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BOVINE ADRENAL MEDULLARY DOPAMINE β-HYDROXYLASE: PURIFICATION BY AFFINITY CHROMATOGRAPHY, KINETIC STUDIES AND PRESENCE OF ESSENTIAL HISTIDYL RESIDUES

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SUMMARY

1. Dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase, EC 1.14.17.1) from bovine adrenal medulla has been purified by affinity chromatography. The affinity column was prepared by fixation of tyramine to *p*-aminobenzoamidoethyl–Sepharose *via* the aromatic ring of tyramine. Not complete retention, but sufficient retardation, of the enzyme on the column was observed. The enzyme has been obtained pure as judged by the criterion of polyacrylamide gel electrophoresis.

2. With this highly purified dopamine β-hydroxylase, attempts were made to dissociate the native protein.

3. Catalase was a protective agent against irreversible denaturation. Kinetic studies were performed in order to determine the kinetic parameters modified by this denaturation.

4. The kinetic properties of the enzyme purified by affinity chromatography are the same as those described by other authors using different methods of preparation.

5. Diethylpyrocarbonate, a specific reagent for histidine residues, was used in order to confirm the presence of essential histidyl residues. Treatment of the enzyme with diethylpyrocarbonate was associated with both spectra modifications and loss of activity of dopamine β-hydroxylase. Moreover, this inhibition appeared to be different from the inhibition obtained with a copper-chelating agent.

INTRODUCTION

In 1965, Friedman and Kaufman[1] described a revised and improved procedure for the isolation and purification of dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating) EC 1.14.17.1) from

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bovine adrenal medulla. With this multistep method, large amounts of essentially pure enzyme could be obtained. In connection with our studies on animal and organ specificities and antigenicity of dopamine β-hydroxylase, we felt the need for a more rapid and simplified procedure. To achieve this aim, we have investigated the use of affinity chromatography, a method which has proved valuable for the rapid and selective purification of a large number of enzymes from partially purified mixtures[2,3].

Studies reported by Bridgers and Kaufman[4] indicate that the structural characteristics of the substrates of dopamine β-hydroxylase are an aromatic ring with a side chain of two or three carbon atoms terminating in a free amino group. Based on these considerations, we have developed a method which allows rapid, reproducible and convenient purification of dopamine β-hydroxylase, using an affinity chromatography column in which tyramine with its free amino group is linked to an insoluble support via the aromatic ring. The enzyme was purified not by a complete retention but by a sufficient retardation which depended on the column characteristics.

MATERIALS

Sepharose-4B was obtained from Pharmacia (Sweden). Cyanogen bromide, *p*-nitrobenzoylazide, acrylamide and bis-acrylamide were purchased from Eastman Kodak. Sodium dithionite, *N*,*N*′-dimethylformamide and ascorbate were obtained from Merck (Darmstadt, Germany). Fumaric acid and tyramine hydrochloride were obtained from Sigma (Minneapolis, U.S.A.). [β-^{14}C]tyramine hydrochloride was purchased from the Radiochemical Centre (Amersham, England), of a specific activity of 42 Ci/mole. Ethylenediamine was purchased from Fluka (Buchs, Switzerland). Catalase from beef liver (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) was purchased from Boehringer (Mannheim, Germany), of a specific activity of 39 000 units/mg. Dithiothreitol, octopamine, and bovine serum albumin were obtained from Calbiochem (Los Angeles, U.S.A.). Diethylpyrocarbonate was a Bayer product (Baycovin ®).

METHODS

Preparation of chromaffin granules

The method for the preparation of chromaffin granules from bovine adrenal medulla was based on that of Friedman and Kaufman[1]. About 1–1.5 kg of beef adrenals were obtained fresh from a local slaughterhouse and immediately placed in cold 0.32 M sucrose. The adrenals were defatted, and 50–75 g of medulla, which were obtained by fine dissection, were suspended in 5 vol. of 0.32 M sucrose. The mixture was placed in a bucket and partially blended for 30 s at 5000 rev./min in an homogenizer apparatus with double-bladed knives (Equipements Industriels, Paris). The mixture was finely homogenized in a Potter–Elvehjem type homogenizer with about 15 strokes of the teflon pestle and then centrifuged for 15 min at 750 × *g*.

The supernatant was passed through cheese cloth to remove floating fats and the precipitate was homogenized again in 2 vol. of 0.32 M sucrose and centrifuged under the same conditions as above. The supernatant fluids were mixed and then centrifuged at 11 500 × *g* for 20 min. The supernatant was discarded and the particles were suspended in 0.32 M sucrose (1 ml of sucrose for 2 g of starting

material). The suspension was layered on 1.6 M sucrose (10 ml of suspension on 15 ml of 1.6 M sucrose in Swing SW-25 tubes). The tubes were centrifuged for 90 min at 100 000 × *g*. The pellet was resuspended in 10 mM potassium phosphate buffer (pH 6.5).

Preparation of the affinity column

Ethylenediamine–Sepharose. Sepharose-4B was activated with cyanogen bromide according to the procedure of Cuatrecasas[5]. The quantity of cyanogen bromide used was 300 mg per ml of gel. The pH of the Sepharose–CNBr mixture was immediately adjusted to pH 11 and kept constant by the addition of 8 M NaOH.

After 12 min the agarose was washed rapidly with 1500 ml of 0.1 M borate buffer (pH 9.5), and the damp gel was added to 100 ml of 2 M ethylenediamine solution adjusted to pH 10 by 6 M HCl. The mixture was stirred gently for 3 h at 4 °C and kept overnight at 4 °C without stirring. The gel was washed with water and then with 2000 ml of 0.2 M sodium borate (pH 9.3).

p-Aminobenzamidoethyl–Sepharose. To the aminoethyl–Sepharose solution, 0.175 M *p*-nitrobenzoylazide in *N*,*N*′-dimethylformamide was added until the dimethylformamide concentration reached 40%. The mixture was stirred gently for 1 h at room temperature.

The *p*-nitrobenzoamidoethyl–Sepharose was washed extensively with 50% dimethylformamide. The gel was suspended in 0.5 M $NaHCO_3$ (pH 8.5), and the suspension was treated with 0.1 M sodium dithionite at 40 °C for 40 min. The *p*-nitrobenzoamidoethyl–Sepharose was reduced to *p*-aminobenzoamidoethyl–Sepharose. The reduced gel was then washed thoroughly with water and suspended in 0.5 M HCl.

Coupling of tyramine. The washed *p*-aminobenzoamidoethyl–Sepharose suspended in HCl was diazotized with 0.1 M sodium nitrite at 4 °C for 7 min. The diazotized derivative was washed with water and then with saturated sodium borate. Tyramine was added to the mixture to give a concentration of 10 mM. The pH was adjusted to 10 with 1 M NaOH. The suspension was allowed to stand overnight at 4 °C with gentle stirring. After coupling, the mixture was washed with water and then with 10 mM potassium phosphate buffer (pH 6.5).

To establish the extent of coupling, [β-^{14}C]tyramine was used. Samples of substituted agarose were centrifuged and washed extensively with water. An aliquot of the packed gel was added to 10 ml of toluene containing 1 ml of 0.4% omnifluor. The ^{14}C content determined by scintillation spectrometry indicated a tyramine content of 8–10 μmole per ml of Sepharose in the preparations used routinely.

Protein determinations

Protein was estimated by the method of Lowry *et al.*[6] using bovine serum albumin as standard.

Enzyme activity determinations

Enzyme activity was determined with tyramine as the substrate by an adaptation of the spectrophotometric assay of Pisano *et al.*[7] as we have previously described[8].

Electrophoresis

Electrophoresis on polyacrylamide gels was performed according to Davis[9]. The separating gel was 6% in polyacrylamide. The pH of the gel was 8.3. The pH of 0.05 M Tris–0.1 M glycine buffer solution was 8.3.

Samples were prepared for electrophoresis by mixing 1 ml of the enzyme preparation with 0.2 ml of saturated sucrose and 0.1 ml of tracking dye (0.01% Bromophenol blue in the electrophoresis buffer). A 50-μl-150-μl aliquot of this mixture, containing 10 μg–200 μg of protein was layered onto the gel.

Electrophoresis was performed at 0.5 mA per tube until the tracking dye had moved 10–15 mm into the gel. 1 mA per tube was then applied until the tracking dye had moved 100 mm.

The gels were stained for 2 h with Coomassie brilliant blue (0.05%, w/v, in a solution containing 75 ml of methanol, 9 ml of acetic acid, 4.75 ml of glycerol and 100 ml of water). The gels were then destained in a solution containing 60 ml of methanol, 15 ml of acetic acid, 7.5 ml of glycerol and 217.5 ml of water. The gels were stored in this solution after destaining.

When the enzyme was localized in the gel, the gels were removed from the tubes and cut vertically with a slicer. Each segment was 2 mm thick and divided into four sections. The four small pieces from each segment were transferred into tubes containing 500 μl of 0.2 M sodium acetate buffer and 100 μg of catalase was then added. The tubes were allowed to stand at 4 °C for 1 h. Then, ascorbate and fumarate were added as described before[8] and the tubes were preincubated at 37 °C for 20 min. [β-^{14}C]tyramine (specific activity 0.25 Ci per mole at a concentration of 10 mM) was next added and the enzymatic reaction was allowed to proceed for 30 min.

The method of Weber and Osborn[10] has been used for the estimation of molecular weight. Reference proteins were catalase, lactate dehydrogenase, bovine serum albumin, α-chymotrypsin, carboxypeptidase, phosphoglucoisomerase, glycerate kinase and pyruvate kinase. In order to achieve rotal reduction of all disulphide bonds, the improved procedure described by Virella and Parkhouse[11] has been used.

Treatment of the enzyme with diethylpyrocarbonate

The enzyme was treated with diethylpyrocarbonate following the procedure described by Huc *et al.*[12].

RESULTS

Preparation of the enzyme

Solubilization of dopamine β-hydroxylase. To the suspension of chromaffin granules 1% Triton X-100 in 10 mM potassium phosphate buffer was added to a final concentration of 0.1%. The mixture was allowed to stand for 45 min with gentle stirring. Then the mixture was centrifuged for 15 min at 1000 $\times$ *g*. The pellet was discarded and the supernatant was again centrifuged at 100 000 $\times$ *g* for 20 min. The pellet was again discarded. The supernatant was diluted to 500 ml with 10 mM potassium phosphate buffer (pH 6.5), and the mixture was ultrafiltered in a Diaflo cell through XM-100A membrane under compressed N_2 (pressure about 1.5 bar)*.

* 1 bar = 10^5 Newtons/m^2.

When the volume was reduced to 50 ml, the solution was again diluted to 500 ml with the same buffer and filtered under the same conditions to a final volume of 7.5 ml.

The mixture was divided into three aliquots of 2.5 ml each and stored frozen at −180 °C in liquid N_2. This preparation could be stored frozen for several weeks at this temperature without any loss of activity.

Affinity chromatography. Affinity chromatography was performed at 4 °C. Columns were equilibrated with 10 mM potassium phosphate (pH 6.5). A 2.5-ml portion of the solubilized enzyme was layered onto the surface of the column (1 cm × 15 cm) and passed through the column at a rate of about 7 ml/h. The elution buffer was 10 mM potassium phosphate (pH 6.5). The elution pattern that was routinely obtained is presented in Fig. 1.

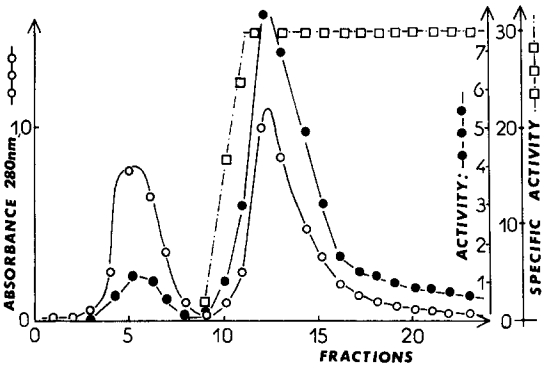

Fig. 1. Chromatography of dopamine β-hydroxylase on affinity column. Column volume: 12.5 ml, internal diameter: 10 mm. 2.5 ml of partially purified enzyme (6 mg/ml) obtained by solubilization with Triton X-100 and extensive washing in a Diaflo cell through an XM-100A membrane (see Methods) were applied. At a flow rate of 7 ml/h, the peak containing high dopamine β-hydroxylase activity was retarded from the flow-through peak which contained about 5% of dopamine β-hydroxylase activity. The fraction volume was 2 ml. Activities were determined at pH 5.5 and are expressed in μmole of octopamine formed/30 min per fraction. Specific activities are expressed in μmole of octopamine formed/30 min per mg protein.

After elution, the tubes containing enzyme activity with a constant specific activity were pooled. The mixture was concentrated in a Diaflo cell through a XM-100A membrane until the volume reached 5 ml.

At this stage the purified enzyme was stored at −30 °C. A typical purification is given in Table I.

Stability

The purified enzyme in 10 mM potassium phosphate buffer (pH 6.5) may be stored frozen at −30 °C for at least one month without loss of activity. When the enzyme was stored in such a buffer at 0–4 °C, the purified enzyme lost about 10–15% of its activity in 24 h.

Polyacrylamide gel electrophoresis; molecular weight

The purified enzyme moved as a single band on polyacrylamide gel electrophoresis at pH 8.3. When enzyme activity was directly tested in the gel, the

TABLE I

TYPICAL PURIFICATION OF DOPAMINE β-HYDROXYLASE BY AFFINITY CHROMATOGRAPHY

The preparation obtained after the ultrafiltration step was divided into three aliquots of 2.5 ml each which were applied to the affinity column. Results given here are the total recovery obtained after the passage through the column of all three aliquots. From Fig. 1, the specific activity of each fraction was 30 μmoles/30 min per mg protein. After concentration of pooled fractions in the Diaflo cell, the specific activity increased as the protein concentration was increased up to a final value. It is this final value which is reported here. This phenomena has been observed for a large number of enzymes (dilution effect).

Fraction	*Volume (ml)*	*Protein (mg/ml)*	*Activity (μmoles/30 min per ml)*	*Total activity (μmoles/30 min)*	*Specific activity (μmoles/30 min per mg protein)*	*Yield (%)*
Chromaffin granules	5.5	28	232	1276	8.28	100
Solubilization by Triton	15	6.25	58	870	9.28	68
Ultrafiltration	7.5	6.02	103	772	17.1	60.5
Affinity chromatography–ultrafiltration	60	0.0875	10	600	114.3	46.5

recovered activity corresponded to the single band (Fig. 2). When electrophoresis was carried out at + 4 °C in the presence of 0.01% sodium dodecylsulphate the enzyme again migrated as a single band but no activity could be detected. From calibrated gels with proteins of known molecular weights, the molecular weight of dopamine β-hydroxylase was estimated to be 280 000 (±1%). Some aggregation of the enzyme occurred when electrophoresis was carried out at 25 °C. Treatment of the enzyme with 2% sodium dodecylsulphate and 50 mM dithiothreitol at 37 °C for 30 min resulted in a partial dissociation of the enzyme: two bands with molecular weights of 155 000 and 145 000 appeared.

The effect of stronger conditions for the reduction of disulphide bonds was also investigated[11]. The enzyme was treated with 2% sodium dodecylsulphate and 50 mM dithiothreitol, then heated at 60 °C for 30 min, and immediately loaded onto polyacrylamide gels. The electrophoresis pattern showed the disappearance of the 280 000 band and the appearance of two major bands with calculated molecular weights of 77 000 and 60 000. The ratio of their absorbances was about 3:1, respectively. Moreover, two faint bands occurred with molecular weights of 36 000 and 25 000. These data are summarized in Table II.

Protective action of catalase

Catalase is known to be a protective agent of dopamine β-hydroxylase[13]. Consequently, catalase was added in all incubation mixtures in order to determine dopamine β-hydroxylase activities. With our enzyme preparation, the same phenomenon has been observed. Fig. 3 shows this protective action of catalase. No loss of activity was observed when dopamine β-hydroxylase was preincubated and incubated at 37 °C with catalase. The preincubation with albumin or inactivated catalase instead of catalase resulted in a slight loss of activity. The loss of activity reached 90% after 20 min preincubation of dopamine β-hydroxylase without any protein. The loss of activity was irreversible since 100% activity could not be recovered after the addition of catalase to the assay mixture. When catalase was

TABLE II

EFFECT OF EXPERIMENTAL CONDITIONS ON THE DISSOCIATION OF DOPAMINE β-HYDROXYLASE

Enzyme treatment	*% gel*	*Temperature (°C)*	*Additional conditions*	*Molecular weight*	*Results*
None	6	+4	—	280 000	One band with enzymatic activity
Sodium dodecylsulphate (0.01 %)	6	+4	Sodium dodecylsulphate (0.01%) in gels and buffers	280 000	One band without enzymatic activity
Sodium dodecylsulphate (0.01%)	6	+25	Sodium dodecylsulphate (0.01%) in gels and buffers	280 000	One major band Several bands (aggregation)
Sodium dodecylsulphate (2%) + dithiothreitol (50 mM)	10	+25	Gels with 0.01% sodium dodecylsulphate	280 000	Band of native protein
			Buffers bubbled with N_2 gas	155 000 140 000	Partially dissociated native enzyme (two bands) due either to partial structural change of one or to the presence of two different great subunits
Sodium dodecylsulphate (2%) + dithiothreitol (50 mM)	10	+25	Sodium dodecylsulphate (0.01%) in gels Enzyme heated at 60 °C for 0.5 h	77 000 60 000	Two bands reproducible with different intensities in absorbance (3:1)
			Buffers bubbled with N_2 gas	36 000 25 000	Two bands reproducible but very faint

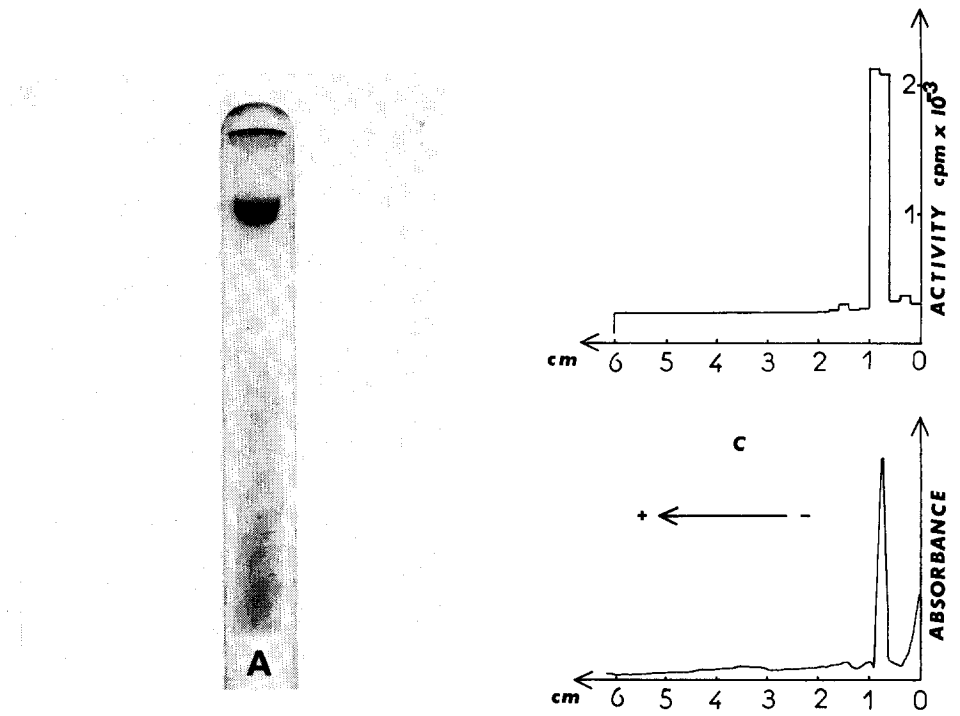

Fig. 2. Gel electrophoresis of dopamine β-hydroxylase purified by affinity chromatography. 50 μg of protein were applied and the run was performed at 4 °C at 1 mA/tube. Gels were stained by Coomassie brilliant blue (A). (Lower right) Densitometer profile of the proteins after coloration of the gel. (Upper right) Localization of the enzyme activity as described in Methods.

replaced by horseradish peroxidase, no loss of activity was observed. In some experiments, tyramine or ascorbate were added during the preincubation and the reaction was started by the addition of the second substrate. In this case, neither protection or supplementary inactivation of the enzyme activity was observed.

The extent of inactivation is not temperature dependent. Separate experiments demonstrated that preincubations without catalase at several temperatures from 10–50 °C (50 °C is the optimal temperature for dopamine β-hydroxylase) resulted in a loss of 56% of the activity recovered when preincubations were carried out in the presence of catalase.

The inactivation of the enzyme resulted in modification of the kinetic parameters, as seen in Fig. 4. In Fig. 4B, the modification of the maximum velocity to tyramine is simply due to the destruction of a part of active dopamine β-hydroxylase, whereas the modification of the Michaelis constant for ascorbate (Fig. 4A) seems to indicate that catalase avoids the decrease of the affinity of the enzyme for ascorbate.

Kinetics of the dopamine β-hydroxylase reaction

Dopamine β-hydroxylase activity was linear with respect to time and enzyme concentration in our experiments.

The kinetic curves are presented in Fig. 5. The only parameters which were varied were the concentrations of ascorbate and tyramine.

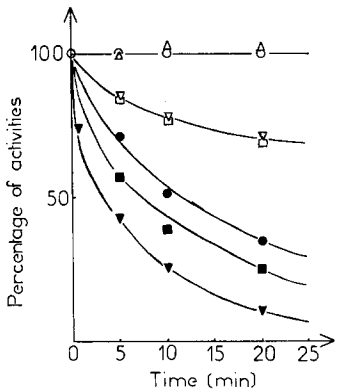

Fig. 3. Influence of the preliminary incubation time on dopamine β-hydroxylase activity. 10 μg of enzyme were preincubated at 37 °C in 0.8 ml containing 100 μmoles of potassium phosphate buffer (pH 6.5), 20 μmoles of fumarate, and 100 μg of catalase (○—○), 100 μg of horseradish peroxidase (△—△), 100 μg of bovine serum albumin (□—□) or 100 μg of inactivated catalase (▽—▽) (obtained by exposure at 80 °C for 5 min). Assays were initiated by the addition of 10 μmoles of tyramine and 10 μmoles of ascorbate in a final volume of 1.0 ml. In three experiments, enzyme was preincubated in the same medium, but assays were initiated by the addition of 10 μmoles of tyramine and 10 μmoles of ascorbate and carried out in the presence of 100 μg of catalase (●—●), 100 μg of albumin (■—■), or no proteins (▼—▼). Recovered activities are expressed as % of the total activities recovered when the enzyme was preincubated with catalase.

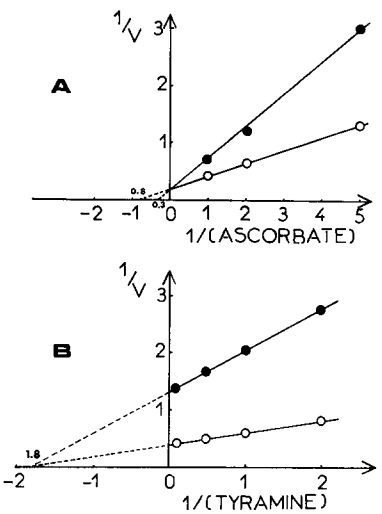

Fig. 4. Effect of catalase on kinetic parameters. A, reciprocal rates were plotted *versus* reciprocal concentration of ascorbate according to Lineweaver and Burk[14]. Mixtures contained 10 μg of enzyme, 100 μmoles of potassium phosphate (pH 6.5), 20 μmoles of fumarate, variable amounts of ascorbate and 100 μg of catalase (○) or no catalase (●) in a total volume of 0.9 ml. Preincubation time was 10 min at 37 °C. Assays were initiated by the addition of 10 μmoles of tyramine. Final volume was 1.0 ml. B, reciprocal rates were plotted *versus* reciprocal concentration of tyramine. Mixtures contained tyramine instead of ascorbate and assays were initiated by the addition of 10 μmoles of ascorbate. Concentrations are expressed in mM and velocities in μmole of octopamine formed/30 min per ml enzyme solution.

Studies were carried out in which the concentration of tyramine was held at various constant levels and v determined as a function of the varied concentrations of ascorbate (Fig. 5A), and in which ascorbate was held constant at various levels and v determined as a function of tyramine concentration (Fig. 5B). When the tyramine concentration was varied, the K_m values for ascorbate, calculated as the negative reciprocals of the x-axis intercepts of extrapolations in Fig. 5A, changed as a function of tyramine concentration, and the lines were parallel. The K_m values for tyramine in the enzymatic reaction, similarly calculated, were also affected by variations in the concentration of ascrobate, as indicated in Fig. 5B and the lines were parallel. These results indicate a part of the mechanism of the reaction; firstly that there is an order in the addition of the substrates to the enzyme and secondly that there are two

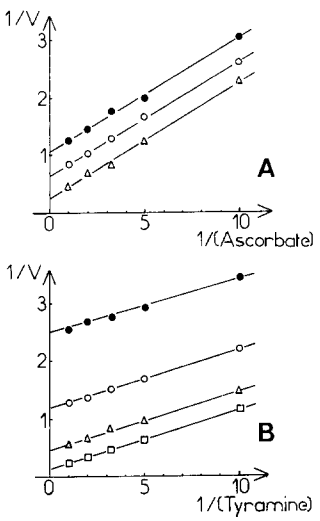

Fig. 5. Lineweaver–Burk plot[14] of dopamine β-hydroxylase activity. (A) Initial velocity study with ascorbate as the varied substrate. Preincubation media contained the following components in a total volume of 0.9 ml: 100 μmoles of potassium phosphate buffer (pH 6.5), 20 μmoles of fumarate, 100 μg of catalase and ascorbate as indicated. Preincubation time was 10 min at 37 °C. Assays were initiated by addition of tyramine: (●—●), 0.1 μmole; (○—○), 0.2 μmole; (△—△), 1 μmole. Final volume was 1.0 ml. (B) Initial velocity study with tyramine as the varied substrate. Preincubation media was as in (A), tyramine as indicated instead of ascorbate. Assays were initiated by addition of ascorbate: (●—●), 0.1 μmole; (○—○), 0.2 μmole; (△—△), 0.75 μmole and (□—□), 10 μmoles. Velocities are expressed in μmole of octopamine formed/30 min per ml of enzyme solution. Concentrations are expressed in mM. The lines in the graph were calculated by the regression procedure using a computer programme.

oscillating forms of the enzyme. Friedman and Kaufman[1] have shown that dopamine β-hydroxylase contains copper atoms, which are reduced by ascorbate to cuprous atoms. Thus data are in good agreement with a "ping-pong" mechanism according to Cleland's nomenclature[15].

In Fig. 6, the variation of v obtained from Fig. 5 has been plotted *versus* the reciprocal substrate concentration. From this data, real kinetic constants have been determined for tyramine and ascorbate. At pH 6.5, the real Michaelis constant for tyramine was 0.55 mM, for ascorbate 1.25 mM and the real maximum velocity was 33 μmoles of octopamine formed/30 min per mg of enzyme.

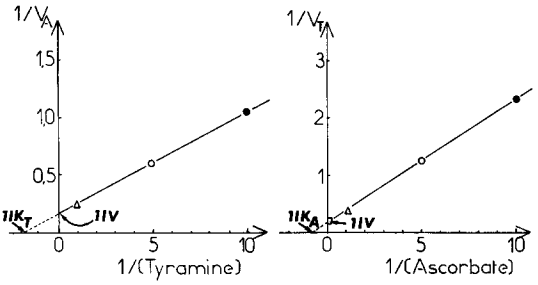

Fig. 6. Determination of real kinetic constants of dopamine β-hydroxylase for tyramine and ascorbate. Reciprocal maximum velocities for one substrate ($1/V_A$, ascorbate and $1/V_T$, tyramine) determined from Fig. 5 were plotted versus the reciprocal concentration of the other substrate. According to Cleland[15], proportionality is expected in view of a ping-pong mechanism. Moreover, $1/V$ is identical for tyramine and ascorbate (at pH 6.5, 33 μmoles of octopamine formed/30 min per mg enzyme). K_T is the real Michaelis constant for tyramine (0.55 mM at pH 6.5) and K_A the real Michaelis constant for ascorbate (1.25 mM at pH 6.5).

Inactivation by diethylpyrocarbonate

Diethylpyrocarbonate is able to react specifically with histidyl residues[16–18]. When dopamine β-hydroxylase was incubated at 0 °C with diethylpyrocarbonate, the difference spectrum between the native enzyme and the modified enzyme showed an absorption peak at 242 nm (Fig. 7A). This absorption is indicative of a carbethoxylated protein via the imidazole ring. The carbethoxylation was a function of time as seen in Fig. 7B. The spectral modification was concomitant with a loss of enzyme activity (Fig. 7C).

In order to determine the type of inhibition, the carbethoxylated enzyme was tested with either tyramine or ascorbate as the varied substrate (Fig. 8). From Fig. 8A, the inhibition appeared to be of the non-competitive type when tyramine was the varied substrate. The inhibition appeared to be of the competitive type when ascorbate was the varied substrate (Fig. 8B). Therefore, a replot of the slopes calculated from the Lineweaver–Burk plots versus inhibitor concentration was attempted. The curves obtained appeared to be hyperbolic.

Inactivation by diethyldithiocarbamate

Dopamine β-hydroxylase is a copper enzyme[1], and thus is sensitive to copper-chelating agents, such as diethyldithiocarbamate[1]. In copper proteins the metal may be bound to the protein by nitrogenous ligands from histidyl residues[19]. Diethylpyrocarbonate could react with such residues and consequently displace copper.

Diethyldithiocarbamate provided an inhibition of the non-competitive type when either tyramine or ascorbate was the varied substrate.

DISCUSSION

The isolation procedure yields a preparation of dopamine β-hydroxylase with a specific activity of 100–120 μmoles of octopamine formed per 30 min per mg of protein (Table I). Friedman and Kaufman[1] have prepared a purified enzyme with a specific activity of 60–80 μmoles of octopamine formed/30 min per mg of protein.

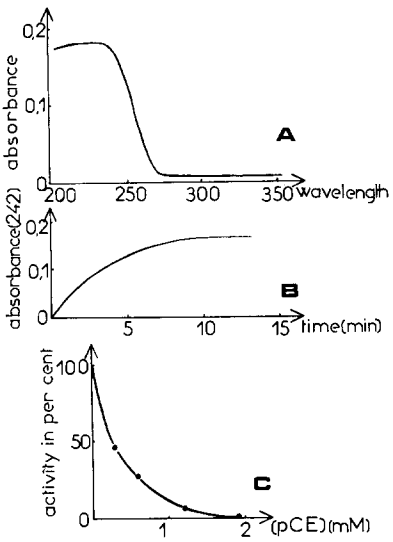

Fig. 7. Interaction of diethylpyrocarbonate and dopamine β-hydroxylase. (A) Difference spectrum between the native protein and the carbethoxylated protein. The protein concentration was 0.3 mg/ml in 0.1 M potassium phosphate buffer (pH 6.2). 10 μl of 0.12 M diethylpyrocarbonate in absolute ethanol was added to 1 ml of enzyme solution. The spectrum was recorded 15 min after the addition of reagent. (B) Kinetics of the carbethoxylation reaction. 50 μl of 0.12 M diethylpyrocarbonate solution in absolute ethanol was added to 5 ml of 0.3 mg/ml protein solution. Reaction was performed at 0 °C. Aliquots were pipetted and absorbance was determined at 242 nm against native protein at the same concentration and treated in a similar manner but without diethylpyrocarbonate as reference. (C) Effect of diethylpyrocarbonate on enzyme activity. 0.3 mg of protein in 1 ml of potassium phosphate buffer were incubated at 0 °C with 10 μl of 0.03, 0.06, 0.12 and 0.18 M of diethylpyrocarbonate solution in absolute ethanol. After 15 min, 10-μl aliquots of this mixture were added to 1 ml of the assay mixture. The extent of inhibition was calculated by comparing the modified enzyme to references treated in a similar manner but without diethylpyrocarbonate.

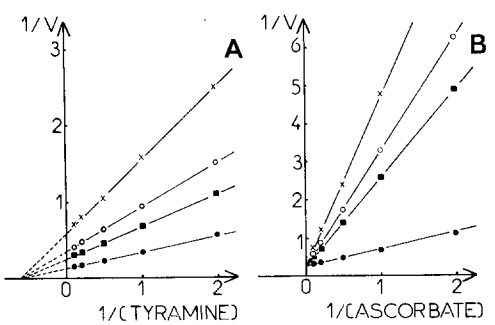

Fig. 8. Kinetics of the carbethoxylated dopamine β-hydroxylase with tyramine (A) or ascorbate (B) as the varied substrate. (A) Mixtures contained in μmole: 500 μmoles of sodium acetate buffer (pH 5.5), 20 μmoles of fumarate, 10 μg of catalase, 10 μmoles of ascorbate and tyramine as indicated in a final volume of 10 ml. (B) Mixtures contained in μmole: 500 μmoles of sodium acetate buffer (pH 5.5), 20 μmoles of fumarate, 100 μg of catalase, 10 μmoles of tyramine and ascorbate as indicated in a final volume of 1.0 ml. Assays were initiated by the addition of 10 μg of dopamine β-hydroxylase (●), 10 μg of enzyme preliminary treated with diethylpyrocarbonate at a concentration of 0.3 mM (■), 0.6 mM (○) and 1.2 mM (×). Dilution of native and treated enzyme was 1/100. Substrate concentrations are expressed in mM and velocities as μmole of octopamine formed/30 min per ml of enzyme solution.

The difference is due to the pH of the medium chosen for dopamine β-hydroxylase activity measurements[20].

Hörtnagl *et al.*[21] have reported a method of purification based on the selective precipitation of proteins of chromaffin granules by *N*-cetylpyridinium chloride. By this procedure, pure enzyme could be obtained but the preparation very quickly loses its activity. The earlier multistep method decribed by Foldes *et al.*[22] leads to an enzyme preparation with a specific activity of 52 μmoles of octopamine formed/30 min per mg of protein. In fact specific activities of our preparation and those of other authors[1,22] were similar when the difference in the pH at which the measurements were performed is taken into account[20].

Affinity chromatography resulted in a highly purified enzyme obtained in one step after the isolation of chromaffin granules. The preparation appeared to be homogeneous as judged by specific activity and polyacrylamide gel electrophoresis. The preparation was free of monoaminoxidase activity and of the endogenous inhibitors[23] since dopamine β-hydroxylase isolated by our procedure appeared not to be activated but inhibited by relatively small amounts of copper (unpublished results).

The molecular weight of dopamine β-hydroxylase has been estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate to be 280 000 which is in good agreement with values reported by authors using polyacrylamide gel electrophoresis[21,24] and sedimentation velocity ultracentrifugation[1,22]. The investigation of the subunit structure of dopamine β-hydroxylase appeared to be fraught with difficulties, the major problem being the complete reduction of disulphide bonds. Actually, the method that we have adopted has been used and improved by many others[11,25,26] for different kinds of proteins. The treatment with dithiothreitol alone is not sufficient. It is necessary to heat the protein at 60 °C for 30 min in the presence of 2% sodium dodecylsulphate and 50 mM dithiothreitol. Thus dopamine β-hydroxylase seemed to be dissociated since bands with intermediary molecular weights (145 000 and 155 000) did not appear on the gel. Two major bands with a molecular weight of 77 000 and 60 000 in a ratio of 3:1 were detected. Therefore, a definite subunit structure cannot be definitely ascribed to dopamine β-hydroxylase. It is possible to attribute a composition of three subunits each with a molecular weight of 77 000 and one subunit with a molecular weight of 60 000. If one assumes that the 60 000 subunit is the result of a slight decomposition of the 77 000 subunit in particular as two faint bands with molecular weights of 36 000 and 25 000 were also present, it may be acceptable to postulate a composition of four subunits each with a molecular weight of 77 000. During the preparation of this manuscript, Foldes *et al.*[27] have reported that the exposure of dopamine β-hydroxylase to 8 M urea leads to a dissociation of the enzyme into components with a molecular weight each of 100 000. Thus, these authors concluded that the enzyme is composed of three subunits. However, it is not possible to assume that disulphide bonds were completely reduced in the conditions described by them.

Dopamine β-hydroxylase purified by more involved procedures is sensitive to endogenous peroxide compounds. Complete protection takes place when catalase is present in the media[13,22]. Our enzyme appeared to be equally sensitive to peroxide compounds. The addition either of catalase or of horse radish peroxidase prevents the decrease of activity. A slight decrease occurs when albumin or inactivated catalase are present in the medium. Peroxide compounds present in the medium could modify

the active centre thus preventing the hydroxylation of tyramine and modifying the affinity for ascorbate since it has been shown that ascorbate is complexed via the copper atom of the active site during the reaction[20]. Therefore, H_2O_2 is an inhibitor of dopamine β-hydroxylase[13] and its presence does not lead to modifications of the affinity for ascorbate[8]. Moreover, ascorbate is itself an H_2O_2-generating agent[28]. Thus, catalase or horse radish peroxidase could have two functions: the prevention of the oxidation of dopamine β-hydroxylase by endogenous peroxide compounds and then the prevention of an oxidation of ascorbate which can occur when traces of metals are present.

Our enzyme preparation follows a "ping-pong mechanism" in good agreement with the proposition previously reported by Goldstein *et al.*[29].

In a previous report[20], the free enzyme and the enzyme–tyramine complex show three pK values. One pK value of 6.6 has been reported, and attributed to an histidyl residue involved in the hydroxylation reaction. The work presented here gives an additional argument.

The carbethoxylation of the enzyme by diethylpyrocarbonate leads to a spectral modification. The increase of absorption at 240 nm, indicative of carbethoxylated proteins, is concomitant with the loss of activity. The lack of absorption changes at 280 nm shows that tyrosine residues are not modified by the reagent. Diethylpyrocarbonate prevents the hydroxylation of tyramine. When ascorbate was the varied substrate, the inactivation appeared to be hyperbolic and competitive. The carbethoxylated protein–ascorbate complex converts ascorbate to the product at the same rate, but the presence of the carbethoxyl group affects the affinity of the enzyme for ascorbate.

What role do the histidyl residue(s) actually play in dopamine β-hydroxylase? Histidine is assumed to serve as a proton source[20], but the exact mechanism cannot be defined yet.

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