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STRUCTURAL ANALYSIS OF THE SOLUBLE FORM OF BOVINE DOPAMINE BETA-HYDROXYLASE

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ABSTRACT: *Dopamine beta-hydroxylase (DBH) is a neurotransmitter biosynthetic enzyme which catalyzes the conversion of dopamine to norepinephrine. Although structural studies of the mature form of this enzyme have been extensive, the culmination of these finding had been hightly controversial and contradictory. In this study, biochemical approaches were taken to characterize the structure of mature DBH. Soluble bovine DBH was purified from aderenal medulla. Three bands of 69 kDa, 72 kDa and 75 kDa which were physically separable and similar in structure were observed by SDS-PAGE. Furthermore, gas phase sequence analysis revealed that the 72 kDa band consists of two polypeptides which are present at equimolar concentrations and differed in that one had three extra amino acids at the N-terminus. Taken together, the soluble form of DBH exist in at least four forms, three identified by SDS-PAGE, one of which consists of two polypeptides as identified by N-terminal sequence analysis. The significance of these forms and their possible biosynthetic mechanisms are discussed.*

Keywords: *Dopamine beta-hydroxylase, norepinephrine, chromaffin granules*

INTRODUCTION

The enzyme dopamin beta-hydroxylase (DBH; 3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (beta-hydroxylating), EC 1.14.17.1) catalyzes the conversion of dopamine to norepinephrine, the third step in the catecholamine biosynthetic pathway (Friedman and Kaufman, 1965). The enzyme is present in the catecholamine-containing vesicles of noradrenergic and adrenergic neurons as well as adrenal medullary cells (Laduron and Belpaire, 1968). In the vesicle, DBH exists as both membrane-bound and soluble forms, the latter form being released by exocytosis upon stimulation of the cell (Viveros *et al.*, 1968; Weinshilboum *et al.*, 1971). DBH is one of

copper-containing mixed function monooxygenases, whose structural and mechanistic characteristics have not been fully elucidated and thus this enzyme has been considered to be an ideal model for such class of enzymes.

Because of its role in catecholamine biosynthesis as well as its potential to be a model with which protein targetting to secretory granules and the characteristics of copper-containing mixed function monooxygenases can be studied, the structure of mature native DBH has been the interest of many investigators. Although the first purification of DBH dates back to 1965 (Friedman and Kaufman) and a considerable amount of structural studies on DBH had been done since, the structure of DBH had not been well-delineated prior to the initiation of the present study. This stemmed mainly from the structural complexity of the protein as well as the apparent species-specific differences which have made interpretation of data difficult. Recently the primary structure of bovine DBH has become available from the cDNA sequence data (Taljanidsz *et al.*, 1989; Lewis *et al.*, 1990) as well as the protein sequence data (Robertson *et al.*, 1990). The present report discusses the relationship between the primary structure and the structure of mature DBH and the possible posttranslational processing mechanisms which would produce the apparent structural complexity of the mature protein.

PURIFICATION OF DBH

As a first step in characterization of DBH, the soluble form was purified to apparent homogeneity from fresh bovine adrenal medulla. Briefly, chromaffin granules, which are the catecholamine storage vesicles in the adrenal medulla, were first isolated by obtaining the P_2 fraction in isotonic solution (0.32M sucrose) and then taking the preparation through 1.6M sucrose. All solutions contained 25 μg/ml PMSF (phenylmethylsulfonyl fluoride) to inhibit degradation by proteases. The soluble fraction of the vesicle was obtained by lysing the chromaffin granules in hypotonic solution and subsequently taking the supernatant from high speed centrifugation. DBH was purified by subjecting the preparation through ammonium sulfate fractionation, DEAE column chromatography (Fig. 1) and finally Biogel P200 gel filtration (Fig. 2). The summary of a typical purification is shown in Table 1. From 400 g of starting tissue, approximately 5 mg of highly purified enzyme was obtained. This preparation was demonstrated to be apparently homogeneous as determined by native polyacrylamide gel electrophoresis followed by silver nitrate staining (Fig. 3). With both 3 and 6 hour electrophoresis, only one band of protein was apparent and this band corresponded with DBH activity. Typical of glycoproteins, the band was broad, presumably due to charge heterogeneity produced by the carbohydrate moieties.

MOLECULAR WEIGHT OF DBH

Molecular weight of DBH had been reported to be in the range of 290 kDa to 300 kDa as determined by sedimentation equilibrium analysis (Hogue-Angeletti, 1977) and approximately 75 kDa as demonstrated by SDS-PAGE (Craine *et al.*, 1981), sug-

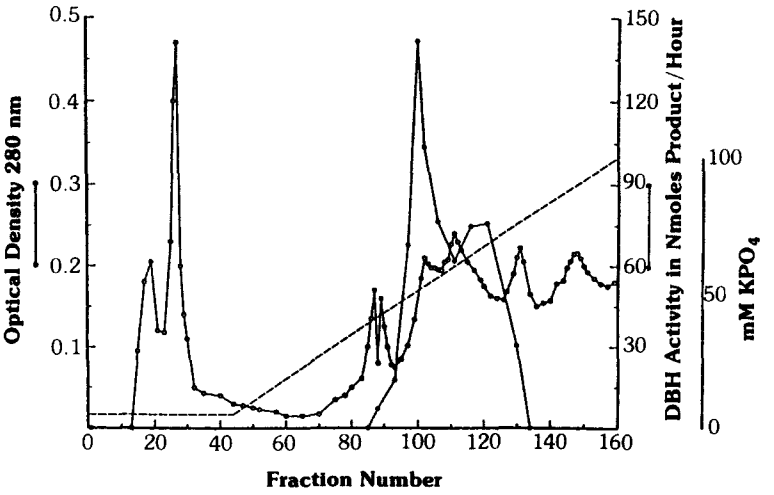

Fig. 1. DEAE-celluose column chromatography of soluble bovine DBH.

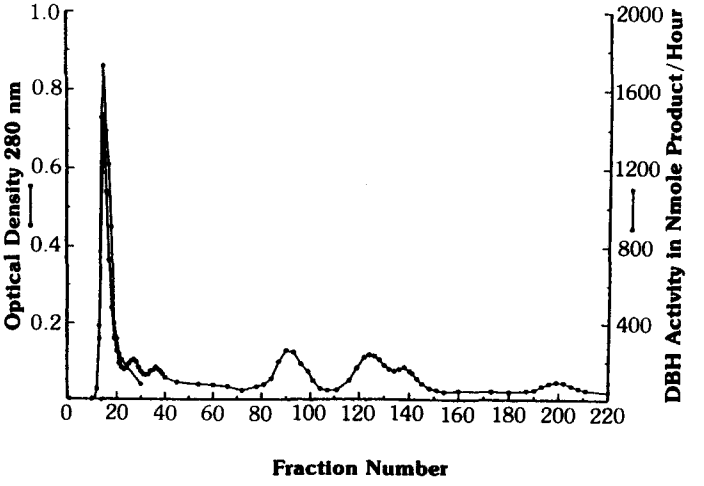

Fig. 2. Biogel P-200 column chromatography of soluble bovine DBH.

gesting the enzyme may be a tetramer. Previously, the tetramer was believed to be composed of apparently identical subunits (Ocuno and Fujisawa, 1984; Long *et al.*, 1981) although that this may not be the case was also suggested (Saxena *et al.*, 1985; Duong *et al.*, 1985). In order to clarify this contradicting results, the purified DBH preparation was subjected to SDS-polyacrylamide gel electrophoresis for determination of its molecular weight. A diffuse triplet of 75, 72, and 69 kDa bands were observed (Fig. 4) with the 72 kDa band present in the highest concentration. Similar results

Table 1. Purification of dopamine b-hydroxylase from bovine adrenal medulla[a]

Purification Step	Total Protein (mgs)	Total Activity (units)[b]	Spec. Activity (units/mg)	Purification (fold)	Yield
Homogenate[c]	110,000	,00,000	4	—	100%
Chromaffin Granules	5,400	250,000	47	12	62%
Granule Lystate (100,000 × g)	2,500	140,000	56	14	35%
Ammonium Surfate (25-50%)	360	64,800	180	45	14%
DEAE-Cellulose	25	32,500	1300	325	8.1%
Biogel P200	5	12,500	2500	625	3.1%

[a]Starting material was 400 grams bovine adrenal medulla.
[b]One unit of activity corresponds to one nmole synephrine formed per hour at 37°C.
[c]Refers to the pellet from 16,000 × g centifugation.

Fig. 3. Native PAGE of purified bovine DBH illustrating the purity of the preparation. Both 3 hour and 6 hour electrophoresis show a single protein band.

had been reported by Speedie *et al.*, (1985). However, since glycoproteins, such as DBH, electrophorese as a diffuse band, it was still necessary to ascertain that they are unequivocally distinct proteins and not a diffuse band of glycoproteins. For this, preparative SDS-PAGE was performed and the protein bands visualized by sodium acetate shadowing technique (Higgins and Dahmus, 1979), and the bands cut out separately followed by electroelution and precipitation. When each preparation was then subjected to SDS-PAGE, the 75, 72 and 69 kDa bands were resolved without contamination (Fig. 5) demonstrating that these DBH-associated bands are physically separable and that they are distinct.

To confirm that all three bands represented DBH and not contaminating proteins, peptide mapping analysis was performed. As shown in Fig. 6, the three bands yielded peptide patterns very similar to each other with both V8 protease and chymotrypsin proteolysis, indicating that the three bands are structurally similar and that they represent different forms of DBH. Thus we unequivocally demonstrated that the soluble form of DBH exist in multiple forms.

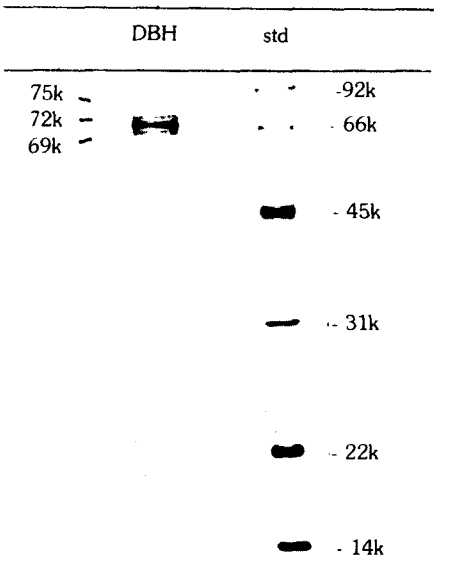

Fig. 4. SDS-PAGE of purified DBH stained with silver nitrate. Lane 1: DBH, separating into three bands: 75, 72 and 69 kDa; lane 3; molecular weight standards.

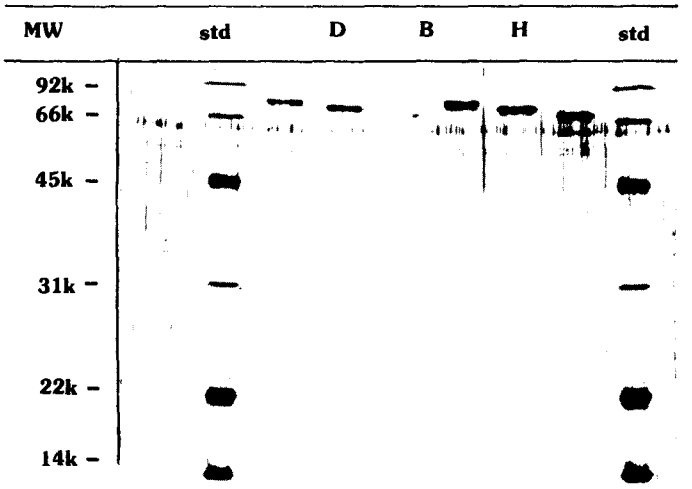

Fig. 5. SDS-PAGE of the excised DBH protein band stained with silver nitrate. Samples were loaded in two concentrations. Lanes 2 and 5: 75 kDa; lanes 3 and 6: 72 kDa; lanes 4 and 7: 69 kDa; lanes 1 and 8: molecular weight standards.

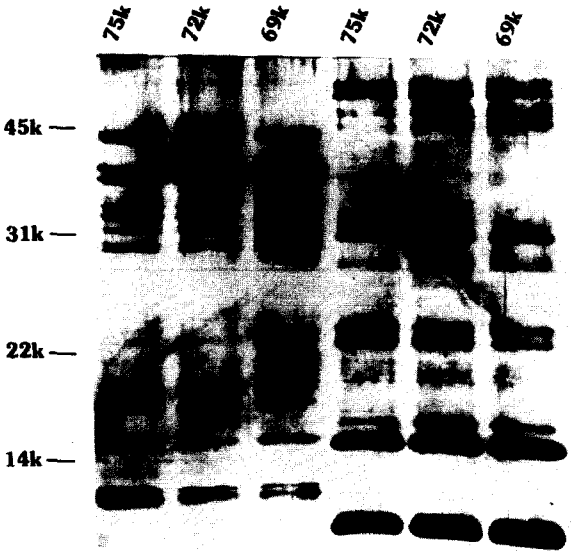

Fig. 6. Comparative peptide mapping analysis of the excised DBH protein bands. Lanes 1 through 3: proteolysis by V8 protease; lanes 4 through 6: proteolysis by chymotrypsin.

N-TERMINAL SEQUENCES OF DBH

For further characterization of the different DBH proteins, the preparations were subjected to gas phase sequence analysis. While the 75 and 69 kDa proteins were too low in concentration to obtain reliable sequence data, the 72 kDa protein, surprisingly, consisted of two polypeptides presenting in equimolar concentrations (Fig. 7). The two polypeptides were identical in the regions sequenced except that one had three extra amino acids at its N-terminus. The difference in size by 3 amino acids would of course not have been detected or resolved by 10% SDS-PAGE. These data were in disagreement with the report by Hogue-Angeletti *et al.* (1977) that DBH is blocked at its N-terminus, and confirmed the data of Skotland *et al.* (1977) which had awaited confirmation due to the technical limitations at the time of their experiments. With a few modifications and combination, we obtained the definitive N-terminal sequences of the soluble DBH. These data were later confirmed by protein sequence analyses (Taylor *et al.*, 1989; Robertson *et al.*, 1990) as well as the deduced sequence from cDNA sequence (Taljanidisz *et al.*, 1989; Lewis *et al.*, 1990).

Thus, contrary to the previous reports that soluble bovine DBH consisted of four identical subunits (Skotland *et al.*, 1977; Wallace *et al.*, 1973), the present data demonstrated that the structure of DBH was highly complex. In addition to existing as previously defined soluble and membrane bound forms, the soluble form is now found

to consist of 4 different structurs. Three bands are separated by SDS-PAGE, 75, 72, and 69 kDa. Furthermore, the 72 kDa band consists of two different polypeptide chains. The fact that they were present at equimolar concentrations and that serine protease inhibitor PMSF was used throughout the purification process lessens the possibility that they were produced by non-specific proteolysis during purification.

Ser-Ala-Pro-Ala-Glu-Ser-Pro-Phe-Pro-Phe-His-Ile-Pro-Leu-Asp-

Ala-Glu-Ser-Pro-Phe-Pro-Phe-His-Ile-P ro-Leu-Asp-

Fig. 7. N-terminal sequences of the 72 kDa protein of soluble bovine DBH.

DISCUSSION

The present data demonstrated that in contrast to most of the previous report that DBH tetramer consists of identical subunits, soluble DBH exists in multiple forms. Three forms of 75, 72, and 69 kDa were identified on SDS-polyacrylamide gel with the 72 kDa band existing in the highest concentration. In addition, the 72 kDa band itself consisted of two polypeptides that were slightly different at their N-termini. Since DBH is glycosylated, some degree of its structural complexity might derive from glycosylation. We have biochemical evidence (not shown) that the three different molecular weight forms of DBH originate from differential glycosylation. Which one(s) of the four potential glycosylation sites identified on DBH primary structure are actually glycosylated in the native tetramer of DBH is not known. On the other hand, the observed variation in N-terminal sequences also suggested a possiblity that additional complexity might originate from the primary structure.

It is likely, then, that DBH is composed of two types of polypeptide chains which are identical except for the N-termini. That the Ser- and Ala-polypeptide chains are present in equal concentrations suggest that mature DBH is a heterotetramer consisting of two Ser-polypeptide chains and two Ala-polypeptide chains. The alternative, of course, is that DBH is composed of two distinct tetrameric forms: a Ser-polypeptide and a Ala-polypeptide homotetramer. This seems unlikely since the equimolar concentration of the polypeptides would require equal amount of each tetramer.

The two polypeptides chains of DBH may be generated by differential posttranslational proteolytic processing of a common precursor by two distinct mRNA's. The fact that DBH cDNA's isolated so far do not exhibit variations in their coding regions and that all cDNA's include both Ser-form and Ala-form in their sequences strongly suggest that the former is the case. Being a vesicular protein, the primary structure of DBH contains an N-terminal signal sequence that is presumed to be responsible for targetting of DBH to the catecholamine storage vesicles (Taljanidisz *et al.*, 1989; Lewis

et al., 1990). In addition, a good consensus site for signal peptidase (Von Heijne, 1984) is present, the cleavage at which site will produce one of the presently determined N-terminal amino acid sequences (Ser-Ala-Pro-Ala-). The second polypeptide chain which starts three amino acid residues behind (Ala-Glu-Ser-Pro-) is also found. Because of the presence of proline residue at the -1 position, however, this is not a cleavage site of a knwon peptidase. It is possible that this cleavage is carried out by a peptidase yet to be identified and further analysis is necessary to test this hypothesis.

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