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# 4-HYDROXYPHENYLPYRUVATE DIOXYGENASE APPEARS TO DISPLAY α-KETOISOCAPROATE DIOXYGENASE ACTIVITY IN RAT LIVER.

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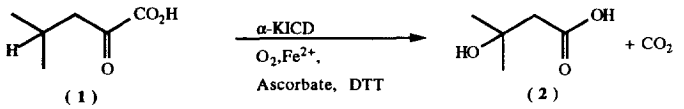
**Abstract**

Isolation and sequence determination of a protein exhibiting α-ketoisocaproate dioxygenase activity from rat liver revealed that the protein was identical to the liver-specific rat F antigen which is believed to be a species variant of 4-hydroxyphenylpyruvate dioxygenase.

α-Ketoisocaproate dioxygenase (α-KICD) is an internal keto-acid dependent dioxygenase responsible for the oxidative decarboxylation and hydroxylation of α-ketoisocaproate **1** to β-hydroxyisovaleric acid (3-hydroxy-3-methyl-butyric acid)[1] **2** (Scheme 1).

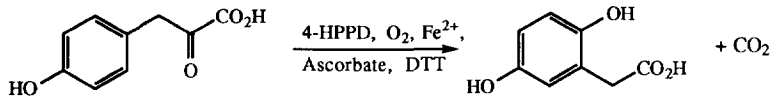



(1) (2)

Scheme 1

This enzyme, which is found in the cytosol of liver cells, has been purified from various sources of liver including rat[2,3], human, mouse, rabbit, guinea pig, cow and chicken[4]. The co-substrate and co-factor requirements of α-KICD are typical for keto-acid dependent dioxygenases i.e. molecular oxygen as co-substrate, ferrous iron as co-factor and ascorbate and dithiothreitol for maximal activity[5]. SDS-polyacrylamide gel electrophoresis gives an approximate molecular weight for the protein of 46KDa but no data relating to the primary sequence has been published. Mechanistic studies with purified enzyme preparations have investigated the origin of the hydroxyl oxygen in the hydroxylated product[6]. Thus incubations performed under an atmosphere of $^{18}O_2$ resulted in the incorporation of label into the hydroxyl group and one labelled oxygen into the carboxylate group, indicating that both are derived from molecular oxygen. Overall it has been shown that the stoichiometry of the reaction involves the consumption of one molecule of dioxygen for each molecule of $CO_2$ released.

At present, only one other example of an internal keto-acid dependent dioxygenase is known, 4-hydroxyphenylpyruvate dioxygenase (4-HPPD). This enzyme catalyses the oxidative decarboxylation and hydroxylation of 4-hydroxyphenylpyruvate **3** to homogentisate (2,5-dihydroxyphenylacetate)[7] **4** (Scheme 2) and is an important enzyme in the metabolism of the aromatic amino acid, tyrosine.




(3) Scheme 2 (4)

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4-HPPD has been partially purified from pig[8], cow[9], dog[10], rat[11], human[7,12], and frog[13] liver and the co-factor and co-substrate requirements for this protein appear to be essentially the same as those of α-KICD. 4-HPPD from human liver[14] and from *Pseudomonas* sp. strain P.J.874[15] have been sequenced and cloned and molecular weight determinations indicate that the enzyme from human liver is a homodimer with sub-units of ca 46 KDa. It is interesting to note that α-KICD, from rat liver has essentially the same reported molecular weight i.e. ca 46KDa as determined by SDS-PAGE[3].

We have systematically studied the bio-organic chemistry of α-ketoglutarate dependent dioxygenases involved in the biosynthesis of β-lactam containing secondary metabolites[16] and, due to probable mechanistic similarity between these two groups of enzymes, extended our research to include these internal keto-acid dependent dioxygenases. Initially a crude rat liver extract was prepared according to the protocol of Sabourin and Bieber[1]. α-Ketoisocaproate dioxygenase enzyme activity was assayed by monitoring the release of $^{14}CO_2$ from [1-$^{14}$C]-2-ketoisocaproate[3], prepared by incubating L-[1-$^{14}$C]-leucine with L-amino acid oxidase[17]. Further purification was achieved by a G75 gel filtration desalting step, DEAE ion exchange chromatography, phenyl sepharose hydrophobic interaction chromatography, Superdex S75 gel filtration, and Mono-Q ion exchange chromatography (see Table 1).

| Fraction | Volume (ml) | Protein ($mg.ml^{-1}$) | Activity ($nmol.min^{-1}$) | Recovery | Specific Activity ($nmol.min^{-1}.mg^{-1}$) | Purification |
|---|---|---|---|---|---|---|
| Crude | 200 | 36.3 | 2550 | 100 | 0.35 | 1 |
| G75 | 672 | 4.15 | 1530 | 60 | 0.55 | 1.6 |
| DEAE | 450 | 1.01 | 900 | 35 | 1.99 | 5.7 |
| Phenyl Sepharose | 200 | 0.26 | 290 | 11 | 6.60 | 19 |
| Superdex 75 | 20 | 0.39 | 280 | 11 | 35.0 | 100 |
| Mono-Q | | | | | | |
| First Peak | 5 | 0.48 | 19 | 0.7 | 8.0 | 23 |
| Second Peak | 3 | 0.60 | 34 | 1.3 | 18.7 | 53 |
| Third Peak | 7 | 0.31 | 68 | 2.7 | 30.9 | 88 |

Table 1. Purification of α-Ketoisocaproate Dioxygenase

Mono-Q ion exchange chromatography resolved the α-KICD activity into three major bands. A sample of the protein eluting in the third peak of activity was subjected to N-terminal sequencing but yielded no data and was assumed to be blocked. A tryptic digest was therefore performed on further material and the products purified by reverse phase HPLC. Selected peptides were sequenced and their sequences compared against the Protein Identification Resource (PIR) package (version 4.0). All the peptides showed total identity with liver specific rat F-antigen[18], which has recently been recognised as a species variant of 4-hydroxyphenylpyruvate dioxygenase[14] (see Figure 1).

This result was surprising not least because our purification protocol was based on the oxidative decarboxylation of α-ketoisocaproate. In an attempt to clarify the situation we re-assayed all the fractions obtained from the individual purification steps with [1-$^{14}$C]-4-hydroxyphenylpyruvate (obtained by treating L-[1-$^{14}$C]-tyrosine with L-amino acid oxidase according to the procedure of Lindstedt *et al*)[12] using the same incubation conditions we had employed in assaying the α-KICD activity. It can be seen from the figures (Figure 2 ) that the two activities coincide very closely.

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1     Y W D K G P K P E R G R F L H F H S V T F W V G N A K Q A A
31    S F Y C N K M G F E P L A Y K G L E T G S R E V V S H V I K
61    Q G K I V F V L C S A L N P W N K E M G D H L V K H G D G V
91    K D I A F E V E D C E H I V Q K A R E R G A K I V R E P W V
121   E E D K F G K V K F A V L Q T Y G D T T H T L V E K I N Y T
                        F A V L Q T Y G D T T H T L V E K
151   G R F L P G F E A P T Y K D T L L P K L P S C N L E I I D H
181   I V G N Q P D Q E M E S A S E W Y L K N L Q F H R F W S V D
211   D T Q V H T E Y S S L R S I V V A N Y E E S I K M P I N E P
241   A P G R K K S Q I Q E Y V D Y N G G A G V Q H I A L R T E D
                  S Q I Q E Y V D Y N G G A G V Q H I A L
271   I I T T I R H L R E R G M E F L A V P S S Y Y R L L R E N L
                              G M E F L A V P S S Y Y R
301   K T S K I Q V K E N M D V L E E L K I L V D Y D E K G Y L L
                        E N M D V L E E L K
331   Q I F T K P M Q D R P T L F L E V I Q R H N H Q G F G A G N
361   F N S L F K A F E E E Q A L R G
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Figure 1. Data Base Sequence of Rat Specific Antigen F and Sequence of Fragments Obtained by Tryptic Digest of a Protein Possessing α-Ketoisocaproate Dioxygenase Activity.

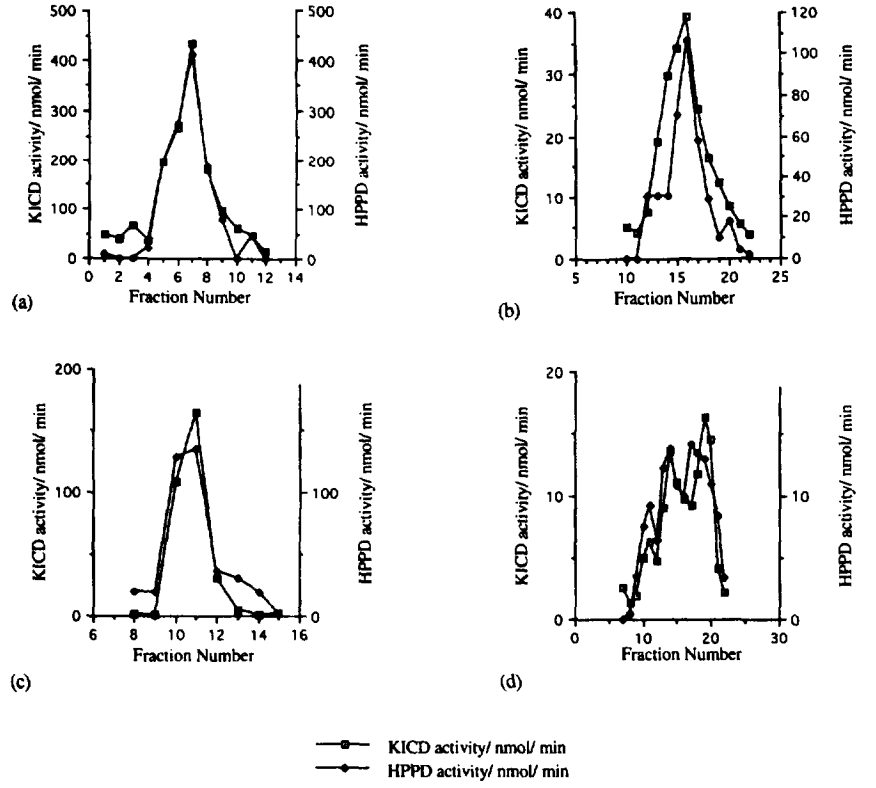


Figure 2: Purification of Protein Displaying α-Ketoisocaproate Dioxygenase and 4-Hydroxyphenylpyruvate Dioxygenase Activity; (a) DEAE Ion-exchange Chromatography; (b) Phenyl Sepharose Hydrophobic Chromatography; (c) Superdex 75 Gel-filtration; and (d) Mono-Q Ion-exchange Chromatography.

Examination of the α-KICD and 4-HPPD activities displayed by the fractions from the Mono-Q column (Figure 2d) indicates that the protein or proteins responsible for either or both activities were resolved into three bands, each of which retained both activities. Further analysis of the two activities by comparison of the ratio of the relative specific activities between the peaks obtained from the Mono-Q ion exchange column reveals that these ratios are quite similar, as one would expect for a single protein exhibiting two activities (see Table 2).

| Peak | Fraction Numbers | Total Protein ($mg.ml^{-1}$) | KICD Specific Activity ($nmol.ml^{-1}mg^{-1}$) | 4-HPPD Specific Activity ($nmol.ml^{-1}mg^{-1}$) | Relative Ratio of KICD Specific Activity | Relative Ratio of 4-HPPD Specific Activity |
|---|---|---|---|---|---|---|
| 1 | 8 - 12 | 2.4 | 8.0 | 11.3 | 1 | 1 |
| 2 | 13 - 15 | 1.8 | 18.7 | 20.4 | 2.3 | 1.8 |
| 3 | 16 - 22 | 2.2 | 30.9 | 33.3 | 3.9 | 3.0 |

Table 2. Comparison of α-KICD and 4-HPPD Activities Displayed by peaks 1, 2 and 3 from Mono-Q Ion Exchange Chromatography.

In our opinion, it is unlikely that two separate proteins responsible for two distinct activities should behave in this manner when subjected to purification as described. It is noteworthy that the purified human 4-HPPD was also resolved into three bands by Mono-Q chromatography[12].

To confirm these results, α-ketoisocaproate and 4-hydroxyphenylpyruvate were incubated in separate experiments with two samples of the same purified enzyme batch and their conversion to their respective products observed by 500 MHz proton NMR. In the case of α-ketoisocaproate, the product was esterified to the phenacyl ester derivative and purified by normal phase HPLC. The product was then characterised by $^1$H-NMR (500MHz, $CDCl_3$) and mass spectroscopy[19]. In the case of 4-hydroxyphenylpyruvate the crude incubation mixture was examined by $^1$H-NMR (500MHz, $D_2O$, HOD suppressed) (Figure 3a) and formation of homogentisate in the incubation mixture was confirmed by comparison with a $^1$H-NMR spectrum of authentic homogentisate (purchased from Aldrich Chemical Company). In addition, a sample of the crude incubation mixture was doped with authentic homogentisate and the $^1$H-NMR spectrum (500MHz, $D_2O$, HOD suppressed) of the resulting mixture recorded (Figure 3b).

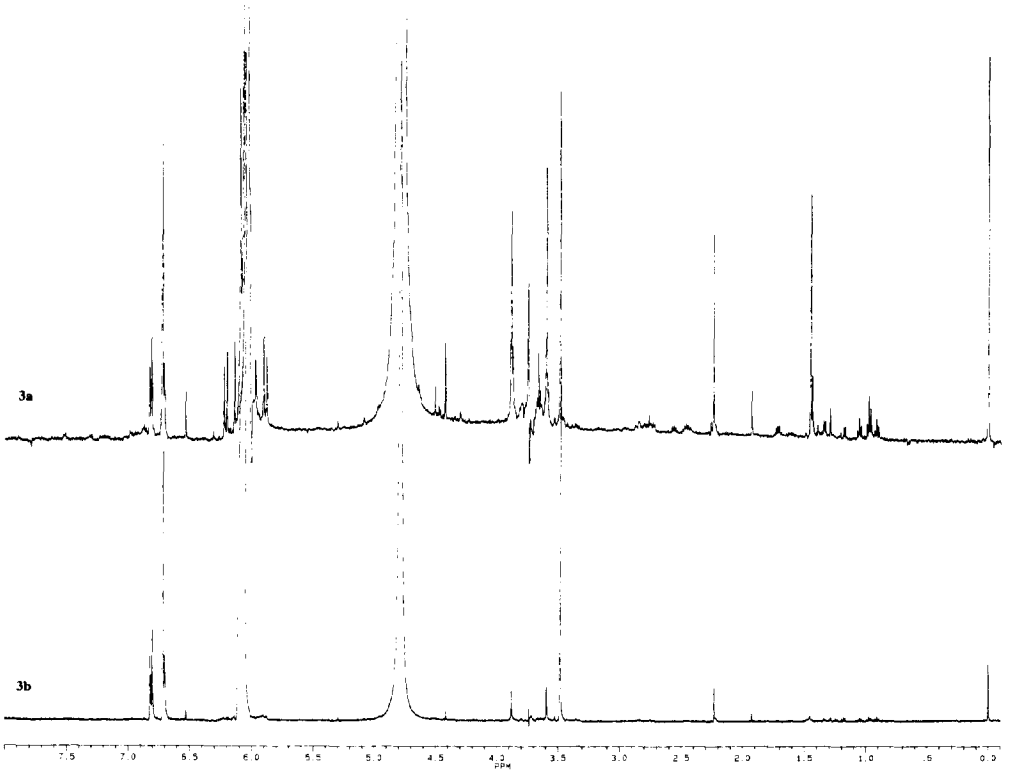


Figure 3. (a) 500MHz $^1$H-NMR Spectrum of a Crude Incubation Mixture of 4-Hydroxyphenylpyruvate with 4-HPPD and (b) 500MHz $^1$H-NMR Spectrum of Incubation Doped with Authentic Homogentisate.

The results presented here indicate that 4-HPPD is capable of converting both 4-hydroxyphenylpyruvate **3** to homogentisate **4** and α-ketoisocaproate **1** to 3-hydroxy-3-methylbutyric acid **2**. In the light of these findings, it remains unclear whether previous publications describing the purification of proteins possessing α-ketoisocaproate dioxygenase activity were infact due specifically to α-KICD or to the purification of 4-HPPD which possesses α-KICD activity and thus whether a single enzyme, 4-HPPD, is responsible for the total observed cytosolic α-KICD activity. We are continuing our studies on 'these enzymes' in an attempt to resolve the questions raised by the results presented here and will report our findings in due course.

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19 β-Hydroxyisovalerate, phenacyl ester; $^1$H-NMR (500MHz, $CDCl_3$) 1.37 (6H, s, $C(C\underline{H}_3)_2$), 2.67 (2H, s, $C\underline{H}_2C(OH)Me_2$), 5.42 (2H, s, $PhC\underline{H}_2$), 7.48-7.53 (2H, m, *m*-CH of $\underline{Ph}CH_2$), 7.61-7.64 (1H, m, *p*-CH of $\underline{Ph}CH_2$), 7.91-7.93 (2H, m, *o*-CH of $\underline{Ph}CH_2$). *m/z* (C.I., $NH_3$) 236 (100%, M+), 237 (41, M+1), 238 (8, M+2).

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