PRELIMINARY NOTES

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A stereospecific 2-keto-4-hydroxyglutarate aldolase from *Escherichia coli*

2-Keto-4-hydroxyglutarate, an intermediate in mammalian[1–3], in bacterial[4], and possibly in plant[5,6] metabolism, has one asymmetric carbon atom and hence two isomeric forms. 2-Keto-4-hydroxyglutarate aldolase, previously detected in extracts of animal tissues[7–9] and in an unidentified soil bacterium[10], catalyzes the reversible cleavage of 2-keto-4-hydroxyglutarate yielding pyruvate and glyoxylate. All preparations of 2-keto-4-hydroxyglutarate aldolase studied so far have the very striking and unusual property of being nonstereospecific toward the two optical antipodes of 2-keto-4-hydroxyglutarate; the D and L isomers of 2-keto-4-hydroxyglutarate are cleaved and formed enzymically to essentially the same extent. This peculiar characteristic of 2-keto-4-hydroxyglutarate aldolase has been demonstrated with the enzyme obtained in partially purified (10-fold) form from a soil bacterium grown on α-ketoglutarate as the carbon source[10], in partially (70-fold)[7] or highly purified (400–500-fold)[8] form from rat liver, and in homogeneous (1300-fold purified) form from extracts of bovine liver[9]. In contrast, other highly purified aldolases (like fructose-1,6-diphosphate aldolase[11], 2-keto-3-deoxy-6-phosphogluconate aldolase[12], and 2-deoxyribose-5-phosphate aldolase[13]) have strict optical isomer specificities. Just recently, we obtained 2-keto-4-hydroxyglutarate aldolase in homogeneous form from extracts of *Escherichia coli*; we report here the ability of the pure aldolase from this source to preferentially utilize one of the two isomers of 2-keto-4-hydroxyglutarate as substrate.

DL-2-Keto-4-hydroxyglutarate, L-2-keto-4-hydroxyglutarate and D-2-keto-4-hydroxyglutarate were prepared by nonenzymic transamination of *threo*-DL-γ-hydroxyglutamate, *threo*-L-γ-hydroxyglutamate and *erythro*-L-γ-hydroxyglutamate, respectively, according to the procedure of MAITRA AND DEKKER[3]; the convention is followed that D-malic acid is formed from D-2-keto-4-hydroxyglutarate and L-2-keto-4-hydroxyglutarate yields L-malic acid by oxidative decarboxylation[2,3]. *threo*-γ-Hydroxy-DL-glutamic acid was synthesized chemically by a modification of the procedure of BENOITON AND BOUTHILLIER[14]; *threo*- and *erythro*-L-γ-hydroxyglutamate were prepared enzymically by reductive amination of DL-2-keto-4-hydroxyglutarate with NADH and NH_4^+ in the presence of glutamate dehydrogenase and by subsequent resolution of the two diastereoisomers of the hydroxyamino acid on a column of Dowex-1 (acetate) resin[15]. *Escherichia coli* K-12 was grown in nutrient broth medium and was harvested in late log phase; extracts were prepared by sonic oscillation. 2-Keto-4-hydroxyglutarate aldolase activity was determined by measuring the formation of glyoxylate[7]. A 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 refers to units of enzyme activity per mg of protein. Protein was estimated by the method of LOWRY *et al.*[16].

We succeeded in purifying 2-keto-4-hydroxyglutarate aldolase activity from *E. coli* extracts over 2000-fold by procedures including ammonium sulfate fractionation, controlled heat denaturation, addition of protamine sulfate, gel filtration on Sephadex G-100 and column chromatography on DEAE-cellulose; the pure enzyme

has a specific activity of approx. 100. Disc polyacrylamide gel electrophoresis of the final enzyme preparation at three different pH values shows a single protein band which coincides with aldolase activity. We have found that pure 2-keto-4-hydroxyglutarate aldolases from *E. coli* and bovine liver are quite similar in their divalent metal ion requirement, sulfhydryl group requirement and mechanism involving Schiff base formation with substrates[9,17] indicating that 2-keto-4-hydroxyglutarate aldolase from this bacterium is a Class I and not a Class II aldolase (classification according to RUTTER[18]).

E. coli 2-keto-4-hydroxyglutarate aldolase, however, is strikingly different from the liver enzyme in two respects. Both rat liver[8] and bovine liver[9,17] 2-keto-4-hydroxyglutarate aldolases have molecular weights of about 120 000; in contrast, the molecular weight of our pure bacterial aldolase is estimated to be approx. 62 000 by Sephadex G-200 gel filtration[19] and by sucrose density gradient centrifugation[20]. In addition, and quite surprisingly, 2-keto-4-hydroxyglutarate aldolase from *E. coli* exhibits a strong preferential utilization of L-2-keto-4-hydroxyglutarate, as shown in Table I.

TABLE I

SUBSTRATE STEREOSPECIFICITY OF *E. coli* 2-KETO-4-HYDROXYGLUTARATE ALDOLASE

The reaction mixture contained 100 mM Tris–HCl buffer (pH 8.4), 5 mM reduced glutathione, 0.7 μg of purified aldolase, and substrate as indicated. Water was added to a final volume of 1 ml. After incubating for 20 min at 37°, the reaction was terminated by adding 0.4 ml of 12% metaphosphoric acid and 1-ml aliquots were removed for the determination of glyoxylic acid.

Substrate tested	*Initial concn. (mM)*	*Glyoxylate formed (μmoles/mg protein)*
DL-2-Keto-4-hydroxyglutarate	5.0	97
L-2-Keto-4-hydroxyglutarate	2.5	97
	5.0	121
D-2-Keto-4-hydroxyglutarate	2.5	5
	5.0	9

This result was exactly reproducible with four different preparations of the purified aldolase. For the *E. coli* enzyme, the K_m value for D-2-keto-4-hydroxyglutarate is about 10 times greater than that for L-2-keto-4-hydroxyglutarate (25 mM *vs.* 2.4 mM, respectively), whereas for pure bovine liver 2-keto-4-hydroxyglutarate aldolase these two values are nearly equal (about 0.1 mM). Also, L-2-keto-4-hydroxyglutarate is preferentially formed when *E. coli* 2-keto-4-hydroxyglutarate aldolase catalyzes the condensation of glyoxylate with pyruvate. Direct tests demonstrated that the minor extent to which the D isomer is utilized as substrate is due neither to a small contamination of D-2-keto-4-hydroxyglutarate with L-2-keto-4-hydroxyglutarate nor to the presence of racemase activity in the aldolase preparations.

This finding of *E. coli* 2-keto-4-hydroxyglutarate aldolase with markedly different properties from the liver 2-keto-4-hydroxyglutarate aldolases previously studied presents several interesting possibilities. For example, since the molecular weight of the bacterial aldolase is essentially one-half that of rat and bovine liver

aldolases, it would appear that 2-keto-4-hydroxyglutarate aldolase could have several molecular forms; we have also detected in bovine liver preparations a species which, in order of magnitude, has a molecular weight of about 240 000 (ref. 17). Furthermore, the substrate to enzyme binding ratio (moles of substrate bound per 120 000 g protein) so far determined for rat[8] and bovine liver[9] 2-keto-4-hydroxyglutarate aldolase may actually be greater than the value of 1; we have found with the enzyme from *E. coli* that 1 mole of substrate is bound per 62 000 g of protein. In addition, having 2-keto-4-hydroxyglutarate aldolase in pure form from both *E. coli* (mol. wt., 62 000) and bovine liver (mol. wt., 120 000) extracts, with the former being highly stereospecific for L-2-keto-4-hydroxyglutarate and the latter virtually nonstereospecific toward the two optical antipodes of 2-keto-4-hydroxyglutarate, would seem to provide an interesting system for studies which correlate enzymic properties with molecular structure.

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