Biochem. J. (1979) **181**, 231–237
Printed in Great Britain

Immunochemically Identical Hydrophilic and Amphiphilic Forms of the Bovine Adrenomedullary Dopamine β-Hydroxylase

By Ole J. BJERRUM*, Karen B. HELLE† and Elisabeth BOCK*
**The Protein Laboratory, University of Copenhagen, Sigurdsgade 34, DK-2200 Copenhagen N, Denmark, and †Institute of Physiology PKI, University of Bergen, Årstadvei 19, N-5000 Bergen, Norway*

(*Received* 28 *December* 1978)

By means of a monospecific antibody, dopamine β-hydroxylase was monitored immunoelectrophoretically in various extracts of chromaffin granules. Approximately one-third of the dopamine β-hydroxylase present was located in the membrane fraction and could only be liberated with detergent. The dopamine β-hydroxylases of the buffer and membrane fractions were antigenically identical, but differed in their amphiphilicity, as demonstrated by the change in precipitation patterns on removal of Triton X-100 from the gel, on charge-shift crossed immunoelectrophoresis and on crossed hydrophobic interaction immunoelectrophoresis with phenyl-Sepharose. Furthermore, immunoelectrophoretic analysis in the presence of Triton X-100 plus the cationic detergent cetyltrimethylammonium bromide indicates additional heterogeneity of the membrane-bound dopamine β-hydroxylase. By limited proteolysis with chymotrypsin and thermolysin the amphiphilic form could be converted into its hydrophilic counterpart.

Chromaffin granules, the catecholamine-storage granules of the adrenal medulla, contain, in addition to the low-molecular-weight hormones, a number of specific proteins and enzyme activities (Helle & Serck-Hanssen, 1975; Winkler, 1976). Among the latter, the noradrenaline-forming enzyme, dopamine β-hydroxylase (EC 1.14.17.1) is unique in several respects; it is a constituent of both the aqueous phase of the core and the membrane fraction of the granule (Kaufmann & Friedmann, 1965; Viveros *et al.*, 1968; Helle, 1971; Hørtnagl *et al.*, 1972). When purified from these two sources there appears to be no difference in enzymic and immunological properties between the two forms (Aunis *et al.*, 1975; Ljones *et al.*, 1976; Hogue-Angeletti, 1977; Helle *et al.*, 1977, 1978). Nonetheless, the soluble enzyme has been obtained in a trypsin-resistant form, lower in hydrophobic amino acid residues than most purified preparations of this enzyme (Helle *et al.*, 1977), and a dissociation of the two forms has been seen during crossed immunoelectrophoresis of the Triton-solubilized chromaffin granules (Helle *et al.*, 1978). Differences in apolar regions in the enzyme may distinguish the membrane form from its soluble counterpart. When solubilized from the granule membrane the enzyme seems to be associated with a lipid moiety that cannot be dispersed from the protein and that affects activation energy and the enzyme kinetics (Aunis *et al.*, 1977). As a difference in rate of synthesis of the membrane and soluble form of β-hydroxylase has also been obtained (Winkler, 1977), these findings can be explained best if one postulates that the membrane and soluble forms of dopamine β-hydroxylase differ with respect to a hydrophobic 'tail' that anchors the membranous form in the lipid bilayer of the granule membrane. Such a hydrophobic 'tail' would not be expected to contribute to the antigenicity of this carbohydrate-containing enzyme (Bjerrum, 1977) and would thus account for the immunological identity repeatedly observed for the two forms (Helle, 1971; Helle & Serck-Hanssen, 1975; Winkler, 1976; Helle *et al.*, 1978). Furthermore, a common synthetic pathway could still exist for the two forms, if one serves as a precursor for the other and is converted, e.g. by specific proteolysis, into the secreted form, as has been suggested as a common scheme for synthesis and maturation of zymogens (Blobel & Dobberstein, 1975).

A number of electrophoretic techniques have been developed to characterize membrane proteins with respect to their amphiphilic properties (Tanford & Reynolds, 1976; Helenius & Simons, 1977; Bhakdi *et al.*, 1977; Bjerrum & Bhakdi, 1977; Bjerrum, 1978). Two of these methods, charge-shift crossed immunoelectrophoresis (Bhakdi *et al.*, 1977) and crossed hydrophobic-interaction immunoelectrophoresis with phenyl-Sepharose (Bjerrum, 1978), have been used in the present study to characterize the amphiphilic properties of dopamine β-hydroxylase in various granule fractions and to elucidate how these properties can be modified by various degrees of limited proteolysis.

Materials and Methods

Bovine adrenals

These were obtained from the local slaughterhouse, and were transferred to ice about 30 min *post mortem*. The dissected medullae were homogenized in 0.3 M-sucrose containing 10^{-5} M-pargyline (1 : 5, w/v) and the 'large granules', i.e. chromaffin granules, mitochondria and lysosomes, were obtained as the $3\times 10^5 g_{av.}$-min pellet of the homogenate after removal of the unbroken cells, nuclei and debris at $6.4\times 10^3 g_{av.}$-min (Helle & Serck-Hanssen, 1975; Winkler, 1976). Chromaffin granules were separated from the lysosomes and mitochondria by sedimentation of the resuspended 'large granules' through 1.6 M-sucrose in 10^{-5} M-pargyline at $7.2\times 10^6 g_{av.}$-min. Partial lysis of the granules was obtained by resuspension of the pellet in 18 mM-$CaCl_2$, 50 mM-Tris/HCl, pH 7.5, and storage at −20°C until required. After dilution 1 : 5 in the Tris buffer to a final protein concentration of 12–20 mg/ml the granules were subjected to a further cycle of freezing and thawing before mixing with equal volumes of the following buffers: (A) 100 mM-sodium phosphate, pH 7.4; (B) 100 mM-glycine, 38 mM-Tris, pH 8.7; or (C) 100 mM-glycine, 38 mM-Tris, pH 8.7, containing 2% (v/v) Triton X-100 {α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxypoly-(oxy-1,2-ethanediyl), scintillation grade; BDH Chemicals, Poole, Dorset, U.K.}.

Soluble fractions

Soluble fractions of the granule suspension in buffer A or in buffer B, left at room temperature (21°C) for 10 min, were obtained as the supernatant after centrifugation at $4.7\times 10^6 g_{av.}$-min.

Membrane fractions

These were solubilized from the pellet obtained from buffer B with the detergent-containing Tris buffer (buffer C), left at room temperature for 10 min and centrifuged at $4.7\times 10^6 g_{av.}$-min.

Total solubilized fractions

These were obtained as the supernatant of whole granules suspended in Triton-containing buffer (buffer C) after centrifugation at $4.7\times 10^6 g_{av.}$-min.

Suspended chromaffin granules and proteins extracted by detergent from the membrane fractions (Triton X-100; 1% v/v final concentration) were enzymically digested at a range of 0.5–6 μl of enzyme/mg of protein at 37°C with three proteinases active at neutral pH; two serine proteinases, trypsin and chymotrypsin (EC 3.4.21.1, oral grade, 855 NF/g), both from Novo, Copenhagen, Denmark, and the microbial metalloenzyme, thermolysin (EC 3.4.24.4, grade A, 8080 proteolytic units/mg at 35°C) from Calbiochem, San Diego, CA, U.S.A. These proteinases differ with respect to preferential cleavage sites, trypsin cleaving at Arg and Lys (Walsh, 1970), and chymotrypsin and thermolysin preferentially cleaving at hydrophobic residues such as Phe and Leu (Wilcox, 1970; Matsubara, 1970). Proteins of the soluble granule fraction were incubated with the respective proteinases in concentrations up to 30 μg/mg of granule protein. A suspension of 'large granules' in the sucrose medium (5 mg of protein/ml) was lysed by one cycle of freezing and thawing and incubated at 37°C. Portions taken at 2, 6, 24 and 80 h were subjected to immunoelectrophoretic analysis as described above.

Dopamine β-hydroxylase (dopamine β-mono-oxygenase, EC 1.14.17.1)

This was monitored with a monospecific antibody raised in rabbits against the water-soluble enzyme by using crossed immunoelectrophoresis as previously described (Helle *et al.*, 1978). Also an antibody raised against dopamine β-hydroxylase from whole granules, by the method of immunization described by Harboe & Ingild (1973), was used in some experiments.

Protein was determined by the method of Lowry *et al.* (1951). To all samples NaOH (0.5 M) was added, thus eliminating the interference of catecholamines up to a concentration of 10 nmol/mg of protein in the samples.

Crossed, tandem-crossed and crossed-line immunoelectrophoresis

These techniques were performed essentially as described by Axelsen *et al.* (1973). Two electrophoresis buffers were used: 73 mM-barbital and 25 mM-Tris (pH 8.6) or 100 mM-glycine and 38 mM-Tris (pH 8.7). The gels normally contained 0.5% Triton X-100. Identical precipitation patterns were observed for the two buffer systems.

Charge-shift crossed immunoelectrophoresis

This was performed as described by Bhakdi *et al.* (1977). The method is based on the observation that amphiphilic detergent-binding proteins exhibit bidirectional migration changes when electrophoresed in the presence of Triton plus an anionic (e.g. deoxycholate) or a cationic (e.g. cetyltrimethylammonium bromide) detergent, compared with their migration in Triton alone (Helenius & Simons, 1977).

Crossed hydrophobic-interaction immunoelectrophoresis

This was performed with phenyl-Sepharose CL-4B (40 μmol/ml of gel bed; Pharmacia Fine Chemicals Uppsala, Sweden) by the method of Bjerrum (1978). The first-dimension electrophoresis was carried out in a 1.5 mm thick 1% (w/v) agarose gel (type HSA; Litex, Glostrup, Denmark) containing the electrophoresis buffer and 33% (v/v) phenyl-Sepharose gel matrix, obtained by mixing of

2% melted agarose gel with preheated (56°C) washed gel slurry in the ratio 1 : 1 (gel bed diluted 1.5 times). The electrophoresis was normally performed at 10 V/cm until a Bromophenol Blue-stained albumin marker had migrated 4 cm. Gel slabs (0.5 cm wide) containing the separated proteins were then cut out and transferred to glass plates (5 cm × 7 cm) as described by Axelsen *et al.* (1973). The antibody containing gel plus 1% (v/v) Triton X-100 was cast to a thickness of 1 mm. Second-dimension electrophoresis was performed at 3 V/cm overnight. Conventional crossed immunoelectrophoresis with detergent present in the second-dimension gel was employed as a control.

Results

Crossed immunoelectrophoresis of dopamine β-hydroxylase

Aqueous and detergent-extracted fractions of the chromaffin granules from the bovine adrenal medulla were compared for their contents of immunoreactive dopamine β-hydroxylase (Fig. 1; Helle *et al.*, 1978). The precipitate of the Triton-extracted granules was asymmetrical with a shoulder on the cathodic side of the precipitate (Fig. 1*c*). The front peak had a position similar to the dopamine β-hydroxylase of the water-soluble fraction (Figs. 1*a* and 1*b*). Examination with crossed-tandem immunoelectrophoresis by using both types of antibodies revealed no antigenic differences between the enzymes of the two extracts. Since standardized extraction conditions were used, the areas delimited by the precipitates corresponded to the amounts of enzyme protein extracted with the three different solvents. No difference was observed between the areas of the two buffer extracts, and these were each smaller than the area obtained with the Triton-containing buffer extract. On the basis of a quantification of the areas, the buffer-extractable form of dopamine β-hydroxylase was estimated to comprise approx. two-thirds of the total enzyme protein in the chromaffin-granule preparation (see also Fig. 2*e*).

When detergent was omitted from the agarose gel the crossed immunoelectrophoresis of the detergent-extracted enzyme showed less heterogeneity, appearing as a nearly symmetrical peak of migration velocity equal to the front peak (Fig. 1). The total area of the detergent-solubilized enzyme was the same in the presence or absence of detergent in the agarose gels, indicating that the detergent-dependent form of dopamine β-hydroxylase migrated in the gel.

Dopamine β-hydroxylase in charge-shift crossed immunoelectrophoresis

The reaction of dopamine β-hydroxylase with the non-ionic detergent could be interpreted as the

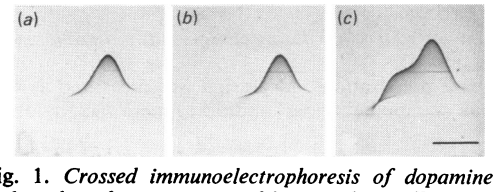

Fig. 1. *Crossed immunoelectrophoresis of dopamine β-hydroxylase from extracts of bovine chromaffin granules*
The granules were extracted with (*a*) 0.1 M-sodium phosphate buffer, pH 7.4, (*b*) 0.1 M-glycine and 0.038 M-Tris, pH 8.7 and (*c*) in the same buffer as (*b*) plus 2% (v/v) Triton X-100. Portions (20 μl) of the supernatants obtained from 200 μg of granule protein were electrophoresed in the presence of 0.5% Triton X-100. The first dimension was performed at 10 V/cm for 35 min and the second-dimension electrophoresis was performed at 3 V/cm for 18 h into gels containing 1 μl of a monospecific antibody to dopamine β-hydroxylase/cm². An antibody-free gel was inserted to increase the resolution. Staining was with Coomassie Brilliant Blue. The bar represents 1 cm.

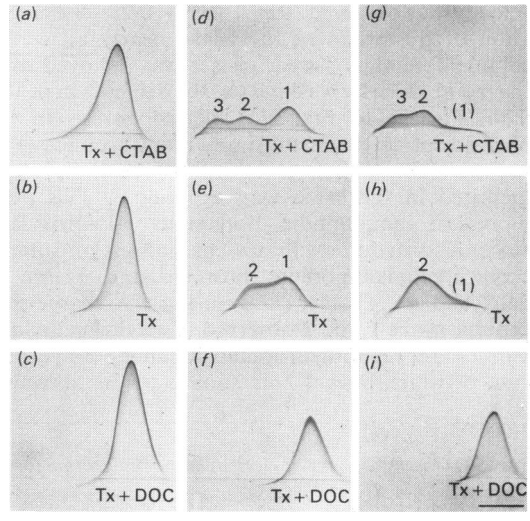

Fig. 2. *Charge-shift crossed immunoelectrophoresis of dopamine β-hydroxylase of bovine chromaffin granules*
(*a*), (*b*) and (*c*) show 40 μl of water-soluble fraction (phosphate buffer, pH 7.4, corresponding to Fig. 1*a*); (*d*), (*e*) and (*f*) show 20 μl of totally solubilized fraction (Triton X-100 extract, pH 8.7, corresponding to Fig. 1*c*); (*g*), (*h*) and (*i*) show 40 μl of water-insoluble membrane fraction (Triton X-100 extract, pH 8.7). First-dimension electrophoresis was performed in Triton plus cetyltrimethylammonium bromide (Tx+CTAB; *a*, *d* and *g*), Triton (Tx; *b*, *e*, and *h*) or Triton plus deoxycholate (Tx+DOC; *c*, *f* and *i*). Haemoglobin migration was 20 mm. Second-dimension electrophoresis was performed in gel containing Triton and 0.5 μl of monospecific antibodies to dopamine β-hydroxylase/cm². The peaks are numbered from the anodic side. The bar represents 1 cm.

presence of two forms of the enzyme, one a hydrophilic water-soluble form and the other an amphiphilic detergent-binding form. For such a characterization charge-shift crossed immunoelectrophoresis was used (Bhakdi *et al.*, 1977). Fig. 2 shows the results of such an investigation of dopamine β-hydroxylase in various extracts. In previous studies (Bhakdi *et al.*, 1977) the limit for a significant charge-shift was found to be ±5mm under the electrophoretic conditions employed. Thus the water-soluble dopamine β-hydroxylase did not reveal any amphiphilic detergent-binding property, since its migration was not significantly influenced by the various detergents (Table 1). The detergent-solubilized dopamine β-hydroxylase showed, however, the presence of amphiphilic molecules (Figs. 2*d*–2*i*). For total dopamine β-hydroxylase three peaks were observed in cetyltrimethylammonium bromide (Fig. 2*d*). Two of these peaks showed migration velocities corresponding to those seen in Triton alone (peaks 1 and 2), whereas peak 3 showed a decreased migration. In deoxycholate where all three peaks fused, a faster migration velocity was observed (Fig. 2*f*). For a closer examination of the origin of the peaks nearly all water-soluble dopamine β-hydroxylase was removed from the membranes by washing (cf. the decrease in peak 1 from Fig. 2*e* to Fig. 2*h*). Electrophoresis in the presence of cetyltrimethylammonium bromide (Fig. 2*g*) now shows that it is part of peak 2, which migrated in the same way as peak 3. This peak represents amphiphilic dopamine β-hydroxylase, since this fraction exhibits a charge-shift of 6mm in cetyltrimethylammonium bromide and 12mm in deoxycholate. (Table 1). By using the charge-shift criteria peaks 1 and 2 observed in cetyltrimethylammonium represent hydrophilic dopamine β-hydroxylase, of which peak 1 corresponds to the dopamine β-hydroxylase seen in the soluble fraction of the granules. Thus dopamine β-hydroxylase seems to exist in two forms depending on their origin of membranes or soluble core fractions.

Interaction between dopamine β-hydroxylase and phenyl-Sepharose

The presence of apolar domains in dopamine β-hydroxylase could also be shown by crossed hydrophobic-interaction immunoelectrophoresis with phenyl-Sepharose. In this method where the first-dimension electrophoresis is performed in the presence of the hydrophobic matrix, only amphiphilic proteins are totally retarded in the gel (Bjerrum, 1978). Such

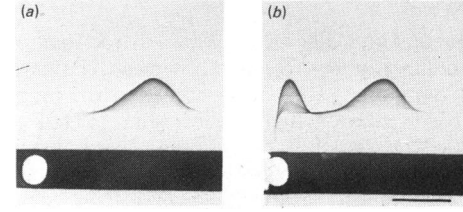

Fig. 3. *Crossed hydrophobic-interaction immunoelectrophoresis with phenyl-Sepharose of dopamine β-hydroxylase of bovine chromaffin granules*

(*a*) shows 20μl of water-soluble fraction (phosphate-buffer extract, pH7.4); (*b*) shows 20μl of totally solubilized fraction (Triton X-100 extract of granules, pH8.7). First-dimension electrophoresis was performed at 10V/cm in detergent-free gels. Second-dimension electrophoresis was performed at 3V/cm for 18h in gels containing 1% (v/v) Triton X-100 and 1μl of monospecific antibodies to dopamine β-hydroxylase/cm². An antibody-free gel was inserted to increase the resolution. The bar represents 1cm.

Table 1. *Observed range of electrophoretic migration for bovine dopamine β-hydroxylase during charge-shift crossed immunoelectrophoresis*

The range of values was for three different dopamine β-hydroxylase preparations. Migration was measured as the distance in mm from the front of the well to the maximum of the peak or shoulder under conditions where haemoglobin had migrated 20mm.

Buffer composition during first dimension electrophoresis	Migration distance for dopamine β-hydroxylase (mm)		
	Membrane fraction		Water-soluble fraction
	Peak 2	Peak 3	Peak 1
Triton X-100 (non-ionic detergent)	10–11	—	19–22
Triton X-100 + cetyltrimethylammonium bromide (cationic detergent)	10–11	4	19–22
Triton X-100 + deoxycholate (anionic detergent)		21–25	22–25

a retardation was not seen for water-soluble dopamine β-hydroxylase (Fig. 3*a*). When the detergent-extracted enzyme of a non-washed granule preparation was examined with the same technique, two forms of dopamine β-hydroxylase were clearly demonstrated (Fig. 3*b*), of which the retarded peak represents the amphiphilic form. All dopamine β-hydroxylase extracted from the washed membrane fraction was totally retarded on the phenyl-Sepharose matrix (results not shown).

The addition of Triton X-100 to the second-dimension gel was essential for the liberation of the amphiphilic form from the phenyl-Sepharose matrix. Under these conditions the recovery of the two forms of dopamine β-hydroxylase was examined by analysis of various mixtures of water-soluble/water-insoluble enzyme preparations (100:0, 3:1, 1:1, 1:3, 0:100). Measurements of the areas delimited by the two precipitates in the various mixtures show the following recoveries: 100:0, 66:23, 35:46, 13:91, 0:100, indicating that the liberation of the matrix-bound enzyme is almost complete. Thus a semi-quantitative estimation of the proportion between the two forms of the enzyme was possible from such immunoelectrophoretograms, indicating that the hydrophobic form accounts for one-third of the total enzyme protein of the granules (Fig. 3*b*). This agrees with the results obtained from crossed immunoelectrophoresis (Fig. 2*c*).

Effects of proteolysis on the two forms of dopamine β-hydroxylase

Mild treatment of the chromaffin granules suspended in Tris buffer with trypsin (2–3 μg/mg of granule protein at pH 7.5) caused cleavage of all acidic chromogranins within 30 min at 37°C without loss of enzyme activity of dopamine β-hydroxylase compared with the undigested controls, in keeping with previous findings (Helle *et al.*, 1977). Crossed immunoelectrophoresis alone or combined with charge-shift and hydrophobic interaction revealed no, or only a very slight, difference in the relative proportions of hydrophilic and hydrophobic forms of dopamine β-hydroxylase between the control preparations and digested preparations. Either an increase in trypsin concentration or the use of other proteinases such as chymotrypsin or thermolysin (2–10 μg/mg of

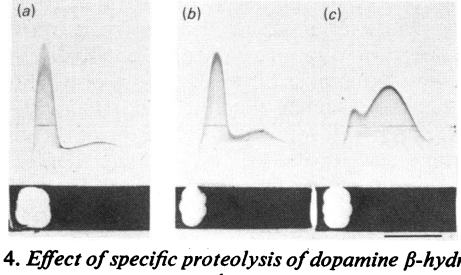

Fig. 4. *Effect of specific proteolysis of dopamine β-hydroxylase*

Crossed hydrophobic interaction immunoelectrophoresis with phenyl-Sepharose of 20 μl of Triton extract of water-insoluble membrane fraction. (*a*) shows the native extract; (*b*) shows the same extract after incubation for 60 min at 37°C with thermolysin (1 μg/ml) and (*c*) 10 μg of thermolysin/ml. Otherwise the experimental conditions correspond to those described in the legend to Fig. 3.

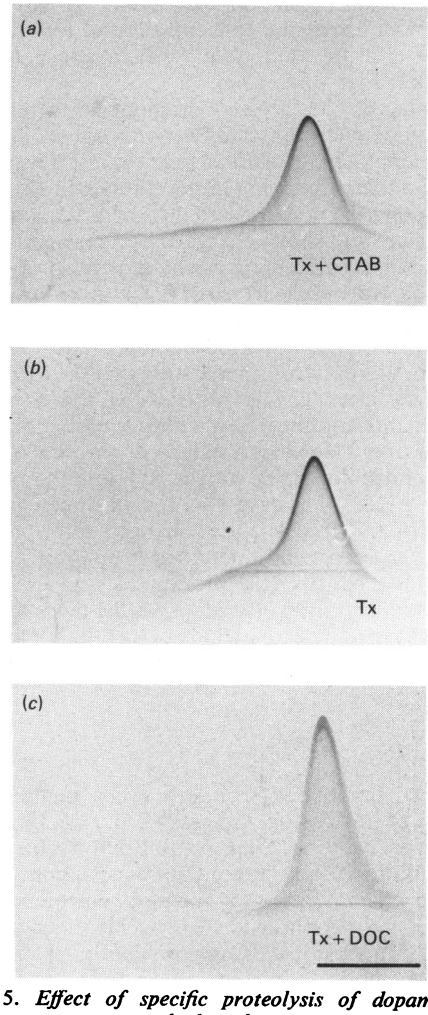

Fig. 5. *Effect of specific proteolysis of dopamine β-hydroxylase*

Charge-shift crossed immunoelectrophoresis of 20 μl of Triton extract of water-insoluble membrane fraction, which has been incubated with chymotrypsin (10 μg/ml) for 60 min at 37°C. Otherwise the experimental conditions and the abbreviations on the Figure correspond to those described in the legend to Fig. 2. The bar represents 1 cm.

protein for 1 h at 37°C in Tris/glycine buffer, pH 8.7) changed the patterns.

However, after solubilization with Triton X-100 a conversion of the amphiphilic into the hydrophilic form of dopamine β-hydroxylase could be achieved. Chymotrypsin and thermolysin (which cleave at hydrophobic residues) were especially effective, whereas trypsin also changed the precipitation pattern (see below). The crossed hydrophobic-interaction immunoelectrophoresis experiments of Fig. 4 show how the amphiphilic Triton-solubilized membrane dopamine β-hydroxylase (Fig. 4*a*) is converted into the hydrophilic form on incubation for 60 min at pH 8.7 with increasing concentration of the thermolysin (1 and 10 μg/ml, total protein concentration 1.5 mg/ml; Figs. 4*b* and 4*c*).

In charge-shift crossed immunoelectrophoresis experiments such degraded forms do not exhibit any bidirectional charge-shifts (Fig. 5). However, the small shoulder appearing in the cathode in Fig. 5(*b*) representing the non-converted form of dopamine β-hydroxylase still shows charge-shifting properties.

No antigenic differences could be demonstrated between the hydrophilic forms of dopamine β-hydroxylase after this type of proteolysis, as examined by double-diffusion and crossed-line and crossed tandem immunoelectrophoresis, indicating that the moderate proteolytic cleavage did not destroy the antigenic determinants confined to the hydrophilic moiety of the enzyme. However, extensive proteolysis of water-soluble dopamine β-hydroxylase (30 μg/mg of protein, pH 8.7, 1 h) gave rise, for all three enzymes investigated, to a slightly changed precipitation pattern in crossed immunoelectrophoresis. The dopamine β-hydroxylase precipitate appeared at a more anodic position (10–20%), with a more narrow shape and with a decreased area (10–20%). Furthermore the staining intensity of the precipitate was fainter.

Discussion

Crossed immunoelectrophoresis is a quantitative method of increasing importance for analyses of membrane proteins after solubilization (Bjerrum, 1977). Without any preceding purification many valuable characteristics of membrane-protein antigens can be established, such as identification of intrinsic proteins possessing amphiphilic properties. When membranes are solubilized by non-ionic detergents, lipids bound to apolar domains are replaced by detergent molecules (Helenius & Simons, 1975; Tanford & Reynolds, 1976). These properties are not known to be shared by any hydrophilic protein studied to date (Helenius & Simons, 1975; Tanford & Reynolds, 1976; Bhakdi *et al.*, 1977).

Proteins exhibiting such detergent binding can be detected indirectly by means of charge-shift electrophoresis (Helenius & Simons, 1977). In combination with crossed immunoelectrophoresis the charge-shift principle has been used for demonstration of amphiphilic properties for many proteins in complex mixtures: erythrocyte membrane proteins (Bhakdi *et al.*, 1977), serum lipoproteins and serum arylesterase (Bhakdi *et al.*, 1977; Bøg-Hansen *et al.*, 1978), synaptosomal proteins (Jørgensen, 1977) and intestinal brush-border aminopeptidase (Sjöström *et al.*, 1978). By exploiting the binding properties of proteins to hydrophobic gel matrices during electrophoresis it has also been possible to obtain a similar classification of proteins to that obtained on the basis of charge-shift electrophoresis (Bjerrum, 1978).

The molecular heterogeneity of dopamine β-hydroxylase of the bovine chromaffin granules observed in crossed immunoelectrophoresis (Helle *et al.*, 1978) could imply that some of the dopamine β-hydroxylase behaves like an integral membrane protein. Formation of vesicles during lysis of the granules could capture some of the soluble dopamine β-hydroxylase (Helle, 1973) and, if so, would give similar results, but such an interpretation was not supported by the influence of detergent on the immunoelectrophoretic-precipitation pattern (cf. Bjerrum, 1977) observed in the present paper.

That part of the solubilized dopamine β-hydroxylase in the Triton extract of granules was indeed amphiphilic was proved by the charge-shift electrophoresis experiments (cf. Fig. 2). Presence of further molecular heterogeneity of the membrane-bound form of dopamine β-hydroxylase was indicated by the observation of an intermediate peak (peak 2) in the cetyltrimethylammonium bromide-containing buffer. A similar heterogeneity has been observed for pig intestinal brush-border aminopeptidase, which is composed of subunits (Helenius & Simons, 1977; Sjöström *et al.*, 1978). However, peak 2, which does not show any charge-shifting properties, differs in migration velocity from the soluble form of dopamine β-hydroxylase and cannot simply be a result of dissociation of subunits with different hydrophobic properties. Other possible explanations of this finding are either the existence of an intermediate form in the cleavage process leading to the secretion of the soluble dopamine β-hydroxylase or various degrees of glycosylation or protein complexing. It is not a result of unsaturation of the Triton micelles with cetyltrimethylammonium bromide, since a doubling in the cetyltrimethylammonium bromide/Triton ratio gave rise to the same electrophoretic distribution of dopamine β-hydroxylase.

The crossed hydrophobic-interaction immunoelectrophoresis experiments further verified the existence of amphiphilic dopamine β-hydroxylase. However, the intermediate form of dopamine β-hydroxylase observed in the cetyltrimethylammonium bromide-containing buffer was found to be totally

retarded on the phenyl-Sepharose, indicating some hydrophobic properties of the intermediate form. Furthermore, the method also allows a semiquantitative determination of the ratio of the membrane-bound and the soluble form of dopamine β-hydroxylase because of their antigenic similarity. Approximately one-third of the total granule dopamine β-hydroxylase was found to possess amphiphilic properties in agreement with determinations based on activity measurements. Such an estimation cannot be performed with charge-shift crossed immunoelectrophoresis, because the ionic detergents gave the antigens different migration velocities and may be bound to the antibodies during the electrophoresis (cf. Bjerrum, 1977).

Many membrane-associated enzymes are bound through an apolar peptide 'tail' inserted in the lipid matrix. The 'tails' of such enzymes can be cleaved by limited proteolysis (Tanford & Reynolds, 1976; Sjöström *et al.*, 1978). Our experiments on solubilized membrane material from granules with the proteolytic enzymes thermolysin and chymotrypsin show that cleavage indeed takes place for dopamine β-hydroxylase. Like the soluble dopamine β-hydroxylase, the cleaved form did not show any amphiphilic properties.

These cleavage studies, the demonstrated amphiphilicity and the immunochemical identity taken together with the reported similarity in molecular weight (Helle, 1971; Hørtnagl *et al.*, 1972) of membrane-bound and soluble forms of dopamine β-hydroxylase and the apparent homogeneity of the total dopamine β-hydroxylase by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Ljones *et al.*, 1976; Wallace *et al.*, 1973) strongly suggest the presence of an extra, small hydrophobic and poorly antigenic sequence of peptides, or 'tail', in the membrane-bound form of the enzyme. Furthermore in a search for the existence of 'natural' enzyme systems capable of converting the membrane-bound form of dopamine β-hydroxylase into the hydrophilic counterpart, 'large granules' were lysed and incubated at 37°C. Crossed immunoelectrophoresis of portions taken at various times showed that a conversion gradually took place and was complete after 24h. The converted dopamine β-hydroxylase showed changes resembling those obtained with extensive proteolysis with specific enzymes. However, further studies are necessary for determination of the nature, specificity, localization and activation of the dopamine β-hydroxylase-converting system(s) to elucidate their role in the sequestration of dopamine β-hydroxylase during granule biogenesis. However, already it is clear that the existence of the membrane-bound and soluble forms of dopamine β-hydroxylase need not be an expression of a difference in the rate of synthesis (Winkler, 1977).

We thank Dr. Guldborg Serck-Hanssen and Dr. Greta Bolstad for supplying us with some of the granule preparations. We also thank Mrs. Lena Baron, Mrs. Kirsten Slange, Mrs. Ranvig Knudsen and Miss Karen E. Pihl for their skilful technical assistance. Financial support from Bloddonorernes Forskningsfond (to O.J.B.) and from the Norwegian Research Council for Science and the Humanities (to K. B. H.) is gratefully acknowledged.

References

Aunis, D., Allard, D., Miras-Portugal, M. T. & Mandel, P. (1975) *Biochim. Biophys. Acta* **393**, 284–295
Aunis, D., Bouclier, M., Pescheloche, M. & Mandel, P. (1977) *J. Neurochem.* **297**, 439–447
Axelsen, N., Krøll, J. & Weeke, B. (eds.) (1973) *Scand. J. Immunol.* **2**, *Suppl.* **1**
Bhakdi, S., Bhakdi-Lehnen, B. & Bjerrum, O. J. (1977) *Biochim. Biophys. Acta* **470**, 35–44
Bjerrum, O. J. (1977) *Biochim. Biophys. Acta* **472**, 135–195
Bjerrum, O. J. (1978) *Anal. Biochem.* **90**, 331–348
Bjerrum, O. J. & Bhakdi, S. (1977) *FEBS Lett.* **81**, 151–156
Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851
Bøg-Hansen, T. C., Bhakdi, S., Back, U. & Hanel, K. M. (1978) *Prot. Biol. Fluids* **25**, 251–254
Harboe, N. & Ingild, A. (1973) *Scand. J. Immunol.* **2**, *Suppl.* **1**, 161–164
Helenius, A. & Simons, K. (1975) *Biochim. Biophys. Acta* **415**, 29–79
Helenius, A. & Simons, K. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 529–532
Helle, K. B. (1971) *Biochim. Biophys. Acta* **245**, 94–104
Helle, K. B. (1973) *Biochim. Biophys. Acta* **318**, 167–180
Helle, K. B. & Serck-Hanssen, G. (1975) *Mol. Cell. Biochem.* **6**, 127–146
Helle, K. B., Serck-Hanssen, G. & Reichelt, K. L. (1977) *Int. J. Biochem.* **8**, 693–704
Helle, K. B., Serck-Hanssen, G. & Bock, E. (1978) *Biochim. Biophys. Acta* **533**, 396–407
Hogue-Angeletti, R. (1977) *Arch. Biochem. Biophys.* **184**, 364–372
Hørtnagl, H., Winkler, H. & Lochs, H. (1972) *Biochem. J.* **129**, 187–195
Jørgensen, O. S. (1977) *FEBS Lett.* **79**, 42–45
Kaufmann, S. & Freidmann, S. (1965) *Pharmacol. Rev.* **17**, 71–100
Ljones, T., Skotland, T. & Flatmark, T. (1976) *Eur. J. Biochem.* **61**, 525–533
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Matsubara, H. (1970) *Methods Enzymol.* **14**, 642–650
Sjöström, H., Noren, O., Jeppesen, L., Stavn, M., Svensson, B. & Christiansen, L. (1978) *Eur. J. Biochem.* **88**, 503–511
Tanford, C. & Reynolds, J. (1976) *Biochim. Biophys. Acta* **457**, 133–170
Viveros, O. H., Arqueros, L., Connett, R. J. & Kirshner, N. (1968) *Mol. Pharmacol.* **5**, 60–68
Wallace, E. F., Krantz, M. J. & Lovenberg, W. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 2233–2255
Walsh, K. A. (1970) *Methods Enzymol.* **14**, 41–63
Wilcox, P. E. (1970) *Methods Enzymol.* **14**, 84–108
Winkler, H. (1976) *Neuroscience* **1**, 65–80
Winkler, H. (1977) *Neuroscience* **2**, 657–683