Biochimica et Biophysica Acta, 334 (1974) 287–296

BBA 67141

PIG LIVER MONOAMINE OXIDASE I:

ISOLATION AND CHARACTERIZATION

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(Received July 23rd, 1973)

SUMMARY

1. Monoamine oxidase has been isolated from pig liver and crystallized from an $(NH_4)_2SO_4$ solution.

2. This enzyme has a molecular weight of 1 200 000 with subunits of approximately 146 000 which readily polymerize to the higher molecular weight form.

3. The pH of maximum activity is 9.0. Its activity increases with temperature over the range 25–40 °C.

4. The apparent K_m at 25 °C is $1.28 \cdot 10^{-6}$ M at pH 9.0 (0.05 M glycine) and $1.74 \cdot 10^{-5}$ M at pH 7.2 (0.2 M phosphate).

5. The enzyme contains approximately eight copper(II) ions per 1 200 000 molecular weight. This metal can be removed and replaced by other divalent metal ions: Cu^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+} all produce a catalytically active form of this enzyme; but replacement with Mn^{2+} did not restore catalytic activity.

INTRODUCTION

Liver mitochondrial amine oxidase has been isolated from a number of sources including rat [1–4], human [5], beef [6–8], guinea pig [9] and pig [10] liver. The enzyme catalyzes the oxidative deamination of amines in the following general reaction:

$$RCH_2NHR' + O_2 + H_2O \rightarrow RCHO + R'NH_2 + H_2O_2 \quad (1)$$

The classification of amine oxidases into monoamine and diamine oxidases has been used throughout the literature despite numerous exceptions as documented by Kapeller-Adler [11]. Another method of classification involves the reaction of the enzyme to carbonyl and other reagents [12]. Recent reports by Gorkin et al. [13–16] indicate that these separate enzymes may be intra-convertable through modification of sulfhydryl groups by either oxidized oleic acid, glutathione or H_2O_2. If this latter assertation is established, then the general term amine oxidase will suffice.

In a previous study concerning the isolation of a pig liver [10], an extraction procedure using 2-butanone was reported. The investigators expressed doubt regard-

ing the use of detergents in amine oxidase preparations and indicated that the enzyme solutions obtained while using such techniques are either micellular or polydispersed particulate systems. In this investigation we have developed an alternate method using Triton X-100 which leads to the preparation of a similar enzyme in a highly purified state. Furthermore we have proceeded to partially characterize this enzyme with regard to its various physical properties. Our results compare favorably with the original report [10] and suggest an inconsistency in the more recent version [17].

METHODS AND MATERIALS

Assay methods

The enzyme assay with benzylamine as a substrate was carried out at 25 °C as described by Tabor et al. [18] at 250 nm. Either 0.2 M phosphate (pH 7.2) or 0.05 M glycine (pH 9.0) containing 1 mM EDTA was used as the buffer system. One unit of activity is defined as the amount of enzyme catalyzing a change of 0.001 absorbance per min per mg of protein at 25 °C. pH values were determined on a Corning Model 7 pH meter. Protein concentrations were determined by the biuret method using bovine albumin from Sigma as a standard. A value of 10.9 for $E^{1\%}_{280\,nm}$ was established.

Chemicals

Benzylamine from Sigma was distilled under vacuum just before use in all kinetic studies. Sephadex was obtained from Pharmacia and deoxyribonuclease (beef pancreas) was purchased from Sigma. All chemicals used in this study were reagent grade. Collodion membranes were obtained from Schleicher and Schuell, Inc.

Isolation procedure

Liver obtained from a freshly slaughtered pig was cut into 1-cm cubes and frozen overnight. The liver was thawed at room temperature and 50-g amounts were homogenized in a Waring Blendor with 150 ml of water for 120 s. The resulting solution was filtered through cheese cloth to remove large particles, and centrifuged at 6000 × *g* for 1 h at 4 °C. The precipitate was discarded and $(NH_4)_2SO_4$ (21 g/100 ml) was slowly added with gentle stirring. The solution was then allowed to stand for at least 4 h at 4 °C. The final suspension was centrifuged at 10 000 × *g* for 30 min at 4 °C, and the supernatant discarded. The precipitate was treated with Triton X-100 (4 drops Triton X-100/3 ml precipitate) in a tissue homogenizer and ground by hand for approx. 2 min.

The resulting suspension was increased in volume by 50% using 0.2 M phosphate buffer (pH 7.0) and was stored overnight at 4 °C. The solution was then centrifuged at 20 000 × *g* for 1 h at 4 °C. Any suspended material and the precipitate were discarded. The suspended matter was removed from the supernatant by filtering through nylon mesh or other porous material.

At this point, 20 mg of deoxyribonuclease (6000 Kunitz units) was added and the solution stored at 4 °C for 1 week. As in the isolation of sugar dehydrogenases [20] and catalase [21] from pig liver, the addition of deoxyribonuclease results in an overall increase in enzyme units. This is due to the breakdown of large nucleic acid particles which slowly precipitate from the solution. These large particles were apparently

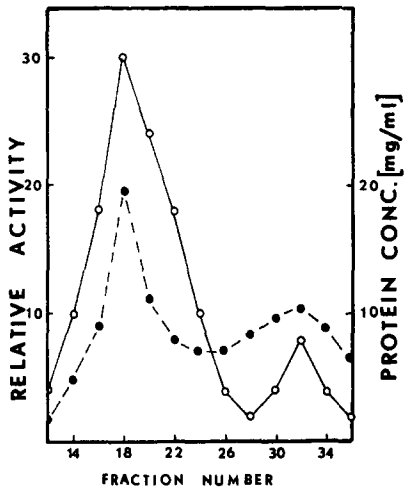

Fig. 1. Relative activity (○) and protein concentration (●) vs fraction number for first chromatographic separation on Sephadex G-200.

complexed with the enzyme of interest, thus reducing the effective number of catalytic sites per mg of total protein. The results in Table I are consistent with our previous [20, 21] results as the number of units is seen to increase with time. At the end of 5 or more days, the solution was centrifuged at 20 000 × *g* for 30 min and the supernatant added in 5-ml quantities to a Sephadex G-200 column (flow rate 0.25 ml/min).

The results of the first G-200 treatment are shown in Fig. 1 and a sample set of data is given in Table I. As is indicated in Fig. 1, there are two main components, one of molecular weight 1 200 000 and the other 146 000 as determined on a stan-

TABLE I

PURIFICATION OF PIG LIVER MONOAMINE OXIDASE*

Assayed at 25 °C, pH 7.2, 0.2 M phosphate buffer. Numbers in parenthesis refer to assay at pH 9.0, 25 °C, 0.05 M glycine buffer.

	Vol. (ml)	Units	Protein (mg)	Specific activity
1. Crude extract after centrifugation at 6000 × *g*	122	29 622	7296	4.06 (10.8)
2. $(NH_4)_2SO_4$ fraction, treated with Triton X-100	55	39 593	1889	21.0 (56)
3. 3 days after adding nuclease and centrifugation at 20 000 × *g*	54	47 765	1409	33.9 (90.4)
4. 2 days later	53	45 712	1325	34.5 (92.0)
5. 1st G-200 column major fraction (150 mg added)	5	4 684	6.94	675 (1800)
6. 2nd G-200 column major fraction (50 mg added)	5	20 240	17.6	1150 (3067)
7. 1st recrystallization from $(NH_4)_2SO_4$ (50 mg used)	10	54 450	45	1210
8. 2nd recrystallization using 27 mg	6	25 300	23	1100

* High (1 200 000) molecular weight component only.

dardized Sephadex G-200 column [20, 22]. The most active fractions of the high molecular weight component with respect to benzylamine, were condensed using collodion membranes and chromatographed on a second Sephadex G-200 column. The column eluent was triply distilled water at pH 7.0 (0.001 M phosphate buffer) containing 1 mM EDTA, and 0.004% NaN_3. Sample results are given in Table I and Fig. 2.

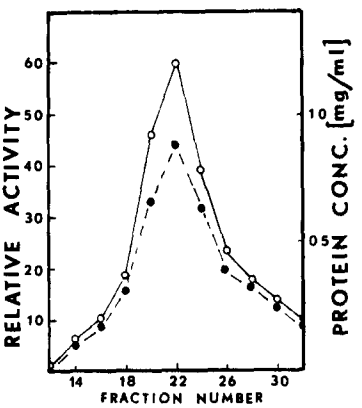

Fig. 2. Relative activity (○) and protein concentration (●) vs fraction number for second chromatographic separation on Sephadex G-200.

Attempts to increase the specific activity beyond that obtained from the second column elution by $(NH_4)_2SO_4$ recrystallization proved fruitless. The crystallization procedure consisted of first subjecting 10-ml aliquots of solution, each containing 45 mg of enzyme to salting out with $(NH_4)_2SO_4$ at 0 °C. This was accomplished at 25% saturation. The precipitate was resuspended in 0.05 M phosphate buffer, pH 7.0, and then $(NH_4)_2SO_4$ was added to 28% saturation. Crystals were seen to form overnight at 5 °C. This technique yielded samples showing little or no improvement over the chromatographed samples. In view of this fact, the majority of the experiments reported herein used the high molecular weight form eluted from the second column.

RESULTS AND DISCUSSION

As we have previously indicated, our isolation procedure results in the identification of two molecular components, the larger of which possesses the greatest specific activity by a factor of 2–3 to 1. Furthermore the ratio of molecular weights suggests that the higher molecular weight form is an octamer of the lower one. Additional evidence to support this view will be presented later on in this report.

In view of the various classification systems, we proceeded to test the behavior of this enzyme with respect to carbonyl and metal-chelating agents. As Table II indicates, this enzyme fails to react to either CN^- or semicarbazide. Unlike other liver monoamine oxidases, however, it is inhibited by NaN_3. The strength of the inhibitors is in the order *o*-phenanthroline > neocuproine > 8-hydroxyquinoline > diethyldithiocarbamate > N_3^- > hydroxylamine. These results are similar to those obtained for beef plasma [23] and liver [24] amine oxidase, with the exception of

TABLE II

INHIBITION BY METAL-CHELATING AGENTS

Chelator	Final concentration	Inhibition (%)
NaCN	$3.3 \cdot 10^{-4}$	0
Neocuproine	$3.3 \cdot 10^{-4}$	61
Hydroxylamine	$3.3 \cdot 10^{-3}$	30
	$3.3 \cdot 10^{-4}$	3
o-phenanthroline	$3.3 \cdot 10^{-4}$	65
Semicarbazide	$3.3 \cdot 10^{-3}$	0
Diethyldithiocarbamate	$3.3 \cdot 10^{-3}$	74
	$3.3 \cdot 10^{-4}$	5
NaN_3	$3.3 \cdot 10^{-3}$	48
	$3.3 \cdot 10^{-4}$	2
EDTA	$3.3 \cdot 10^{-3}$	0
8-hydroxyquinoline	$3.3 \cdot 10^{-4}$	13

CN^-, an inhibitor of the plasma enzyme. Inhibitor studies of the human plasma monoamine oxidase also gave similar results [25].

As Table III indicates, the enzyme activity increases over the range 25–40 °C. This result is identical to that obtained for the high molecular weight (1 280 000) form of beef liver mitochondrial amine oxidase [6].

The results of a pH study are given in Fig. 3 and point out that 0.2 M phosphate buffer is an unwise choice as it not only alters the pH maximum from 9.0 to 8.5, but also decreases the enzymatic activity. We observed linear velocity plots for periods of 20 min or more at all pH values and therefore chose 0.05 M glycine buffer (1 mM EDTA) as our model assay system. We also include $1 \cdot 10^{-4}$ M dithioerythritol to protect sulfhydryl groups as these can be altered in the presence of various oxidizing agents [13–16]. Our pH optimum of 9.0 is also similar to that obtained for the beef liver enzyme [6] of molecular weights 1 280 000 and 405 000. The pig liver enzyme [17] of molecular weight 275 000–290 000 also possesses a similar pH maximum, however its activity is more sharply curtailed at higher and lower pH values.

Having noted the above similarities between our pig liver monoamine oxidase and the beef liver enzyme [6], we undertook two types of polymerization experiments with identical results. First we obtained 8–10-mg quantities of the lower molecular

TABLE III

RELATIVE ACTIVITY VERSUS TEMPERATURE AT pH 9.0 IN 0.05 M GLYCINE BUFFER

Temperature (°C)	Relative activity
25.0	22.8
27.5	23.2
30.0	25.2
32.5	26.5
35.0	27.4
37.5	29.5
40.0	31.2

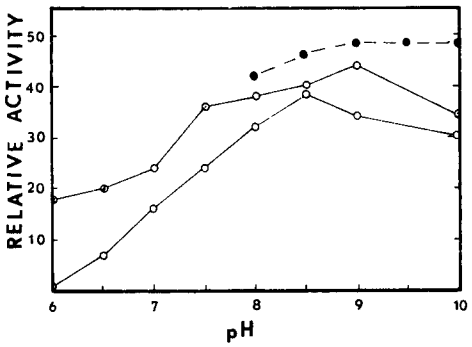

Fig. 3 Enzymatic activity vs pH in 0.05 M glycine (●), 0.05 M phosphate (○), and 0 2 M phosphate (○) buffers at 25 °C.

weight species, adjusted its concentration to 4–5 mg/ml using Collodion membranes, and stored it for 24 h at 4 °C. At the end of this period we centrifuged the sample at 20 000 × *g* and discarded the precipitate which accounted for some 5% of the original sample. We then chromatographed the sample on a standardized Sephadex G-200 column and observed that 70% of the sample had polymerized to the higher molecular weight form.

In a second experiment we used $(NH_4)_2SO_4$ to precipitate the lower molecular weight form and redissolved it in pH 7.0 phosphate (0.002 M) buffer. The solution was centrifuged at 20 000 × *g* and some 20% of the sample discarded as a precipitate. The remainder was passed down the same Sephadex G-200 as the previous sample, and some 80% of the remaining sample appeared as the higher molecular weight form.

The next step was an assay for the presence of Cu(I) and Cu(II). Cu(II) has been found in plasma amine oxidases, but not in pig brain monoamine oxidase [26]. Triplicate samples from different runs were dialyzed against 2 mM EDTA (volume 400:1) for 24 h before analysis. Total Cu was determined by the use of biquinoline, and Cu(I) was determined by use of dithionate and biquinoline [24, 27]. The total Cu content was 0.0434% or 8.19 ± 0.29 atoms Cu per enzyme unit of molecular weight 1 200 000. The amount of Cu(I) was less than 0.50 atom per enzyme molecule and apparently represents a trapped impurity. Once again, the evidence supports a major unit of octameric structure.

At this point we tested for the possible replacement of Cu(II) by other metals. Samples of enzyme were dialyzed against 0.01 M diethyldithiocarbamate for 24 h, at which time there was no enzymatic activity. The samples were then made 0.01 M in various divalent metal ions and stored overnight at 4 °C. These were then dialyzed against 0.01 M EDTA for 24 h at room temperature. The samples were then tested for enzymatic activity and the results are given in Table IV. The results are unusual in this is the first report of general metal substitution in amine oxidases that we are aware of.

Having observed the polymeric condition of pig liver amine oxidase, we assayed both the high and low molecular weight forms for spermadine activity under a variety of conditions. No activity whatsoever was observed during any stage of the preparation, nor was there any spermadine activity with the substituted metal-con-

TABLE IV

RESTORATION OF BENZYLAMINE ACTIVITY BY DIVALENT IONS

Ion	Source	% recovery
Cu^{2+}	$Cu(C_2H_3O_2)_2\ H_2O$	22.1
Co^{2+}	$CoCl_2\ 6H_2O$	43.5
Zn^{2+}	$ZnCl_2$	50.5
Ni^{2+}	$NiCl_2 \cdot 6H_2O$	77.6
Mn^{2+}	$MnSO_4 \cdot H_2O$	0
Hg^{2+}	$Hg(NO_3)_2 \cdot H_2O$	0

taining enzymes. Thus, in view of this and previous evidence, we conclude that this enzyme should be classified as a monoamine oxidase.

As a kinetic test for purity, we determined K_m at 25 °C in pH 7.2 (0.2 M phosphate) and pH 9.0 (0.05 M glycine) buffers. The results are shown in Fig. 4. A K_m of $1.74 \cdot 10^{-5}$ M was determined at pH 7.2 and $1.28 \cdot 10^{-6}$ M at pH 9.0. A value of $4.5 \cdot 10^{-4}$ M at pH 7.2 was reported for the pig liver enzyme of molecular weight 275 000–290 000 [17] and no indication of substrate inhibition was given such as that shown in Fig. 4. These values are considerably larger than ours, despite reported specific activities [17] several times greater than those in Table I.

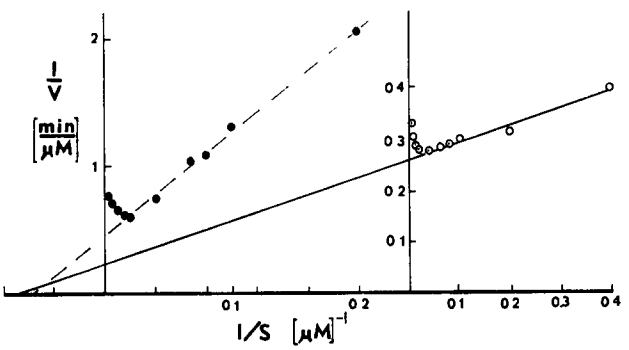

Fig. 4. Michaelis plots at 25 °C at pH values 7.2 (0.2 M phosphate buffer)(●) and 9.0 (0.05 M glycine buffer) (○).

Other workers have reported K_m values of $5 \cdot 10^{-6}$ M (pH 9.0) and $3.0 \cdot 10^{-4}$ M (pH 7.2) for human plasma [28], $8.4 \cdot 10^{-5}$ M (pH 7.8) for rabbit serum [29], $2.5 \cdot 10^{-5}$ M (pH independent) for pig plasma [30] and $2.2 \cdot 10^{-4}$ M (pH 7.4) for beef liver monoamine oxidase [31]. Substrate inhibition with benzylamine is quite evident in human plasma [28] and rabbit serum [29] kinetics. This latter fact fits in well with a subunit model containing allosteric sites and is consistent with our general observations.

The highly purified enzyme possesses a peak at 412 nm and a shoulder at approx. 530 nm in pH 9.0 glycine buffer. Upon reduction with dithionite, the peak at 412 nm decreases in intensity and the peak at 530 nm disappears. Addition of benzylamine results in a decrease in absorption of both the 412 and 530 bands, as is shown in Fig. 5. This decrease is proportional to the concentration of benzylamine added and to the time after addition.

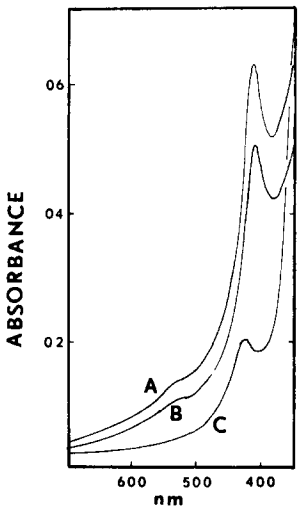

Fig. 5. Spectra of monoamine oxidase: A, 4 5 mg/ml at 25 °C; B, A + 3.3 mM benzylamine; C, A + 10^{-4} M dithionite.

No evidence of cytochrome oxidase was observed [32], however a small amount of catalase activity was detected [33]. Catalase activity of 0.695 units/mg was observed, and represents 0.001 % activity as compared to the crystalline pig liver enzyme [21]. It is apparent that the heme content of Oreland's [17] form of pig liver monoamine oxidase is due to a catalase impurity. This is to be expected as catalase will be necessary in the mitochondria if any evolved H_2O_2 is to be converted back into water and O_2. Should this impurity be present in even small amounts, its high absorption at 406 nm will account for a high percentage of the absorption observed at 410 nm [17].

CONCLUSION

In this investigation we have isolated a highly purified form of pig liver monoamine oxidase. This enzyme of molecular weight 1 200 000 is highly polymeric and is made up of subunits which are similar in molecular weight, but not necessarily identical in either structure or function. This latter question will be considered in a future report.

We find that this enzyme is similar to that isolated from beef liver [6] of molecular weight 1 200 000 although the beef liver subunits are considerably larger (405 000) than ours. In the original report on pig liver monoamine oxidase [10] values of 400 000 and 100 000 were given as possible molecular weights. More recently [17] the molecular weights given were 275 000–290 000 and 108 000–117 000 for this same enzyme. Methylethyl ketone was compared [17] with Triton X-100 in that Triton X-100 produced a similar enzyme with a K_m of approximately three times the value (0.45 mM) obtained for the ketone-extracted enzyme. Obviously, any useage of Triton X-100 which fails to produce a high molecular weight form of monoamine oxidase is somewhat surprising.

An attempt was made in our laboratory to observe the effect of methylethyl ketone upon a sample of our high molecular weight enzyme. All efforts at extraction of possible lipid group resulted in denaturation of this enzyme. Attempts to regenerate activity with cardiolipin were also negative.

Coupling the above facts with the low K_m values obtained with the high molecular weight enzyme, we can offer only two solutions. First, there is the distinct possibility that separate forms of this enzyme do exist within the mitochondria. Secondly, the use of methylethyl ketone may have removed a portion of this lipoprotein, thus altering its size, binding and catalytic properties. Obviously more needs to be done if we are to establish which concept is correct. Temporarily, we propose the latter conclusion until more convincing evidence is forthcoming.

ACKNOWLEDGEMENTS

This research was supported in part by Biomedical Sciences Support Grant 5 S05 RR07121-02 from the General Research Support Branch, Division of Research Resources, Bureau of Health Professions, Education and Manpower Training, National Institutes of Health. We gratefully acknowledge a PHS Special Research Fellowship (to W. R. C., 1F0 3MH 52945-01 BLS) from the National Institute of Mental Health and a PHS Career Award (to D. F. M., 5-K04-GM42569-04) from the National Institute of General Medical Sciences.

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