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SHORT COMMUNICATION

Purification and Properties of Dopamine β-Hydroxylase from Human Pheochromocytoma

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SUMMARY

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Dopamine β-hydroxylase (EC 1.14.17.1) was isolated as a pure protein from a human pheochromocytoma. The tumor enzyme has chemical and physical properties similar to those of the enzyme isolated from bovine adrenal medulla. Both enzymes are glycoproteins and have similar amino acid compositions. Both enzymes have subunit molecular weights of 75,000–77,000 determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by equilibrium centrifugation in 6 M guanidine hydrochloride after reduction and carboxymethylation. Equilibrium centrifugation in the presence of 6 M guanidine hydrochloride in the absence of reducing agents gives subunit molecular weights of about 150,000 for both enzymes. Studies of the native enzyme obtained from the human tumor show that, in contrast to the bovine enzyme, human dopamine β-hydroxylase has an unusual tendency to dissociate and aggregate; however, its behavior on Sephadex G-200 suggests a molecular weight of 300,000, the same as that of the bovine enzyme.

Purification of the enzyme dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase, EC 1.14.17.1) from bovine adrenal medulla was first reported by Levin *et al.* in 1960 (1). Viveros, Arqueros, and Kirshner (2) later demonstrated that the enzyme is released simultaneously with the catecholamines from the adrenal medulla, and others (3) reported the concomitant discharge of dopamine β-hydroxylase and catecholamines upon stimulation of sympathetic neurons. The detection of dopamine β-hydroxylase in human plasma led to the proposal that its activity in plasma may provide an index of sympathetic nervous system function in man (4), and alterations of plasma dopamine β-hydroxylase activity have been reported for diseases that are associated with aberrations of adrenergic function (5–7).

Recently it was proposed that radioimmunoassay of dopamine β-hydroxylase protein may more accurately reflect sympathetic nerve activity than assay of the plasma enzymatic activity (8). However, those data were derived from a study in

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which heterologous bovine dopamine β-hydroxylase was used for preparation of antiserum; other, more recent investigations, utilizing antisera prepared against the human enzyme, have revealed a high correlation between human dopamine β-hydroxylase protein and plasma enzyme activity (9, 10). For this reason, we have isolated human dopamine β-hydroxylase in order to compare some of its properties with those of the bovine enzyme.

A 29-g pheochromocytoma was obtained as a surgical specimen immediately upon removal. The gland was cleaned of fat and homogenized in 5 volumes of ice-cold 0.3 M sucrose. The homogenate was centrifuged at 800 × *g* for 10 min, and the precipitate was discarded. The supernatant fraction, which contained intact chromaffin vesicles, was centrifuged at 26,000 × *g* for 15 min. The precipitate, containing chromaffin vesicles, was washed twice in ice-cold 0.3 M sucrose, recentrifuged at 26,000 × *g* for 10 min, and homogenized twice in ice-cold water to lyse the vesicles. The resulting homogenate (100 ml) was then centrifuged at 100,000 × *g* for 60 min in order to sediment the particulate dopamine β-hydroxylase fraction. The precipitate was washed with 10 mM phosphate buffer at pH 6.5 and recentrifuged at 100,000 × *g*. The precipitate, containing the particulate enzyme, was frozen for future assay, and the 100,000 × *g* supernatant fractions were combined for assay and the remainder of the isolation.

The supernatant fraction was concentrated by Amicon Diaflo filtration (XM100A membrane) to 30 ml, rediluted with 10 mM phosphate buffer (pH 6.5), and again concentrated to 30 ml. Part (10 ml) of this fraction was applied directly to a column of DEAE-cellulose equilibrated with 10 mM phosphate buffer, pH 6.5, and the remainder (20 ml) was dialyzed for 18 hr against 6000 ml of 10 mM phosphate buffer (pH 6.5) in order to remove catecholamines before application to the column. The enzyme was eluted with a linear salt concentration gradient of 0–0.8 M sodium chloride in 10 mM phosphate buffer (pH 6.5). The activity of the nondialyzed fraction was eluted with the major protein peak between 0.08 and 0.15 M sodium chloride, and the dialyzed fraction was eluted between 0.04 and 0.06 M sodium chloride. Since subsequent procedures yielded a poor recovery of enzyme from the nondialyzed fraction, the dialyzed fraction was used for subsequent determination of the molecular properties.

The fractions with the peak dopamine β-hydroxylase activity were concentrated by Amicon Diaflo filtration (XM100A membrane), and 1 M sodium chloride was added to a final concentration of 0.1 M sodium chloride. This solution was passed through a column of Sephadex G-200 previously equilibrated with 0.1 M sodium chloride containing 10 mM phosphate buffer (pH 6.5). The G-200 column had been calibrated with blue dextran and had a void volume of 130 ml. The active fractions, which were eluted with a symmetrical peak at 145 ml, were combined and dialyzed against 10 mM phosphate buffer (pH 6.5). The solution was then concentrated by lyophilization, dissolved in water, dialyzed, and again concentrated by filtration.

Using 0.5–50 μg of dopamine β-hydroxylase protein per assay, enzyme activity was determined spectrophotometrically by the assay of Nagatsu and Udenfriend (11). Activity is expressed as micromoles of octopamine formed per minute per milligram of protein at 37°; assay conditions were always optimal for enzyme activity. Prior to chromatography, protein concentration was assayed by the method of Lowry *et al.* (12) following precipitation with 0.5 M trichloracetic acid; thereafter protein was measured by determining the optical density at 280 nm.

Disc gel electrophoresis was performed using a standard, pH 8.3, 7.5% acrylamide gel formulation (13). After treatment of the protein with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol, electrophoresis was carried out on 10% polyacrylamide gels in 0.1 M phosphate buffer, pH 7.1, essentially as described by Weber and Osborn (14). The gels were calibrated using protein of known molecular weight as shown in Fig. 2. The gels were stained with Coomassie brilliant blue to reveal the protein bands and with the periodic acid–Schiff reagent to determine the presence of carbohydrate (15).

Sedimentation equilibrium determinations were carried out in a Beckman model E

ultracentrifuge equipped with a photoelectric scanner. Partial specific volume was calculated from the amino acid analysis, and the concentration of guanidine hydrochloride was determined by refractive index measurements at 25°.

Samples were hydrolyzed in 6 N hydrochloric acid at 110° in sealed, evacuated hydrolysis tubes with a crystal of phenol added to prevent destruction of tyrosine. Hydrolyzed samples were analyzed on a Beckman model 121 automatic amino acid analyzer equipped with high-sensitivity cuvettes and an expanded recorder range. Injection reproducibility was monitored by including α-amino-β-guanidinopropionic acid and L-norleucine (20 μM) in the sample application buffer (0.01 N hydrochloric acid).

Dopamine β-hydroxylase was isolated from the chromaffin vesicle lysate of a human pheochromocytoma (Table 1). The final preparation contained 1.5 mg of protein in 3 ml of phosphate buffer (pH 6.5) and catalyzed β-hydroxylation of tyramine at the rate of 5.4 μmoles/min/mg of protein. This represents 80-fold purification of the chromaffin vesicle lysate, with 12% recovery of enzyme activity. Of further interest was the ratio of soluble to particulate enzyme after hypotonic lysis of vesicles and centrifugation at 100,000 × *g*. There was a total activity of 67 units in the supernatant fraction and only 35 units in the precipitate. Despite the fact that the 26,000 × *g* pellet most likely contained some dopamine β-hydroxylase associated with vesicle membranes broken during the preparation, the ratio of soluble to particulate enzyme was higher than that found in adrenal glands of other species (16, 17) and indicates that at least 65% of the enzyme was present in a readily solubilized form.

TABLE 1

Summary of purification procedure

Fraction	Total enzyme activity	Total protein	Specific activity
	units	*mg*	*units/mg*
800 × *g* supernatant	181	3000	0.06
Chromaffin vesicle lysate	67	900	0.07
Concentrated vesicle lysate (Amicon Diaflo filtration with XM100A membrane)	33	445	0.07
Peak from DEAE-cellulose	11.4	13.2	0.86
Concentrated eluate from DEAE-cellulose (Amicon Diaflo filtration with XM100A membrane)	8.2	9.5	0.87
Peak from Sephadex G-200	8.1	1.5	5.40

Disc gel electrophoresis of the purified preparation with aliquots of 12.5, 25, and 50 μg of protein yielded only one sharp band (Fig. 1). Sodium dodecyl sulfate gel electrophoresis displayed one major band and several minor bands (Fig. 1). When the electrophoretic mobility was plotted against the logarithms of the known protein molecular weights, the molecular weight of the dopamine β-hydroxylase subunit was estimated to be 75,000–77,000 (Fig. 2). A sample of the preparation stained with the periodic acid-Schiff reagent demonstrated one pink band, corresponding to the major protein band (Fig. 1).

The subunit molecular weight of human dopamine β-hydroxylase was determined in 6.3 M guanidine HCl by sedimentation equilibrium. Table 2 presents the results, which show that the polypeptide chain from the human enzyme has the same molecular weight as that of the bovine. Two polypeptides are disulfide-bonded in the native molecule.

Ultracentrifugal studies of the native enzyme in 0.1 M sodium chloride–0.5 M sodium acetate buffer (pH 5.0) or in 0.1 M sodium chloride–0.01 M sodium phosphate buffer (pH 6.8) at a variety of rotor speeds (6,000–15,000 rpm) showed reversible aggregation to molecular weights as high as 500,000 at the cell bottom. The lowest molecular weight observed at the meniscus at 15,000 rpm was 145,000, which corresponds to the dimer molecular weight observed in guanidine HCl. The human enzyme appears to exist as the dimer at concentration of 0.011–0.15 mg/ml, in direct contrast to the bovine enzyme, which is a tetramer over the con-

FIG. 1. *Gel electrophoresis of purified dopamine β-hydroxylase*

The first three gels on the left were run according to Weber and Osborn (14); the gel on the right was run according to Ornstein (13). Polyacrylamide gels, from left to right: dopamine β-hydroxylase in 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol stained with Coomassie blue; protein standards [from top to bottom: phosphorylase *a* (94,000), serum albumin (68,000), catalase (60,000), ovalbumin (43,000), chymotrypsinogen (25,700), ribonuclease (13,700)]; enzyme stained with periodic acid–Schiff (PAS) reagent; disc gel stained with Coomassie blue.

centration range 0.01–1.00 mg/ml as determined by sedimentation equilibrium.[3]

Table 3 shows the amino acid composition of human dopamine β-hydroxylase and, for comparison, the compositions of the bovine enzyme previously reported by Craine *et al.* (18), Foldes *et al.* (19), and Hörtnagl *et al.* (20). The composition of the human enzyme reported here was computed from an average of two values for all residues except threonine, serine, valine, and isoleucine. Values for half-cystine and tryptophan, reported for bovine dopamine β-hydroxylase (20), have been omitted from all calculations as they were not determined for the human enzyme. A partial specific volume of 0.72 ml/g was calculated from the amino acid composition of the human enzyme reported in Table 3.

Dopamine β-hydroxylase was isolated from a human pheochromocytoma tumor by techniques similar to those described for the bovine enzyme (21). To our knowledge this report represents the first detailed description of the purification and structural properties of the human enzyme. The final protein, which was eluted as a single, symmetrical peak from the Sephadex G-200 column, appeared to be homogeneous on disc gel electrophoresis and in the analytical ultracentrifuge. The explanation for the minor accessory bands in the sodium dodecyl sulfate gels is not obvious at the present time, but the extremely faint staining and proximity to the single major band make it attractive to hypothesize that these represent degradation products of the native protein. This hypothesis is supported by the data from the analytical ultracentrifuge, which indicate that contamination did not exceed 5%.

These analyses suggest that the human enzyme may differ from the bovine in several ways. First, the observed tendency of the native protein to aggregate in the analytical ultracentrifuge at a modest protein concentration is unique to the human enzyme. In our experience, the bovine enzyme remains as a 300,000 mol wt tetramer irrespective of enzyme protein concentration. In addition, the ultracentrifugation data suggest that the human enzyme may exist as a dimer when present at low protein concentration (within the range of concentrations detected in human plasma). Further-

[3] J. A. Reynolds and N. Kirshner, unpublished observations.

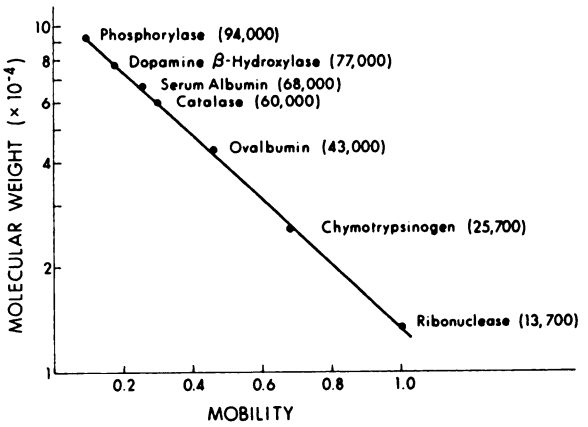

FIG. 2. *Determination of molecular weight of dopamine β-hydroxylase subunit from sodium dodecyl sulfate gels.*

The mobilities are plotted relative to ribonuclease.

TABLE 2

Subunit structure in analytical ultracentrifuge

Solvent	Mol wt	Rotor speed
		rpm
6.3 M guanidine HCl	149,000	20,000
6.3 M guanidine HCl, reduced + alkylated	74,000	20,000

more, the specific activity of the purified human enzyme (5.4 units/mg of protein) is less than the 15 units/mg of protein recently reported for the bovine enzyme (22). The lower specific activity may be due to the presence of inactive enzymes, perhaps as a result of aggregation. We have noted that storage of pure bovine dopamine β-hydroxylase for several months at −20° results in the formation of a protein precipitate and partial loss of activity.

Common properties of human dopamine β-hydroxylase and the bovine enzyme are the presence of a carbohydrate moiety, the 75,000–77,000 molecular weight of the subunit, the 149,000 molecular weight of the dimer, and the amino acid composition discussed below. The fact that the native human enzyme was eluted from a Sephadex G-200 column in the same volume as the native bovine enzyme suggests that the native human enzyme also has a molecular weight of approximately 300,000. Wallace, Krantz, and Lovenberg (22) recently reported that carbohydrate constitutes 4% of the bovine dopamine β-hydroxylase molecule. Any contribution of this portion of the molecule to enzyme localization *in vivo* and/or activity must be regarded as not yet established.

The amino acid composition of human dopamine β-hydroxylase reported in Table 3 is very similar to that reported by Hörtnagl et al. (20) for the bovine enzyme (Table 3, bovine "C"). The composition of the bovine enzyme reported by Foldes *et al.* (19), bovine "B" in Table 3, differs significantly in some residues from both the human and bovine compositions but is similar in over-all amino acid content. The amino acid composition of bovine dopamine β-hydroxylase recently reported by Craine *et al.* (18) (bovine "A," Table 3) is strikingly different from the other data presented in Table 3. The high arginine to lysine ratio, the extremely low valine and isoleucine content, and relatively high methionine content reported by those authors are at extreme variance with the other compositions reported for the bovine enzyme and the composition of human dopamine β-hydroxylase reported here. It seems unlikely that contamination by chromogranins could be responsible for these differences in amino acid compositions, as the chromogranins have a much higher

TABLE 3

Amino acid compositions of soluble dopamine β-hydroxylases

Amino acid	Human pheochromocytoma[a]	Bovine		
		A[b]	B[c]	C[d]
	moles 10^5 g protein			
Lysine	51	19	39	45
Histidine	25	20	30	23
Arginine	40	53	50	44
Aspartic acid	83	86	82	81
Threonine	58[e]	60	53	59
Serine	87[e]	62	52	76
Glutamic acid	113	123	107	105
Proline	67	66	65	56
Glycine	83	67	84	79
Alanine	62	72	75	66
Valine	58[f]	18	71	61
Methionine	13	38	0	16
Isoleucine	35[f]	11	42	35
Leucine	80	111	95	76
Tyrosine	32	35	26	35
Phenylalanine	33	46	46	39

[a] Determined as described in the text.
[b] Calculated from Craine *et al.* (18).
[c] Calculated from Foldes *et al.* (19).
[d] Calculated from Hörtnagl *et al.* (20).
[e] Corrected for destructive loses by time course analysis.
[f] Taken from 72-hr hydrolysate.

glutamate content (23) than shown in any of the compositions in Table 3.

The enzyme utilized in this study was isolated from a human tumor, and differences may exist between this protein and enzyme isolated from a normal human adrenal gland or sympathetic nerve. The close similarity in amino acid content and subunit structure of the bovine and human tumor enzymes strongly suggests a similar close relationship, if not identity, between the tumor enzyme and the enzyme present in normal tissues. Despite the similarities, the tumor and bovine enzymes differ in some physical properties and have different immunological specificities, and such differences may also exist between the enzymes isolated from a pheochromocytoma and from normal tissue.

Although acute alterations in plasma dopamine β-hydroxylase activity may not be specific for altered adrenergic function (24), quantitation of the enzyme in plasma may indeed reflect a value that is representative of sympathetic activity over a prolonged time period (7). Radioimmunoassay of dopamine β-hydroxylases from human pheochromocytoma (10) and human autopsy adrenal glands (9) appears to show excellent correlation with assays that measure enzyme activity. However, immunological assays of plasma dopamine β-hydroxylase, which utilize antisera raised to antigen from heterologous species (cow and sheep), correlate poorly with enzyme activity in human sera (9). The different molecular properties of the human enzyme mentioned above might at least partially explain the observed poor immunological cross-reactivity with antisera to dopamine β-hydroxylases from other species.

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