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CRYSTALLINE L-AMINO-ACID OXIDASE FROM THE SOLUBLE FRACTION OF RAT-KIDNEY CELLS

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SUMMARY

1. L-Amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) has been crystallized from the soluble fraction of rat-kidney cells and compared with crystalline L-amino-acid oxidase isolated earlier from extracts of rat-kidney mitochondria. The immunochemical and physicochemical properties of the crystalline enzyme from the soluble fraction are essentially the same as those of crystalline mitochondrial oxidase.

2. L-Amino-acid oxidase is present in the soluble fraction of rat-kidney cells in concentrations some 4-fold greater than those isolated from mitochondrial extracts.

3. Crystalline L-amino-acid oxidase obtained from either the soluble fraction or from mitochondrial extracts is completely precipitable with antienzyme and the resulting enzyme–antienzyme complex shows full oxidase activity.

INTRODUCTION

Mammalian L-amino-acid oxidase (L-amino-acid: O_2 oxidoreductase (deaminating), EC 1.4.3.2) has been crystallized from rat-kidney mitochondria in this laboratory[1]. In a preliminary study of the intracellular distribution of L-amino-acid oxidase, we found that the soluble fraction of rat-kidney cells is rich in L-amino-acid oxidase.

The data herein presented indicate that the soluble fraction obtained from rat-kidney cells is a better source of active oxidase than that present in the mitochondrial fraction from the same tissue. The crystallization of mammalian L-amino-acid oxidase from the soluble fraction of rat-kidney cells is described with physicochemical and immunochemical evidence establishing the identity of this oxidase with mitochondrial L-amino-acid oxidase.

EXPERIMENTAL PROCEDURE

Enzyme assays

L-Amino-acid oxidase was assayed by incubation of the enzyme preparation at 37° in 6.25 mM α-amino acid or α-hydroxy acid (12.5 mM DL configuration). The

system was buffered with 3.13 mM sodium phosphate (pH 7.9) and contained 0.31 mM EDTA and 40 μg of catalase in a final vol. of 1.6 ml of the standard reaction mixture. The keto acid formed from the aliphatic amino or the α-hydroxy acid was then measured by a modification of the method of WALDMAN AND BURCH[2] (Method 1); that formed from the aromatic amino acid or from the α-hydroxy acid was assayed colorimetrically (absorption spectrum of the enol–borate complex[3]; Method 2). The unit selected was the quantity of enzyme required for the formation of 1 mμmole of the keto acid from the amino acid in 60 min or that obtained from the α-hydroxy acid in 20 min (in air). Specific activity was expressed in units/mg protein. The concentration of protein was determined by the phenol–biuret method[4]. The reduction of 2,6-dichlorophenolindophenol was employed in measuring the oxidation of α-hydroxy acids by the purified enzyme[5] (Method 3).

Preparation of the antibody

Crystalline L-amino-acid oxidase obtained from rat-kidney mitochondria was injected into a New Zealand rabbit (body wt., 4.3 kg). On the first day 2.4 mg of the enzyme in 0.5 ml of 0.02 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl (buffered saline) emulsified in Freund's adjuvant (General Biochem. Inc.) were given, half intraperitoneally and half subcutaneously, into a foot pad. Five days later 30 mg of the enzyme in buffered saline were injected into an ear vein and 4.5 mg of the enzyme emulsified in Freund's adjuvant were administered subcutaneously in the area of the scapula. Six days later 1.1 mg of the enzyme emulsified in the same adjuvant were administered subcutaneously into a foot pad. A 50 ml of blood was obtained by cardiac puncture on the 16th day following the initial injection followed by an 80-ml blood sample on the 20th day. Precipitin activity was high in both samples of sera (approx. 1:200 000). Sera from the two bleedings were pooled and the γ-globulin fraction was concentrated by fractionation with 50% aqueous ethanol according to the method of NICHOL AND DEUTSCH[6]. The precipitate was suspended in a small volume of buffered saline and dialyzed against 3 l of buffered saline for 20 h at 4°. It was found that the antibody could be stored at −30° for at least 8 months without loss of activity.

Titration assay

The enzyme and the antibody were mixed and incubated first for 30 min at

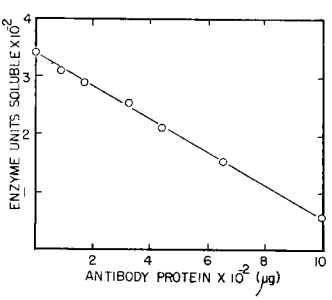

Fig. 1. Determination of antibody units. Each tube contained the crystalline enzyme preparation (25.2 μg) with specific activity of 10 620 units/mg protein as α-hydroxyisocaproate oxidase, antibody in the protein concentrations indicated and buffered saline added to a final vol. of 1.0 ml. The assay conditions are described in the section entitled *Titration assay*.

23° and then for 18 h at 4°. The mixture was then centrifuged at 15 000 × *g* for 20 min at 0–4° and the supernatant saved for the enzyme assay. The precipitate was washed twice with buffered saline (pH 7.4) and then resuspended in buffered saline. Aliquots of the supernatant fluid and the resuspended precipitate were used for the enzyme assays. The antibody preparation was also standardized by this method, using crystalline L-amino-acid oxidase obtained from rat-kidney mitochondria.

The activity unit was defined as that amount of antibody preparation which removed 1 unit of enzyme from the purified solution of enzyme (Fig. 1).

RESULTS

Enzyme purification

Kidneys from 20 adult male Sprague–Dawley rats weighing 400–430 g were used in a typical experiment; all steps were carried out at 0–4°. The kidneys were homogenized with 4 volumes of 0.25 M sucrose in a motor-driven Teflon homogenizer and centrifuged at 700 × *g* for 10 min to remove cells, nuclei and tissue debris. The supernatant fluid was than centrifuged for 10 min at 8500 × *g* to collect the mitochondria for the preparation of the mitochondrial amino-acid oxidase[1]. The supernatant fluid from the latter separation was then centrifuged for 120 min at 25 000 × *g* and the clear supernatant was designated as the 'soluble fraction'. This soluble fraction was found to contain approx. 75% of α-hydroxy-L-isocaproate oxidase activity or L-leucine oxidase activity in the rat-kidney homogenate.

The subsequent six steps (first $(NH_4)_2SO_4$ fractionation, first DEAE-cellulose column chromatography, second $(NH_4)_2SO_4$ fractionation, second DEAE-cellulose column chromatography, gel filtration on Sephadex G-200 and crystallization) were carried out by the methods described for the purification of mitochondrial L-amino-acid oxidase[1]. Table I and Fig. 2 illustrate a typical procedure. At each stage oxidase

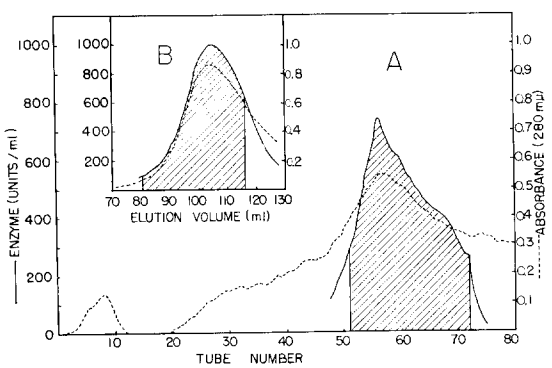

Fig. 2. A. Elution pattern of enzyme from DEAE-cellulose column. A total of 100 mg of protein (specific activity, 476) were added to the column (1.5 cm × 17 cm) and eluted with 0.005 M sodium phosphate buffer containing 10^{-3} M EDTA (300 ml) and 0.1 M sodium phosphate buffer with 10^{-3} M EDTA (300 ml) followed by linear gradient elution. The contents of each tube were assayed for leucine oxidase activity and protein. The enzyme activity was measured by Method 1, using L-leucine as substrate. B. Gel Filtration of L-amino-acid oxidase from Sephadex G-200. A total of 22 mg of protein (specific activity, 1540) were added to the column (2.5 cm × 45 cm) and eluted with 0.005 M sodium phosphate buffer containing 10^{-3} M EDTA. The enzyme activity was measured by Method 1, using L-leucine as substrate.

TABLE I

SUMMARY OF PURIFICATION OF OXIDASE FROM RAT KIDNEY*

Stage	*Fraction*	*Volume (ml)*	*Protein*		*Enzyme activity*		*Recovery (%)*	*Ratio***
			(mg/ml)	*(total mg)*	*Specific*	*Total*		
1	Soluble fraction	234.0	12.50	2925.0	69	201 825		3.8
2	First $(NH_4)_2SO_4$ precipitation	44.0	27.00	1188.0	91	108 110	53.5	5.3
3	First column eluate	77.0	3.75	289.0	264.0	76 230	37.5	6.0
4	Second $(NH_4)_2SO_4$ precipitation							
	0–35%	14.0	4.30	59.5	37.0			
	35–40%	15.4	3.10	47.4	365.0	49 000	24.5	6.2
	40–45%	16.0	3.50	56.0	566.0			
	45–50%	16.0	2.30	37.0	135.0			
5	Second column eluate	72.0	0.31	22.0	1490.0	32 780	16.1	6.2
6	Gel eluate	40.0	0.25	10.0	2030.0	20 300	10.1	6.2
7	Crystals				1980.0			6.1

* Kidneys (56 g wet wt.) from 20 rats used in this experiment.

** Specific activity of α-hydroxyisocaproate oxidation / Specific activity of L-leucine oxidation.

activity was determined according to Method 1 with L-leucine and α-hydroxy-L-isocaproate as substrates. The ratio of specific activity (α-hydroxyisocaproate oxidation/L-leucine oxidation) remained essentially constant at each step following the initial elution of the enzyme from a DEAE-cellulose column. The low recovery of oxidase after Step 1, *viz.* the first $(NH_4)_2SO_4$ fractionation (see above), might be attributable to contamination with other enzyme(s) catalyzing L-leucine deamination or α-hydroxyisocaproate dehydrogenation. This possibility is supported by two sets of findings. Thus, only 50–60% of the α-hydroxyisocaproate dehydrogenation activity present in soluble fraction of rat kidney, but nearly 100% of the dehydrogenation activity in the mitochondrial extract or in purified L-amino-acid oxidase from both mitochondrial and soluble fractions (see below), could be recovered in the precipitate obtained after the incubation of each preparation with an excess of the antibody. Furthermore, the substrate specificity of the soluble fraction is strictly different from that of purified oxidase.

The chromatogram of the partially purified oxidase on DEAE-cellulose (Fig. 2A) resembled that of the L-amino-acid oxidase obtained from the mitochondrial extract at the same stage of the purification[1]. On gel filtration of the enzyme the elution volume for attainment of peak enzyme activity was approx. 105 ml and enzyme activity and protein concentration were distributed symmetrically in a single peak (Fig. 2B). These results are identical with those obtained with the mitochondrial oxidase, suggesting that both enzymes have essentially the same molecular weight. Yield of the ultracentrifugically pure oxidase from the soluble fraction was found to be approx. 4 times greater than that from the mitochondrial fraction of rat kidney.

Physicochemical properties of oxidase from the soluble fraction (cytoplasmic oxidase)

Data obtained with L-amino-acid oxidase prepared from both the soluble and the mitochondrial fractions are summarized in Table II. These suggest that the enzymes obtained from different cellular fractions of rat kidney are identical with

TABLE II

PHYSIOCHEMICAL PROPERTIES OF L-AMINO ACID OXIDASE FROM SOLUBLE FRACTION AND MITOCHONDRIAL FRACTIC

Optical property		*Oxidase*					
		Soluble fraction			*Mitochondrial fraction**		
Crystalline enzyme	$\lambda_{max.}$ (mμ)	275	358	455	275	358	455*
	$\varepsilon_{M,max.}$ ($10^3 \cdot M^{-1} \cdot cm^{-1}$)	87.4	10.9	12.6	85.0	10.7	12.7*
	$A_{280\ m\mu}/A_{450\ m\mu}$		6.7			6.58	*
	$A_{410\ m\mu}/A_{450\ m\mu}$		0.56			0.58	*
	Protein/$A_{280\ m\mu}$		0.55			0.59	*
Prosthetic group	$\lambda_{max.}$ (mμ)	267	375	450	267	375	450*
	$\varepsilon_{M,max.}$ ($10^3 \cdot M^{-1} \cdot cm^{-1}$)	29.6	11.1	11.05	29.5	11.2	11.05*
FMN content (%)			0.98			0.925	*
Ultracentrifugal property		Single homogenous peak $s^0_{20,w}$ = 10.5 S			Single homogenous peak* $s^0_{20,w}$ = 10.5 S		
Crystal form		Octagonal, yellowish			Octagonal, yellowish*		
Specific activity	L-Leucine oxidase	1 900/60 min per mg			1 980/60 min per mg		
	α-Hydroxy-L-isocaproate oxidase	11 500/20 min per mg			10 000/20 min per mg		

* Data are taken from our previous report[1].

regard to their physicochemical properties. Furthermore, within the limits of experimental error, the substrate specificity of L-amino-acid oxidase obtained from the soluble fraction appears to be identical with that of the mitochondrial L-amino-acid oxidase (Table III).

Immunochemical properties

As shown in Fig. 3, titration of the antibody with crystalline L-amino-acid

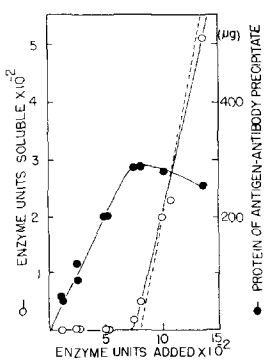

Fig. 3. Titration of the antibody with crystalline L-amino-acid oxidase. Crystalline L-amino-acid oxidase in buffered saline was incubated with antibody (800 units) as described in text. After centrifugation, enzyme activities in both the supernatant fluid and the precipitate were assayed by Method 1, using α-hydroxyisocaproate as the substrate. Protein concentration of the precipitate was measured by the phenol–biuret method. Broken line represents the theoretical oxidase activity in the supernatant fluid.

TABLE III

SUBSTRATE SPECIFICITY

Substrate	*Oxidase*	
	Soluble fraction	*Mitochondrial fraction****
Amino acid (monoamino and monocarboxylic)	*% of leucine oxidation**	*% of leucine oxidation*
Leucine	100.0	100
Methionine	74.2	87.5
Tryptophan	34.1	35.4
Phenylalanine	33.0	32.0
Isoleucine	14.0	9.0
Glycine	0	0
α-Hydroxy acid	*% of α-hydroxyvalerate oxidation***	*% of α-hydroxyvalerate oxidation*
Hydroxyvaleric	100.0	100.0
Hydroxyisocaproic	130.0	142.0
Hydroxycaproic	72.0	89.0
p-Hydroxyphenyllactic	36.0	40.0
Phenyllactic	33.0	35.0
Lactic	20.0	30.0
Hydroxyisovaleric	28.0	28.0
Mandelic	145.0	185.0
Hydroxyisobutyric	0.0	0.0

* Crystalline enzyme (180 units as leucine oxidase) was incubated with α-amino acid listed here under standard conditions. The activity was determined by Method 1 for aliphatic amino acid oxidation and by Method 2 for aromatic amino-acid oxidase.

** α-Hydroxy acid was added to the reaction mixture containing crystalline oxidase (320 units as α-hydroxyisocaproate oxidase) and 2,6-dichlorophenolindophenol; activity was determined by Method 3.

*** Data are taken from the previous report[1] for comparison.

oxidase obtained from the soluble fraction yields a typical precipitin curve in which antigen (enzyme) excess is indicated by a decrease in the amount of precipitable antigen–antibody complex. Assays of the supernatant of these mixtures for enzyme activity provide an equivalence point for oxidase–antibody interaction. This equi-

TABLE IV

OXIDASE–ANTIOXIDASE REACTION

Each enzyme was incubated with 5 times excess of the antibody for 18 h at 4°. The mixture was then centrifuged at 15 000 × *g* for 20 min at 4°; the resulting precipitate and supernatant fluid were employed in the assay of oxidase activity as described in text.

Crystalline oxidase	*Substrate*	*Enzyme activity recovered in*	
		Supernatant	*Precipitate*
Soluble fraction	α-Hydroxyisocaproate	6 ± 3	103 ± 2
	Leucine	7 ± 3	108 ± 5
Mitochondrial fraction	α-Hydroxyisocaproate	8 ± 4	102 ± 7

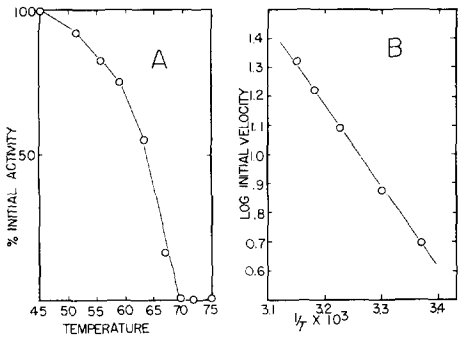

Fig. 4. A. The heat stability of oxidase. Pure oxidase, 30 μg in 35 mM sodium phosphate buffer containing 0.35 mM EDTA (total vol., 1.4 ml), was heated at the indicated temperature for 5 min, cooled, and assayed for oxidase activity by Method 1 using α-hydroxyisocaproate as substrate. B. The activation energy of the oxidase reaction. Standard mixtures containing 30 μg of pure oxidase were employed. Each mixture was incubated at the indicated temperature for 5 min, and the activity assayed by Method 1. The initial velocity is expressed as mμmoles of α-ketoisocaproate formed per min, and T is the absolute temperature.

valence point is essentially identical with that of the precipitin assay, indicating that the oxidase is the only component reacting with this antibody. The results were the same when mitochondrial L-amino-acid oxidase was used as the antigen. It was also found that, in the presence of an excess of antibody, the oxidase from the soluble fraction as well as the mitochondrial oxidase could be precipitated completely with the antibody. Both enzyme–antibody complexes manifested full enzyme activity (Table IV), suggesting that the catalytic site of the enzyme either is not involved directly in conjugation or is not blocked by combination with its antibody.

Effect of temperature on enzyme reaction

The enzyme was heated at various temperatures during 5-min intervals. The free enzyme retained full activity at 45°, but inactivation started at 51° and at 71° the activity was completely lost (Fig. 4A). Interpolation of the curve reveals that 50% of the enzyme can be expected to be inactivated when heated for 5 min at 64°. When the initial velocity of the enzyme reaction (k = mμmoles of α-ketoisocaproate formed from α-hydroxyisocaproate per min), measured at less than 45° was plotted against the reciprocal of the absolute temperature ($1/T$), a straight line was obtained (Fig. 4B), in agreement with the Arrhenius equation:

$$\log k = -\frac{E}{2.302\,R} \times \frac{1}{T} + \log S$$

where E is the heat of activation, R is the gas constant, and S is a constant. From the slope of the line, a value of 12 600 cal/mole was obtained.

The effects of various intervals of heat are shown in Fig. 5A. The velocity fell virtually to zero after 20 min at 56°, whereas the reaction rate remained constant during maintenance of the temperature at 26° and 37° for 20 min. Preparations heated at 56° without substrate also lost all of their enzyme activity in the course of 20 min (Fig. 5B). However, the enzyme–antibody complex did not lose any of its activity after 20 min at 56°; after 60 min at this temperature only one-third of the

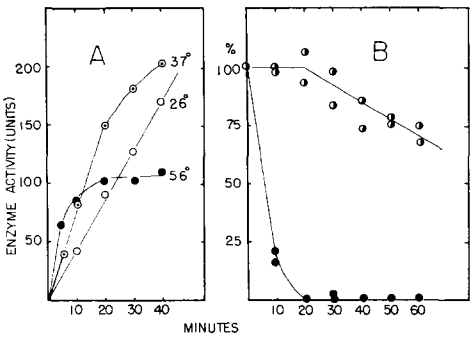

Fig. 5. A. Progress curves of enzyme reaction at various temperatures. Pure enzyme (15 μg) was incubated under the conditions described in the legend to Fig. 4B, and assayed for oxidase at the time indicated. B. Heat stability of the free enzyme and the enzyme–antienzyme complex. Pure enzyme (15 μg protein) (●) or enzyme–antienzyme complex (corresponding 15 μg pure enzyme) (◑) dissolved or suspended, respectively, in 0.8 ml of 0.02 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.4), was heated at 56° for the time indicated and assayed for oxidase activity by Method 1, after the addition of 40 μg of catalase, 0.5 ml of 0.1 M sodium phosphate buffer containing 10^{-3} M EDTA and of α-hydroxyisocaproate to 6.25 mM (total vol., 1.6 ml).

initial activity had been lost. From these findings it is evident that the substrate does not protect the enzyme from heat inactivation under these conditions, and that the stability of the enzyme is magnified by conjugation of the antibody and the enzyme.

Inhibition

Several sulfhydryl reagents were examined as possible inhibitors of the oxidase. Crystalline oxidase (25 μg) was incubated under the standard conditions with α-hydroxyisocaproate in the presence of varying concentrations of each reagent without added EDTA. Enzyme activity was measured by Method 1. Under these conditions the concentration of inhibitor corresponding to 50% of full activity was found to be $2.8 \cdot 10^{-6}$ M for *p*-chloromercuribenzoate, $1.4 \cdot 10^{-1}$ M for benzenarsonic acid. In contrast to the findings of BLANCHARD *et al.*[7], $(NH_4)_2SO_4$ at a concentration of $1.3 \cdot 10^{-2}$ M did not inhibit L-leucine oxidase activity.

DISCUSSION

BLANCHARD *et al.*[7] have reported that purified but not crystallized L-amino-acid oxidase obtained from whole rat kidney consists of three ultracentrifugally different protein molecules. On the other hand, crystalline L-amino-acid oxidase prepared in this laboratory from rat-kidney mitochondria was found to be a single protein, judging by the ultracentrifugal pattern and behavior on starch-gel electrophoresis[1].

The data herein presented confirm the finding that crystalline L-amino-acid oxidase from the soluble fraction of rat-kidney cells is essentially the same as that obtained from rat-kidney mitochondria, since their physicochemical and immunological properties are identical.

Since L-amino-acid oxidase is largely located in the soluble fraction of rat-kidney cells (the concentrations are approx. 4 times those found in the mitochondrial fraction), the total yield of crystalline L-amino-acid oxidase (or ultracentri-

fugally pure oxidase) from both the mitochondrial and soluble fractions would not be expected to be significantly different from that of the enzyme from whole kidney cells.

The purification procedure used by us was based on the isolation of the enzymatically most active fraction and elimination of less active moieties with impurities. Hence it cannot be concluded that the naturally occurring mammalian L-amino-acid oxidase in the mitochondria and in the soluble fractions of kidney cells is a single protein identical with the oxidase obtained in crystalline form. The purified oxidase obtained from whole kidney by BLANCHARD *et al.*[8] was estimated to consist of approx. 70% of crystalline L-amino acid, judging from its flavin content, corresponding perhaps to the eluate we obtained from the second DEAE-cellulose column in our purification procedure. However, in our studies gel filtration of this column eluate showed a single flavoprotein peak with high specific activity for oxidase. On the basis of these findings, it is suggested that the purified oxidase obtained by BLANCHARD *et al.* represented an aggregation of a single protein formed during purification.

The L-amino-acid oxidase–antienzyme complex manifested full enzyme activity and in this respect resembles several other enzyme–antibody systems[9–12]. It would appear, therefore, that the catalytic site of the L-amino-acid oxidase is either not involved directly in conjugation or is not blocked by the combination of enzyme with its antibody.

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