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MOLECULAR FORMS OF L-α-HYDROXY ACID OXIDASE FROM RAT KIDNEY

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SUMMARY

L-α-Hydroxy acid oxidase (L-amino acid:oxygen oxidoreductase, EC 1.4.3.2) has been purified from "heavy" and "light" mitochondrial subfractions and from the soluble fraction of rat kidney homogenates. Studies by gel electrophoresis, column chromatography and stability to heating indicated that the enzyme from "heavy" mitochondria differs from that of "light" mitochondria and cytosol. They are two distinct molecular forms with different net charge but similar molecular size. Effect of substrate and product upon these forms has been studied with a variety of α-hydroxy acids and α-keto acids. α-Hydroxy valerate and -isocaproate produced inhibition of enzymatic activity at concentrations higher than 50 mM. Certain α-keto acids act as non-competitive inhibitors of the enzymes, α-keto isocaproate being the most active. Catalytic behavior and pH optima for the different molecular forms were very similar.

INTRODUCTION

Blanchard *et al.*[1] were the first to demonstrate the existence of an enzyme which oxidizes L-α-amino acids and L-α-hydroxy acids to their corresponding keto acids. The enzyme was found in total extracts of rat kidney and designated L-α-amino acid oxidase or L-α-hydroxy acid oxidase (L-amino acid:oxygen oxidoreductase, EC 1.4.3.2). It is a flavoprotein, with FMN as prosthetic group.

Later, Nakano *et al.*[2,3] purified the enzyme from mitochondria and soluble fractions of rat kidney homogenates and studied the reactivity against an ample variety of substrates. Their findings confirmed those of Blanchard *et al.*[1]. The enzyme shows higher activity *in vitro* with α-hydroxy acids than with α-amino acids; for this reason, some authors prefer the name L-α-hydroxy acid oxidase. Kinetic properties of the enzyme isolated from mitochondria and cytosol were identical.

In 1967, Domenech and Blanco[4] showed that the enzymes from mitochondrial extracts and the soluble phase had different electrophoretic mobility. Crude prepa-

rations of those enzymes presented slight differences in reactivity against substrates. These evidences indicated that both enzymes were different molecular forms of the α-hydroxy acid oxidase.

As this observation did not agree with those of other authors, we decided to carry out studies with purified preparations. This paper presents the results of a study of some properties of the L-α-hydroxy acid oxidase from different fractions of rat kidney homogenates.

METHODS

Cellular fractionation

Adult male albino rats weighing about 300 g were killed by decapitation. The kidneys were removed and rinsed in cold 0.25 M sucrose solution. Kidneys were pooled, minced with scissors and homogenized in 3 parts of 0.25 M sucrose solution (1:3, w/v) in glass homogenizers fitted with a motor driven Teflon pestle for 2 min.

Cellular fractionation was performed with the procedure of de Duve *et al.*[5]. Nuclear sediment was discarded and the following fractions were kept for studies: (A) "heavy" mitochondria (the pellet obtained by centrifuging the supernatant remaining after separation of nuclei and tissue debris at 3300 × *g* for 10 min); (B) "light" mitochondria (the sediment obtained by centrifuging the supernatant from the previous step at 25 000 × g_{med} for 10 min); (C) microsomes; and (D) soluble phase.

Approximately 11% of the total L-α-hydroxy acid oxidase activity contained in the supernatant remaining after separation of nuclei and debris was recovered in the fraction of "heavy" mitochondria; 16%, in "light" mitochondria, and 70% in the soluble fraction. The activity of the microsomal fraction was less than 2% of the total.

Extraction and purification of the enzyme

Mitochondrial sediments were suspended and homogenized in 2 ml 0.25 M sucrose per g of original tissue, frozen at −30 °C and thawed at room temperature. Freezing–thawing was repeated once and finally the suspension was centrifuged at 44 000 × g_{med} for 120 min. To maintain comparable conditions, the soluble fractions were submitted to the same freezing–thawing–centrifugation treatment.

Other preparations of mitochondrial subfractions were extracted with: (a) distilled water; (b) 0.01 M sodium phosphate–HCl buffer, pH 7.4; or (c) 1% solution of detergents like Triton X-100 or digitonin, instead of 0.25 M sucrose.

Purification of the enzyme was performed with the method proposed by Nakano and Danowski[2], except that the first fractionation in DEAE-cellulose was carried out by eluting with 0.15 M phosphate buffer instead of 0.1 M when working with preparations from "heavy" mitochondria.

The specific activity of the crude extracts of "heavy" mitochondria was 12.9 units/mg protein and that of soluble fraction was 25.3 units/mg, while that of the purified enzymes was 840.5 and 1612 units/mg, respectively. Enzyme activity was assayed with 20 mM α-hydroxy butyrate as substrate.

A simplified method was also used to attain partial purification of the enzyme molecular forms. It included fractionation with ammonium sulfate as in the procedure

of Nakano and Danowski, followed by separation on DEAE-cellulose in a single step with a linear gradient of sodium phosphate pH 7.9 (see legend of Fig. 2). A 30–50-fold purification was attained with this method. The results obtained with the enzymes isolated with this technique were identical to those revealed by purer enzymes.

Enzyme assay

The determination of enzymatic activity was performed with a method modified from that of Nachlas *et al.*[6] for lactate dehydrogenase. The reagent mixture contained: (A) 0.5 ml of 0.3 M sodium phosphate buffer, pH 7.9 containing 0.006 M EDTA; (B) 0.6 ml of nitro blue tetrazolium 2 mg/ml; (C) 0.3 ml of gelatin 1 mg/ml; (D) α-hydroxy acid (final concentrations will be indicated in each case); (E) water, an amount necessary to bring the total volume up to 2.5 ml. Reagents were mixed and incubated in the dark in a water bath at 37 °C for 15 min. Then, 0.3 ml of phenazine methosulfate (0.2 mg/ml) was added and the reaction started by addition of 0.2 ml of enzyme solution. Formazan generated by the reduction of nitro blue tetrazolium was determined continuously by reading at 540 nm every minute for 10 min with the cells maintained at 37 °C. 1 unit of enzyme is defined as the amount needed to reduce 1 μg of nitro blue tetrazolium per min in the conditions of the assay.

Electrophoresis

Starch gel electrophoresis was carried out with the vertical technique of Smithies[7]. Polyacrylamide gel electrophoresis was performed with the technique proposed by Davis[8].

Study of possible differences in molecular size between the enzyme fractions was carried out with the method of Hedrick and Smith[9]. For this analysis, the enzyme preparations were diluted with 50% glycerol solution in 0.05 M imidazole–HCl buffer, pH 5.7 to obtain a final concentration of 0.4 mg/ml.

After electrophoresis, α-hydroxy acid oxidase activity was localized in gels by staining using the method of Blanco *et al.*[10] for lactate dehydrogenase, except that NAD was omitted in the reagent mixture. Occasionally, other α-hydroxy acids were used as substrate.

Proteins were stained in gels with Amido Black.

RESULTS

Electrophoretic patterns

After electrophoresis and staining for α-hydroxy acid oxidase activity, extracts of the different fractions showed a single band migrating toward the anode (Fig. 1). The bands present in the soluble fraction and extracts of "light" mitochondria exhibited identical velocity of migration. The enzyme from "heavy" mitochondria showed higher mobility than that of the other fractions. There was no detectable activity in microsomal fraction extracts.

The difference in migration rate between preparations of "heavy" mitochondria and soluble or "light" mitochondria was the same for crude extracts or purified enzymes and was not modified by the extraction media used. Identical results were obtained with starch or polyacrylamide gel as supporting media.

Electrophoresis of a mixture of extracts of "heavy" and "light" mitochondria,

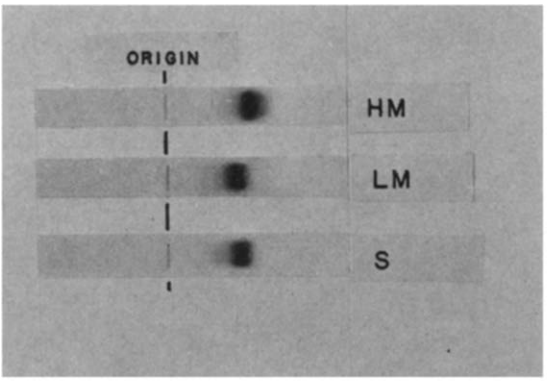

Fig. 1. Starch gel electrophoretic patterns of α-hydroxy acid oxidase from "heavy" mitochondria (HM), "light" mitochondria (LM) and soluble fraction (S) of rat kidney. All samples were run simultaneously in the same starch block. Stained for enzymatic activity using lactate as substrate (see text).

or of "heavy" mitochondria and soluble fraction, gave always two clearly separated bands (Fig. 3).

Analysis of the velocity of migration of purified enzymes by the method of Hedrick and Smith[9] indicated that the α-hydroxy acid oxidases from "heavy" mitochondria and the soluble phase have similar molecular sizes. The slopes of curves obtained by plotting logarithm of relative migration rate against concentration of polyacrylamide gel was the same for both enzymes.

Chromatographic profiles

The enzyme preparation obtained from "heavy" mitochondria gave a single

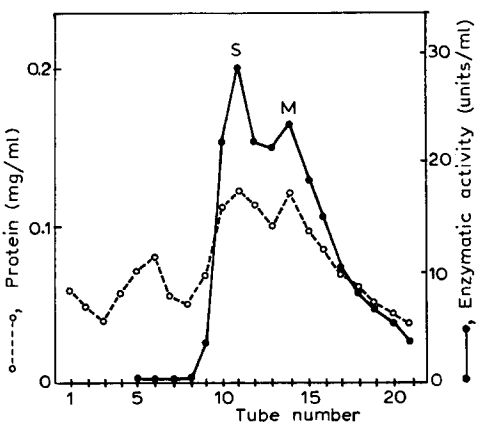

Fig. 2. Chromatographic profile of a mixture of α-hydroxy acid oxidase isolated from "heavy" mitochondria and soluble fractions of rat kidney. S corresponds to the peak of the enzyme from the soluble fraction; M, to that of "heavy" mitochondria. The mixture of enzymes containing about 150 mg of protein in a volume of 30 ml was poured into a 17 cm × 1.5 cm column of DEAE-cellulose previously equilibrated with 0.005 M sodium phosphate buffer (pH 7.9) containing 0.001 M EDTA. After addition of the enzyme, the column was washed with 200 ml of the same buffer and then developed with 500 ml of a linear gradient from 0.005 to 0.3 M sodium phosphate (pH 7.9) .The rate of flow was 0.8 ml/min and fractions of 10 ml were collected from the time the gradient was started.

peak with enzymatic activity that was eluted at higher concentrations of phosphate than the peak of L-α-hydroxy acid oxidase from the soluble phase or "light" mitochondria. This result is in agreement with the observed difference in electrophoretic migration of those enzymes.

Chromatography of a mixture of equal parts of "heavy" mitochondria and "soluble" enzymes gave two peaks of activity (Fig. 2).

Electrophoresis of effluent fractions corresponding to the first peak showed a band with the mobility of the enzyme from "light" mitochondria or the soluble fraction. Eluates of the second peak contained enzyme with a migration rate identical to that of "heavy" mitochondria. Mixtures of eluates containing equal activity of

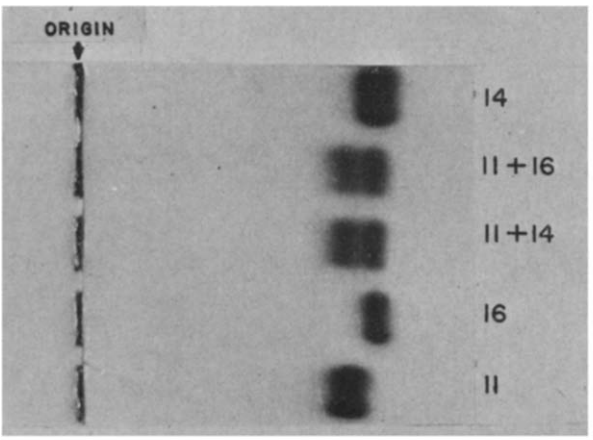

Fig. 3. Starch gel electrophoretic patterns of eluates from a DEAE-cellulose column (Expt of Fig. 2). Numbers indicate those of effluent fractions. 11 + 16 and 11 + 14 are mixtures of equal amounts of the corresponding fractions. Stained for enzymatic activity using lactate as substrate.

each of the enzymes gave the electrophoretic patterns shown in Fig. 3, with two distinct bands of L-α-hydroxy acid oxidase activity.

Effect of pre-heating upon activity of L-α-*hydroxy acid oxidase forms*

Purified enzymes were subjected to 45, 50, 55, 60, 65 and 70 °C for 5 min in a water bath. The enzyme was diluted with 0.1 M sodium phosphate buffer pH 7.9 containing 0.001 M EDTA, to obtain the same protein concentration for all samples (0.2 mg/ml). After heating, the preparations were kept on ice and enzymatic activity was assayed with 50 mM α-hydroxy butyrate and 15 mM α-hydroxy isocaproate.

The average of results from six determinations on different samples indicated that the enzyme from "heavy" mitochondria is more labile than the other form. Remaining activity after heating at 60 °C and 65 °C for the "heavy" mitochondrial enzyme was 63% and 16%, respectively, while that of "soluble" enzyme was 93% and 45%. For both differences $P < 0.001$. Essentially the same results were obtained with α-hydroxy butyrate or α-hydroxy isocaproate.

Catalytic properties of L-α-*hydroxy acid oxidase*

Effect of substrate concentration upon L-α-*hydroxy acid oxidase activity.* Sodium

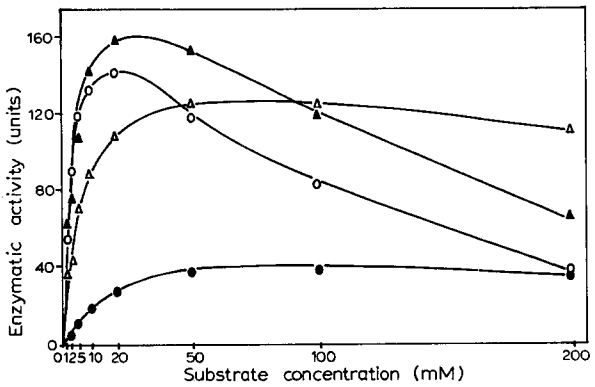

Fig. 4. Effect of substrate concentration upon the activity of α-hydroxy acid oxidase from soluble fraction of rat kidney. ▲, DL-α-hydroxy valerate; ○, DL-α-hydroxy isocaproate; △, DL-α-hydroxy butyrate; and ●, L-lactate. Each point represents average of six determinations on different samples.

salts of L-lactic, DL-α-hydroxy butyric, DL-α-hydroxy valeric and DL-α-hydroxy isocaproic acids were used as substrates. Results obtained with L-α-hydroxy acid oxidase from the soluble fraction are presented in Fig. 4 by plotting enzymatic activity against substrate concentration. The enzymes from "heavy" and "light" mitochondria gave identical results to those presented in Fig. 4.

The curves for all substrates used were of the Michaelian type. Two of the substrates, α-hydroxy valerate and -isocaproate produced a marked inhibition when used at concentrations higher than 50 mM. This effect of α-hydroxy isocaproate had been previously referred by Nakano *et al.*[11] for the L-α-hydroxy acid oxidase from rat liver.

Values of K_m for a variety of substrates were determined by plotting substrate concentration over velocity of reaction (s/v) against substrate concentration (s) (ref. 12). The L-α-hydroxy acid oxidase showed higher affinity with C_5 and C_6 hydroxy acids. Results for the different forms were similar (Table I).

Relative V, taking as 100% the value obtained with α-hydroxy butyrate, were the same for the enzymes of cytosol and "light" mitochondria, but showed some

TABLE I

K_m AND RELATIVE V FOR L-α HYDROXY ACID OXIDASE OF DIFFERENT CELLULAR FRACTIONS FROM RAT KIDNEY*

Substrate	*K_m (mM)*		*% V*	
	"Heavy" mitochondria	*Soluble*	*"Heavy" mitochondria*	*Soluble*
L-Lactate	5.7	5.6	28	50
DL-α-Hydroxy butyrate	2.8	2.4	100	100
DL-α-Hydroxy isovalerate	0.7	0.6	17	25
DL-α-Hydroxy valerate	0.7	0.6	54	96
DL-α-Hydroxy isocaproate	0.6	0.6	53	107
DL-α-Hydroxy caproate	0.9	0.8	44	71

* Values indicate averages of six determinations on different preparations.

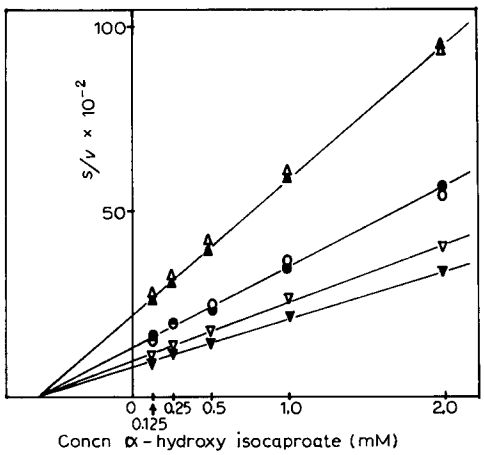

Fig. 5. Effect of α-keto acids upon oxidation of DL-α-hydroxy isocaproate catalysed by the α-hydroxy oxidase from the soluble fraction of rat kidney. Final concentration of added keto acid was 5 mM. △, α-keto caproate; ▲, α-keto isocaproate; ○, α-keto valerate; ●, α-keto isovalerate; ▽, α-keto butyrate; ▼, without added keto acid.

differences when compared with the values for the L-α-hydroxy acid oxidase from "heavy" mitochondria (Table I).

Effect of product. The inhibitory action of α-keto acids upon the L-α-hydroxy acid oxidase had been indicated by Allen and Beard[13]. The effect of α-keto butyrate, -valerate, -isovalerate and -isocaproate was studied by adding the product to the assay mixture before addition of phenazine methosulfate and enzyme.

α-Keto butyrate showed a weaker inhibitory action, while α-keto isocaproate was the most active inhibitor. The reaction with α-hydroxy butyrate was the most sensitive to that inhibition.

Fig. 5 represents the effect of α-keto acids upon the oxidation of α-hydroxy isocaproate catalysed by "soluble" L-α-hydroxy acid oxidase. Isomer keto acids showed identical effects. The inhibition is of the non-competitive type. The same results were

TABLE II

EFFECT OF ADDITION OF 5 mM KETO ACIDS UPON L-α-HYDROXY ACID OXIDASE ACTIVITY*

Substrate	*Inhibitor*			
	α-Keto butyrate	*α-Keto isovalerate*	*α-Keto valerate*	*α-Keto isocaproate*
DL-α-Hydroxy butyrate	25	57	57	77
DL-α-Hydroxy isovalerate	35	65	65	79
DL-α-Hydroxy valerate	31	52	52	78
DL-α-Hydroxy isocaproate	13	35	38	65

* Values express percentual reduction of V, taking as 100% the V without added α-keto acid. Averages of six determinations on different preparations. V was calculated from the slope of curves obtained plotting s/v against s (Fig. 5). Concentrations of substrate used were 0.125, 0.25, 0.5, 1.0 and 2 mM for α-hydroxy isovalerate, -valerate and -isocaproate, and 0.5, 1.0, 5.0 and 10 mM for α-hydroxy butyrate.

obtained with the enzymes from both mitochondrial subfractions. By using α-hydroxy valerate, -isovalerate, -caproate and -butyrate as substrates, the inhibitory effect of α-keto acids was similar to that observed for the reaction with α-hydroxy isocaproate.

Percentage of reduction of V for several substrates produced by 5 mM keto acids are shown in Table II.

Effect of pH

Determination of pH effect was performed using 33 mM Tris–HCl buffer in the reagent mixture instead of phosphate.

When saturating concentrations of substrate at all pH values between 7.0 and 9.6 were used, *e.g.* 100 mM α-hydroxy butyrate and 20 mM α-hydroxy isocaproate, the optimum was between 8.6 and 8.9 for the first substrate and between 8.9 and 9.4 for the second. No difference in pH effect was found between L-α-hydroxy acid oxidase from mitochondria and the soluble fraction.

DISCUSSION

The subcellular distribution of L-α-hydroxy acid oxidase from rat kidney had been studied by Baudhuin *et al.*[14], de Duve and Baudhuin[15], Allen and Beard[13,16,17], and Shnitka and Talibi[18], who reported the association of the enzyme to peroxisomes or microbodies. Nakano *et al.*[2,3,19] demonstrated the presence of L-α-hydroxy acid oxidase in the cytosol and the mitochondrial fraction (a mixture of mitochondria, peroxisomes and lysosomes) of rat kidney. Data from these authors and from Domenech and Blanco[4] indicated that the enzyme is not exclusively located in a particular type of organelles, but is distributed in different fractions.

With the procedure of de Duve *et al.*[5] used in the present study, the peroxisomes are concentrated in the fraction of "light" mitochondria.

Nakano *et al.*[2,3] pointed out that the enzymes obtained from mitochondria and cytosol have identical physico-chemical and immunological properties and suggested that they are the same molecule. Evidence presented here indicates that there are two different molecular forms of L-α-hydroxy acid oxidase in rat kidney. A dense subfraction of the mitochondrial moiety ("heavy" mitochondria) contains a molecular form of the enzyme which differs in electrical charge and stability to heating from the enzyme present in other cellular fractions.

With the available data it is not possible to substantiate the nature of differences at the molecular level between those two L-α-hydroxy acid oxidases. Analysis of electrophoretic mobility at different concentrations of polyacrylamide gel indicated that the molecular weights of both forms must be similar and that they could be classified as "charge isomers" according to the nomenclature proposed by Hedrick and Smith[9]. This precludes the possibility that they are polymers of different degree of the same polypeptide unit. Differences in electrical charge could be accounted for by the binding or splitting of low weight molecular groups or conformational modifications of the same protein molecule.

The study of catalytic properties revealed that substrate affinity, inhibition by substrate and product and effect of pH are remarkably similar for the enzymes purified from different cellular fractions. Some difference between the enzyme from

"heavy" mitochondria and that from the soluble phase or "light" mitochondria was only apparent when relative V_m against different substrates was compared. From these observations it can be concluded that the molecular forms of L-α-hydroxy acid oxidase are isokinetic. There must be close molecular similarities between the enzymes isolated from different fractions and, probably, their catalytic sites are identical.

The functional role of L-α-hydroxy acid oxidase is unknown. Indirect assumptions were made by de Duve and Baudhuin[15], Müller, Hogg and de Duve[20], Baudhuin[21], and Allen and Beard[13,16,17], who assigned physiological significance to the possible association of the enzyme with peroxisomes.

Keto acids as those formed in the reaction catalysed by L-α-hydroxy acid oxidase may follow the pathway of glyconeogenesis or, some of them, can be intermediates in the biosynthesis of amino acids. But the significance of some α-hydroxy acids against which the enzyme presents its highest activity *in vitro*, or the metabolic origin of those substrates are totally uncertain.

In any case, the effect of product upon the activity of L-α-hydroxy acid oxidase suggests the possibility of a regulatory mechanism. The accumulation of some keto acids, especially α-keto isocaproic, inhibits rather differentially the oxidation of certain substrates. The physiological implications of these observations cannot be ascertained until the metabolic function of the L-α-hydroxy acid oxidase becomes understood.

The demonstration that the molecular forms are distributed in different locations within the cell, suggests that they must be involved in distinct metabolic pathways.

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