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SHORT COMMUNICATIONS

Studies on cytochrome b_{561} in amine storage granules*

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A large fraction of the enzyme activities present in the amine storage granules of the adrenal medulla are bound to the membrane of these organelles. While dopamine-β-hydroxylase (DBO) appears to be a major protein constituent of the membrane, other proteins, including ATPase activity, are localized in the membrane [1]. A detailed investigation by Flatmark *et al.* [2] on the spectral properties of the amine granule membranes has revealed the presence of two electron carrier proteins among the constituents of the membrane. One of these is a flavoprotein while the second, a hemoprotein previously referred to as cytochrome b_{559}, has been shown to have an absorbance maximum at 561 nm (Cyt b_{561}).

The significance of the electron carriers in the amine storage granules is not known. Flatmark *et al.* [2] have suggested that cytochrome b_{561} might function in conjunction with DBO to complete the final step in the amine biosynthesis. Other evidence implicates an electron transport chain of which Cyt b_{561} might be a component in the catecholamine uptake mechanism operating at the amine storage granule membrane [3].

It is apparent then that the participation of Cyt b_{561} in any of the above-mentioned processes is at present hypothetical, and that further studies are required to delineate its role.

In the present investigation, we have studied the capacity of Cyt b_{561} to function as an electron carrier, particularly in relation to the enzymatic oxidation of dopamine. The possible significance of Cyt b_{561} in the amine uptake mechanism was approached by investigating the presence of the cytochrome in the rabbit blood platelet organelles in which the uptake mechanism is remarkably similar to the one of adrenomedullary granules [4].

Chromaffin granules were isolated from the bovine adrenal medulla by the procedure described by Smith and Winkler [5] and further purified by centrifugation through a second sucrose gradient [2]. The final pellet containing amine storage granules was the source of the granule membrane fraction which was obtained as follows. The pellet was resuspended in 10 ml Na-citrate buffer (100 μM, pH 6·5), and after taking a small aliquot (0·5 ml) for various enzyme assays was dialyzed overnight against 1 liter K-phosphate buffer (55 mM, pH 7·4) containing $K_3Fe(CN)_6$, 100 μM, for spectrophotometric determination of cytochrome b_{561}. The membrane fraction was finally separated from the soluble intragranular proteins (liberated by the dialysis in hypotonic buffer) by centrifuging the dialysate at $3{\cdot}3 \times 10^6$ *g*-min in a Beckman rotor 50. The pellet was resuspended in K-phosphate buffer (55 mM, pH 7·4).

The microsomal fraction of the adrenal medulla was isolated by centrifuging the sucrose homogenate (1:5 in 0·3 M sucrose), after removal of chromaffin granules and mitochondria, at 9×10^6 *g*-min. The 5-hydroxytryptamine storage organelles of the rabbit blood platelets were isolated as described by DaPrada and Pletscher [6].

All procedures were carried out at 4°. Proteins were determined by the method of Lowry *et al.* [7]; bovine albumin was used as a standard. Catecholamines (epinephrine and norepinephrine) were determined according to von Euler and Lishajko [8], omitting the purification with Alumina.

Glucose 6-phosphatase activity in chromaffin granules and microsomes was assayed according to Harper [9]; the phosphorus liberated by the enzymatic activity was determined by the procedure of Rockstein and Heron [10]. Dopamine-β-hydroxylase activity in the membrane fraction was assayed according to the procedure of Viveros *et al.* [11].

Difference spectra of membrane preparations were measured in an Aminco–Chance dual wavelength/split beam spectrophotometer. A 3-ml aliquot of membrane fraction suspension, adjusted to a protein concentration of 1 mg/ml, was placed in each cuvette (1-cm light path); the difference absorbance spectra were obtained by adding a reducing agent (Na-dithionite) to the sample cuvette.

The presence of various transport functions in the membrane preparations was detected by their ability to promote the oxidation of NADH when incubated with artificial electron acceptors such as $K_3Fe(CN)_6$, dichlorophenolindophenol and cytochrome *c*. The oxidation of NADH was measured with a Hitachi double-beam spectrophotometer by following the disappearance of NADH at 340 nm for all electron acceptors used as well as the decrease in absorbance at 420 nm of $K_3FE(CN)_6$ and 550 nm of cytochrome *c* upon their reduction.

The membrane fraction of purified chromaffin granules contained appreciable amounts of NADH oxido-reductase activities. The results of Table 1 show that the ability to catalyze the oxidation of NADH varied according to the electron acceptor used; there was a good stochiometric rela-

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Table 1. NADH—(Acceptor) oxidoreductase activities of amine storage membrane*

Electron acceptor	NADH oxidized (μmoles/mg protein/min)	Acceptor reduced (μmoles/mg protein/min)
$Fe(CN)_6^=$	0·490 ± 0·035	0·890 ± 0·048
DCPIP	0·022 ± 0·003	
Cyt c	0·57 ± 0·007	0·33 (0·30–0·36)

* Electron acceptors dissolved in K-phosphate buffer (55 μM, pH 7·4) were added in 10-μl aliquots to give the following final concn: $K_3Fe(CN)_6$, 100 μM; DCPIP (dichorophenolindophenol), 50 μM; cytochrome *c*, type VI horse heart (Sigma), 100 μM. NADH concentration was 85 μM. Protein concentration of the membrane suspension varied between 0·022 and 0·056 mg/ml. The total volume was 3 ml.

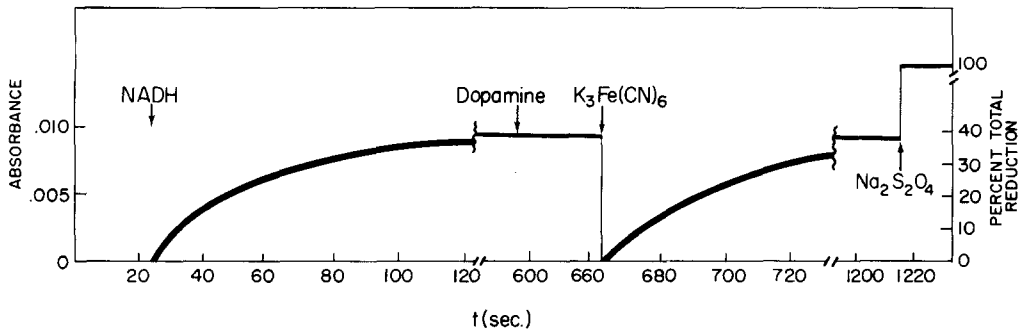

Fig. 1. Effect of various agents on the redox state of membrane-bound cytochrome b_{561}. Absorbance increases at 561 nm as the cytochrome becomes reduced. Agents were present in the following final concentrations: NADH, 141 μM; dopamine HCl, 110 μM; $K_3Fe(CN)_6$, 100 μM; Na-dithionite, 14 mM. Protein concentration was approximately 1 mg/ml. Oxygen concentration measured after reducing with dithionite revealed an aerobic condition in the cuvette.

tionship between the amount of NADH oxidized and the amount of single electron acceptor reduced as typified by the results with ferricianide.

The NADH oxido-reductase activities measured in this study were in good agreement with those reported by Flatmark *et al.* [2], with the exception of the experiments with $K_3Fe(CN)_6$ for which a considerably greater value was observed with the present preparation.

These findings confirm the presence of electron transfer functions in the membrane of chromaffin granule and emphasize the high catalytic properties of this preparation.

The reduced minus oxidized difference spectra of suspensions of chromaffin granule membranes revealed the presence of a hemoprotein with a characteristic absorbance maximum of 561 nm. In addition to this peak, other absorbance maxima occurred at 532 and 430 nm. The granule membrane preparations were free of mitochondrial contamination as witnessed by the absence of an absorbance band in the 605 nm region characteristic of mitochondrial cytochrome oxidase. Moreover, the granule membrane preparation as well as the medullary microsomal preparation appeared to be free of cortical microsomes as evidenced by the absence of CO-binding pigments in the recorded spectra.

That Cyt b_{561} bound to granule membrane can function as a redox protein was demonstrated by its ability to be alternately reduced and oxidized by Na-dithionite and $K_3Fe(CN)_6$. In agreement with observations by other workers [2, 11] we found that NADH could also reduce cytochrome b_{561}; the extent of reduction, when measured with the Aminco–Chance spectrophotometer in the dual wavelength mode at 561 nm with the reference wavelength set at 575 nm (isosbestic point) never exceeded 32 per cent of the maximum reduction attained with Na-dithionite (Fig. 1). The NADH-reduced cytochrome b_{561} could be returned to the oxidized state in an essentially instantaneous reaction by adding $K_3Fe(CN)_6$ to the cuvette. Reduction of the membrane-bound Cyt b_{561} was also observed in a few experiments with Na-ascorbate (100 μM); as was the case for NADH, ascorbate could only partially reduce the cytochrome.

At variance with results presented by Flatmark *et al.* [2], addition of dopamine to the NADH-reduced cytochrome did not induce the reoxidation of the hemoprotein with our experimental conditions (Fig. 1). In fact, if DBO is functionally linked to Cyt b_{561}, dopamine would be expected to promote the formation of oxidized DBO which, in turn, should oxidize the reduced form of the cytochrome. In view of this finding, Cyt b_{561} does not appear to be directly linked to enzymatic hydroxylation of dopamine in the membrane of chromaffin granules. It should be noted that the membrane fraction isolated under the conditions described above had considerable dopamine-β-hydroxylase activity, as shown by its ability to hydroxylate tyramine (38·5 ± 6·2 nmoles octopamine formed/mg of protein/hr at 37°).

Another indication that Cyt b_{561} may not be closely linked to DBO is evidenced by the different distribution pattern of the two activities within the storage organelles. In fact, among the soluble proteins of lysed chromaffin granules, of which DBO is a major constituent [1], the presence of Cyt b_{561} could not be detected.

Recently, Terland and Flatmark [13] postulated the presence in chromaffin granules of a cyanide-sensitive oxidase, localized between Cyt b_{561} and dopamine-β-hydroxylase, which might function as an electron transfer protein. Our data do not permit any conclusion as to the validity of this model for electron transport; they suggest, however, that the electron transfer function in chromaffin granule membrane is more complex than the one previously proposed by Flatmark *et al.* [2].

The presence of Cyt b_{561} was not confined to the membrane fraction of chromaffin granules (Table 2). The adrenomedullary microsomes contained 0·72 nmole Cyt b_{561}/mg of protein, a value remarkably similar to that obtained by

Table 2. Distribution of cytochrome b_{561} and other constituents in two subcellular fractions of bovine adrenal medulla

Fraction	Cytochrome b_{561}* (nmoles ± S.E./ mg protein)	Glucose 6-phosphatase (μmoles Pi/60 min/ mg protein)	Catecholamine (nmoles epinephrine/ mg protein)
Amine storage granules	1·37 ± 0·42	0·04 ± 0·002	719 ± 70
Microsome	0·72 ± 0·04	0·27 ± 0·03	103 ± 9·5

* Amount of cytochrome b_{561} was calculated using a molar extinction coefficient of $1{\cdot}8 \times 10^4\ M^{-1}\ cm^{-1}$; this value represents an average extinction coefficient for the α-band region of *b*- and *c*-type cytochromes.

Ichikawa and Yamano [12] who estimated the hemoprotein content by subtracting from the total protoheme content that contributed by cytochrome b_5 and P_{450}.

The catecholamine present in the microsomal fraction (Table 2) indicated contamination by intact chromaffin granules; their occurrence, along with disrupted chromaffin granules which lost their amine content during fractionation, might have partially contributed to the level of Cyt b_{561} found in the microsomal preparation used in these studies.

It is our estimate, however, that contamination by chromaffin granules, both intact and disrupted, never exceeded 25 per cent of the total protein in the microsomal fraction.

It is thus clear that Cyt b_{561} is a constituent of both adrenomedullary microsomes and chromaffin granules, a reflection of the origin of chromaffin granules in the endoplasmic reticulum from which microsomes are derived [14].

Since the studies of Flatmark *et al.* [15] revealed the presence of Cyt b_{561} in chromaffin granules as well as in the norepinephrine-storing granules found in adrenergic nerves, it was considered of interest to investigate the occurrence of the cytochrome in the amine-storing organelles of the rabbit blood platelets which have a mechanism for amine uptake strikingly similar to the one of adrenergic nerve organelles.

However, Cyt b_{561} could not be detected by light absorption studies on suspensions of serotonin (5-HT) organelles isolated from rabbit blood platelets, even when high concentrations of organelle membrane protein were used. In view of the well known similarities in amine uptake mechanism of chromaffin granules and rabbit blood platelet organelles, the presence of Cyt b_{561} in the latter organelles would have provided some evidence for the involvement of Cyt b_{561} in the amine uptake process. The negative finding is inconsistent with a participation of Cyt b_{561} in this process; however, it is possible that in the platelet organelle another mechanism might subserve this function.

In analogy with the classic interactions between drugs and the microsomal cytochrome P_{450}, the ability of various inhibitors of amine uptake in chromaffin granules to alter the spectral properties of Cyt b_{561} was investigated.

Since changes of the light absorption spectra (recorded between 350 and 600 nm) of a suspension of chromaffin granule membranes could not be detected by adding reserpine, guanethidine or *N*-ethylmaleimide in concentrations varying from 10^{-6} M to 10^{-3} M, we conclude that the interactions between granule membranes and these drugs are not amenable to the same type of spectral analysis applied to microsomal cytochrome P_{450}.

The data presented in this paper demonstrate that membranes of bovine chromaffin granules possess enzymes which may be involved in electron transport. The electron transfer activity of Cyt b_{561} does not appear to be integrated with the biosynthetic step of catecholamines occurring at the membrane level, i.e. the hydroxylation of dopamine by DBO.

Thus the physiological role of the electron transfer chain in the chromaffin granule membrane remains, at present, obscure.

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The biotransformation of 1-hexadecene to carcinogenic 1,2-epoxyhexadecane by hepatic microsomes

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n-1-Olefins with eight, ten or twelve carbon atoms are common sources for plasticizers and with fourteen, sixteen or eighteen carbon atoms for sodium alkyl sulfate detergents. One of the major metabolic pathways of these compounds in mammalian liver is now assumed to be oxidation to the corresponding epoxides by microsomal epoxidase followed by conversion of the products to glycols by hepatic microsomal epoxide hydrolase (epoxide hydrase) [1–3] or to α-hydroxyalkyl-*S*-glutathione conjugates by soluble exposide-*S*-glutathione transferase [3]. However, except for a few cases such as epoxides of polychlorinated cyclodiene insecticides, heptachlor and related toxicants [4, 5], cyclohexene [6], indene [6], styrene [6], and stilbene [7], epoxides formed *in vitro* are labile to the hydrolase and are rapidly converted to glycols, so that it is very difficult to detect them without using epoxide hydrolase inhibitors, such as various epoxides [1–3] and certain aziridines [8]. The initial evidence for epoxide intermediates in the microsomal metabolism of ole-