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SHORT COMMUNICATIONS

Properties of dopamine β-hydroxylase in soluble and particulate fractions of bovine adrenal medulla

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IT HAS been reported that dopamine β-hydroxylase in the homogenate of bovine adrenal medulla is localized not only in chromaffin granules, but also in the soluble fraction.[1,2] The activity of dopamine β-hydroxylase in the crude adrenal preparations was found to be inhibited by the presence of endogenous inhibitors[3] which are sulfhydryl compounds.[1,4] The enzyme activity appears in the soluble fraction by inactivating the endogenous inhibitors by adding either *N*-ethylmaleimide[4] or copper.[1] Some part of the particle bound dopamine β-hydroxylase could be easily solubilized either by freeze-thawing or by sonication.[2,5] The rest of the firmly bound activity could be solubilized by the detergent Cutscum.[2] These results suggest that the dopamine β-hydroxylase in the soluble fraction may be originally particle bound but released into the soluble supernatant during homogenization. Another possibility would be that dopamine β-hydroxylase in soluble and particulate fractions may be isoenzymes. In order to see whether dopamine β-hydroxylase in soluble and particulate fractions may be isoenzymes or not, the dopamine β-hydroxylase from either soluble fraction or Cutscum-treated particulate fraction was compared to each other by using Sepharose 6B column chromatography and further by electrophoresis. Tyrosine hydroxylase and dopa decarboxylase activities in soluble and particulate fractions were also examined.

Bovine adrenal medulla (10 g) was homogenized by using an Ultra Turrax homogenizer in 30 ml of 0·25 M sucrose containing 0·02 M potassium phosphate buffer, pH 7·0.[6] After centrifuging the homogenate for 10 min at 700 *g* to remove cell debris and the nuclear fraction, the supernatant was centrifuged for 60 min at 100,000 *g*. Most of the chromaffin granules were recovered in the precipitate. The precipitate was washed with the sucrose medium and centrifuged again. The precipitate was resuspended in 15 ml of the 0·02 M phosphate buffer, pH 7·0, and a 5% solution of Cutscum (detergent containing isooctylphenoxypolyethoxyethanol) was added dropwise to a final concentration of 1·2%.[3] After centrifuging the suspension for 90 min at 44,000 *g*, the supernatant was fractionated with 2 vol. of neutralized and saturated ammonium sulfate. On centrifugation, the precipitate rose to the surface. The precipitate was quantitatively removed and dissolved in 0·02 M potassium phosphate buffer, pH 7·0. After dialysis against 0·02 M potassium phosphate buffer, pH 7·0, and centrifugation, the supernatant which contained the enzyme activities was layered on a Sepharose 6B column (column size 2·5 × 82 cm). Sepharose 6B had been equilibrated with 0·02 M potassium phosphate buffer, pH 7·0, containing 0·1 M sodium chloride. The same buffer was used for elution. The sample volume applied on the Sephadex 6B column was 5 ml corresponding to about 5 g of the tissues. The fraction volume was 2 ml. An aliquot of each fraction was used for the assay of the enzyme activities. The high-speed supernatant of the homogenate was also fractionated with 2 vol. of neutralized and saturated ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 0·02 M potassium phosphate buffer, pH 7·0. After dialysis and centrifugation, the sample was subjected to a Sepharose 6B column chromatography as described above.

Dopamine β-hydroxylase in the Sepharose eluate was further examined by electrophoresis. Cellulose acetate film electrophoresis was carried out at 5° for 2 hr at 3 mA/2 cm using barbiturate buffer ($\mu = 0{\cdot}06$), pH 8·6, and phosphate buffer ($\mu = 0{\cdot}1$), pH 6·5, for the identification of dopamine β-hydroxylase of soluble and particulate fractions of bovine adrenal medulla. After electrophoresis, the film was cut off at intervals of 2 mm from the starting line.

Dopamine β-hydroxylase activity was measured spectrophotometrically by the method of Creveling *et al.*[7] The incubation mixture (final volume 1·0 ml) contained 300 μmoles of potassium phosphate buffer, pH 5·5, 20 μmoles of tyramine (substrate), 10 μmoles of ascorbate, 10 μmoles of fumarate, 0·3 μmole of harmaline, 30 μmoles of *N*-ethylmaleimide, enough catalase to give maximum stimulation and the enzyme. The reaction mixture was incubated in air for 30 min at 37° and 0·2 ml of 3 M trichloroacetic acid was added to stop the reaction. The mixture was centrifuged, and the supernatant was passed through an Amberlite IR-CG-120-H^+ column (0·5 × 3·0 cm). After washing

the column with 10 ml of water, the norsynephrine formed from tyramine was eluted with 3·0 ml of 4 N NH_4OH. The norsynephrine formed was measured spectrophotometrically after periodate oxidation to *p*-hydroxybenzaldehyde at 330 mμ.

Tyrosine hydroxylase activity was determined by the formation of [^{14}C]L-dopa from [^{14}C]L-tyrosine as substrate.[8] The incubation mixture contained 200 μmoles of acetate buffer, pH 6·0, 0·1 μmole of L-tyrosine containing 0·05 μc of U-[^{14}C]L-tyrosine (substrate), 100 μmoles of mercaptoethanol, 1 μmole of 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine, and the enzyme in a total volume of 1·0 ml. Samples were incubated for 15 min at 30° and the reaction was stopped by the addition of 0·1 ml of 30% trichloroacetic acid. [^{14}C]Dopa was isolated by an alumina column and counted.

Dopa decarboxylase activity was measured fluorometrically by the method of Lovenberg *et al.*[9] Incubation mixture (final volume 1·0 ml) contained 100 μmoles of potassium phosphate buffer, pH 7·0, 1 μmole of L-dopa, 700 mμmoles of pyridoxal 5-phosphate, 30 mμmoles of harmaline (a monoamine oxidase inhibitor) and the enzyme. The reaction mixture was incubated for 3 min at 37°, and the reaction was stopped by heating. After the dilution of the reaction mixture with 4·0 ml of water, the diluted sample was passed through an Amberlite IRC-50-Na^+ column (0·5 × 3·0 cm) which had been buffered at pH 6·5. The resin was washed with 5 ml of 0·1 M potassium phosphate buffer, pH 6·5, and the dopamine was eluted with 5·0 ml of 0·5 N HCl. The fluorescence of the acid eluate containing the dopamine was directly read in an Aminco–Bowman spectrophotofluorometer at 330 mμ excited at 280 mμ.

The enzyme activities were expressed in IUB milliunits (mU, mμmoles/min) of the formed products. Protein amount was assayed by the method of Lowry *et al.*[10]

The activities of dopamine β-hydroxylase, tyrosine hydroxylase, and Dopa decarboxylase in soluble and particulate fractions of bovine adrenal medulla are shown in Table 1. Most of the dopamine β-hydroxylase activity in the particulate fraction could be solubilized by adding Cutscum. In contrast, only a part (9 per cent) of tyrosine hydroxylase activity in the particulate fraction could be solubilized by adding Cutscum. Dopa decarboxylase activity was found only in the soluble fraction.

TABLE 1. INTRACELLULAR DISTRIBUTION OF THE ACTIVITIES OF DOPAMINE β-HYDROXYLASE, TYROSINE HYDROXYLASE, AND DOPA DECARBOXYLASE AND THE EFFECT OF CUTSCUM ON SOLUBILIZATION OF THE ACTIVITIES*

Enzyme	Enzyme activity mU/g tissue		
	Soluble fraction	Particulate fraction	
		Before Cutscum treatment	Supernatant after Cutscum treatment
Dopamine β-hydroxylase	5586	6953	6202
Tyrosine hydroxylase	4·26	7·19	0·62
Dopa decarboxylase	121	0	0

* Soluble and particulate fractions were separated by differential centrifugation, and the enzymes in the particulate fraction were solubilized by using Cutscum, as described in the text.

The elution patterns of the enzymes from Sepharose 6B column chromatography are shown in Fig. 1 a and b. Tyrosine hydroxylase, dopa decarboxylase and dopamine β-hydroxylase activities were present in the soluble fraction of bovine adrenal medulla. In contrast, only tyrosine hydroxylase and dopamine β-hydroxylase activities were detected in the solubilized supernatant from the particulate fraction. The void volume which was determined by blue dextran was at fraction 60 in Fig. 1. Molecular weights of the three enzymes, tyrosine hydroxylase, dopamine β-hydroxylase and dopa decarboxylase were approximately 400,000, 250,000 and 87,000 respectively. The reported molecular weights of tyrosine hydroxylase are 155,000 for the native form,[11] 34,000 for the trypsin digested form,[11] and 40,000 for the chymotrypsin digested form.[12] The molecular weight of dopamine β-hydroxylase was reported to be 290,000.[6] The peak of tyrosine hydroxylase activity from the particulate fraction and the soluble fraction was at fraction 124 in Fig. 1 a and b. The peak of dopamine β-hydroxylase activities from either soluble or particulate fraction of bovine adrenal medulla was at fraction 134

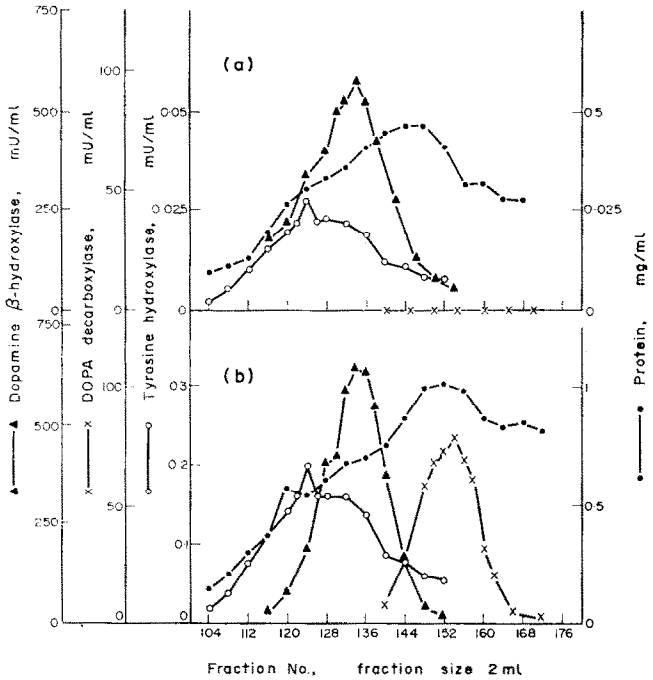

FIG. 1. Chromatography of the enzymes of biosynthesis of catecholamines in the particulate fraction (a) and the soluble fraction (b) of bovine adrenal medulla. The particle-bound enzymes were solubilized by using the detergent Cutscum and subsequently concentrated by ammonium sulfate precipitation and dialyzed. The soluble enzymes were concentrated by ammonium sulfate precipitation and dialyzed. The Sepharose 6B column (2·5 × 82 cm) was equilibrated with 0·02 M potassium phosphate buffer, pH 7·0, containing 0·1 M sodium chloride. The sample applied was 66 mg of protein from the particulate fraction (a) and 142 mg of protein from the soluble fraction (b), each from 5 g of the adrenal medulla in 5·0 ml of the buffer. The sample was eluted with the same buffer.

The elution pattern of dopamine β-hydroxylase activity was quite similar to that of the soluble and particle-bound enzymes. This result suggests that molecular weights of dopamine β-hydroxylase in both soluble and particulate fractions are almost identical. The estimated molecular weight (250,000) is similar to the value of 290,000 reported by Friedman and Kaufman.[6]

Dopamine β-hydroxylase in fraction 134 (3 μl each) from both soluble and particulate fractions was further compared by cellulose acetate film electrophoresis. A single peak of dopamine β-hydroxylase activity was detected at the cathode side with both soluble and particle-bound dopamine β-hydroxylase. The patterns and the mobilities of both soluble and particle-bound dopamine β-hydroxylase in electrophoresis either at pH 8·6 or at pH 6·5 were quite similar. It should be considered that the presence of the detergent Cutscum, which binds rather tenaciously to protein, may alter the mobility of the enzyme. However, after the Sepharose 6B column chromatography, the enzyme precipitated when ammonium sulfate was added, suggesting that most of the detergent could be removed by the previous treatments. These results suggest that particle-bound dopamine β-hydroxylase and the soluble enzyme may be identical and may not be isoenzymes, and that there may exist a loosely bound enzyme and a firmly bound enzyme in the particles of bovine adrenal medulla. Only loosely bound enzymes may be released into soluble fraction by homogenization or by other simple physical treatments.

Another possibility which should be considered is the problem of aggregation, particularly of tyrosine hydroxylase.[11] Musacchio *et al.*[11] reported that the native form of tyrosine hydroxylase had a molecular weight of 155,000 and that the trypsin digestion form had a molecular weight of 34,000. Shiman *et al.*[12] reported that a highly purified tyrosine hydroxylase by chymotrypsin digestion from chromaffin particles had a molecular weight of 40,000. These reports suggest that either trypsin or chymotrypsin may cause depolymerization of the enzyme. The higher molecular weight shown in

our results either of the soluble enzyme or of the particulate enzyme which was solubilized by using a detergent, suggests that each enzyme may be a polymerized form and have a similar molecular weight. The difference in the molecular weight between the value (155,000) reported by Musacchio *et al.*[11] and that in our present report (400,000) suggests that there may exist various polymerized forms of tyrosine hydroxylase. The problem as to which molecular form is the natural form remains for further investigation. The detergent Cutscum appears to solubilize the enzyme without causing the depolymerization of the particulate tyrosine hydroxylase.

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Department of Biochemistry,
School of Dentistry,
Aichi-Gakuin University,
Nagoya 464, *Japan*

H. KUZUYA
T. NAGATSU

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Intracellular distribution of endogenous inhibitors of dopamine β-hydroxylase in bovine adrenal medulla

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THE EXISTENCE of some endogenous inhibitors of dopamine β-hydroxylase which are probably sulfhydryl compounds was reported.[1–3] We had previously reported that endogenous inhibitors of dopamine β-hydroxylase are present in homogenates of the adrenal medulla and other sympathetically innervated organs, and in every subcellular fraction of bovine adrenal medulla including supernatant and chromaffin granules.[4] We had reported an assay procedure of dopamine β-hydroxylase in crude adrenal preparations by including *N*-ethylmaleimide into the reaction mixture. *N*-ethylmaleimide did not inhibit the purified dopamine β-hydroxylase at high concentrations. A maximum activity was obtained at 10^{-2} M of *N*-ethylmaleimide. Copper could be used also for the inactivation of endogenous inhibitors. However, since copper inhibits dopamine β-hydroxylase itself, it was necessary to find an optimum concentration of copper by preliminary titration experiments.