# AN UNUSUAL GLYOXYLATE-ENZYME COMPLEX AND β-DECARBOXYLASE ACTIVITY OF BEEF LIVER 2-KETO-4-HYDROXYGLUTARATE ALDOLASE*

Rodger D. Kobes** and Eugene E. Dekker
Department of Biological Chemistry,
The University of Michigan, Ann Arbor, Michigan 48104

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2-Keto-4-hydroxyglutarate aldolase catalyzes the reversible cleavage of KHG[1], an intermediate in hydroxyproline breakdown by animals, into pyruvate and glyoxylate. We recently reported (Kobes and Dekker, 1966) purifying this enzyme from beef liver extracts to near homogeneity. Results outlined then showed that KHG-aldolase is unique in that it (1) cleaves both optical isomers of KHG, and (2) binds either pyruvate or glyoxylate (1 mole/mole enzyme) via azomethine linkages at apparently the same site (lysyl residue) in the enzyme molecule. These properties of KHG-aldolase contrast markedly with those of other aldolases, such as FDP-aldolase (Horecker, 1959; Grazi et al., 1962; Horecker et al., 1963), 2-deoxyribose-5-phosphate aldolase (Hoffee et al., 1965), and KDPG-aldolase (Grazi et al., 1963; Ingram and Wood, 1965). These three enzymes all show strict optical isomer specificities and bind as Schiff bases only one (dihydroxyacetone phosphate, acetaldehyde, or pyruvate, respectively) of the two substrates involved in the aldol condensation reaction.

All Class I aldolases (classification of Rutter, 1964) function via Schiff base mechanisms; the mechanism of action of β-decarboxylases also involves the formation of an azomethine linkage between the substrate and the ε-amino group of a lysyl residue in the enzyme, as shown for acetoacetate decarboxylase (Warren et al., 1966). In both instances (aldolases and β-decarboxylases), the enzyme substrate complexes are readily inactivated when reduced by $NaBH_4$. The loss of enzymic activity when enzyme and substrate are incubated together in the presence of cyanide has also been used to detect Schiff base intermediates; this technique was used with acetoacetate decarboxylase (Westheimer, 1963) and just recently with FDP-aldolase (Cash and Wilson, 1966).

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[1] Abbreviations used: KHG, 2-keto-4-hydroxyglutarate; FDP, fructose 1,6-diphosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; OAA, oxaloacetate.

Unusual Schiff-base binding of glyoxylate by KHG-aldolase is confirmed by our latest findings that (1) azomethine formation with the enzyme is highly specific for pyruvate but not for glyoxylate, and (2) the aldolase is completely inactivated when treated with glyoxylate + $CN^-$ (1 mole of glyoxylate is bound/mole of enzyme) but not with pyruvate + $CN^-$ or with $CN^-$ alone. In addition, we report here the novel ability of this purified aldolase to catalyze the β-decarboxylation of OAA.

## EXPERIMENTAL AND RESULTS

Materials and Methods - Beef liver KHG-aldolase (1300-fold purified) was prepared and assayed as outlined previously (Kobes and Dekker, 1966). Protein was estimated by the method of Murphy and Kies (1960). 1-$^{14}$C-Pyruvate and 1-$^{14}$C-glyoxylate were purchased from Nuclear Chicago Corp. 2-$^{14}$C-KHG was prepared by nonenzymatic transamination of 2-$^{14}$C-γ-hydroxyglutamate (Maitra and Dekker, 1963). β-Decarboxylation of OAA was measured with a Gilford spectrophotometer equipped with a digital absorbance meter; the rate of formation of pyruvate in a modified lactate dehydrogenase assay (Kornberg, 1955) was followed.

Substrate Analog Binding by the Aldolase - We studied the specificity of azomethine formation by treating KHG-aldolase with a large number of substrate analogs in the presence of $NaBH_4$; the existence of a Schiff base intermediate is indicated by loss of enzymic activity. As shown in Table I, the aldolase binds a number of analogs of glyoxylate but is highly specific for pyruvate.

Cyanide Adduct of the Glyoxylate-Aldolase Imine - Cash and Wilson (1966) found that FDP-aldolase is inactivated by $CN^-$ in the presence of dihydroxyacetone phosphate; by analogy, KHG-aldolase activity should be lost when the pyruvate-enzyme imine (Schiff base) is exposed to $CN^-$. When tested in this regard, KHG-aldolase shows no loss of activity when incubated with either pyruvate or glyoxylate alone (Exp. 1,2) or when treated with $CN^-$ alone (Exp. 3) (Table II). In the absence of $CN^-$, negligible amounts of $^{14}$C-pyruvate or $^{14}$C-glyoxylate are bound to the enzyme (Exp. 1,2). Surprisingly, KHG-aldolase is not inactivated when treated with $CN^-$ in the presence of either $^{14}$C-pyruvate or $^{14}$C-KHG and no radioactivity is stably bound to the protein (Exp. 4,5). In contrast, nearly all KHG-aldolase activity is lost when the enzyme is incubated with $CN^-$ + $^{14}$C-glyoxylate; radioactivity is bound to the enzyme in the ratio of 1 mole of glyoxylate to 120,000 gm of protein (Exp. 6). The observed inactivation of the enzyme and binding of $^{14}$C-glyoxylate (Exp. 6) cannot be reversed by dilution or by exhaustive dialysis.

β-Decarboxylase Activity of KHG-Aldolase with OAA - Since the mechanism of action of β-decarboxylases involves Schiff base formation (Warren et al.,

TABLE I

Azomethine Formation with Analogs of Pyruvate or Glyoxylate

| Exp. | Additions to Enzyme | Percent of Initial Enzymic Activity |
|---|---|---|
| Controls | None<br>$NaBH_4$ alone<br>Any analog listed alone | 97-100 |
| Pyruvate Analogs† | Pyruvate | 0 |
| | α-Ketobutyrate | 43 |
| Glyoxylate Analogs | Glyoxylate | 0 |
| | Glyoxal | 4 |
| | Formaldehyde | 19 |
| | Acetaldehyde | 30 |
| | Glycolaldehyde | 43 |

† Other pyruvate analogs tested which were found to be ineffective (less than 7% inactivation of enzymic activity) included hydroxypyruvate, acetone, monohydroxyacetone, dihydroxyacetone, DL-glyceraldehyde, DL-glyceraldehyde-3-phosphate, and D-erythrose.

The reaction mixtures (0.30 ml) contained 50 μmoles potassium phosphate buffer, pH 6.3; 9 μmoles of each substrate analog; and 0.2 mg of purified aldolase (sp. act., 150). Each reaction mixture was treated as described in the legend to Table III of our previous report (Kobes and Dekker, 1966). Aldolase activity was determined before and after reduction with $NaBH_4$.

1966) and since OAA resembles pyruvate, a substrate for KHG-aldolase, as well as α-ketobutyrate (which was shown to form an azomethine with the enzyme, Table I), we decided to test KHG-aldolase for β-decarboxylase activity. Under the conditions listed in the legend to Figure 1, KHG-aldolase catalyzes the loss of $CO_2$ from OAA with formation of pyruvate at approximately 49% the rate of KHG cleavage. Figure 1 shows that (1) the reaction is not catalyzed by enzyme alone, by OAA alone, nor by OAA plus boiled enzyme, (2) the rate of DPNH oxidation is proportional to the amount of enzyme added, and (3) β-decarboxylase activity is not lost when the aldolase is treated with $NaBH_4$ alone but only when the enzyme is incubated with $NaBH_4$ in the presence of either pyruvate or glyoxylate.

## DISCUSSION

The results reported here support our earlier findings (Kobes and Dekker, 1966) and those of Rosso and Adams (1966) that KHG-aldolase is unusual in that it binds glyoxylate via an azomethine linkage. Not only is glyoxylate bound in this manner but so also are a number of structural analogs of this compound

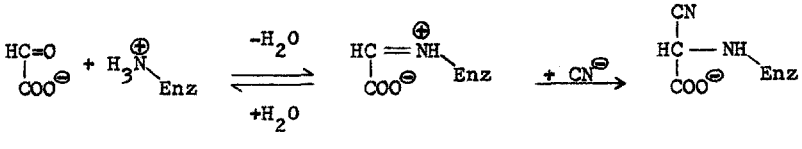
TABLE II

Enzymic Activity and Substrate Binding after NaCN Treatment

| Exp. | Additions to Enzyme | Percent of Initial Enzymic Activity | Radioactivity Bound | Substrate Bound |
|---|---|---|---|---|
| | | | cpm/120,000 μg protein | moles/120,000 gm protein |
| 1. | $^{14}$C-Pyruvate | 100 | $0.08 \times 10^6$ | 0.01 |
| 2. | $^{14}$C-Glyoxylate | 107 | $0.16 \times 10^6$ | 0.03 |
| 3. | NaCN | 100 | 0 | 0 |
| 4. | $^{14}$C-KHG + NaCN | 100 | 0 | 0 |
| 5. | $^{14}$C-Pyruvate + NaCN | 90 | $0.06 \times 10^6$ | 0.01 |
| 6. | $^{14}$C-Glyoxylate + NaCN | 9 | $4.52 \times 10^6$ | 0.96 |

The reaction mixtures (0.20 ml) contained the following components (in μmoles), as indicated: 50, potassium phosphate buffer, pH 7.4; 0.725 sodium 1-$^{14}$C-pyruvate ($6.51 \times 10^6$ cpm/μmole); 1.14, sodium 1-$^{14}$C-glyoxylate ($4.69 \times 10^6$ cpm/μmole); 1.26, 2-$^{14}$C-KHG ($0.103 \times 10^6$ cpm/μmole). Purified aldolase (0.22 mg; sp. act., 150) was used uniformly. Each reaction mixture, except 1 and 2, was treated at 25° C with 0.1 ml (8 μmoles) of NaCN solution for 15 minutes. The enzyme was then precipitated with 168 mg of $(NH_4)_2SO_4$, centrifuged, and washed 4 times with 0.4 ml of 80% saturated $(NH_4)_2SO_4$ solution. The washed precipitates were dissolved in 0.3 ml of 0.05 M Tris-HCl buffer, pH 7.4, plus 0.005 M 2-mercaptoethanol and the resulting solutions dialyzed against the same buffer mixture for 24 hours. The dialyzed solutions were used to determine aldolase activity and protein content; aliquots were also plated for radioactivity measurements. Levels of radioactivity were determined with a Nuclear Chicago thin-window gas flow counter. No differences in results were obtained when the time of incubation with NaCN was increased to 120 minutes (Exp. 5) or decreased to 2 minutes (Exp. 6).

(Table I); in contrast, a very high degree of specificity is shown for pyruvate in this respect. Further evidence for the existence of a unique glyoxylate-aldolase imine is the finding that enzymic activity is lost only when the aldolase is treated with $CN^-$ in the presence of glyoxylate; this loss of activity is concurrent with the binding of one mole of $^{14}$C-glyoxylate per 120,000 gm of protein. It is postulated that cyanide adds to the glyoxylate-aldolase imine yielding an inactive form (aminonitrile) of the enzyme:



(imine) (aminonitrile)

There is no obvious explanation for the observation that KHG-aldolase is not

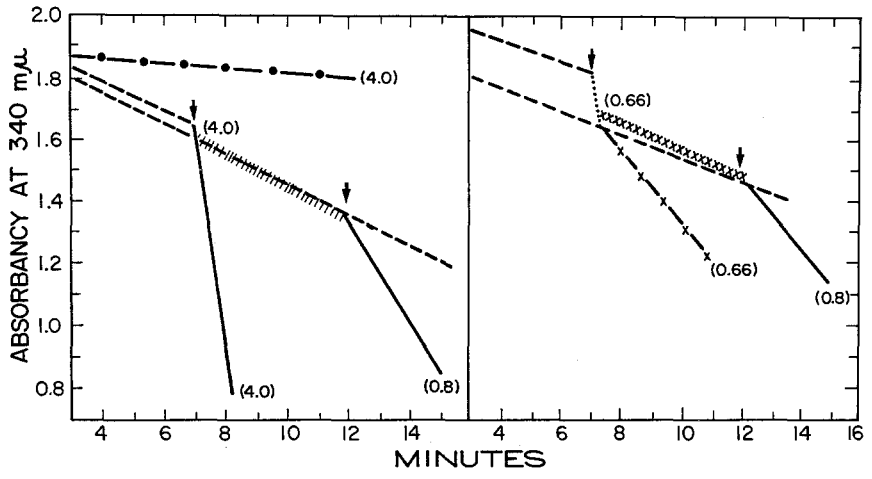



Fig. 1. β-Decarboxylation of OAA by KHG-aldolase. The reaction mixtures (1.0 ml) contained (in μmoles): 100, Tris-HCl buffer, pH 8.1; 0.33, DPNH; 3.3, OAA; 0.1 mg of lactate dehydrogenase; and varying amounts of aldolase added at 7 or 12 minutes. Units of aldolase added are indicated in parentheses; a unit is the amount that liberates 1.0 μmole of glyoxylate in the aldolase assay at 26° C, pH 8.1. For heat inactivation, the aldolase was boiled for 5 minutes. Reduction with $NaBH_4$ was carried out as described before (Kobes and Dekker, 1966). Complete (———); aldolase alone, no OAA (—•—•—); OAA alone, no aldolase (– – – –); complete with boiled aldolase (IIIIIIIIIIII); complete with $NaBH_4$-treated aldolase (x—x—x); complete, aldolase treated with $NaBH_4$ in the presence of pyruvate or glyoxylate (xxxxxx).

inactivated when incubated (as long as 120 minutes) with $CN^-$ plus pyruvate; possible suggestions are that either the aminonitrile-enzyme derivative is not formed in this case or that, if formed, this adduct is unstable. In contrast, the aminonitrile formed by addition of $CN^-$ to the glyoxylate-aldolase imine is quite stable since the observed inactivation cannot be reversed by dialysis or by dilution.

The comparatively high ratio of OAA-decarboxylase to aldolase activity we find for the purified enzyme is most striking. This observation coupled with Ingram and Wood's report (1966) that KDPG-aldolase catalyzes the formation of pyruvate from OAA at approximately 0.5% the rate of KDPG cleavage lends increasing support to the phylogenetic concept of Rutter (1964) that Class I aldolases may structurally resemble a pre-existing enzyme such as a β-decarboxylase. The fact that the β-decarboxylase activity of purified KHG-aldolase is destroyed by a prior incubation of the enzyme with either pyruvate or glyoxylate plus $NaBH_4$ indicates that the same active site catalyzes the two processes (decarboxylation and aldol cleavage or condensation). Using the assay procedure of Fridovich and Westheimer (1962), we do

not observe enzymic decarboxylation of acetoacetate by KHG-aldolase. This absence of acetoacetate decarboxylase activity in preparations of KHG-aldolase agrees with our finding that acetone is not bound via an azomethine linkage by the aldolase (Table I).

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