Reduction of membrane-bound dopamine β -hydroxylase from the cytoplasmic surface of the chromaffin-granule membrane

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Resealed bovine chromaffin-granule 'ghosts' were used for assaying the membrane-bound form of dopamine β -hydroxylase. Hydroxylation of the substrate tyramine is dependent on its accumulation within the 'ghosts', where the active site of the enzyme is located. Free tyramine in the medium is at a low concentration, low ionic strength and a relatively high pH (7.0), so that even in the presence of a reducing agent (co-reductant) the unaccumulated amine is hydroxylated at a negligible rate. 'Ghosts' contain an endogenous co-reductant, which is shown to be catecholamine remaining in the membrane itself after granule lysis. Catecholamine that is free in solution in the medium or in the interior of the 'ghosts' is not effective as co-reductant, nor is ascorbate, in contrast with the situation with soluble dopamine β -hydroxylase. Ferrocyanide is an active co-reductant, however, giving a hydroxylation rate approximately equal to the tyramine accumulation rate: it does not enter the 'ghosts', nor does the enzyme appear to utilize ferrocyanide sealed inside the 'ghosts'. A mechanism must therefore exist for transferring electrons across the membrane from the cytoplasmic surface to the matrix surface. NADH is not an electron donor for the enzyme, nor is cytochrome *b*-561 involved.

Dopamine β -hydroxylase (dopamine β -mono-oxygenase, EC 1.14.17.1) catalyses the conversion of dopamine into noradrenaline. It is unique among the enzymes in the catecholamine-biosynthetic pathway in that it is located within the amine-storage vesicles, i.e. synaptic vesicles in the nervous system and chromaffin granules in the adrenal medulla. This location is found even in those cells of the adrenal medulla that store and release adrenaline rather than noradrenaline, in which the terminal methylating enzyme phenylethanolamine *N*-methyltransferase (EC 2.1.1.28) is cytoplasmic.

The enzyme is a mixed-function oxidase, requiring molecular oxygen and a co-reductant in addition to dopamine, the products being the hydroxylated amine and water. The enzyme's catalytic site is on the interior surface of the vesicle membrane, or in the vesicle matrix, as pointed out first by Kirshner (1962), who showed that dopamine transport to the interior of a chromaffin granule was

a prerequisite for hydroxylation; this was confirmed by latency studies (Laduron, 1975). In synaptic vesicles (Klein *et al.*, 1977; Kirksey *et al.*, 1978) and in the chromaffin granules of many species [e.g. the rat (Ciaranello *et al.*, 1975; Helle *et al.*, 1979)] dopamine β -hydroxylase is recovered largely as a component of the vesicle membrane. The best-characterized granules, however, are those of bovine origin, and in this case about 50% of the enzyme activity is recovered as a soluble component of the granule matrix (Hörtlagl *et al.*, 1972). It has proved very difficult to distinguish the membrane-bound and soluble enzymes by physical, chemical or immunological criteria; this similarity was resolved by a study (Bjerrum *et al.*, 1979) showing that the membrane form is distinguished by containing an extra hydrophobic domain, and that it can be converted into the soluble form by limited proteolysis. Studies on the release of the enzyme from stimulated glands have suggested that *in vivo* most of the enzyme may in fact be in the membrane-bound form (Dixon *et al.*, 1975; Edwards *et al.*, 1980), since less activity is released together with catecholamine than is found when isolated granules are broken open by hypo-osmotic lysis.

In spite of this, most studies of dopamine

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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β -hydroxylase have utilized the soluble enzyme, since this is easily purified from granule lysates by adsorption on concanavalin A-Sepharose (Rush *et al.*, 1974; Wallace & Lovenberg, 1974). The enzyme is known to have a broad substrate specificity (dopamine, tyramine, epinine) and to accept electrons from a variety of co-reductants (e.g. ascorbate, ferrocyanide, pteridines, catechols) (Friedman & Kaufman, 1965). The most effective reductant *in vitro* is ascorbate; this is converted into semidehydroascorbate, which may dismutate to ascorbate and dehydroascorbate (Ljones & Skotland, 1979; Skotland & Ljones, 1980; Diliberto & Allen, 1981).

Bovine chromaffin granules contain about 20 mM-ascorbate (Ingebretsen *et al.*, 1980). Although dehydroascorbate can cross the membrane, presumably by diffusion, it has not been possible to demonstrate any mechanism for ascorbate transport (Tirrell & Westhead, 1979), even though this must presumably be constantly replenished if it is used as the co-reductant by dopamine β -hydroxylase. We therefore decided to investigate whether membrane-bound dopamine β -hydroxylase is reduced *in situ* in a manner analogous to that inferred from studies of the soluble enzyme. We report an assay for this form of the enzyme that depends on transporting a substrate, tyramine, to the interior of a resealed membrane 'ghost', and show that the enzyme's utilization of co-reductants differs markedly from that of the soluble form.

Experimental

Materials

[7- 14 C]Tyramine hydrochloride was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and [7- 14 C]octopamine hydrochloride was from C.E.A., Gil-sur-Yvette, France. Catecholamines, purified soluble dopamine β -hydroxylase, Hepes and other biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K., and general chemicals were from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-50 (medium grade) was from Pharmacia, Hounslow, Middlesex, U.K., and cellulose nitrate filters (0.45 μ m pore size) were from Sartorius, Göttingen, Germany.

All solutions were buffered with 10 mM-Hepes, pH 7.0: the pH of 1 M-Hepes (free acid) was adjusted with 4 M-NaOH. Ascorbate and ferrocyanide solutions were always prepared just before use.

Methods

Resealed chromaffin-granule 'ghosts' were prepared from bovine adrenal glands as described previously (Apps *et al.*, 1980b). [14 C]Tyramine accumulation was followed in media containing 0.3 M-sucrose, 10 mM-Hepes buffer, pH 7.0, 5.5 mM-

ATP, 2 mM-MgCl₂, 1.7 mg of bovine serum albumin/ml, 48 μ M-[14 C]tyramine (28 mCi/mmol; 50 mCi/mmol in Table 3) and 'ghosts' (generally 200–300 μ g of protein/ml). Incubations (37°C) were terminated by diluting samples (50 μ l) in 2 ml of ice-cold buffered sucrose (or in 10 mM-Hepes buffer lacking 0.3 M-sucrose for samples subjected to osmotic shock). The samples were then passed immediately through cellulose nitrate filters, which were washed with 2 $\times$ 2 ml of cold buffered sucrose, dried and counted for radioactivity in scintillation fluid containing 2,5-diphenyloxazole (5 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3 g/l) in toluene. Standards were prepared on filters and their radioactivities were counted in the same way.

Other samples (50 μ l) were removed at different times from these incubations for assay of tyramine conversion into octopamine by dopamine β -hydroxylase activity in the 'ghosts'. These samples were added to 100 μ l of ice-cold 20% (w/w) trichloroacetic acid to stop the reaction. [14 C]Octopamine formed was assayed by periodate oxidation by a method developed from that of Friedman & Kaufman (1965). A 50 μ l volume of 35% (v/v) ammonia solution was added to the samples at room temperature, followed by 25 μ l of 2% (w/v) NaIO₄. After 4 min 0.3 ml of 10% (v/v) glycerol containing 4 M-HCl was added, followed by 0.5 ml of toluene. Samples were mixed vigorously for 30 s, centrifuged briefly and a sample of the toluene layer was taken for determination of radioactivity. The efficiency of the assay was assessed under all experimental conditions by using known concentrations of commercial [14 C]octopamine.

Simultaneous oxidation of endogenous catecholamine was followed by adding samples (100 μ l) to 2 ml of 3.5 M-NaOH containing 2% (v/v) ethylenediamine and 0.2% (w/v) ascorbic acid. The formation of fluorescent lutines was assayed within 1 h as described by von Euler & Lishajko (1961).

Catecholamine assays were also performed by this method, with freshly prepared adrenaline and noradrenaline bitartrate solutions as standards. Catecholamine outside the 'ghosts' (approx. 20 μ g of protein in 100 μ l) was assayed by oxidizing with 0.2 mM-K₃Fe(CN)₆ in buffered 0.3 M-sucrose (0.9 ml). Additional catecholamine inside the 'ghosts' was determined by adding a similar volume of the 'ghosts' to a solution containing 10 mM-Hepes buffer, pH 7.0, and 0.2 mM-K₃Fe(CN)₆, but no sucrose. Other assays (see the text below) contained, in addition, 0.2% (v/v) Triton X-100. Sephadex G-50 columns were 10 cm $\times$ 0.8 cm diameter.

Catecholamine leakage from 'ghosts' was assessed by two methods. The 'ghosts' (100–250 μ g of protein/ml) were either incubated in buffered sucrose containing 0.2 mM-K₃Fe(CN)₆, samples being removed at intervals to alkali as described

above but with an extra 1 ml of 10 mM-Hepes buffer added; alternatively the 'ghosts' were incubated in the absence of $K_3Fe(CN)_6$ but samples (100 μ l) were removed at intervals to 1 ml of buffered sucrose containing 0.2 mM- $K_3Fe(CN)_6$. After 30 s alkali was added and fluorescent lutines were assayed.

In some experiments ascorbate or $K_4Fe(CN)_6$ was sealed inside 'ghosts'. This was done as follows: the 'ghosts' were divided into two portions, one of which was diluted into 0.3 M-sucrose containing 10 mM-Hepes buffer, pH 7.0, and reducing agent (control). The other was diluted into buffer containing the reducing agent but lacking sucrose. After 5 min at 0°C, sucrose was added to 0.3 M, and both portions of 'ghosts' were maintained at 0°C for a further 1 h before use.

Protein was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Results

Tyramine accumulation and hydroxylation by chromaffin-granule 'ghosts'

Chromaffin granules were subjected to hypo-osmotic lysis to release their content of small molecules (catecholamines, ATP) and protein (chromogranin A, soluble dopamine β -hydroxylase),

and the resealed 'ghosts' were purified. Catecholamine transport by these membrane vesicles has been well characterized (Apps *et al.*, 1980b). It is clear that, in order to use 'ghosts' in a study of dopamine β -hydroxylase, one must consider not only the hydroxylation reaction, but also the accessibility of the substrate to the enzyme and the function of membrane carriers, as well as changes of the internal environment of the 'ghosts' (such as pH, electrostatic potential and ionic strength). We therefore first characterized tyramine accumulation.

Incubation of 'ghosts' in a medium containing buffered sucrose and 48 μ M- $[^{14}C]$ tyramine leads to an incorporation of radioactivity into particulate material (Fig. 1). This is relatively slow (initial rate about 1 nmol/min per mg of protein), and reaches an equilibrium value of about 3 nmol/mg of protein within about 10 min. It is non-saturable and is not inhibited by catecholamines in the medium or by reserpine; it does not occur at 0°C. About 80–90% of the accumulated radioactive amine is released if the 'ghosts' are subjected to hypo-osmotic shock. This accumulation that occurs in the absence of ATP is assumed to result from tyramine diffusion through the membrane, as has been shown to occur in pH-jump experiments with 'ghosts' (Scherman & Henry, 1980). Its accumulation [the nominal internal concentration is about 700 μ M if the 'ghost' volume is

Fig. 1. Dopamine β -hydroxylase activity of chromaffin-granule 'ghosts'

'Ghosts' (320 μ g/ml) were incubated with 48 μ M- $[^{14}C]$ tyramine, and amine accumulation (●) and conversion into $[^{14}C]$ octopamine (○) were assessed. ATP (5.5 mM) and $MgCl_2$ (2 mM) were added after 29 min of incubation (arrowed). For full experimental details see the text.

about 4 μM /mg of protein, as found by Phillips & Allison (1978)] of about 15-fold across the membrane is assumed to occur because of loose binding to internal membrane sites and possibly to residual internal protein. There is no pH gradient across these membranes.

If MgATP is added to the medium, there is a stimulation of both rate and extent of tyramine accumulation (Fig. 1), owing to the generation of both pH and potential gradients across the membrane. A steady-state internal concentration of about 4 mM-tyramine is reached when the external concentration is 48 μM . This uptake, which is inhibited by catecholamines and by reserpine, is mediated by the catecholamine transporter in the membrane (Apps *et al.*, 1980b).

The tyramine accumulated by the 'ghosts' is hydroxylated to yield octopamine, in the absence of added reducing agent (Fig. 1). The hydroxylated product was measured by withdrawing samples from the incubation and subjecting them to periodate oxidation; the efficiency of the assay procedure was assessed by adding known concentrations of [^{14}C]octopamine. Although the rate of hydroxylation is relatively low, it continues linearly for more than 1 h, suggesting that the steady-state concentration of ^{14}C -labelled amine measured in the 'ghosts' is an equilibrium, with octopamine leaking into the medium and being replaced by new tyramine.

When MgATP is added to the medium the steady-state hydroxylation rate approximately doubles, after a brief lag (Fig. 1). This is partly due to the increase in tyramine concentration within the 'ghosts': the steady-state internal tyramine concentration in the absence of MgATP in the experiment shown in Fig. 1 is approx. 0.7 mM, less the internal concentration of octopamine (the proportion of octopamine that is inside the 'ghosts' is unknown). As shown in Fig. 1, the internal tyramine concentration increases rapidly on addition of MgATP. The apparent K_m for tyramine of membrane-bound dopamine β -hydroxylase is 0.29 mM (Miras-Portugal *et al.*, 1976). The short lag period observed may be due to the time taken for establishing steady-state pH and potential gradients across the membrane. Soluble dopamine β -hydroxylase has a pH optimum of 6.0, with ferrocyanide as reductant and in the presence of sodium fumarate (Rosenberg *et al.*, 1980), and that of the membrane enzyme may be even lower (Miras-Portugal *et al.*, 1976). The increase in steady-state rate may therefore also be explained by the decrease in internal pH of the 'ghosts' that results from ATP hydrolysis (Phillips & Allison, 1978).

Location of the dopamine β -hydroxylase

The hydroxylation shown in Fig. 1. could be

catalysed by enzyme located within the 'ghosts', as assumed above, or by enzyme attached to broken membrane fragments, or by soluble enzyme contaminating the 'ghost' preparation. In the last two cases, the enzyme would be utilizing as substrate the 48 μM -tyramine provided in the medium.

We added purified soluble dopamine β -hydroxylase to the incubations to test for the latter possibility. We assumed that the enzyme could be maximally 20% of the 'ghost' protein (Winkler & Westhead, 1980) and added an equivalent amount of soluble enzyme. There was no significant increase of the activity measured in Fig. 1. This experimental fact is the consequence of: (i) the high apparent K_m for tyramine of the soluble enzyme, about 2.0 mM (Miras-Portugal *et al.*, 1976); (ii) the low ionic strength of the medium; (iii) its high pH (the pH optimum of the soluble enzyme decreases in the absence of sodium fumarate); (iv) the absence of added reducing agent.

The stimulatory effect of MgATP, which is known to affect the internal environment of the 'ghosts', is evidence that the substrate and the enzyme are internal, and that the enzyme is utilizing the internal tyramine. Further evidence that substantiates this point is given further on in the present paper.

Nature of the endogenous co-reductant

The hydroxylation activity is proportional to the 'ghost' concentration in the medium over the range 40–800 $\mu\text{g}/\text{ml}$. It is unaffected by adding superoxide dismutase (EC 1.15.1.1) to the incubations, making it unlikely that superoxide is acting as reductant for the reaction under these conditions (Henry *et al.*, 1978).

The 'ghosts' were passed down a Sephadex G-50 column, or were dialysed against buffered 0.3 M-sucrose. Neither treatment lowered the hydroxylation activity, suggesting that any small-molecule reductant must be either inside the 'ghosts' or located within the membrane.

The stoichiometry of the reaction catalysed by dopamine β -hydroxylase in these experiments is:

If the hydroxylation (Fig. 1) is followed for a prolonged time course, it finally ceases when about 20% of the initial tyramine in the medium (48 μM) has been converted into octopamine, equivalent to about 40 nmol/mg of protein. If a two-electron donor equivalent to this amount of substrate is present within the 'ghosts', its internal concentration would have to be about 10 mM [if the internal volume of the 'ghosts' is 4 $\mu\text{L}/\text{mg}$ (Phillips & Allison, 1978)]. One therefore cannot explain the endogenous hydroxylation activity by invoking the presence of residual ascorbate, which is present at only about 20 mM in intact granules (Ingebrechtsen *et al.*, 1980).

and is almost completely removed by lysis. The most likely reductant is therefore catecholamine (Levin *et al.*, 1960), the location of which we have investigated by using a ferricyanide oxidation technique.

Catecholamine compartmentation

Catecholamines can be assayed as fluorescent lutine derivatives (von Euler & Lishajko, 1961), which are formed by alkali treatment of the products formed by ferricyanide oxidation (Harley-Mason, 1950). Although it is well known that ferricyanide cannot cross mitochondrial membranes (Klingenberg, 1970; Miller *et al.*, 1979), we checked that it could not cross chromaffin-granule membranes by investigating 'ghost' swelling in high concentrations of potassium ferricyanide in the presence and in the absence of valinomycin: no swelling was observed. Marked swelling is observed under these conditions if permeant anions are present (Phillips, 1977).

Intact bovine chromaffin granules contain about 10–15 μ mol of catecholamine/mg of membrane protein (Winkler, 1976). Between 97 and 99% of this is removed during the preparation of the resealed 'ghosts', the present preparations containing 100–300 nmol/mg of protein. Catecholamine outside the 'ghosts' was determined after oxidation by ferricyanide in the presence of 0.3 M-sucrose: this reaction was complete in less than 0.5 min at either 0 or 20°C and was independent of ferricyanide concentration in the range 0.03–13 mM.

Results for a typical 'ghost' preparation are given in Table 1. If the ferricyanide oxidation was performed in the absence of sucrose (second column of Table 1), more catecholamine was revealed than when the assay was performed in a sucrose medium: under such conditions 'ghosts' lyse and internal catecholamine becomes accessible to ferricyanide. Even more catecholamine is seen if 0.2% Triton X-100 is also added to solubilize the membrane components, suggesting that there is a third com-

partment, localized within the membrane. Passing 'ghosts' down a column of Sephadex G-50 equilibrated with 0.3 M-sucrose removed 75% of the external catecholamine, but did not affect the internal or 'membrane-bound' pools. If the column is equilibrated with water containing only 10 mM-Hepes, however, the 'ghosts' lyse and both internal and external pools are lost; the membrane-bound catecholamine is unaffected (see analysis in Table 2).

If 'ghosts' are incubated with potassium ferricyanide at 22°C in 0.3 M-sucrose, the initial rapid appearance of aminochrome (oxidation of external catecholamine) is followed by a slow formation of additional oxidation product over about 3 h (Fig. 2). This is not found at 0°C, and we ascribe it to a slow leakage of catecholamine from the interior of the 'ghosts'. In quantity the extent of this leakage is always equal to the size of the internal compartment, as defined in Table 2. The 'membrane-bound' compartment does not seem to be affected, since it can still be released by solubilization with Triton. Identical results are obtained if the incubation is performed in the absence of ferricyanide; in this case, ferricyanide is added to samples of the 'ghost' suspension at intervals, and the resulting aminochrome measured. The external catecholamine pool is found to increase slowly, at a rate identical with the rate of aminochrome formation shown in Fig. 2. The oxidation observed in Fig. 2 is therefore not due to penetration of the 'ghosts' by ferricyanide, which remains entirely in the external medium.

Catecholamines as reductants in the hydroxylation

If contaminating catecholamines act as reductants for the hydroxylation of tyramine, the quinone product is likely to be spontaneously converted into the corresponding aminochrome in the absence of an exogenous reducing agent (Levin & Kaufman, 1961). This compound can be detected and assayed by conversion into the fluorescent lutine in the

Table 1. *Oxidation of catecholamines in chromaffin-granule 'ghosts' by ferricyanide*

Catecholamines were assayed fluorimetrically after oxidation by 0.2 mM- $K_3Fe(CN)_6$ under the conditions shown. For full experimental details see the text. Total catecholamines are given, the percentages of adrenaline being shown in parentheses.

	Total catecholamine oxidized (nmol/mg of protein)			
	In sucrose (0.3 M)	No sucrose	In sucrose (0.3 M) plus 0.2% Triton X-100	No sucrose, plus 0.2% Triton X-100
Treatment of 'ghosts'				
'Ghosts' in sucrose	120 (74%)	198 (74%)	247 (75%)	245 (74%)
'Ghosts' after Sephadex filtration in the presence of sucrose (0.3 M)	31 (81%)	113 (73%)	163 (78%)	165 (78%)
'Ghosts' after Sephadex filtration in the absence of sucrose (lysis)	29 (76%)	35 (80%)	80 (75%)	78 (74%)

Table 2. *Compartmental analysis of catecholamines in chromaffin-granule 'ghosts'*
Data from Table 1 were used.

	Total catecholamines (nmol/mg of protein)		
	External compartment	Internal compartment	Membrane compartment
'Ghosts' in sucrose	120	78	49
'Ghosts' after Sephadex filtration in the presence of sucrose (0.3 M)	31	82	50
'Ghosts' after Sephadex filtration in the absence of sucrose (lysis)	29	6	45

presence of alkali. We therefore measured [^{14}C]octopamine formation from [^{14}C]tyramine and aminochrome formation in the same experiment by subjecting samples to alkali treatment (Fig. 3). In the absence of tyramine there was no aminochrome formation, but in its presence the rates of catecholamine oxidation and of tyramine hydroxylation were identical, strongly suggesting that catechol oxidation accompanies dopamine β -hydroxylase-catalysed hydroxylation. The rates of both processes decreased when about 45 nmol/mg had been converted. Interestingly, of the aminochrome formed in the experiment of Fig. 3 and in similar experiments, 90–95% was derived from adrenaline, although this comprises only about 75% of the total catecholamine present (Table 1).

The 'ghosts' were subjected to a second lysis procedure on a water-equilibrated Sephadex G-50 column. This removes most of the free catecholamine present in the 'ghost' preparation both inside and outside the 'ghosts' (Table 2). Under these conditions the rate of tyramine hydroxylation is unaltered. A similar result is obtained if the 'ghosts' are dialysed. We conclude that membrane-bound catecholamine is the endogenous reductant.

Ascorbate is not a co-reductant

If ascorbate is added to the incubation medium, hydroxylation accompanies the tyramine uptake (Fig. 4), but the rate of hydroxylation does not exceed the rate found when endogenous catecholamines were acting as reductant. Indeed, after a short period of incubation, ascorbate was found to inhibit the endogenous hydroxylation.

The rate observed in the presence of ascorbate varied considerably from one experiment to another, as did endogenous rates (ten 'ghost' preparations were tested; rates varied by a factor of 2–3, as discussed below). However, in the presence of ascorbate, the rate was always less than or equal to the endogenous rate. We were not able to decide whether catecholamine or ascorbate was the reductant under these conditions, since ascorbate

Fig. 2. *Oxidation of 'ghost' catecholamine by ferricyanide*

'Ghosts' (100 $\mu\text{g}/\text{ml}$) were incubated with 0.2 mM- $\text{K}_3\text{Fe}(\text{CN})_6$ at 22°C, and samples were withdrawn at intervals for alkali treatment and lutine determination (Δ). Another portion of 'ghosts' was passed down a column of Sephadex G-50 equilibrated with 0.3 M-sucrose, and was then incubated (180 $\mu\text{g}/\text{ml}$) with 0.2 mM- $\text{K}_3\text{Fe}(\text{CN})_6$ at 22°C ($\bullet$) or at 0°C ($\circ$). For full experimental details see the text.

reduces any aminochrome formed (Levin & Kaufman, 1961), making it impossible to assay for this product by lutine fluorescence. It is possible that preincubation of the 'ghosts' with ascorbate affects the observed hydroxylation rate, but we have not investigated this in detail.

Since ascorbate cannot cross the chromaffin-granule membrane (Tirrell & Westhead, 1979), we lysed 'ghosts' and resealed them in the presence of 7 mM-ascorbate so that this reductant should have direct access to the dopamine β -hydroxylase. It still failed to stimulate hydroxylation (Fig. 4). This result

Fig. 3. Catecholamines as reductants for dopamine β -hydroxylase

'Ghosts' (250 μ g/ml) were incubated in the presence of 48 μ M-[14 C]tyramine and MgATP. Formation of [14 C]octopamine was assessed (O). At the same time samples were removed for assay of total adrenochrome and noradrenochrome (●). Similar assays were performed on a sample of 'ghosts' incubated without tyramine (Δ). For full experimental details see the text.

Fig. 4. Dopamine β -hydroxylase activity of chromaffin-granule 'ghosts'

'Ghosts' (200 μ g/ml) were incubated with 48 μ M-[14 C]tyramine and MgATP. In one experiment octopamine formation was followed with no reducing agent in the medium (●), with 7 mM-ascorbate (O) or with 0.25 mM- $K_4Fe(CN)_6$ (□). In the other experiment the 'ghosts' were subjected to re-lysis and resealing in the presence (▲) or absence (Δ) of 7 mM-ascorbate, and octopamine formation was assayed with 7 mM-ascorbate present in the external medium. For full experimental details see the text.

was quite unexpected, since ascorbate greatly stimulated soluble dopamine β -hydroxylase. We checked that it did not interfere with the assay of [14 C]octopamine; furthermore, 'ghosts' were passed down Sephadex G-50 columns equilibrated in the presence of sucrose or of water in order to remove possible low-molecular-weight inhibitors from the medium or from the 'ghost' interior, without effect.

Ferrocyanide as co-reductant

The membrane-bound dopamine β -hydroxylase is, however, stimulated by potassium ferrocyanide, as shown in Fig. 4, in which the same 'ghost' preparation has been incubated in media containing either ferrocyanide or ascorbate.

The use of ferrocyanide as reductant is illustrated in more detail in Fig. 5 (a typical result from 20 separate experiments). All solutions were initially at 37°C, and uptake of [14 C]tyramine was initiated by adding 'ghosts' to a medium containing MgATP in the presence or in the absence of ferrocyanide. The low endogenous hydroxylation rate, with catecholamines as reductants, is initiated after a brief lag. The ferrocyanide-stimulated reaction shows a pronounced lag followed by a steady-state rate, which persists for only about 5 min before decreasing. The total extent of hydroxylation greatly exceeds the equilibrium concentration of 14 C-labelled amine inside the 'ghosts'.

In a separate experiment (also shown in Fig. 5 for convenience), 'ghosts' were loaded with [14 C]tyramine in the presence of MgATP at 37°C for 5 min, thus initiating the endogenous hydroxylation reaction. Ferrocyanide was then added. It was found that, although the lag phase still persisted after the addition of ferrocyanide, the steady-state rate was linear over a long period.

This experiment suggests that the lowering of the rate of ferrocyanide-dependent hydroxylation after 10 min is a consequence of MgATP-induced changes in the interior environment of the 'ghosts'. Such changes, affecting the dopamine β -hydroxylase, are virtually complete within 10 min of adding MgATP, so that when ferrocyanide is added after 5 min the lower steady-state rate is reached immediately.

In contrast, the lag cannot be caused by MgATP-induced changes such as (i) a steadily increasing internal tyramine concentration on addition of MgATP, as in this case the lag should have been abolished by the preincubation with tyramine and MgATP, or (ii) changes in internal pH and membrane potential, which are greatest in the first few minutes after MgATP addition (Phillips & Allison, 1978). It appears to be, instead, a consequence of ferrocyanide addition.

One possibility is that the lag represents a phase of ferrocyanide penetration of the 'ghosts'. This was tested by sealing ferrocyanide inside the 'ghosts' as

Fig. 5. Dopamine β -hydroxylase activity in the presence of ferrocyanide 'Ghosts' (250 μ g/ml) were incubated with 48 μ M-[14 C]tyramine and MgATP. Uptake of amine into the 'ghosts' was followed by filtration ($\square$, broken line). Octopamine formation was followed in incubations with no added reductant ($\bullet$) or with 0.25 mM- $K_4Fe(CN)_6$ added at 0 min ($\circ$) or at 5 min (arrowed; Δ). For full experimental details see the text.

well as having it in the medium. This treatment has no effect on the hydroxylation reaction: results exactly similar to those in Fig. 5 were obtained, the lag phase being completely unaltered.

This result with internal ferrocyanide is particularly noteworthy since soluble dopamine β -hydroxylase, which can also utilize ferrocyanide as a reductant (Ljones *et al.*, 1976), does not show any lag. This therefore suggests that the membrane-bound enzyme cannot use ferrocyanide in the same way, but can only use ferrocyanide supplied in the medium outside the 'ghosts'. The lag in this case might be accounted for by a rate-limiting transfer of electrons across the membrane to the active site.

Variation of ferrocyanide concentration

The ferrocyanide concentration of incubations was varied between 2 μ M and 200 μ M in the presence and in the absence of MgATP, and the linear hydroxylation rates following the lag period were determined. Rates of hydroxylation were constant for about 12 min with all concentrations used: presumably the catecholamine present in the medium acts as a regenerating system to reduce ferricyanide formed in the reaction. A Hanes plot of a typical experiment is shown in Fig. 6. The plot departs from linearity at low ferrocyanide concentrations (below 10 μ M), where rates are higher than expected owing to the significant endogenous rate.

Fig. 6. Hanes plot for ferrocyanide-stimulated hydroxylation 'Ghosts' (200 μ g/ml) were incubated with [14 C]tyramine in the presence of various concentrations of $K_4Fe(CN)_6$, and the steady-state hydroxylation rate (v) was assessed (nmol/min per mg of protein). For full experimental details see the text.

Apparent K_m values between $8\mu\text{M}$ and $15\mu\text{M}$ were measured for ferrocyanide, with V_{max} between 1.3 and $1.8\text{nmol/min per mg}$ at 37°C . We found that V_{max} values varied from one 'ghost' preparation to another, however, in some instances being as much as twice as high as these values. Endogenous rates (catecholamine as reductant) were also variable. It is probable that this is accounted for by variability in the copper content of the preparations, since the enzyme is extremely sensitive to this element (Rosenberg *et al.*, 1980).

Variation of tyramine concentration

The apparent K_m for tyramine for membrane-bound dopamine β -hydroxylase is 0.29mM (Miras-Portugal *et al.*, 1976). We investigated the dependence of the ferrocyanide-stimulated hydroxylation rate on tyramine concentration in the medium, up to 0.1mM . If a substantial proportion of the enzyme activity measured is due to enzyme that is membrane-bound, but facing the medium (e.g. membrane fragments, or inside-out 'ghosts') one would expect to see proportionality between the rate and the tyramine concentration in the medium.

Results are given in Table 3. In the upper part are incubations conducted in the presence of MgATP: it can be seen that the internal amine concentration reached at plateau is roughly constant. The small rise in enzyme activity parallels the rise in internal concentration, rather than the concentration in the medium. It must be remembered that a substantial proportion of the ^{14}C -labelled amine measured within the 'ghosts' is in fact ^{14}C octopamine rather than ^{14}C tyramine, since the steady-state hydroxyl-

ation rate roughly equals the tyramine accumulation rate (the rates of octopamine leakage and re-uptake by the 'ghosts' are unknown but are presumably fairly similar to the rates for tyramine), so that the enzyme is probably not quite saturated with substrate.

In the lower part of Table 3 are rates for incubations conducted in the absence of MgATP with 'ghosts' that have been lysed twice to remove as much internal protein as possible. With such 'ghosts' the internal tyramine concentration is about 5 times higher than the external concentration. It is clear that under these conditions the hydroxylation rate is essentially proportional to the substrate concentration (either inside or outside the 'ghosts').

Effect of Triton X-100 on hydroxylation

Purified Triton X-100 has little effect on the activity of soluble dopamine β -hydroxylase (Terland *et al.*, 1977). In the presence of sodium fumarate at pH 7.0 we have found an inhibition of up to 20% of the activity of pure soluble enzyme when ferrocyanide is used as reductant (Fig. 7). In contrast, the ferrocyanide-stimulated hydroxylation catalysed by the dopamine β -hydroxylase in the 'ghosts' was almost completely inhibited by concentrations of Triton X-100 as low as 0.03% (Fig. 7). Tyramine accumulation by the 'ghosts' is virtually unaffected by 0.01% Triton X-100, but is completely abolished by 0.03%, so we explain the inhibition of hydroxylation as being due to the decreased tyramine concentration at the active site of the enzyme [apparent K_m , 0.29mM (Miras-Portugal *et al.*, 1976); concentration of tyramine in the medium, 0.048mM].

Table 3. Variation of tyramine hydroxylation with tyramine concentration

In the upper section, 'ghosts' ($250\mu\text{g/ml}$) were incubated in 0.3M -sucrose with ^{14}C tyramine, MgATP and 0.25mM - $\text{K}_4\text{Fe}(\text{CN})_6$; the steady-state rate of tyramine hydroxylation was measured. Internal tyramine concentration was calculated assuming that the internal volume of the 'ghosts' is $4\mu\text{l/mg}$ of protein (Phillips & Allison, 1978). In the lower section, 'ghosts' were re-lysed on a column of Sephadex G-50 equilibrated with water and then incubated ($800\mu\text{g/ml}$) with ^{14}C tyramine and $\text{K}_4\text{Fe}(\text{CN})_6$ but without MgATP or sucrose. For full experimental details see the text.

	External tyramine (mM)	Internal ^{14}C -labelled amine (mM)	Dopamine β -hydroxylase activity (nmol/min per mg)	Relative external tyramine concn.	Relative internal amine concn.	Relative dopamine β -hydroxylase activity
MgATP present	0.024	5.3	3.8	1.0	1.0	1.0
	0.048	5.2	3.8	2.0	1.0	1.0
	0.096	6.7	4.6	4.0	1.3	1.2
MgATP absent	0.024	0.11	0.53	1.0	1.0	1.0
	0.048	0.21	1.3	2.0	1.9	2.4
	0.096	0.48	2.2	4.0	4.4	4.1

Fig. 7. Inhibition of dopamine β -hydroxylase by Triton X-100

Soluble dopamine β -hydroxylase (10 μ g/ml) was incubated with 48 μ M-[14 C]tyramine and 0.25 mM- $K_4Fe(CN)_6$ in the presence of 50 mM-sodium fumarate (●). 'Ghosts' (200 μ g/ml) were incubated similarly except that fumarate was omitted (○). For full experimental details see the text.

Discussion

We wish to establish the following three points: (i) that the enzymic activity we are investigating results from enzyme located on the inner surface of right-side-out resealed chromaffin-granule 'ghosts'; (ii) that ferrocyanide is a non-permeant reductant; (iii) that the redox site for the dopamine β -hydroxylase is associated with the membrane and is not accessible to the aqueous environment of the interior of the 'ghost'.

Location of the dopamine β -hydroxylase

The enzymic activity measured is not caused by contaminating soluble enzyme resulting from the granule lysis step. This has been shown by adding purified soluble dopamine β -hydroxylase at 10–20% of the protein concentration of the 'ghost' membranes. The increase in the measured activity is within the experimental error of the endogenous (catecholamine-dependent) activity or within 10% of the activity found in the presence of ferrocyanide; this low activity is due to the low tyramine

concentration outside the 'ghosts' and the high pH of the medium compared with the enzyme's pH optimum (the pH inside the 'ghosts' is about 6.0 in the presence of MgATP). Furthermore, the lack of ascorbate stimulation of the hydroxylation activity substantiates this point, since ascorbate is well known to be the best electron donor for the soluble enzyme (Levin *et al.*, 1960).

The catalytic activity depends on the ability of the 'ghosts' to concentrate the substrate, as shown by the effect of varying the external tyramine concentration, or as shown by making the membranes leaky with Triton X-100. The effect of MgATP in stimulating the hydroxylation rate (Fig. 1) can also be explained by its effect on substrate concentration at the enzyme active site. We therefore believe that the enzymic activity observed results from utilization of internal tyramine and is due to membrane-bound dopamine β -hydroxylase in right-side-out sealed vesicles. Clearly the preparation may well be contaminated by broken membrane fragments, but under the assay conditions their activity is negligible.

It is noteworthy that the ferrocyanide-stimulated hydroxylation rates observed are rather similar to the tyramine accumulation rate, suggesting that the transport process may in fact be rate-limiting in our assay.

Endogenous co-reductant

The transport of tyramine to the interior of the 'ghosts' is accompanied by its hydroxylation at a low rate, utilizing an endogenous co-reductant. This appears to be residual catecholamine, which contaminated the 'ghost' preparation. Such a process is unlikely to occur *in vivo*, since a catecholamine molecule would be lost for every dopamine molecule hydroxylated, and in any case the intact granules contain ascorbate, which is known to inhibit the utilization of catechols as reductants (Levin & Kaufman, 1961).

The ferricyanide oxidation technique allows us to distinguish three pools of catecholamine in the 'ghost' preparation. A surprisingly large proportion of the total amine appears to be closely associated with the membrane, and only to be revealed by membrane solubilization (Table 2). Although different preparations of 'ghosts' were contaminated to various extents with catecholamines (in the range 100–300 nmol/mg of protein), the content of the membrane-associated pool was comparatively constant (43 ± 5 nmol/mg, mean $\pm$ s.d. for five preparations). This is too much to be catecholamine associated with the translocator protein, and presumably represents material dissolved in the membrane itself. The phospholipid/catecholamine ratio is about 50:1, since the membrane contains 2.5 μ mol of phospholipid/mg of protein (Winkler, 1976).

The external catecholamine remaining after Sephadex gel filtration was found to be in the range 30–40 nmol/mg, suggesting that this amount is probably bound to the surface of the 'ghosts', though accessible to ferricyanide. It can presumably be replenished by the interior catecholamine (Fig. 2) in a process that does not involve the main membrane-associated pool, or that leaves this undpleted. Scherman & Henry (1981) report values of 6.0 nmol/mg and 6.5 nmol/mg for the internal and external compartments respectively of their 'ghost' preparation; these low values were, however, obtained for 'ghosts' that had been lysed and centrifuged twice in the presence of bivalent cations, and had been preincubated at 37°C, procedures that clearly decrease the catecholamine contamination.

The catecholamine involved in donating electrons is unlikely to be that in the external medium, since the hydroxylation rate is constant for 'ghost' concentrations in the range 40–800 μ g/ml, corresponding to 5–100 μ M external catecholamine, and it is unlikely that the amine would have a K_m as a reductant as low as 1 μ M. Furthermore, dialysis and gel filtration to remove 75% of the external catecholamine do not affect the rate. Secondly, re-lysis of the 'ghosts' to remove internal catecholamine (Table 2) also has no effect on the hydroxylation rate. We therefore conclude that the membrane-bound pool of catecholamine is that which is involved and that the aqueous catecholamine is not available to the enzyme. This conclusion is strengthened by the fact that the hydroxylation ceases when an amount of catecholamine equal to that in the membrane pool has been consumed. The soluble enzyme, in contrast, is able to utilize catecholamine in the medium (Levin & Kaufman, 1961).

This result is surprising, since dopamine β -hydroxylase is a large, relatively hydrophilic, glycoprotein [a tetramer with subunit molecular weights of 75 000 (Wallace *et al.*, 1973)], which is expected to protrude into the aqueous matrix of the granule from its point of anchorage in the membrane. The results with ascorbate in Fig. 4 are also consistent, however, since it appears that, unlike the soluble enzyme, the membrane form cannot utilize ascorbate in the aqueous medium as a co-reductant. It is therefore unlikely that this is the reductant *in vivo*.

Mode of action of ferrocyanide

In contrast with ascorbate, ferrocyanide is an excellent co-reductant. Either it must be able to cross the membrane to donate electrons to the enzyme or there must be a redox site accessible to the external medium on the cytoplasmic surface of the membrane. The former seems unlikely, since the ion is highly negatively charged and is well known not to cross other biological membranes (Miller *et al.*,

1979). We have been unable to detect entry of either ferrocyanide or ferricyanide into chromaffin-granule 'ghosts' by swelling experiments in the presence of high concentrations (50 mM) of the ions (M. Grouselle & J. H. Phillips, unpublished work): in fact, of course, the concentration used in the hydroxylation assays does not exceed 0.25 mM. The compartmental analysis of catecholamines is consistent with ferricyanide being non-permeant (Fig. 2), and it is likely that this conclusion can be extended to the more highly charged ferrocyanide ion.

We do not know whether electrons are donated by ferrocyanide directly to the enzyme in the membrane or whether instead there is an intermediate electron carrier. The latter possibility would account for the unexpected selectivity of the membrane enzyme (as compared with the soluble form) for aqueous ferrocyanide compared with aqueous ascorbate or catecholamine. If there is such an intermediate, whether it is a protein or a small molecule, its existence might account for the lag in the hydroxylation that is seen when ferrocyanide is added to the medium (Fig. 5).

Two possible intermediates that have been suggested in the literature seem, nevertheless, to be unlikely. (i) NADH:acceptor oxidoreductase (EC 1.6.99.3) can be assayed in the membrane if a suitable acceptor (e.g. ferricyanide) is present (Terland & Flatmark, 1973; Hollenbeck *et al.*, 1975). We have been completely unable, however, to stimulate tyramine hydroxylation with NADH. (ii) Cytochrome *b*-561 is a particularly abundant haemoprotein in the membrane (Apps *et al.*, 1980a; Winkler & Westhead, 1980). We have found that the cytochrome can be reduced by ascorbate (7 mM), as found by Flatmark & Terland (1971), but cannot be reduced by up to 0.8 mM-ferrocyanide. This is consistent with the redox potential, which was measured by Flatmark & Terland (1971) to be +140 mV. It is thus extremely improbable that the cytochrome has a role in mediating electron transfer to dopamine β -hydroxylase, as was also concluded by Hollenbeck *et al.* (1975), who showed that dopamine addition failed to re-oxidize reduced cytochrome.

We conclude, therefore, that chromaffin-granule dopamine β -hydroxylase can receive electrons across the membrane from a cytoplasmic reductant. At present the nature of this reductant is completely unknown.

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