydroxyglutarate ⇋ pyruvate + glyoxylate) catalyzes the cleavage and the formation of both optical isomers of 2-keto-4-hydroxyglutarate at the same rate and to the same extent.

2. The specificity of azomethine (Schiff base) formation with this enzyme was studied. Of some forty compounds tested, inactivation in the presence of $NaBH_4$ occurs (in order of decreasing effectiveness) with 2-keto-4-hydroxy-4-methylglutarate, 2-ketoglutarate, 2-keto-4-hydroxybutyrate, 2-keto-3-deoxy-6-phosphogluconate, fructose 1,6-diphosphate, 2-keto-4,5-dihydroxyvalerate, 2-keto-3-deoxygluconate, and 5-keto-4-deoxyglucarate (among 2-keto-4-hydroxyglutarate analogs); only with bromopyruvate and 2-ketobutyrate (among pyruvate analogs); and also with glyoxal, formaldehyde, acetaldehyde, and glycolaldehyde (glyoxylate analogs). In this regard, therefore, a high degree of specificity is shown for pyruvate but not for glyoxylate; this aldolase is also quite specific for analogs of 2-keto-4-hydroxyglutarate having a pyruvate-like structure on one end of the molecule.

3. 2-Keto-4-hydroxyglutarate aldolase actually catalyzes the cleavage of only 2-keto-4,5-dihydroxyvalerate, 2-keto-4-hydroxy-4-methylglutarate, 5-keto-4-deoxy-glucarate, 2-keto-3-deoxy-6-phosphogluconate, and 2-keto-4-hydroxybutyrate at 33%, 8%, 3%, 2% and 1%, respectively, the rate of 2-keto-4-hydroxyglutarate cleavage. For certain substrate analogs, therefore, there is a dissociation of azomethine formation from a concurrent cleavage of that compound.

4. In addition, this purified aldolase was found to catalyze the β-decarboxylation of oxaloacetate at 50% the rate of 2-keto-4-hydroxyglutarate cleavage.

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INTRODUCTION

Recent studies with Class I aldolases (classification according to RUTTER[1]), such as fructose 1,6-diphosphate aldolase, 2-keto-3-deoxy-6-phosphogluconate aldolase, and 2-deoxyribose 5-phosphate aldolase, suggest that they have a common mechanism involving at least four steps: (1) reversible formation of an azomethine linkage between the substrate (fructose 1,6-diphosphate, 2-keto-3-deoxy-6-phosphogluconate, or 2-deoxyribose 5-phosphate, respectively) and the ε-amino group of one or more specific lysyl residues in the enzyme, (2) concurrent proton elimination and cleavage of the azomethine yielding glyceraldehyde 3-phosphate (in all three cases) plus the carbanion of the other product (dihydroxyacetone phosphate, pyruvate, or acetaldehyde, respectively), (3) uptake of a proton by the enzyme-bound carbanion and (4) hydrolysis of the product (dihydroxyacetone phosphate, pyruvate, or acetaldehyde, respectively) from the enzyme[2-4].

The three aldolase-catalyzed reactions mentioned above are very stereospecific in that all compounds formed by condensation as well as those subject to aldol cleavage in step (2) have fixed configurations[2,5-12]. In the condensation reaction, fructose 1,6-diphosphate aldolase is completely specific for dihydroxyacetone phosphate, whereas glyceraldehyde 3-phosphate can be replaced by a large number of aldehydes[2,7]. Likewise, 2-deoxyribose 5-phosphate aldolase is highly specific for acetaldehyde (only propionaldehyde has been reported to substitute for acetaldehyde[12]), but in this instance also a variety of aldehydes can take the place of glyceraldehyde 3-phosphate[2].

Similarly, 2-keto-3-deoxy-6-phosphogluconate serves as the only substrate for significant dealdolization by 2-keto-3-deoxy-6-phosphogluconate aldolase; of a number of compounds tested, only 2-keto-4-hydroxyglutarate and 2-keto-3-deoxygluconate are cleaved at negligible rates (0.1 and $<0.1\%$, respectively)[11]. This asymmetric nature of each of the reactions catalyzed is thought to be due to the requisite enzyme which, in each case, either forms a stereospecific carbanion with one of the substrates or governs the asymmetric removal of the aldehyde from (or attack on) the enzyme-bound carbanion in step (2) of the overall reaction[2,8].

The specificity for azomethine formation in steps (1) and (4) can be examined by incubating various α-carbonyl compounds with the aldolase in the presence of $NaBH_4$ and, subsequently, determining the loss of enzymic activity; the extent of inactivation is a measure of azomethine formation. 2-Keto-3-deoxy-6-phosphogluconate aldolase was found to be relatively nonspecific in this respect forming azomethines with analogs of both pyruvate and 2-keto-3-deoxy-6-phosphogluconate[11]. The fact, however, that a number of structural analogs did form Schiff bases but were not actually cleaved was taken to indicate a separation of the process of azomethine formation from the process of carbon–carbon bond cleavage (*i.e.* supportive evidence for portions of the postulated four-step mechanism).

Proof that this type of mechanism applies not only to enzymic aldol-type reactions but also to certain enzyme-catalyzed β-decarboxylations comes from studies with acetoacetate decarboxylase. Enzymic activity is extensively lost when acetoacetate decarboxylase is incubated with acetoacetate in the presence of $NaBH_4$, and acetone (formed by decarboxylation of the substrate) was found to be bound to the ε-amino group of a lysyl residue in the stabilized substrate–protein adduct[13,14].

Cyanide addition to a combination of enzyme *plus* acetoacetate also caused inactivation of acetoacetate decarboxylase, presumably due to aminonitrile formation by virtue of cyanide adding to the azomethine linkage[13,15]. Further suggestive evidence for a common mechanism being involved in both aldol-type cleavage and β-decarboxylation reactions is the report that 2-keto-3-deoxy-6-phosphogluconate aldolase catalyzes the decarboxylation of oxaloacetate at 0.5% the rate of 2-keto-3-deoxy-6-phosphogluconate cleavage[11].

In previous papers[16,17], we reported that highly-purified (1300-fold) bovine liver 2-keto-4-hydroxyglutarate aldolase resembles Class I aldolases in a number of properties and it functions *via* a mechanism involving the formation of Schiff-base intermediates with 2-keto-4-hydroxyglutarate and pyruvate. A unique characteristic of this enzyme is that it also forms what appears to be an "abortive" azomethine with glyoxylate; 2-keto-4-hydroxyglutarate aldolase, therefore, is capable of binding as a Schiff base any one of its three normal substrates. We wish to report here studies concerning (a) the stereospecificity of purified bovine liver 2-keto-4-hydroxyglutarate aldolase toward the D- and L-isomers of 2-keto-4-hydroxyglutarate, (b) the selectivity of the enzyme for azomethine formation and dealdolization, as well as (c) the β-decarboxylase activity of this aldolase toward oxaloacetate.

MATERIALS AND METHODS

Materials

DL-2-Keto-4-hydroxyglutarate and bovine liver 2-keto-4-hydroxyglutarate aldolase (1300-fold purified; specific activity 120–150) were prepared and assayed as outlined previously[16]. *threo*-γ-Hydroxy-L-glutamic acid was isolated from 70% ethanol extracts of *Phlox decussata* leaves[18]; *erythro*-γ-hydroxy-L-glutamic acid was prepared enzymically from hydroxy-L-proline by the procedure of ADAMS AND GOLDSTONE[19,20]. L-2-Keto-4-hydroxyglutarate and D-2-keto-4-hydroxyglutarate were prepared by the nonenzymic transamination of *threo*-γ-hydroxy-L-glutamate and *erythro*-γ-hydroxy-L-glutamate, respectively, according to the procedure of MAITRA AND DEKKER[21]. The convention is being followed (*cf.* refs. 22 and 23) that D-2-keto-4-hydroxyglutarate forms D-malic acid and L-2-keto-4-hydroxyglutarate yields L-malic acid by oxidative decarboxylation. 2-Keto-4-hydroxybutyrate and 2-keto-4-hydroxy-4-methylglutarate were synthesized by published procedures[24,25] and acetoacetate was obtained by hydrolysis of ethyl acetoacetate[26]. The following materials were purchased from the companies indicated: [1-^{14}C]pyruvate from Nuclear Chicago Corporation, Des Plaines, Ill. (U.S.A.); $NaBH_4$ from Metal Hydrides, Beverly, Mass. (U.S.A.); NaCN from Mallinckrodt Chemical Works, St. Louis, Mo. (U.S.A.); sodium pyruvate, sodium glyoxylate and oxaloacetate from Nutritional Biochemicals Corporation, Cleveland, Ohio (U.S.A.); lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and glutamate dehydrogenase from Calbiochem, Los Angeles, Calif. (U.S.A.); NADH from P–L Biochemicals, Milwaukee, Wisc. (U.S.A.); and alcohol dehydrogenase from C. F. Boehringer and Sons, New York, N.Y. (U.S.A.).

Gift samples of the following compounds were generously provided as noted: 5-keto-4-deoxyglucarate, 2-keto-3-deoxy-6-phosphogluconate, 2-ketogluconate, 5-ketogluconate, methyl-2-ketogluconate, and 2-keto-L-gulonate from Dr. W. A. Wood at Michigan State University, East Lansing, Mich. (U.S.A.); *N*-acetylneuraminate

and 2-keto-3-deoxygalactonate from Dr. W. Schuster at Case Western Reserve University, Cleveland, Ohio (U.S.A.); 2-keto-4,5-dihydroxyvalerate from Dr. R. Abeles of Brandeis University, Waltham, Mass. (U.S.A.). 2-Keto-3-deoxygluconate was prepared from 2-keto-3-deoxy-6-phosphogluconate by treatment with alkaline phosphatase[27].

Methods

Protein was estimated either by the method of MURPHY AND KIES[28] or by the method of LOWRY *et al.*[29]. Spectrophotometric measurements were made at 26° with a Gilford Model 2000 automatic spectrophotometer equipped with a thermostated cuvette holder and a digital absorbance meter; the rate of formation of pyruvate due to β-decarboxylation of oxaloacetate was followed in a modified lactate dehydrogenase assay[30]. A thin window gas flow counter was used for assaying radioactive samples on planchets. Quantitative ninhydrin determinations were performed according to the method of ROSEN[31].

The ability (or inability) of 2-keto-4-hydroxyglutarate aldolase to utilize a large number of compounds as substrates was tested in a variety of ways. Cleavage of the following compounds (2-keto-4-hydroxyglutarate analogs) was measured by coupling the formation of pyruvate with lactate dehydrogenase: 2-keto-4-hydroxyglutarate, 2-keto-4,5-dihydroxyvalerate, 2-keto-4-hydroxy-4-methylglutarate, 5-keto-4-deoxyglucarate, 2-keto-3-deoxy-6-phosphogluconate, 2-ketoglutarate, 2-keto-3-deoxygluconate, 2-ketoisovalerate, 2-ketovalerate, 2-keto-3-deoxygalactonate, and *N*-acetylneuraminate. The assay mixtures contained the following components: 100 μmoles of Tris–HCl buffer (pH 8.1), 0.2 μmole of NADH, 0.025 mg of lactate dehydrogenase, 10 μmoles of substrate, and 2-keto-4-hydroxyglutarate aldolase (78 μg) in a final volume of 1.0 ml. In some cases, the mixtures were supplemented with 5 μmoles of glutathione and 5 μmoles of $MgCl_2$. The decrease in absorbance at 340 nm was measured at 26° in each case.

The aldol cleavage of 2-keto-4-hydroxybutyrate was assayed by incubating 78 μg of 2-keto-4-hydroxyglutarate aldolase with a mixture of 100 μmoles of Tris–HCl buffer (pH 8.1), 5 μmoles of $MgCl_2$, 5 μmoles of glutathione, and 10 μmoles of 2-keto-4-hydroxybutyrate in a volume of 1.0 ml for 20 min at 26°. The reaction was stopped by adding 0.4 ml of metaphosphoric acid; any formaldehyde released was determined by reaction with chromotropic acid[32]. Enzymic cleavage of fructose 1,6-diphosphate was followed by a modification of the procedure of TAYLOR[33]. These components (in μmoles) were incubated at 26° with 78 μg of 2-keto-4-hydroxyglutarate aldolase in a volume of 1 ml: fructose 1,6-diphosphate, 15; Na_3AsO_4, 17; glycine, 27; glutathione, 20; NAD^+, 0.5; and 0.05 mg of glyceraldehyde 3-phosphate dehydrogenase. An increase in absorbancy at 340 nm was taken as a measure of the amount of glyceraldehyde 3-phosphate formed by cleavage of fructose 1,6-diphosphate. Acetoacetate decarboxylation was measured at 26° by observing the decrease in absorbancy at 270 nm (ref. 15). The reaction mixture (1.0 ml) contained 100 μmoles of potassium phosphate buffer (pH 6.0), 15 μmoles of acetoacetate, and 78 μg of 2-keto-4-hydroxyglutarate aldolase. A modified assay for isocitrate lyase[34] was used to detect the cleavage of isocitrate. 2-Keto-4-hydroxyglutarate aldolase (78 μg) was incubated at 30° for 10 min with 80 μmoles of Tris–HCl buffer (pH 7.4), 10 μmoles of glutathione, 3 μmoles of $MgCl_2$, and 4 μmoles of isocitrate in a final volume of 1.0 ml. The reaction

was terminated by adding 0.4 ml of metaphosphoric acid and the reaction mixture then assayed for glyoxylate as described before[35]. Degradation of 2-deoxyribose or of 2-deoxyribose 5-phosphate with liberation of acetaldehyde was measured by incubating 78 μg of 2-keto-4-hydroxyglutarate aldolase at 26° in a volume of 1.0 ml with 100 μmoles of potassium phosphate buffer (pH 7.4), 0.2 μmole of NADH, 0.15 mg of alcohol dehydrogenase, and 10 μmoles of either one of the two substrates. The decrease in absorbancy at 340 nm was observed. Citrate cleavage was determined by incubating 78 μg of 2-keto-4-hydroxyglutarate aldolase at 26° with 170 μmoles of potassium phosphate buffer (pH 7.0), 3 μmoles of $MgCl_2$, and 10 μmoles of citrate. The amount of oxaloacetate formed in the 1.0 ml reaction mixture was measured by observing the increase in absorbancy at 285 nm, according to a modified assay procedure for citritase[36]. With L-threonine as substrate, the reaction mixture (1.0 ml) contained 100 μmoles of potassium phosphate buffer (pH 7.4), 0.01 μmole of pyridoxal 5'-phosphate, 0.2 μmole of NADH, 66 μmoles of L-threonine, 0.15 mg of alcohol dehydrogenase, and 78 μg of 2-keto-4-hydroxyglutarate aldolase. Any acetaldehyde formed at 26° was detected by following the decrease in absorbancy at 340 nm[37].

All acidic compounds used as substrates were neutralized with KOH solution just before use. Controls containing either no enzyme or no substrate were routinely included in all of these experiments. If 2-keto-4-hydroxyglutarate aldolase was found to catalyze the cleavage of any given compound, varying amounts of enzyme were tested to make sure that the rate of reaction was linear with increasing protein concentration. If no reaction was observed (*i.e.* the test substrate was not cleaved by 2-keto-4-hydroxyglutarate aldolase), a known amount of expected product was added to the reaction mixture to ensure that the assay was operational as set up.

RESULTS

Optical isomer specificity

As shown in Table I, bovine liver 2-keto-4-hydroxyglutarate aldolase is nonstereospecific in that it cleaves both optical isomers of 2-keto-4-hydroxyglutarate. A slight preference for the L-isomer is indicated, but a very significant degree of reactivity is observed in both cases. When the reaction was allowed to proceed to completion, it was observed that both optical antipodes of 2-keto-4-hydroxyglutarate were cleaved to essentially the same extent, with the L-isomer again utilized slightly better. In view of this unique property, the following experiment was carried out to ascertain whether this aldolase is also nonstereospecific in forming 2-keto-4-hydroxyglutarate. Sodium glyoxylate (10 μmoles), 10 μmoles of sodium pyruvate, approx. 0.4 μmole of [1-^{14}C]pyruvate ($1.86 \cdot 10^6$ counts/min), 10 μmoles of $MgCl_2$, 10 μmoles of 2-mercaptoethanol, and 200 μmoles of Tris–HCl buffer (pH 8.4), in a final volume of 2.0 ml, were mixed and incubated for 4 h at 37°. The reaction was stopped by heating at 100° for 5 min. The 2-keto-4-hydroxyglutarate formed by condensation of [^{14}C]pyruvate with glyoxylate was subsequently converted to γ-hydroxyglutamate. For this purpose, the supernatant fluid of the previous reaction mixture was incubated for 2 h at 37° with 40 μmoles of NH_4Cl, 20 μmoles of NADH, and 0.4 mg of glutamate dehydrogenase in a final volume of 3.0 ml. This reaction was terminated by heating as before. Theoretically, if both optical isomers of 2-keto-4-hydroxy[^{14}C]glutarate were formed by the condensation of [^{14}C]pyruvate with glyoxylate, the L- and D-isomers of

TABLE I

SPECIFICITY OF BOVINE LIVER 2-KETO-4-HYDROXYGLUTARATE ALDOLASE FOR OPTICAL ISOMERS OF 2-KETO-4-HYDROXYGLUTARATE

The incubation mixtures contained the usual components except in those cases where DL-2-keto-4-hydroxyglutarate was replaced by the individual isomers of 2-keto-4-hydroxyglutarate, as indicated. The stereochemical conventions applied to the isomers of 2-keto-4-hydroxyglutarate are explained in the section entitled *Materials*. Purified 2-keto-4-hydroxyglutarate aldolase (0.6 μg of protein per assay mixture) was used. Aldolase activity was measured by the usual glyoxylate assay; turnover numbers are based on a molecular weight of 120 000 for the enzyme.

Compound tested	*Initial concentration (μmoles/ml)*	*Turnover number (moles KHG cleaved per min per mole of enzyme)*
DL-2-Keto-4-hydroxyglutarate	2.5	810
DL-2-Keto-4-hydroxyglutarate	5.0	810
L-2-Keto-4-hydroxyglutarate	2.5	840
	5.0	780
D-2-Keto-4-hydroxyglutarate	2.5	702
	5.0	672

2-keto-4-hydroxyglutarate should yield *threo*-γ-hydroxy-L-[^{14}C]glutamate and *erythro*-γ-hydroxy-L-[^{14}C]glutamate, respectively. These two diastereoisomers can be separated by elution from a Dowex-1 (acetate) ion-exchange resin column, as described by BENOITON *et al.*[38]. With this in mind, 50 μmoles of the *erythro*- and of the *threo*-isomers of γ-hydroxy-L-glutamate were added as carrier to the final deproteinized reaction mixture. This solution was then applied to a column (1 cm × 20 cm) of Dowex-50 (H^+) ion-exchange resin. The resin was first washed with ten column volumes of water; thereafter, the amino acids were removed with 2M aqueous ammonia. This solution was concentrated three times *in vacuo* to eliminate the ammonia, the pH finally adjusted to 7.0, and it was then passed over a Dowex-1 (acetate) ion-exchange

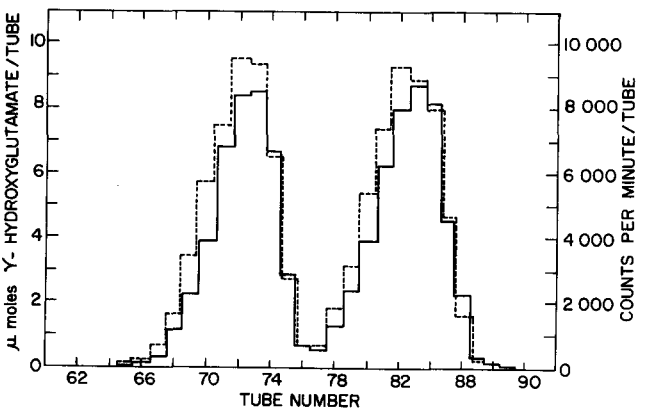


Fig. 1. Elution pattern of the diastereoisomers of γ-hydroxyglutamate derived from 2-keto-4-hydroxyglutarate which had been formed enzymically by condensation of glyoxylate with pyruvate. The radioactive and ninhydrin-positive peak on the left corresponds to the *erythro*-isomer of γ-hydroxy-L-glutamate, and the peak on the right to the *threo*-L-isomer. ———, amino acid content; — — —, radioactivity.

resin column (1.3 cm × 77 cm). The resin was eluted with 0.5 M acetic acid; fractions were collected and each tube was assayed for ninhydrin-positive material as well as for radioactivity. The elution pattern shown in Fig. 1 was obtained. The material eluted first corresponds to the *erythro*-isomer of γ-hydroxy-L-glutamate, and the compound eluted last is the *threo*-isomer[38]. These results show that both diastereoisomers of γ-hydroxy-L-glutamate were present and, therefore, that both D- and L-isomers of 2-keto-4-hydroxyglutarate are formed by the enzymic condensation (catalyzed by 2-keto-4-hydroxyglutarate aldolase) of [^{14}C]pyruvate with glyoxylate. Calculation of the relative amounts of D-2-keto-4-hydroxyglutarate and L-2-keto-4-hydroxyglutarate on the basis of the radioactivity present in the isomers of γ-hydroxy-L-glutamate shows that there is a slight preference (1.1:1) for the formation of L-2-keto-4-hydroxyglutarate in the condensation reaction. This value is in good agreement with the slight preference exhibited by 2-keto-4-hydroxyglutarate aldolase for the cleavage of the L-isomer of 2-keto-4-hydroxyglutarate (Table I).

Substrate analog binding by 2-keto-4-hydroxyglutarate aldolase

We studied the specificity of azomethine formation by treating 2-keto-4-hydroxyglutarate aldolase with a large number of substrate analogs ($3 \cdot 10^{-2}$ M) in the presence of $NaBH_4$; the existence of a Schiff-base intermediate (azomethine) is indicated by enzyme inactivation. As shown in Table II, the aldolase binds a number of analogs of glyoxylate but it is highly specific for pyruvate. Glycolate, a molecule lacking a carbonyl group necessary for Schiff-base formation, was used as a negative control.

Table II also shows the extent to which certain analogs of 2-keto-4-hydroxyglutarate are bound *via* azomethine linkages (values in the left column) by 2-keto-4-hydroxyglutarate aldolase and also the extent to which the enzyme catalyzes the cleavage of those compounds (the values in the column on the right). The first value listed in these studies shows that this aldolase is almost completely inactivated when incubated with 2-keto-4-hydroxyglutarate in the presence of $NaBH_4$; the rate of cleavage of this substrate is arbitrarily taken as 100%. Analogs of 2-keto-4-hydroxyglutarate listed in Group B are cleaved to a limited extent and also cause some inactivation of the enzyme. The compounds in Group C, however, cause inactivation of the aldolase when incubated together with sodium borohydride, but are not cleaved to any measurable extent. In a final group (Group D), some analogs of 2-keto-4-hydroxyglutarate neither inactivate 2-keto-4-hydroxyglutarate aldolase in the presence of $NaBH_4$ nor are they subject to enzymic cleavage. The enzyme, therefore, has a high degree of specificity toward those structural analogs of 2-keto-4-hydroxyglutarate which will undergo dealdolization. The process of azomethine formation with certain analogs of 2-keto-4-hydroxyglutarate can also be clearly dissociated from a concurrent cleavage of those compounds. This is especially true for 2-ketoglutarate and 2-keto-3-deoxygluconate.

β-Decarboxylase activity of 2-keto-4-hydroxyglutarate aldolase with oxaloacetate

Since the mechanism of action of a β-decarboxylase, namely acetoacetate decarboxylase, involves Schiff-base formation[13–15] and since oxaloacetate resembles pyruvate, a substrate for 2-keto-4-hydroxyglutarate aldolase, as well as 2-ketobutyrate (which was shown to form an azomethine with the enzyme, Table II), we tested

TABLE II

AZOMETHINE FORMATION WITH VARIOUS SUBSTRATE ANALOGS AND DEALDOLIZATION OF ANALOGS OF 2-KETO-4-HYDROXYGLUTARATE

The reaction mixtures (0.30 ml) contained 50 μmoles of potassium phosphate buffer (pH 6.3), 9 μmoles of each substrate analog, and 0.20 mg of purified bovine liver 2-keto-4-hydroxyglutarate aldolase (specific activity, 150). Each reaction mixture was treated at 4° with four 0.01 ml portions of 1 M $NaBH_4$ added alternately with four 0.005-ml portions of 2 M acetic acid over a period of 30 min. The enzyme was then precipitated with 168 mg of $(NH_4)_2SO_4$, centrifuged, and washed two times with 1 ml of 80% satd. $(NH_4)_2SO_4$ soln. The washed ppts. were dissolved in 0.3 ml Tris–HCl buffer (pH 7.4), *plus* 0.005 M 2-mercaptoethanol, and the resulting solns. tested for aldolase activity. Protein concns. were measured by the method of MURPHY AND KIES[28]. Cleavage of the 2-keto-4-hydroxyglutarate analogs was measured as described in *Methods*.

Experiment	*Addition to enzyme*	*Inactivation cleavage**	
Controls	None	0	
	$NaBH_4$ alone	0	
	Any analog listed alone	0–3	
Pyruvate analogs	Pyruvate	100	
	2-Ketobutyrate	57	
	Bromopyruvate	68	
	Hydroxypyruvate	0	
	Acetone	3	
	Monohydroxyacetone	0	
	Dihydroxyacetone	7	
	D-Glyceraldehyde	7	
	DL-Glyceraldehyde	0	
	DL-Glyceraldehyde 3-phosphate	0	
	D-Erythrose	0	
Glyoxylate analogs	Glyoxylate	100	
	Glyoxal	96	
	Formaldehyde	81	
	Acetaldehyde	70	
	Glycolaldehyde	57	
	Glycolate	0	
2-Keto-4-hydroxyglutarate analogs	A. 2-Keto-4-hydroxyglutarate	93	100
	B. 2-Keto-4,5-dihydroxyvalerate	63	33
	2-Keto-4-hydroxy-4-methylglutarate	99	8
	5-Keto-4-deoxyglucarate	57	3
	2-Keto-3-deoxy-6-phosphogluconate	84	2
	2-Keto-4-hydroxybutyrate	96	1
	C. 2-Ketoglutarate	97	0
	Fructose 1,6-diphosphate	66	0
	2-Keto-3-deoxygluconate	62	0
	2-Ketoisovalerate	24	0
	Acetoacetate	21	0
	2-Ketovalerate	19	0
	Isocitrate	10	0
	2-Deoxyribose	6	0
	D. 2-Deoxyribose 5-phosphate	0	0
	2-Ketogluconate	0	—**
	5-Ketogluconate	0	—
	Methyl-2-ketogluconate	0	—
	2-Keto-L-gulonate	0	—
	2-Keto-3-deoxygalactonate	0	0
	N-Acetylneuraminate	0	0
	Citrate	0	0
	L-Threonine	0	0

* Expressed as percentage of rate of 2-keto-4-hydroxyglutarate cleavage at pH 8.1, 26°.
** Dash (—) indicates the test was not run.

TABLE III

β-DECARBOXYLATION OF OXALOACETATE BY BOVINE LIVER 2-KETO-4-HYDROXYGLUTARATE ALDOLASE

The reaction mixtures (1.0 ml) contained (in μmoles): 100 Tris–HCl buffer (pH 8.1); 0.33 NADH; 3.3 oxaloacetate; 0.1 mg of lactate dehydrogenase; and varying amounts of 2-keto-4-hydroxyglutarate aldolase. A unit of decarboxylase activity is the amount of enzyme that liberates 1.0 μmole of pyruvate in 20 min at 26° (pH 8.1); a unit of aldolase is the amount of enzyme that liberates 1.0 μmole of glyoxylate in the aldolase assay in 20 min at 26° (pH 8.1). For heat inactivation, the aldolase was boiled for 5 min. Reductions with $NaBH_4$ were carried out as described in the legend to Table II. For NaCN treatment of 2-keto-4-hydroxyglutarate aldolase solns., the reaction mixtures (0.20 ml) contained 50 μmoles of potassium phosphate buffer (pH 7.4), 2 μmoles of pyruvate or glyoxylate, and 0.16 mg of purified aldolase (specific activity, 140). Each reaction mixture was treated at 25° with 0.1 ml (8 μmoles) of NaCN soln. for 15 min. The resulting solns. were dialyzed against 0.05 M Tris–HCl buffer (pH 7.4) *plus* 0.005 M 2-mercaptoethanol for 3 h, and then assayed for β-decarboxylase activity and also for protein by the method of MURPHY AND KIES[28]. In all cases, if the addition of treated enzyme solns. did not result in a change in the rate of disappearance of absorbancy at 340 nm, untreated 2-Keto-4-hydroxyglutarate aldolase was added as a check that all of the components required for the assay were indeed present.

Exp. No.	*Additions*	*Units of decarboxylase activity** *Units of 2-keto-4-hydroxyglutarate aldolase added*
1	Boiled aldolase	0
2	Aldolase	0.49
3	Aldolase + $NaBH_4$	0.44
4	Aldolase + $NaBH_4$ + pyruvate	0.02
5	Aldolase + $NaBH_4$ + glyoxylate	0.02
6	Aldolase + NaCN	0.50
7	Aldolase + NaCN + pyruvate	0.51
8	Aldolase + NaCN + glyoxylate	0

* Decarboxylase activities are corrected for the amount of pyruvate formed by nonenzymic decarboxylation of oxaloacetate.

2-keto-4-hydroxyglutarate aldolase for β-decarboxylase activity toward oxaloacetate. The results are listed in Table III. In every instance where the decarboxylation of oxaloacetate is catalyzed by 2-keto-4-hydroxyglutarate aldolase, the rate of NADH oxidation was shown to be proportional to the amount of enzyme added. Using the assay procedure described in the section entitled, *Methods*, no decarboxylation of acetoacetate by 2-keto-4-hydroxyglutarate aldolase is observed.

The results given in Table III are in complete accord with data published in a previous paper[17] concerning the binding of the three substrates of 2-keto-4-hydroxyglutarate aldolase to the enzyme in the presence of $NaBH_4$ as well as with the stable binding of glyoxylate by cyanide.

DISCUSSION

The ability of 2-keto-4-hydroxyglutarate aldolase to utilize both optical isomers of 2-keto-4-hydroxyglutarate as substrates was first observed with enzyme prepared from rat liver extracts[22,23,39,40]. In these experiments, however, aldolase which had been only minimally purified was often used[22,23,39] and even purified rat-liver enzyme tested in this regard still showed several protein bands when subjected to disc gel electrophoresis[40]. The question, therefore, of whether or not such prepara-

tions might yet contain an active racemase for D- and L-2-keto-4-hydroxyglutarate remained unanswered. As reported before[16], we succeeded in developing a procedure for preparing 2-keto-4-hydroxyglutarate aldolase in pure form (1300-fold purified) from extracts of bovine liver*. As clearly shown in Table I and Fig. 1, this pure enzyme exhibits no optical isomer specificity. The nonstereospecific character of bovine liver 2-keto-4-hydroxyglutarate aldolase, either in catalyzing the cleavage or the formation of 2-keto-4-hydroxyglutarate, is without precedent in the study of pure aldolases. Fructose 1,6-diphosphate aldolase[2,5-10], 2-keto-3-deoxy-6-phosphogluconate aldolase[11], 2-deoxyribose 5-phosphate aldolase[2,12], 5-keto-4-deoxyglucarate aldolase[41], isocitrate lyase[42], threonine aldolase[37], β-hydroxyaspartate aldolase[43], and β-phenylserine aldolase[44] all have strict optical isomer requirements. For all these cases, it is postulated that the enzyme must catalyze either a stereospecific formation of an enzyme-bound carbanion or a stereospecific polarization of the aldehyde substrate so that only one stereoisomer is cleaved or formed. The finding that pure bovine liver 2-keto-4-hydroxyglutarate aldolase is not stereospecific suggests that an asymmetric polarization of glyoxylate does not occur in this enzyme-catalyzed reaction. Since carbon atom 3 of pyruvate carries three hydrogen atoms (rather than two, like dihydroxyacetone phosphate), it does not seem likely that 2-keto-4-hydroxyglutarate aldolase might catalyze a stereospecific proton exchange or formation of a stereospecific carbanion. NISHIHARA AND DEKKER[45] have also obtained 2-keto-4-hydroxyglutarate aldolase in pure form from extracts of *Escherichia coli*. The enzyme from this source has about half the molecular weight (62 000) of the liver enzyme (mol. wt. 120 000) and whereas the latter enzyme is nonstereospecific, the former is highly (90% or better) specific toward L-2-keto-4-hydroxyglutarate. Comparative studies with pure 2-keto-4-hydroxyglutarate aldolase from bovine liver and from *E. coli* extracts should be interesting in this regard.

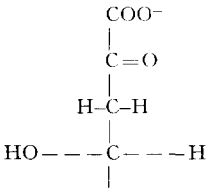
The specificity of azomethine formation with 2-keto-4-hydroxyglutarate aldolase was tested by treating the enzyme with a variety of compounds in the presence of $NaBH_4$; any loss in enzymic activity is taken as a measure of Schiff-base binding of the compound in question. The assumption is that the compounds tested do not form azomethines randomly with any lysyl residue or residues in the protein molecule but, rather, that binding via an azomethine linkage occurs selectively very near or actually at the active-site lysyl residue. This appears valid for two primary reasons. First, azomethine formation proceeds by nucleophilic attack of an amino group which is not protonated (the ε-amino group of the active site lysyl residue of 2-keto-4-hydroxyglutarate aldolase, in this instance) on the carbon atom of a carbonyl group. Since the pK_a value of ε-amino groups of lysyl residues in proteins is normally in the range of 10 and reduction with $NaBH_4$ was carried out at pH 6.3, random azomethine formation by substrate analogs does not appear to be a likely process. Our experimental conditions, in fact, actually accentuate the unusual nucleophilicity of the active-site lysyl residue of 2-keto-4-hydroxyglutarate aldolase. Second, a number of the compounds tested in this regard were found to competitively inhibit the enzymic

* Most preparations of purified bovine liver 2-keto-4-hydroxyglutarate aldolase show two protein-stainable bands, both of which are enzymically active, when subjected to polyacrylamide gel electrophoresis. At times, however, we have also obtained samples of the enzyme which contained only one detectable band. "One-band" or "two-band" aldolase preparations exhibited the same lack of specificity toward the two optical isomers of 1-keto-4-hydroxyglutarate.

cleavage of 2-keto-4-hydroxyglutarate, indicating binding at or near the active site.

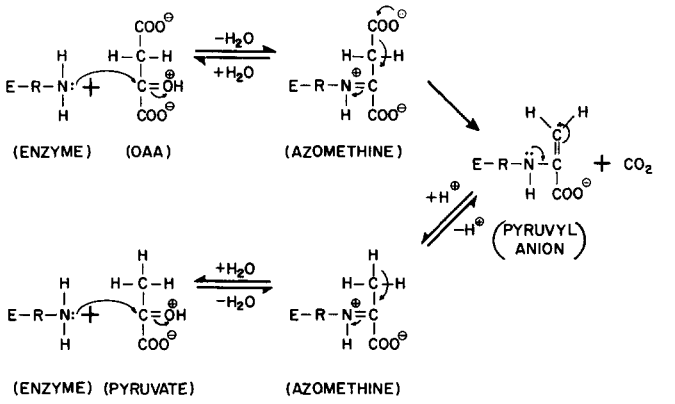
Of the many analogs of 2-keto-4-hydroxyglutarate tested for binding as azomethines with bovine liver 2-keto-4-hydroxyglutarate aldolase, it can be seen that with the exception of fructose 1,6-diphosphate, a rather strict requirement for a pyruvate-like structure ($-CH_2-CO-COO^-$) on one end of the molecule seems to be manifested. Alterations beyond this required segment of the analog molecule (replacing the $-CHOH-COO^-$ portion of 2-keto-4-hydroxyglutarate) cause only minor differences in the extent to which azomethine linkages are formed. Exceptions are 2-keto-3-deoxygalactonate and *N*-acetylneuraminate where certain steric factors may prohibit the binding of these compounds to the aldolase. The requirement of a pyruvyl structure within 2-keto-4-hydroxyglutarate analog molecules (in order for these analogs to be bound as Schiff bases) correlates well with the high degree of specificity shown for pyruvate and the low degree of specificity exhibited for glyoxylate (for azomethine formation).

2-Keto-4-hydroxyglutarate aldolase shows a fairly high degree of specificity toward those compounds which will serve as substrates for actual cleavage. All of the compounds which are cleaved enzymically have the following structure on four carbon atoms at one end of the molecule,



and all compounds having this unit as part of the molecule also form azomethine linkages with 2-keto-4-hydroxyglutarate aldolase. Other 2-keto-4-hydroxyglutarate analogs, namely, 2-ketoglutarate, fructose 1,6-diphosphate, and 2-keto-3-deoxygluconate are strongly bound to 2-keto-4-hydroxyglutarate aldolase in the presence of $NaBH_4$, but these same compounds are not cleaved to any measurable extent. Azomethine formation, therefore, seems to be a prerequisite for enzymic cleavage of the molecule, but cleavage of a molecule is not necessarily a direct result of azomethine formation. As a consequence, it is possible to separate two phases, azomethine formation and carbon–carbon bond cleavage, in the reaction catalyzed by 2-keto-4-hydroxyglutarate aldolase providing additional support for the mechanism of reaction proposed previously[17].

The comparatively high ratio of oxaloacetate decarboxylase to 2-keto-4-hydroxyglutarate aldolase activity we find for the purified enzyme is most striking. The fact that the β-decarboxylase activity of 2-keto-4-hydroxyglutarate aldolase is destroyed by a prior incubation of the enzyme with either pyruvate or glyoxylate *plus* sodium borohydride and by incubation of the enzyme with only glyoxylate and cyanide indicates that the same active site catalyzes the two processes (decarboxylation and aldol cleavage or condensation). Scheme 1 outlines a possible mechanism for the β-decarboxylation of oxaloacetate by 2-keto-4-hydroxyglutarate aldolase. Enzymic decarboxylation of acetoacetate by 2-keto-4-hydroxyglutarate aldolase is not observed. This absence of acetoacetate decarboxylase activity in preparations of



Scheme 1. Proposed mechanism of action for the β-decarboxylation of oxaloacetate (OAA) by 2-keto-4-hydroxyglutarate.

2-keto-4-hydroxyglutarate aldolase agrees with our finding that acetone is not bound via an azomethine linkage by the aldolase (Table II).

Indicating that the β-decarboxylation of oxaloacetate by 2-keto-4-hydroxyglutarate aldolase involves Schiff base formation with an ε-amino group of a lysyl residue in the protein molecule is consonant with the fact that amines[46], lysine[41], and polylysine (or lysine-containing protenoids)[48] selectively catalyze pyruvate formation by decarboxylation of oxaloacetate. Reexamination of model systems of this sort could be of considerable value in studies pertaining to the active sites of decarboxylases and aldolases.

The fact that 2-keto-4-hydroxyglutarate aldolase binds as azomethines and also catalyzes the dealdolization of certain compounds (*i.e.* 2-keto-4,5-dihydroxyvalerate, 2-keto-4-hydroxy-4-methylglutarate, 5-keto-4-deoxyglucarate, and 2-keto-3-deoxy-6-phosphogluconate) which are specifically cleaved by other aldolases indicates the desirability of obtaining all of these enzymes in pure form so that their molecular and catalytic properties can be compared. Furthermore, the observation that 2-keto-4-hydroxyglutarate aldolase readily catalyzes the β-decarboxylation of oxaloacetate, coupled with INGRAM AND WOOD's[11] report that 2-keto-3-deoxy-6-phosphogluconate aldolase catalyzes the formation of pyruvate from oxaloacetate at approximately 0.5% the rate of 2-keto-3-deoxy-6-phosphogluconate cleavage, lends increasing support to the phylogenetic concept of RUTTER[1] that Class I aldolases may structurally and mechanistically resemble a pre-existing enzyme such as a β-decarboxylase. The general validity of this concept awaits comprehensive studies with a broad range of highly-purified aldolases and β-decarboxylases obtained from a wide variety (animals, plants, bacteria) of biological sources.

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