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PURIFICATION AND SOME PROPERTIES OF PORCINE BRAIN MITOCHONDRIAL MONOAMINE OXIDASE B*

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Abstract—Monoamine oxidase was isolated from pig brain mitochondria in a highly purified state by the following procedures: treatment with Ca-phosphate gel, column chromatography on DEAE-cellulose, mercurial-Sepharose, and hydroxyapatite chromatography and gel filtration on Bio-Gel A-1.5m. The properties of the enzyme disclosed that it is a monoamine oxidase of the B-type. The FAD present in the enzyme was covalently attached to the protein and it was shown to react with 3-*N*,*N*-dimethylamino-1-propyne which is indicative of a reaction with the N^5 position of the isoalloxazine ring of FAD. The flavin content from spectral data and from the ^{14}C-content of the enzyme inactivated with labeled 3-*N*,*N*-dimethylamino-1-propyne yielded a FAD content of 1 mole/104,000 g of protein. The subunit molecular weight from SDS-disc electrophoresis yielded a molecular weight of 52,000. Thus, it was concluded that the brain monoamine oxidase probably has two identical subunits, only one of which contained covalently bonded FAD.

There have been several papers published on the purification of monoamine oxidase [1–4] but none of them have described the detailed properties of the highly purified enzyme. The purification of brain monoamine oxidase is a very difficult task due to the high lipid content of the brain and the low amounts of monoamine oxidase present. Tipton [5] has described a purification procedure for the isolation of pig brain monoamine oxidase and some properties of the highly purified enzyme. However, we have not been able to reproduce this purification procedure. Therefore, we have developed our own procedure which is similar to the method that we have used for the purification of the bovine liver monoamine oxidase [6]. Preparations of high purity have been obtained and some interesting new properties of the enzyme have been determined. Our main goal, however, was to know whether or not the FAD in the enzyme was dissociable. The results of these investigations are presented in this publication.

MATERIALS AND METHODS

Mitochondria were prepared by the method of Achee *et al.* [7] from fresh pig brain which was provided by the Island Pork Co., Honolulu. About 20 g mitochondria (dry weight) was obtained from 1 kg of brain. The mitochondrial pellets were then stored in a freezer (−25°). DEAE-cellulose, benzylamine, tyramine, tryptamine, catalase, yeast alcohol dehydrogenase, bovine liver catalase and cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, MO. Ca-phosphate gel was prepared according to the method of Singer and Kearney [8]. Hydroxyapatite was prepared according to the method of Tiselius *et al.* [9]. Mercurial-Sepharose was prepared as described by Sluyterman and Wijdenes [10]. Crystalline bovine serum albumin was purchased from Pentex Inc., Kankakee, IL, and blue dextran 2000 and concanavalin A from Pharmacia, Piscataway, NJ; ferritin was obtained from CalBiochem, LaJolla, CA; bacterial amylase (a gift from Dr. J. Fukumoto, Osaka City University) were used as standard proteins for the molecular weight estimations. The inhibitor, [2-^{14}C]3-*N*,*N*-dimethylamino-1-propyne HCl, was synthesized by Dr. Abeles of Brandeis University and was provided by Dr. T. P. Singer, Veterans Hospital, San Francisco. Rabbit antisera to bovine liver monoamine oxidase was a gift from Dr. B. Hartman, Washington University, St. Louis. Other chemicals were of reagent grade and were purchased from the standard sources.

The spectrophotometric enzyme assay method of Tabor *et al.* [11] was used. It was slightly modified and contained 3.3 mM benzylamine and 0.5% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.4. One unit of enzyme was defined as the amount of enzyme necessary to catalyze an increase of 0.001 absorbance unit/min at 25° using the Cary model 14 spectrophotometer. Specific activity was defined as the number of units/mg of protein.

For part of the substrate specificity investigation the activity was measured by the rate of release of ammonia. Two-chamber porcelain Conway diffusion dishes were filled with 1.0 ml of 2% boric acid, to which 1 drop of brom cresol green indicator was added. In the outer chamber was placed an amount of 0.1 M potassium phosphate buffer (pH 7.4) chosen to make a total of 1.0 ml after the addition of substrate (final concentration of 1×10^{-5} M) and enzyme. Enzyme (0.3 ml) was added and the mixture was allowed to stand under cover for 15 min. Then 0.1 ml of substrate solution (final concentration of 1×10^{-5} M) was added rapidly and the dish was covered with a polished glass

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plate with High Vacuum Silicon grease. After 30 min at 24°, a saturated K_2CO_3 solution (1 ml) was added rapidly, and the tightly covered dish was placed in an oven at 33° for 1 hr. The borate solution was titrated with 7.15×10^{-3} N H_2SO_4. Each determination was run in duplicate. A blank of enzyme and buffer alone was run and this value subtracted from the enzyme run.

Protein concentrations were determined by the method of Lowry *et al.* [12] and bovine serum albumin was used as the standard.

SDS-disc electrophoresis was carried out as described by Weber and Osborn [13] for purity checks and for the estimation of the molecular weight of the enzyme. The gel was stained with 0.5% Coomassie brilliant blue and destained with a methanol–acetic acid–water (50:75:875) solution. The molecular weight of the enzyme was estimated by exclusion-diffusion chromatography using Bio-Gel A-1.5m. The isoelectric point of the enzyme was estimated by the isoelectric focusing technique in which ampholine and sucrose were used to provide pH and density gradients respectively [14].

RESULTS

Purification of pig brain mitochondrial monoamine oxidase

All procedures were carried out at 4° and all centrifugations were performed in the Sorvall Centrifuge (model RC 2B). Buffers used for the purification of the enzyme were KH_2PO_4–K_2HPO_4 buffers which contained 0.005 M β-mercaptoethanol; the concentrations and pH values of the buffers used are indicated in the text.

Step 1. The frozen mitochondrial pellet (60 g dry weight) was thawed, and then washed with 1.5 l. of 0.1 M buffer containing 0.5% Triton X-100, pH 6.8. This is a very important and necessary step which is essential for further purification of the enzyme. After concentration, the washed mitochondrial pellet was suspended in 0.1 M buffer, pH 8.4, which contained 1% Triton X-100 and 0.5% potassium cholate to yield 1.1 l. of suspension. The specific activity of the enzyme at this step was 5.8.

Step 2. After the suspension was stirred gently for 5 hr, the lysate mixture was placed in a freezer for 24 hr. This step is vital for the clarification of the supernatant solution during the next centrifugation step. The frozen lysate was thawed at room temperature and centrifuged at 20,000 *g* for 30 min and yielded 730 ml of a clear solution with a specific activity of 21.5.

Step 3. The resulting supernatant fraction from the previous step was applied to a DEAE-cellulose column (6 × 45 cm) which had been pre-equilibrated with 0.02 M buffer, pH 7.4. Monoamine oxidase was not absorbed but passed through the column along with the Triton X-100. At this step, 730 ml of the enzyme was obtained and its specific activity was 37.5.

Step 4. The enzyme solution from the previous step was diluted with 4 vol. of cold 0.005 M β-mercaptoethanol and the pH was adjusted to 7.4. About 12 g of Ca-phosphate gel (protein to gel ratio was about 1:5) was added to the diluted solution and the mixture was stirred gently overnight at 4° in order to absorb the enzyme on the Ca-phosphate gel. The gel was collected by centrifugation at 5000 *g* for 5 min. The Ca-phosphate gel was washed successively with 1000 ml each of 0.01 M buffer, pH 7.4, and 0.1 M buffer, pH 8.4. The Ca-phosphate gel was re-suspended in 300 ml of 0.1 M buffer, pH 8.4, which contained 0.5% Triton X-100 and cholate and was gently stirred. After 2 hr, the suspension was centrifuged at 5000 *g* for 5 min and the supernatant solution was saved. This elution procedure was repeated a total of three times and about 840 ml of the combined eluate was obtained with a specific activity of 75.5.

Step 5. The eluate from the Ca-phosphate gel was diluted with 4 vol. of cold 0.005 M β-mercaptoethanol and the solution was adjusted to pH 7.4. The diluted eluate was applied to a DEAE-cellulose column (3.5 × 40 cm) which had been equilibrated previously with 0.02 M buffer, pH 7.4. After the column was washed with 800 ml of 0.02 M buffer, pH 7.4, the column was subjected to linear gradient elution with sodium chloride (500 ml of 0.02 M buffer containing 0.5 M sodium chloride, pH 7.4, in the reservoir chamber) to elute the enzyme. The enzyme was eluted at a sodium chloride concentration between 0.18 and 0.22 M prior to the elution of a large amount of inert protein. The active enzyme fractions were collected and used for the further purification of the enzyme. About 350 ml of the eluate was obtained with a specific activity of 256.

Step 6. The eluate from the DEAE-cellulose was chromatographed on a mercurial-Sepharose column (3.5 × 15 cm) which had been washed with an excess of 0.01 M buffer, pH 8.2. After application of the enzyme to the column, the column was washed with 500 ml of 0.1 M phosphate, pH 8.2, containing 0.2 M β-mercaptoethanol. The enzyme was then eluted with 0.1 M phosphate buffer, pH 8.2, which contained 1% Triton X-100, 0.5% cholate and 0.2 M β-mercaptoethanol. The eluate was dialyzed against 0.01 M buffer, pH 8.2, to remove the excess β-mercaptoethanol. At this step, the specific activity of the enzyme was 1000.

Step 7. The dialyzed enzyme from the previous step was diluted with 5 vol. of 0.01 M buffer, pH 8.2, and the diluted enzyme was applied onto a hydroxyapatite column (2.8 × 8 cm) previously equilibrated with 0.01 M buffer, pH 8.2. The column was washed successively with 0.01 M (25 ml) and 0.1 M (60 ml) buffer, pH 8.2, and the monoamine oxidase was eluted with 0.1 M (100 ml) buffer, pH 8.2, which contained 0.25% each of Triton X-100 and cholate. A small amount of enzyme was detected in the 0.1 M buffer eluate but the majority of the enzyme was eluted by the final buffer which contained the detergents. The specific activity of the enzyme (volume 15 ml) at this step was 4000.

Step 8. Finally, after the eluate was carefully concentrated under reduced pressure in a collodion bag, the concentrate (3.5 ml) was applied to a Bio-Gel A-1.5m column (1.8 × 80 cm) in 0.1 M buffer, pH 8.2. The enzyme was eluted as a symmetrical peak and was also separated from a small amount of impurity. The specific activity of the purified enzyme was 6540 and the yield of the activity was 4 per cent. The purified enzyme was used for further studies described in this

Table 1. Summary of the purification data of pig brain mitochondrial monoamine oxidase*

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification
1. Suspension of mitochondria	1100	165,000	28,600	5.8	100	1
2. Extracts with Triton X-100 and cholate	730	102,200	4745	21.5	62	3.7
3. DEAE-cellulose passed through fraction	730	87,600	2336	37.5	53	6.5
4. Ca-phosphate gel eluate	840	69,720	924	75.5	42	13.0
5. DEAE-cellulose eluate	350	40,260	157.5	256	24	44.1
6. Hg-Sepharose eluate	200	22,000	22.0	1000	13	172.4
7. Hydroxyapatite eluate	15	12,000	3	4000	7	689.7
8. Bio-Gel A-1.5m	21	6867	1.05	6540	4	1127.6

* From 60 g (dry weight) of pig brain mitochondria.

report. The results of the purification procedure are summarized in Table 1.

Purity of the enzyme

SDS-disc electrophoresis was performed to check the homogeneity of the purified enzyme (specific activity 6540). As shown in Fig. 1, the purified enzyme was homogeneous by this analysis. However, the enzyme did not penetrate the gels used in ordinary disc electrophoresis, even if 3.75% polyacrylamide gel was used, which suggests that the enzyme, as isolated, has a very high molecular weight.

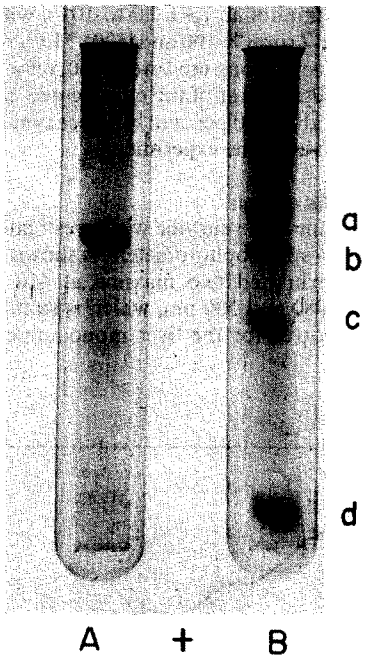

Fig. 1. SDS-disc electrophoresis of the purified monoamine oxidase from pig brain mitochondria. After 25 μg enzyme was incubated with 5% SDS–2% β-mercaptoethanol for 16 hr at 37°, the enzyme was applied to a 7.5% polyacrylamide gel containing 0.1% SDS. Electrophoresis was carried out at an amperage of 8 mA/tube for 4 hr at room temperature using 0.1 M sodium phosphate buffer, pH 7.2, which contained 0.1% SDS. Marker proteins were treated as described above for monoamine oxidase. (A) monoamine oxidase; (B) marker proteins (a, bovine serum albumin; b, bacterial α-amylase; c, yeast alcohol dehydrogenase; and d, cytochrome *c*).

Enzymatic properties of the enzyme

As shown in Fig. 2, the pH optimum of the enzyme was about 9.2 when benzylamine was used as the substrate. The enzyme was stable between pH 7.0 and 9.2 in 0.1 M KH_2PO_4–KOH and 0.1 M $K_4P_2O_7$–KOH buffers for 24 hr at 4°. Also, at temperatures lower than 40°, heating for 15 min in the presence of 0.005 M β-mercaptoethanol, pH 7.4, did not inactivate the enzyme.

Substrate specificity of the enzyme

The substrate specificity of the enzyme was investigated by the peroxidase–*o*-dianisidine assay procedure of McEwen [15]. As shown in Table 2, the enzyme oxidized the following amines in decreasing order (15-min assay): benzylamine, kynuramine, tryptamine and tyramine. However, the enzyme did not oxidize histamine, serotonin and spermidine. The substrate specificity is very similar to the bovine liver enzyme reported previously by Yasunobu *et al.* [6]. Since substrate inhibition occurs at the high substrate concentrations used, a few experiments were conducted, at lower concentrations of substrate, by measuring the rate of release of ammonia. The results are also summarized in Table 2; in this case the enzyme oxidized tyramine the fastest, then benzylamine, and finally tryptamine.

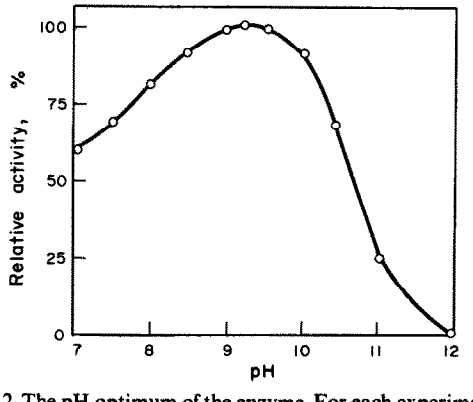

Fig. 2. The pH optimum of the enzyme. For each experiment, 340 units of enzyme was used; the buffers used are described in Materials and Methods.

Table 2. Substrate specificity of the enzyme

Substrate	Relative rate (%)		
	Pig brain*	Pig brain†	Beef liver‡
Benzylamine	100	72	100
Tryptamine	32.1	53	32
Tyramine	29.3	100	30
Kynuramine	43.9	0	52
Histamine	0	0	0
Serotonin	0	0	0
Spermidine	0	0	0

* Each reaction mixture contained 20 units enzyme with specific activity 4900, 3.3 mM of substrate, 0.01% each of peroxidase and *o*-dianisidine in 0.1 M potassium phosphate buffer, pH 7.4. After the mixture was incubated at 25° for 15 min, the developed color was measured at 450 nm.

† Relative rates of deamination were determined as described in Materials and Methods.

‡ Gomes [16].

Estimation of molecular weight of the enzyme

The molecular weight of the enzyme was estimated by exclusion-diffusion chromatography on a Bio-Gel A-1.5m column. The chromatogram is shown in Fig. 3 along with the data for the standard proteins used for calibrating the column. The molecular weight of the enzyme was estimated to be 4.2×10^5 when the enzyme was pretreated with 0.2% Triton X-100 and 0.005 M β-mercaptoethanol. However, the molecular weight was estimated to be 1.2×10^6 in buffers which did not contain Triton X-100 and β-mercaptoethanol. These results suggest that Triton X-100 and β-mercaptoethanol depolymerized monoamine oxidase. The subunit molecular weight was also estimated by SDS-disc electrophoresis and the result obtained is shown in Fig. 4. The molecular weight of the subunit was estimated to be 5.2×10^4.

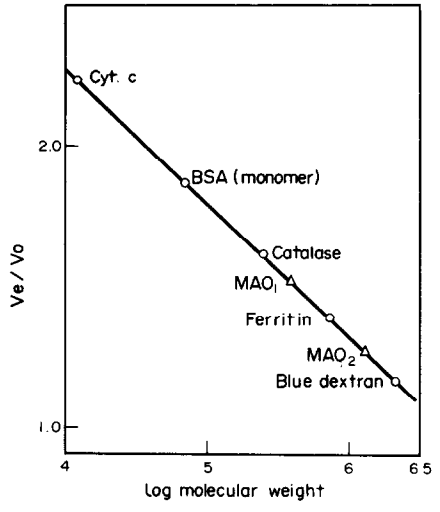

Fig. 3. Estimation of the molecular weight of the enzyme by Bio-Gel A-1.5m exclusion chromatography (1.9 × 120 cm column). For the experiment, 0.05 M potassium phosphate buffer, pH 7.4, which contained 0.01 M β-mercaptoethanol was used. The flow rate was 3 ml/min. MAO_2 represents enzyme as isolated, while MAO_1 represents the molecular weight of monoamine oxidase run in the same buffer which was pretreated with 0.05% Triton X-100 and 0.01 M β-mercaptoethanol solution.

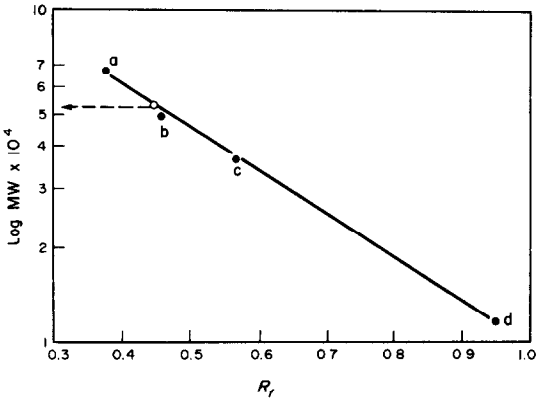

Fig. 4. Estimation of molecular weight of the enzyme by SDS-disc electrophoresis. The experimental conditions are described in the legend for Fig. 1. The protein standards were: a, bovine serum albumin; b, bacterial α-amylase; c, yeast alcohol dehydrogenase; and d, cytochrome *c*. O represents the elution column of monoamine oxidase.

Isoelectric point of the enzyme

The isoelectric point of the enzyme was determined by the isoelectric focusing method. The enzyme from the previous step was applied to a column (143 ml) packed with ampholine, (pH 3.0 to 10.0), which contained 0.005% Triton X-100 and 0.005 M β-mercaptoethanol. The conditions used were 450 V for 48 hr at 2°. The isoelectric point of the enzyme was estimated to be 5.3, and about 90 per cent of the enzyme activity was recovered after the experiment.

Spectrum of the enzyme

The spectrum of the enzyme was investigated in the Cary model 14 spectrophotometer. As shown in Fig. 5, the enzyme exhibited two maxima at 410 nm and shoulders at 480 and 500 nm, which is similar to the spectrum reported for the liver monoamine oxidase [6].

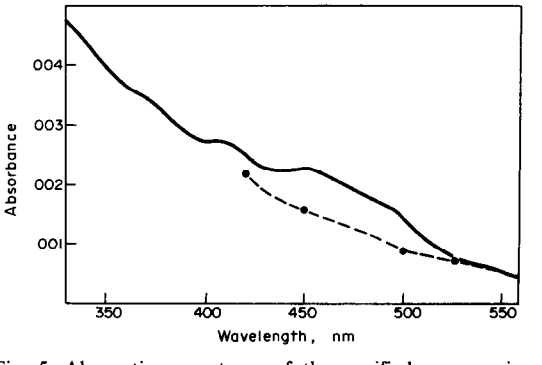

Fig. 5. Absorption spectrum of the purified monoamine oxidase. For the experiment, about 0.23 mg enzyme with specific activity 6540 was dissolved in 0.1 M potassium phosphate buffer containing 0.1% cholate, pH 7.0. The dashed line shows the spectrum of the enzyme reduced by the addition of 1 mg $Na_2S_2O_4$.

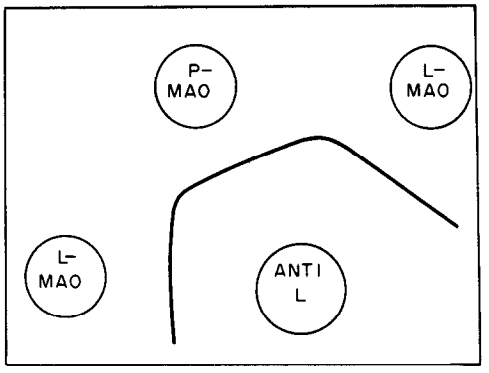

Fig. 6. Reproduction of Ouchterlony double diffusion plates. The plates were prepared using 1% agarose, 0.05 M phosphate buffer, pH 7.0, and sodium azide (20 mg/100 ml). Precipitin lines appeared in 48 hr at 4° but were allowed to develop for 1 week. P-MAO, L-MAO and ANTI-L indicate pig brain enzyme (20 μg), bovine liver enzyme (20 μg), and rabbit antisera to bovine liver enzyme (26 μg).

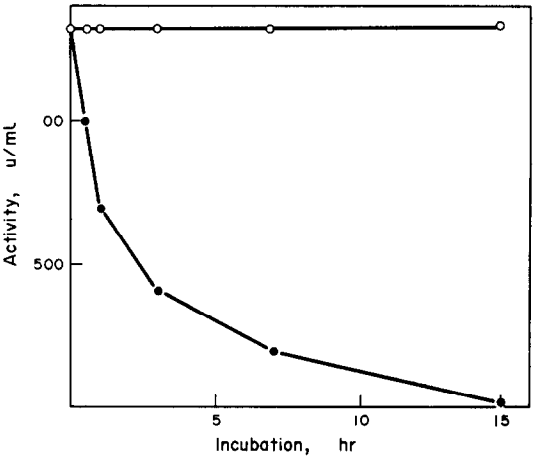

Fig. 7. Inhibition of the enzyme by [2-^{14}C]3-*N*,*N*-dimethylamino-1-propyne HCl. The purified enzyme 1.49 mg (specific activity, 6000) was incubated with 7.5 × 10^{-4} M [2-^{14}C]3-*N*,*N*-dimethylamino-1-propyne HCl in 0.1 M potassium phosphate buffer, pH 7.0, which contained 0.1 M cholate at 25°. The symbols used are: (○) activity without inhibitor, and (●) activity with [2-^{14}C]3-*N*,*N*-dimethylamino-1-propyne HCl added.

Flavin content of the enzyme

The flavin content of the enzyme was determined from the difference spectrum at 450 nm between the oxidized and sodium hydrosulfite reduced forms of the enzyme. The difference molar absorbing index value at 450 nm was assumed to be 11.3 × 10^3 cm^2 $mole^{-1}$ [17]. The flavin content of the enzyme was determined to be 0.92 mole FAD/mole of the enzyme whose molecular weight was 104,000.

Immunological assay

Evidence for the similarity of pig brain and bovine liver monoamine oxidase was derived from the Ouchterlony double immunodiffusion test which is shown in Fig. 6. For this experiment rabbit antisera to bovine liver monoamine oxidase was used. The points of juncture of the precipitin lines showed no spurs and gave the curved juncture which is characteristic of lines of identity. This indicates that there are no antigenic sites present on bovine liver enzyme that are not also present in pig brain monamine oxidase. There must, therefore, be a close structural similarity between the enzyme derived from the two different organs from pig and cow.

Evidence for the presence of covalently linked flavin in the enzyme

The inhibitor, [2-^{14}C]3-*N*,*N*-dimethylamino-1-propyne, has been reported by Maycock *et al.* [18] to react with N^5-position of the isoalloxazine ring of the 8-α-cysteinyl-FAD monoamine oxidase. Figure 7 illustrates the progressive inhibition of the pig brain enzyme with 3-*N*,*N*-dimethylamino-1-propyne. The enzyme was completely inactivated by the inhibitor under the conditions described in Fig. 6. The labeled

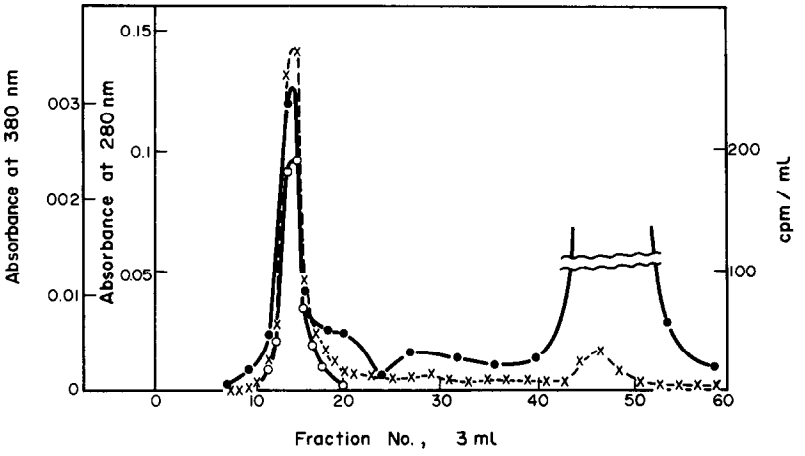

Fig. 8. Exclusion-diffusion chromatography of the 2-^{14}C-labelled enzyme–3-*N*,*N*-dimethylamino-1-propyne complex. For the experiment, 2 ml (1.2 mg) of the enzyme inactivated with labeled 3-dimethylamino-1-propyne HCl, as described in the legend to Fig. 7, was applied to a Bio-Gel A-0.5m column (1.4 × 80 cm) and the column was eluted with 0.1 M potassium phosphate buffer, pH 7.4. Absorbance at 380 nm is due to the 3-dimethylamino-1-propyne. Key: (○) absorbance at 380 nm; (●) radioactivity expressed as cpm; and × absorbance at 280 nm.

Table 3. TCA treatment of the enzyme labeled with [2-^{14}C]3-*N*,*N*-dimethylsmino-1-propyne HCl*

Sample	Protein mg	Protein cpm	Protein cpm/mg	Protein dis./min/mg†	3-Dimethylamino-1-propyne content‡ (μmole/mg protein)	3-Dimethylamino-1-propyne content§ (mole/mole enzyme)
Before TCA treatment‖	0.28	1120	4000	5479	0.978×10^{-2}	0.509
TCA-soluble	ND	52				
TCA-insoluble	0.17	693	4079	5584	0.997×10^{-2}	0.519

* See legend to Fig. 7 for other experimental details.
† Calculated using an efficiency of 73 per cent.
‡ Calculated by assuming dis./min/μmole of [2-^{14}C]3-*N*,*N*-dimethylamino-1-propyne was 5.6×10^5.
§ Calculated by assuming that the molecular weight of the monoamine oxidase was 52,000.
‖ To 2.8 ml of the concentrated labeled enzyme, TCA was added to a final concentration of 10% and the sample was left standing for 8 hr at 8°.
¶ Not determined.

enzyme, as shown in Fig. 6, was applied to a Bio-Gel A-0.5m column to remove excess inhibitor. The chromatographic pattern obtained is shown in Fig. 8. The peaks calculated from the 280 nm absorbance and the 380 nm absorbance due to the enzyme-inhibitor complex were coincident with the radioactive peak. The pooled enzyme fraction was treated with trichloroacetic acid (TCA) as described in Table 3. The mixture was centrifuged and the supernatant fraction, which was still contaminated with a slight amount of insoluble floating material, was removed by filtration. The resulting filtrate is designated as the TCA-soluble fraction. The precipitate, which was dissolved in dilute sodium hydroxide, is designated as the TCA-insoluble fraction. Table 3 summarizes the radioactive contents of both fractions; almost all of the radioactivity was found in the TCA-insoluble fraction. The results indicate that the FAD in the pig brain monoamine oxidase is not dissociable from the protein and is covalently bonded to FAD. The flavin content of the enzyme determined from the ^{14}C content was found to be approximately 1.0 mole/100,000 g of protein, before and after TCA treatment.

DISCUSSION

A few comments concerning the purification of the enzyme deserve special attention. The enzyme activity of pig brain monoamine oxidase in the crude stages could not be measured accurately by the usual spectrophotometric method of Tabor *et al.* [11], in which benzylamine is used as the substrate. The problem that occurred was the appearance of a fine precipitate which caused an increase in the absorbance at 250 nm. However, in the presence of 0.5% Triton X-100, no inhibition of the enzyme nor precipitation of protein was observed and the activity could be determined accurately.

The purification of monoamine oxidase from pig brain mitochondria was very difficult since the mitochondria pellet contained much lipid and the total amount of enzyme present was much lower than in liver or kidney. In the present study, affinity column chromatography (mercurial-Sepharose) was used to purify the enzyme. This affinity column chromatography step is very useful, not only for purification of pig brain monoamine oxidase, but most likely for the purification of monoamine from other sources, also.

The pH optimum of the enzyme (benzylamine as substrate) was found to be about 9.2, which is similar to the pH optimum reported for the beef liver enzyme [19]. However, the pH optimum of our enzyme was different from that of pig brain enzyme reported by Tipton [5], where tyramine was used as the substrate. However, the pH optimum is known to vary with the substrate used. Tipton [5] also reported that the molecular weight of the pig brain enzyme was 102,000. As shown in Fig. 3, the molecular weight of the enzyme obtained in the present study was about 4.2×10^5 in the presence of Triton X-100 and β-mercaptoethanol and 1.2×10^6 in the absence of detergent. The molecular weights determined are similar to those reported for the bovine liver monoamine oxidase [20] which was reported to be a polymer of a monomer, with a molecular weight of about 115,000 [21].

A major discrepancy exists between the findings of Tipton [22] and our results concerning the attachment of FAD to the enzyme. The FAD in the pig brain enzyme was covalently linked to the enzyme, while Tipton [22] and Nagatsu *et al.* [4] have reported that the FAD is dissociable. This difference would have very interesting physiological and enzymatic implications. In the present investigation, [2-^{14}C]3-*N*,*N*-dimethylamino-1-propyne, which has been shown to react with the N^5 moiety of the isoalloxazine ring of the FAD in bovine liver monoamine oxidase [18], was used to show that the pig brain enzyme also contains covalently bonded FAD [23, 24].

Finally, another surprising observation was uncovered. The preliminary investigations reported in this paper suggest that the enzyme is made up of two identical subunits, with a molecular weight of 52,000, which are probably linked by disulfide bond(s). SDS-disc electrophoresis analysis of the purified brain enzyme disclosed a single protein band with a subunit molecular weight of 52,000. The flavin content of the enzyme was approximately 1.0 mole/104,000 g of protein, which is in agreement with the value found for the beef liver enzyme [20]. Thus, all monoamine oxidases, regardless of their source, are enzymes which probably contain two identical subunits which are covalently linked and contain 1 mole of covalently bonded FAD in one of the two subunits.

Immunological studies of bovine brain and liver

monoamine oxidases have shown that the enzymes from these two sources are immunologically identical [25]. The pig brain and bovine liver monoamine oxidases were found, in this study, to be immunologically identical when the enzymes were analyzed by the Ouchterlony double immunodiffusion test. This is not a surprising finding since the amino acid sequences of the bovine and pig cytochrome *c* are identical [26]. Furthermore, from all the data reported here, it is quite apparent that the pig brain enzyme isolated is monoamine oxidase B rather than A [27]. Since bovine liver monoamine oxidase B has been shown to contain 8-α-cysteinyl-FAD [23, 24], the covalently bonded FAD in the pig brain monoamine oxidase B probably contains 8-α-cysteinyl-FAD, also.

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