Mechanism of Biosynthesis of Soluble and Membrane-bound Forms of Dopamine β-Hydroxylase in PC12 Pheochromocytoma Cells*

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Dopamine β-hydroxylase was present as 2 subunit forms (apparent \( M_r = 77,000 \) and 73,000) in the PC12 pheochromocytoma cell line as detected by immunoprecipitation from \([35S]methylone-labeled cultures, and analyzed by sodium dodecyl sulfate gel electrophoresis and fluorography. The \( M_r = 77,000 \) form was present in a crude membrane fraction, while the \( M_r = 73,000 \) form was soluble. Both forms appeared to be present in approximately equal amounts, and both were glycosylated. Treatment of PC12 cells with tunicamycin, a potent inhibitor of core glycosylation in the endoplasmic reticulum, completely inhibited the appearance of the \( M_r = 77,000 \) and \( M_r = 73,000 \) forms, and 2 new immunoreactive polypeptides were obtained (apparent \( M_r = 67,000 \) and 63,000).

Pulse-chase experiments suggested that the \( M_r = 77,000 \) form is initially synthesized (by 5 min) and a portion is converted in 15–90 min to the \( M_r = 73,000 \) form. Thereafter, the ratio between forms remains relatively constant, at least for several hours. Translation of mRNA from bovine and rat adrenals, and immunoprecipitation, indicated that dopamine β-hydroxylase is initially synthesized as a single polypeptide (apparent \( M_r = 67,000 \)). The subcellular site of biosynthesis of dopamine β-hydroxylase was determined by isolation of mRNA from free and membrane-bound polysomes from bovine adrenal medulla. Translation in a cell free system and immunoprecipitation localized the synthesis of dopamine β-hydroxylase on membrane-bound polysomes.

These experiments suggest that both soluble and membrane-bound forms of dopamine β-hydroxylase are synthesized and core glycosylated in the endoplasmic reticulum, and that there probably is a precursor-product relationship between the \( M_r = 77,000 \) and the \( M_r = 73,000 \) subunit forms of dopamine β-hydroxylase.

Dopamine β-hydroxylase (EC 1.14.17.1) is the enzyme which catalyzes the formation of norepinephrine from dopamine (Kaufman and Friedland, 1965) and consequently it is the marker enzyme for the noradrenergic neuronal system (Geffen et al., 1969; Goldstein et al., 1972). In nerve terminals and in adrenal chromaffin cells, dopamine β-hydroxylase is present in both the membrane and soluble content of the noradrenergic vesicles or chromaffin granules, respectively (Smith and Kirshner, 1967; Lagercrantz, 1976; Winkler, 1976). The soluble form of dopamine β-hydroxylase can be secreted with the catecholamines (De Potter et al., 1969; Weinshilboum et al., 1971; Viveros et al., 1968). The soluble and membrane forms in the adrenal have been found to be similar immunochromatically (Slater et al., 1981) and are reported to consist of four glycosylated subunits each with molecular weight of about 75,000 (Park et al., 1976; Fong et al., 1980; Rush and Geffen, 1980).

Differences in the soluble and bound forms of dopamine β-hydroxylase do appear, however, to exist. For instance, subtle differences in these forms were detected through peptide mapping (Slater et al., 1981). Charge-shift crossed immunoelectrophoresis has differentiated between an amphiphilic membrane-bound and a more hydrophilic soluble form (Bjerum et al., 1979). These results have suggested that a small hydrophobic tail may anchor the enzyme in the membrane, and have raised the possibility of a biosynthetic relationship between the two forms. Recently, immature adrenomedullary vesicles in microsomal and Golgi fractions were shown to contain a high proportion of the amphiphilic form of dopamine β-hydroxylase (Helle and Serck-Hanssen, 1981). Pulse-chase studies with perfused adrenals suggested that the kinetics of incorporation of soluble and membrane forms of dopamine β-hydroxylase into vesicles were similar, but different from that of newly synthesized chromogranin (Ledbetter et al., 1978). On the other hand, Winkler et al. (1972) showed in labeling experiments with radioactive amino acids that the membrane proteins of adrenal-medullary granules were labeled considerably later than proteins in the soluble content. The membrane and releasable form of dopamine β-hydroxylase appeared to turn over at different rates, and it was suggested that the two forms are synthesized as different molecules and that there is no appreciable exchange between the membrane-bound and soluble pools of dopamine β-hydroxylase (Gagnon et al., 1976; Winkler, 1977).

We have utilized the PC12 pheochromocytoma cell line to investigate the mechanisms of biosynthesis of the soluble and membrane-bound forms of dopamine β-hydroxylase. Established from a transplantable rat pheochromocytoma, PC12 cells proliferate in serum-containing medium and possess the differentiated properties of chromaffin cells, including the presence of chromaffin granules, and the synthesis, storage, and release of dopamine and noradrenaline (Greene and Tischler, 1976, 1982; Greene and Rein, 1977). These cells have the advantage of providing large amounts of homogeneous material for biochemical analysis.

In this paper, we report that in PC12 cells, subunits of dopamine β-hydroxylase isolated by immunoprecipitation, are present in soluble and membrane forms (apparent \( M_r = 73,000 \)
and 77,000, respectively). Both subunit forms are glycosylated by the tunicamycin-sensitive pathway. Evidence is presented to support a precursor-product relationship between these forms.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following materials were purchased from commercial sources: \(^{35}\)Smethionine (900 Ci/ml), carrier-free \(^{32}\)Porthophosphate, I, 9, 6-H\(^2\)fluoroscein (56 Ci/mmol), and liquifor (New England Nuclear); d: 1, 6-H\(^2\)mannose (45.9 Ci/mmol) and d: 6-H\(^2\)glucose (22.6 Ci/mmol) (Amersham); \(^{14}\)C-labeled molecular weight markers (Bethesda Research Laboratories); bovine liver catalase (17,000 units/mg), and pargyline-HCl (N-Methyl-N-Benzyl-2-propynyl-N-methylamine) (Sigma); protein A-Sepharose (Pharmacia); Tris (Moby Chemicals, New York, NY); tunicamycin (Calbiochem); Triton X-100 (Eastman); RPMI 1640, methionine-free RPMI 1640 (select amine kit), dialyzed horse serum, and dialyzed fetal calf serum (for radioactive labeling) (Gibco); horse serum and fetal calf serum (for characterization of guinea pig antiserum to dopamine \(\beta\)-hydroxylase in the adrenal is known to exist in

**Preparation of mRNA**—The RNA from rat adrenals or bovine adrenal medulla was prepared according to Liu et al. (1979). Free and membrane-bound polysomes were prepared from bovine adrenal medulla according to the procedure developed by Ramsey and Steele (1976, 1977, 1979) for rat liver polysomes. The RNA in the polysome pellet was extracted with phenol, precipitated with ethanol and processed for immunoprecipitation as described above. Homologous antiserum (guinea pig antiserum to dopamine \(\beta\)-hydroxylase) or goat antiserum (bovine dopamine \(\beta\)-hydroxylase) was used with translations of rat or bovine mRNA, respectively.

**RESULTS**

**Subunit Forms of Dopamine \(\beta\)-Hydroxylase in PC12 Cells**—In order to characterize the subunit forms of dopamine \(\beta\)-hydroxylase, PC12 cells were labeled with \(^{35}\)Smethionine for several hours. The cell lysate was immunoprecipitated with antibodies prepared against rat dopamine \(\beta\)-hydroxylase, the immunoprecipitates were analyzed on 6–12% linear gradient polyacrylamide slab gels in SDS, and the radioactive proteins were detected by fluorography. Two specific subunit forms of immunoprecipitated dopamine \(\beta\)-hydroxylase were consistently detected with apparent molecular weights of 73,000 and 77,000 (Fig. 1). These forms are present in approximately equal amounts as indicated by their approximately equal intensity of \(^{35}\)Smethionine label. When cells were labeled for longer periods of time (24 h), these two bands were still present although they were somewhat more diffuse.

The presence of ascorbic acid, which has been found to stabilize dopamine \(\beta\)-hydroxylase against proteolysis in vitro (Wong et al., 1981), had no effect on the distribution of the subunit forms of dopamine \(\beta\)-hydroxylase. Thus, when 1 and 5 mM ascorbic acid was freshly added to PC12 cells during a 2-h preincubiation and a 2-h labeling period, the two forms were still present in near equal amounts.

**Subunit Forms in Soluble and Membrane Fractions**—Dopamine \(\beta\)-hydroxylase in the adrenal is known to exist in membrane and soluble fractions of chromaffin granules. We carried out a crude subcellular fractionation of \(^{35}\)Smethionine-labeled PC12 cells to find the localization of the subunit forms of dopamine \(\beta\)-hydroxylase. Cells were lysed by brief sonication in hypotonic solution in the presence of protease inhibitors. A crude membrane fraction was prepared. The soluble and membrane fractions were brought to 2% sodium}

\(^{1}\) The abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.
storage sites were lysed. Cholamines indicated that the norepinephrine and dopamine were soluble and hence, that under these conditions, the membrane form was greatly enriched in the soluble fraction (Fig. 2). Analysis of the fractions for catecholamines indicated that the norepinephrine and dopamine were soluble and hence, that under these conditions, the storage sites were lysed.

The assay of dopamine β-hydroxylase activity was carried out on the membrane and soluble fractions (Table I). Dopamine β-hydroxylase activity was recovered in both fractions, supporting the view that both subunit forms are indeed active dopamine β-hydroxylase. However, under the conditions used, the membrane form has an almost 40-fold higher specific activity when expressed per mg of total protein. Since the similar labeling of the 73,000- and 77,000-M₄ subunit forms with [³⁵S]methionine suggests that they are present in near equal molar amounts, and since both forms contained similar amounts of protein (Table I), the variation in activity may indicate that the 77,000-M₄ membrane form is intrinsically more active. Alternatively, it may be less susceptible to inactivation during the fractionation.

Since phosphorylation is known to modulate the activity of many enzymes including tyrosine hydroxylase (Raese et al., 1977; Latendre et al., 1977; Joh et al., 1978; Markey et al., 1980; Yamauchi and Fujisawa, 1979; Lazar et al., 1982), we examined whether the two subunit forms might be differentially phosphorylated. PC12 cells were labeled for 2 h with [³²P]orthophosphate, and dopamine β-hydroxylase was immunoprecipitated from the resulting cell homogenates and resolved by SDS-polyacrylamide gel electrophoresis. Radiograph of the gels revealed no detectable phosphorylation of either of the two subunit forms of dopamine β-hydroxylase, even though phosphorylation of many other proteins was readily detectable.

Glycosylation of Dopamine β-Hydroxylase—Dopamine β-hydroxylase is known to be a glycoprotein (Wallace et al., 1973; Ljones et al., 1976; Geissler et al., 1977). It has recently been shown that soluble and membrane-bound dopamine β-hydroxylase in bovine adrenal chromaffin granules have an indistinguishable sugar composition (Fischer-Colbrie et al., 1982). Thus, the PC12 cells were labeled with radioactive dodecyl sulfate and processed for immunoprecipitation with anti-dopamine β-hydroxylase antiserum. The 77,000-M₄ subunit form was greatly enriched in the membrane fraction, while the 73,000-M₄ subunit form was greatly enriched in the soluble fraction (Fig. 2). Analysis of the fractions for catecholamines indicated that the norepinephrine and dopamine were soluble and hence, that under these conditions, the storage sites were lysed.
Mechanism of Biosynthesis of Dopamine β-Hydroxylase

The mechanism of biosynthesis of dopamine β-hydroxylase was studied, particularly focusing on the role of glycosylation. 

**FIG. 3. [3H]mannose-labeling of dopamine β-hydroxylase.** The fluorograph of an SDS gel shows the total [3H]mannose-labeled protein profile obtained from cells treated in the absence (A and C) and presence of 15 μg/ml of tunicamycin (B). The cells were treated with D-[2,6-3H]mannose for 4 h (33 μCi/ml) (A and B) or for 24 h (200 μCi/ml) (C). The former (cells shown in A and B corresponding to 80,000 and 25,000 cpm of trichloroacetic acid-precipitable material respectively) were immunoprecipitated with antibodies to dopamine β-hydroxylase (D and E) and the entire immunoprecipitates were compared to immunoprecipitates from [35S]Met-labeled cells (F). Arrows indicate positions of the two dopamine β-hydroxylase bands.

After treatment for 4 h with [3H]mannose, both subunit forms of dopamine β-hydroxylase were labeled, suggesting that both are glycosylated (Fig. 3). In longer labeling (several days), the immunoprecipitated material was extremely diffuse with electrophoretic mobility corresponding to Mr = 73,000–77,000. Labeling with [3H]fucose also revealed two subunit forms which incorporate similar amounts of [3H]fucose (not shown).

To further delineate the glycosylation of the multiple forms of dopamine β-hydroxylase, PC12 cells were treated with tunicamycin. The latter drug blocks glycosylation by interfering with the formation of dolichol-bound N-acetyl glucosamine derivatives (Struck and Lennarz, 1977; Tkacz and Lampen, 1975). Tunicamycin (6 μg/ml) inhibited incorporation of [3H]glucosamine into protein in PC12 cells by over 80%, while reducing protein synthesis by only 13%, as monitored by incorporation of [35S]methionine into protein. As shown in Fig. 4, the synthesis of the 73,000- and 77,000-Mr subunit forms was almost completely inhibited in the tunicamycin-treated cultures and two new specific immunoreactive polypeptides (apparent Mr = 63,000 and 67,000) were obtained. It should be noted that, while similar numbers of trichloroacetic acid-precipitable counts were used for each immunoprecipitation shown in Fig. 4, the immunoreactive forms in the tunicamycin-treated cells were considerably reduced, suggesting that the nonglycosylated forms may be more rapidly degraded. Quantification of densitometer scans of the results, shown in Fig. 4, showed that in tunicamycin-treated cells the relative amount of immunoreactive dopamine β-hydroxylase in the two new specific polypeptides is 16% of the original forms. These results also showed that the antibodies recognized the nonglycosylated form of dopamine β-hydroxylase. Addition of up to a 5-fold concentration of antiserum did not increase the amount of the Mr = 67,000 and 63,000 polypep-
The cells were incubated further for 0 min (A), 15 min (B), 30 min (C), 45 min (D), 90 min (E), 4 h (F), or 24 h (G). The immunoprecipitated bands are compared to the immunoprecipitates from a 4-h continuous labeling obtained in a separate experiment (H).

**Fig. 5.** Pulse-chase labeling of PC12 cells. The fluorographs of gel electrophoresis of immunoprecipitates of dopamine β-hydroxylase from PC12 cells is shown. Sister cultures were preincubated for 30 min in methionine-free media, treated with 250 μCi of [35S]Met for 5 min, followed by addition of 150 μg/ml of unlabeled methionine. The cells were incubated further for 0 min (A), 15 min (B), 30 min (C), 45 min (D), 90 min (E), 4 h (F), or 24 h (G). The immunoprecipitated bands are compared to the immunoprecipitated from a 4-h continuous labeling obtained in a separate experiment (H).

**Fig. 6.** Site of synthesis of dopamine β-hydroxylase on membrane-bound polysomes. The translation products in a wheat germ system obtained with mRNA from total bovine adrenal medulla (T) and from free (F) and membrane-bound polysomes (B) are shown. The translation products of mRNA from free (F) and bound (B) polysomes, corresponding to 2 × 10⁶ cpm of trichloroacetic acid-precipitable [35S]Met-labeled protein, were immunoprecipitated with antibodies to dopamine β-hydroxylase and analyzed by gel electrophoresis and fluorography.

**DISCUSSION**

**Subunit Forms of Dopamine β-Hydroxylase**—The PC12 cells have been shown to synthesize two subunit forms of dopamine β-hydroxylase with apparent Mr = 73,000 and 77,000. These forms were separated electrophoretically on gradient polyacrylamide-SDS slab gels of immunoprecipitated...
Mechanism of Biosynthesis of Dopamine β-Hydroxylase

Site of Synthesis of Dopamine β-Hydroxylase—We have shown directly that dopamine β-hydroxylase is synthesized exclusively on membrane-bound polysomes of bovine adrenals. While parallel experiments could not be carried out with PC12 cells, it appears likely that a similar mode of synthesis pertains in this system as well. Such a mechanism is consistent with the site of synthesis of a protein destined for secretion (as occurs in a variety of systems) and of most plasma membrane proteins (Palade, 1975; Blobel, 1978; Sabatini and Kreibich, 1976; Sabatini et al., 1982). Thus, dopamine β-hydroxylase would be expected to contain an NH₂-terminal signal sequence which directs vectorial discharge into the endoplasmic reticulum and which is subsequently removed (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975). However, surprisingly the electrophoretic mobility of the translation products is identical to one of the forms in tunicamycin-treated cells. Perhaps, dopamine β-hydroxylase may contain an internal insertion signal similar to ovalbumin (Lingappa et al., 1979) or the major transmembrane erythrocyte glycoprotein, band 3 (Sabbatini et al., 1981; Braess and Lodish, 1982).

The mechanism by which dopamine β-hydroxylase is inserted into membranes should be investigated further.

The exclusive localization of mRNA for dopamine β-hydroxylase on membrane-bound polysomes would indicate, as previously suggested (Gagnon et al., 1976), that the portion of the dopamine β-hydroxylase which is detected in the supernatant during subcellular fractionation is due to leakage from vesicles during the preparation and that dopamine β-hydroxylase is not present in a free form in the cytosol.

There appears to be only one translation product for dopamine β-hydroxylase, with apparent M₁ = 67,000. This value may seem somewhat low since subunits of bovine dopamine β-hydroxylase, with electrophoretic mobility corresponding to apparent molecular weight of 73,000-75,000, is reported to contain about 5% carbohydrate (Wallace et al., 1973; Geissler et al., 1977; Fischer-Colbrie et al., 1982). However, it is not unusual for glycoproteins, and particularly sialoglycoproteins to have somewhat anomalous electrophoretic mobilities.

It should be noted that immunoprecipitation from the translation with anti-dopamine β-hydroxylase antiserum also identifies a polypeptide identical to newly synthesized phenylethanolamine-N-methyltransferase (apparent M₁ = 32,000). Moreover, the polypeptide is much more prominent in immunoprecipitates from free polysomes, and thus, is unlikely to represent a degradation product of dopamine β-hydroxylase. These results are interesting in light of recent suggestions by Joh and co-workers of possible similarities in domains between the enzymes involved in the synthesis of catecholamines (Joh et al., 1981).

The results presented here, as well as those by Helle and Serck-Hansen (1981), suggest post-translational processing of dopamine β-hydroxylase from the membrane to soluble form. We cannot, however, rule out the possibility of formation of the 73,000-M₁ and 77,000-M₁ forms at different rates from a higher molecular weight precursor which is unrecognized by antiserum to native dopamine β-hydroxylase. However, the processing of the subunit form to the 73,000-M₁ form seems the more likely interpretation of the data. This event occurs relatively quickly (within about 15-90 min), and it appears from the pulse-chase data that, if processing does not occur initially, the distribution of the two forms remains relatively intact. Glycosylation does not appear to be necessary for this process, since in tunicamycin-treated cells, two subunit forms (albeit of lower apparent molecular weight) are obtained. These results indicate that the nonglycosylated forms may be less stable since they are reduced by about 6-fold in the tunicamycin-treated cells. Indeed, it has been
suggested for a number of proteins that the function of the carbohydrate moiety is to prevent degradation, and there is evidence that the nonglycosylated forms of several proteins, such as fibronectin or the acetylcholine receptor, are more susceptible to proteolysis (Olden et al., 1982).

In summation, the experiments presented here lead to the following model for the biosynthesis of dopamine β-hydroxylase. The enzyme is synthesized in the endoplasmic reticulum on membrane-bound polyribosomes (M, ~67,000) and rapidly glycosylated to a 77,000–M form. This membrane-bound form can be processed to a 73,000-M soluble form relatively rapidly within 15–90 min. If not converted then, the distribution remains relatively constant, at least for several hours.

These findings on the mechanism of biosynthesis of dopamine β-hydroxylase should be helpful in designing further experiments on the biogenesis of chromaffin granules and neuronal vesicles. In particular, it would be of considerable interest to elucidate the factors which regulate whether do-

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Additions and Corrections

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Page 7818, left column, the last line of text was omitted during printing. The last sentence should read:

In particular, it would be of considerable interest to elucidate the factors which regulate whether dopamine β-hydroxylase will be soluble or membrane-bound.

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