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ISOELECTRIC FOCUSING OF ISOENZYMES OF MONKEY PLATELET MONOAMINE OXIDASE

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Abstract—Monkey platelet monoamine oxidase (MAO) was preferentially found as the B-form of the enzyme as observed from differences in substrate specificities, as well as liver MAO. The isoelectric points and molecular weights of platelet MAO subunits were compared with those of monkey liver using sodium dodecyl sulfate–disc polyacrylamide gel electrophoresis and isoelectric focusing–disc gel electrophoresis. The pI value of monkey liver was a single peak at 6.5, but the pI values of monkey platelets were triple peaks at 5.5, 6.5 and 7.0. The molecular weight of MAO subunits in monkey platelets was similar to that of liver, and was found to be about 60,000. These results indicate that MAO-B of monkey platelets differs from MAO-B of the liver, and that it has different electrophoretic properties.

The existence of multiple forms of monoamine oxidase (MAO) has been demonstrated using several methodological and technical approaches. Recently, MAO was classified into two forms, the A-form and B-form, on the basis of different sensitivities to inhibition by the selective MAO inhibitors clorgyline [1, 2] and deprenyl [2, 3]. Rat organs, such as the spleen and testis, and neuroblastoma or glioma of the mouse [4, 5] and human placenta [6, 7] contain predominantly a single species of the MAO-A. In contrast, MAO-B activity was found in the mouse, in rabbit liver and in human platelets [8, 9]. Since it has been reported that human platelet MAO is a single B-form enzyme and is related to schizophrenia [10], it has become the object of attention of many investigators. Investigators, using several techniques, have reported the existence of two different functional forms of MAO [11, 12].

In the present study, the isoelectric points and the molecular weights of subunits of monkey platelet and liver MAO were compared. Since previous studies have shown, by means of substrate specificity and inhibitor sensitivity, that these tissues contain primarily MAO-B, they were selected for this study.

MATERIALS AND METHODS

Chemicals

Servalyt (pH 2–11) was purchased from Serva Fine Biochemicals Inc. 5-Hydroxytryptamine creatinine sulfate and tyramine hydrochloride were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). β-Phenylethylamine hydrochloride was obtained from the Tokyo Chemical Industries Co., Ltd. (Japan). Radiochemical substrates, 5-[2-^{14}C]hydroxytryptamine binoxalate (5-HT) (51.1 mCi/mmol), [1-^{14}C]tyramine hydrochloride (55.0 mCi/mmol) and β-[1-^{14}C]phenylethylamine hydrochloride (β-PEA) (50.2 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.).

Enzyme preparation

Japanese adult monkey liver and platelets were used in this study. Monkey liver and platelet mitochondria were prepared by centrifugation as described earlier [13, 14]. For preparation of monkey platelets, the blood was collected into a flask containing 3.8% sodium citrate as an anticoagulant. After the blood was centrifuged at 1500 *g* for 10 min to remove the red cells, the supernatant fraction was centrifuged at 3000 *g* for 30 min. The pellet was resuspended in 10 vol. of 0.01 M potassium phosphate buffer (pH 7.4). This suspension was centrifuged at 20,000 *g* for 30 min, and the pellet was resuspended in 3 vol. of 0.01 M potassium phosphate buffer (pH 7.4) and used as the enzyme preparation.

MAO activity

MAO activity was assayed radiochemically, as described earlier [13] with [^{14}C]tyramine, [^{14}C]5-HT and [^{14}C]β-PEA diluted with the respective unlabeled amines as substrates. The reaction was started by adding 20 μL of labeled substrate, and incubation was carried out for 20 min at 37°. Then the reaction was stopped by adding 2 N HCl. Metabolites formed by this enzyme reaction were extracted with an ethyl acetate–benzene mixture (1:1, v/v) saturated with water, and radioactivity in the extract was measured by a Beckman LS-9000 scintillation spectrometer. MAO activity was expressed as nanomoles of product formed per minute per milligram of protein or in terms of disintegrations per minute.

Binding of [^{3}H]*pargyline*

In these experiments, radiolabeled MAO in liver was compared, by electrophoresis to that in platelets. It is known that pargyline, a MAO inhibitor, binds to MAO irreversibly in the molar ratio of 1:1 [15]. [^{3}H]Pargyline was used as a marker to determine the existence of MAO. Mitochondrial preparations of liver and platelets were incubated with 10^{-8} M

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[^{3}H]pargyline in a final volume of 5 mL of 10 mM phosphate buffer, pH 7.4, for 5 hr at 37°. Following pargyline binding, samples were centrifuged at 28,000 *g* for 30 min at 4°. Supernatant fractions were discarded and pellets were stored at −60°.

Electrophoretic analysis of [^{3}H]*pargyline-labeled proteins*

The isoelectric points and molecular weights of MAO were estimated after specific binding of pargyline was corrected for non-specific binding. Non-specific binding of [^{3}H]pargyline was determined by increasing the concentration of unlabeled pargyline to 10^{-4} M before adding [^{3}H]pargyline. Electrophoresis was carried out using [^{3}H]pargyline as a tracer.

Molecular weight determination. Sodium dodecyl sulfate–disc polyacrylamide gel electrophoresis (SDS–disc PAGE) was carried out with modifications of the method of Weber and Osborn [16]. The molecular weights of MAO in monkey liver and platelets labeled with [^{3}H]pargyline were determined by SDS–PAGE (5%) with RNA polymerase B (z = 39,000, α = 42,000, x = 100,000, β = 140,000 and β' = 180,000, SDS–PAGE marker I, as a marker proteins) [14].

Isoelectric focusing (IEF). [^{3}H]Pargyline labeled and unlabeled mitochondrial preparations of monkey liver and platelets were stirred gently with equal volumes of 0.75% (w/v) Triton X-100 in 0.1 M phosphate buffer, pH 7.4, for 20 min at room temperature. After centrifugation at 105,000 *g* for 30 min, these supernatant fractions were used as solubilized enzymes. IEF was performed by the method of Fawcett [17]. The isoelectric focusing gels were composed of 5% acrylamide, 0.2% bisacrylamide, 0.0005% riboflavin, 0.05% *N,N,N',N'*-tetramethylethylenediamine (TEMED), 0.01% ammonium persulfate, and 2% servalyte (pH 2–11). The cathode buffer in the upper tank was 2% NaOH, and the anode buffer in the lower tank, 0.2% H_3PO_4. Electrophoresis was carried out at a constant voltage of 400 V for 16 hr. After electrophoresis, the gels were cut into approximately 3-mm wide slices and shaken with 0.5 mL water. The pH gradients on the gels were determined using a combination microelectrode. The quantitative distribution of radioactivity in each gel was determined as described earlier [18], and the radioactivity was estimated after the specific binding of pargyline was corrected for non-specific binding. Using unlabeled enzyme preparations, the pH gradient and MAO activity of each gel slice were determined. After adjusting the pH to 7.4, MAO activity in each gel slice was assayed radiochemically with β-PEA as substrate. Then, IEF gels were fixed with 7% acetic acid and methanol for fluorography, dried, and exposed to Fuji RX medical X-ray film at − 70°C for 1 month [19].

Protein

Protein contents in the enzyme preparations were assayed by the method of Lowry *et al.* [20] with bovine serum albumin (BSA) as the standard.

RESULTS

Substrate specificities of monkey liver and platelet MAO

The relative rates of oxidation of various substrates by Triton X-100 solubilized monkey liver, and platelet MAO were compared. The results are shown in Table 1. The activity with 5-HT was only 0.3% of that seen with β-PEA using the liver preparation. β-PEA (a B-form MAO substrate) [2] was the best substrate. Monkey platelet MAO showed similar substrate specificity. After incubation at 37° for 40 min with deprenyl (a B-form MAO inhibitor), MAO activity in monkey liver was highly sensitive with β-PEA as substrate, whereas it was less sensitive with clorgyline (an A-form MAO inhibitor). When monkey platelets were used as the enzyme preparation, similar results were obtained (data not shown). These results indicate that liver and platelet MAO in monkey seem to be preferentially a B-form enzyme.

Table 1. Substrate specificities of 0.75% Triton X-100 solubilized monkey liver and platelet MAO

Substrate	MAO activity × 10^{-4} [nmol/min·mg protein)]	
	Liver	Platelet
Tyramine	316.8 ± 11.6	132.4 ± 3.2
5-HT	11.7 ± 2.1	5.3 ± 0.5
β-PEA	3538.5 ± 36.7	849.5 ± 14.8

MAO activity was assayed radiochemically. Experimental conditions are described in Materials and Methods. Substrate concentrations used were: 100 μM tyramine, 100 μM 5-HT and 10 μM β-PEA as a final concentration. MAO activity is expressed as nmol product formed/min·mg protein). Each value is the mean ± SE of triplicate assays.

Separation of [^{3}H]*pargyline bound to MAO in monkey liver and platelets by isoelectric focusing*

After isoelectric focusing (see Materials and Methods), the gel was cut into 3-mm slices from the origin to a position 11 cm from the top of the gel, followed by the monitoring of the radioactivities (Fig. 1, upper). The other gels were treated for fluorography, dried, and exposed to Fuji RX medical X-ray films at −70° for 1 month (Fig. 1, bottom). As shown in Fig. 1, a single band in monkey liver mitochondria was identified by radioactivity and fluorography with an isoelectric point (pI) value of 6.5, whereas in the monkey platelets, triple bands were found at pI 5.5, 6.5 and 7.0. After isoelectric focusing of the solubilized monkey liver and platelets, MAO activity of each gel slice was determined. As shown in Fig. 2, high MAO activity in monkey liver mitochondria was observed at a position 8 cm from the top of the gel with β-PEA as a substrate; the pI value was near 6.5. When monkey platelets were used, three peaks were clearly distinguished from the profile; the pI values were near 5.5, 6.5 and 7.0.

Determination of the molecular weight of MAO-B in monkey liver and platelets

The electrophoretic properties of monkey MAO were compared in two tisues: liver and platelets. [^{3}H]Pargyline was used to label MAO in mitochondrial preparations from monkey liver and platelets. [^{3}H]Pargyline-labeled mitochondria were

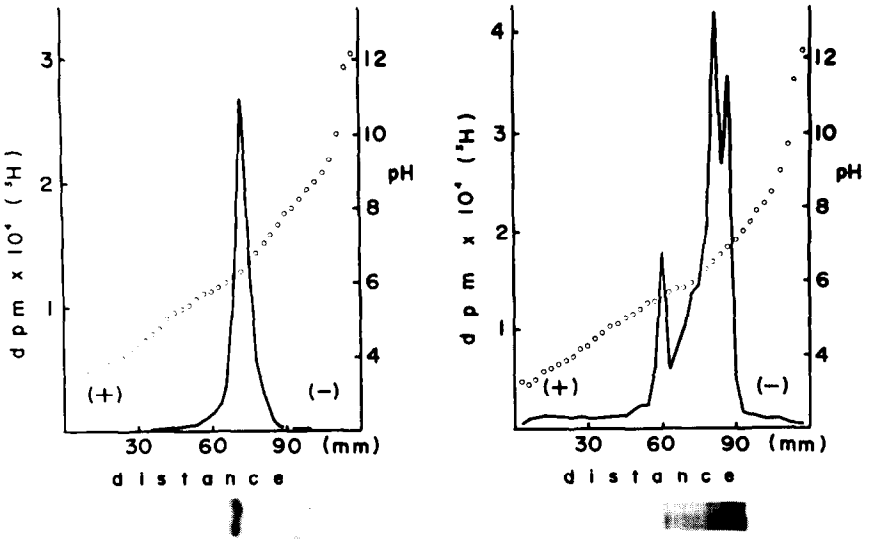

Fig. 1. Estimation of pI values by IEF-disc gel electrophoresis of [^{3}H]pargyline-labeled MAO in monkey liver and platelets. Labeling of 10^{-7} M [^{3}H]pargyline-pretreated monkey liver (left) and platelets (right) was performed. After electrophoresis, the gels were cut into 3-mm wide slices and shaken with 0.5 mL water. The pH gradient on the gels was determined using a combination microelectrode. Each gel was solubilized by NCS-tissue solubilizer, and radioactivity was determined in each slice (upper). Other gels were prepared for fluorography and exposed to Fuji RX medical X-ray film at −70° for 1 month. The labeled bands were visualized by fluorography (bottom). The pI values of MAO in liver (left) and platelets (right) were determined from the corresponding pH. Details of the experimental procedures are described in Materials and Methods.

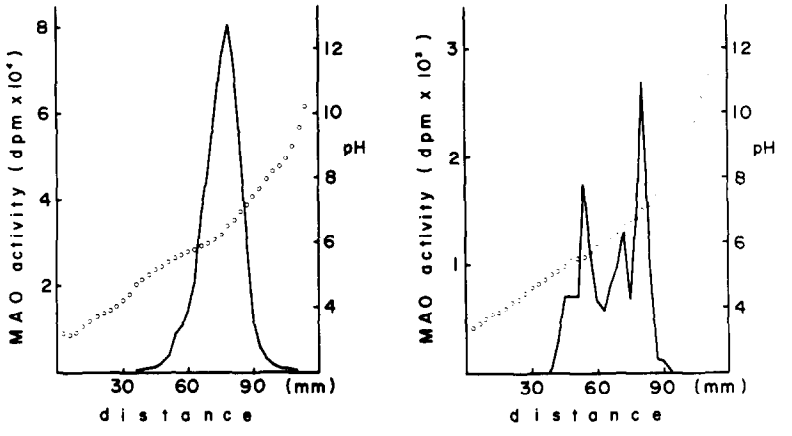

Fig. 2. IEF-disc gel electrophoresis of monkey liver and platelet MAO. Monkey liver mitochondria and platelets were solubilized with 0.75% Triton X-100. After IEF-disc gel electrophoresis, each gel was cut into 3-mm slices, and the pH gradient on the gels was determined using a combination microelectrode. MAO activities were determined in each of the slices with 10 μM β-PEA as a substrate. The pH values of MAO in liver (left) and platelets (right) were determined.

solubilized, and SDS–PAGE was carried out as described in Materials and Methods. The position of the slices from a gel lane relative to the tracer dye front is plotted on the abscissa, and the radioactivity in the gel slices is plotted on the left ordinate (Fig. 3, right). As shown in Fig. 3, the molecular weight of MAO in monkey platelets was found to be about 60,000 daltons, giving a single peak after treatment with 6% SDS. After electrophoresis, the labeled bands were visualized by fluorography using the method of Bonner and Laskey [19]. An apparent molecular weight of about 60,000 daltons for the liver and platelet MAO in monkey was determined by comparing the observed mobilities with proteins of known molecular weights (Fig. 3, left). A single band of labeled material was observed for each preparation.

DISCUSSION

In general, tetrazolium salts are used as histochemical reagents to detect MAO activity after electrophoresis of solubilized MAO preparations. However, the site of enzyme activity detected on PAGE by the histochemical technique does not coincide in all cases with bands of MAO activity

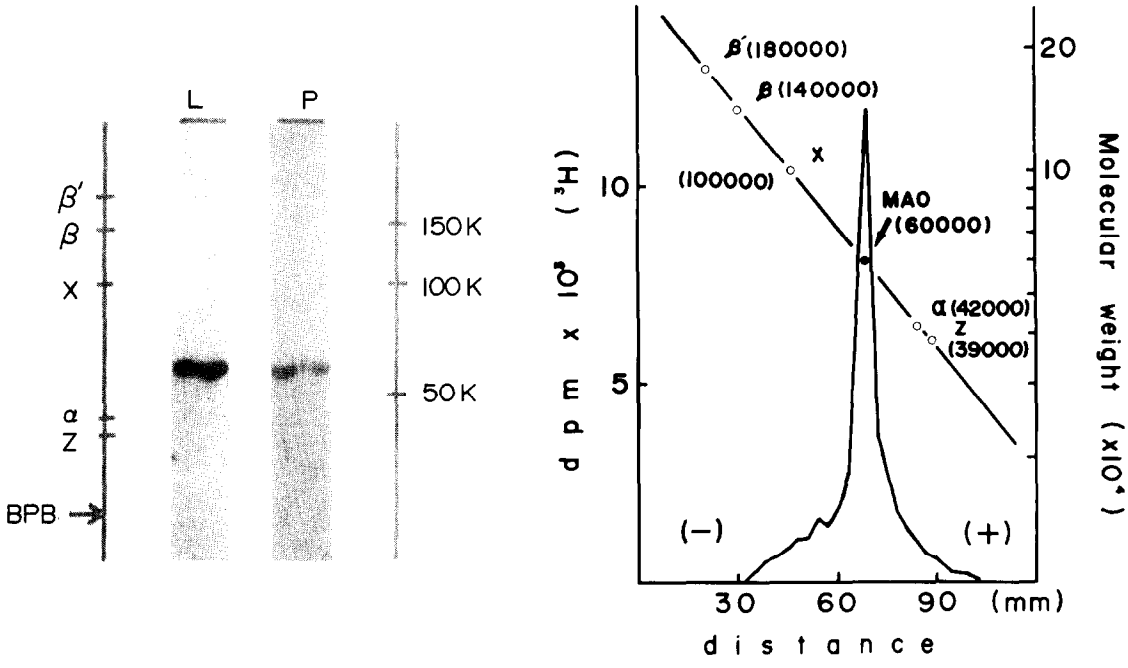

Fig. 3. Estimation of the molecular weight by SDS-disc electrophoresis of [^{3}H]pargyline-labeled MAO from monkey platelet mitochondria. [^{3}H]Pargyline-labeled monkey liver (L) and platelets (P) were used for enzyme preparations. After each enzyme preparation was boiled with 6% SDS–4% β-mercaptoethanol for 3 min, the sample was applied to a 5% acrylamide gel containing 0.1% SDS. After electrophoresis, the labeled bands were visualized by fluorography (left). After fluorography, the gel from the monkey platelets was cut into 3-mm wide slices, and radioactivity was determined in each slice. SDS–PAGE Marker I was used to prepare a calibration curve (polymerase B, mol wt: z = 39,000, α = 42,000, X = 100,000, β = 140,000, β' = 180,000). Molecular weights were determined by comparing the mobilities of marker proteins. The marker proteins were stained with Coomassie brilliant blue in methanol and acetic acid. Then their relative mobilities were determined against the molecular weights.

measured by radioassay. Furthermore, the intensity of tetrazolium reduction does not appear to reflect the radiochemical activity of different forms. It seems likely that these results are related, at least in part, to the inhibition of MAO by tetrazolium salts [21, 22]. In the present experiments, we studied methods to identify the covalently bound flavin moiety in MAO by SDS–PAGE, using proteins labeled with [^{3}H]pargyline, and directing the MAO activity measurement by cutting the gel cylinder. As shown in Figs. 1 and 2, MAOs were detected in the same position on gels by the two above-mentioned methods. The pI value of monkey liver MAO was estimated to be 6.5, whereas with the monkey platelets, triple bands were found at pH 5.5, 6.5 and 7.0. These values are similar to those obtained by Minamiura and Yasunobu [23] in porcine brain mitochondria, suggesting that basic amino acids in the native enzyme may be located inside the molecule. In contrast, Sato *et al.* [24] reported pI values of 8.3 and 8.4 for type A and type B MAO in rat liver mitochondria. These values are similar to those of rat hepatoma cell (pI 7.8) [18] and monkey brain (pI 7.8 and 6.3) [25]. Two forms of MAO may be due to a single enzyme with different allotropic properties conferred by the membrane phospholipid environment [26]. Hence, it has been considered that the multiple forms of MAO shown by IEF may be a procedural artifact. However, we reported previously [14] that pI values of MAO determined by IEF do not depend on the properties of the detergents used to solubilize the enzyme preparations. Our data, obtained by IEF, indicate that three different MAOs occur in monkey platelets, with the same patterns obtained repeatedly (data not shown). It seems likely that distinct pI values have different protein conformations, resulting in a difference in net charge.

In conclusion, it appears that the B-form of MAO of monkey platelets is similar to the B-form of MAO of the liver, but three different MAOs have been shown by IEF. In this study, our results using IEF suggest the possible presence of isoenzymes in monkey platelets. Chen and Weyler [27] also have provided results indicating sequence heterogeneity for human placenta MAO-A. One possibility arising from this speculation is that a variety of enzymes exists in multiple forms and that the isoenzymic pattern in disease [28] may differ from that of the normal individuals. To support this view, however, isoenzymes of platelet MAO need further isolation, purification and characterization, and more detailed testing for the isoelectric focusing of disordered platelets.

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