Biochimica et Biophysica Acta, 597 (1980) 318—330

BBA 78662

OXIDOREDUCTASE ACTIVITIES OF CHROMAFFIN GRANULE GHOSTS ISOLATED FROM THE BOVINE ADRENAL MEDULLA

OLE TERLAND and TORGEIR FLATMARK

Department of Biochemistry, University of Bergen, Årstadvollen, N-5000 Bergen (Norway)

(Received July 16th, 1979)

Key words: Oxidoreductase activity; Cytochrome b-561; (Chromaffin granule ghost)

Summary

1. Based on estimated *s*-values of subpopulations of bovine adrenal chromaffin granules (Bϕdtker-Naess, V., Slinde, E., Terland, O. and Flatmark, T. (1978) Biochim. Biophys. Acta 541, 124—134) a new large-scale procedure is described for the isolation of the total population of chromaffin granules by differential centrifugation in 0.25 M sucrose.

2. Using the total population of chromaffin granules obtained by differential centrifugation, final purification was achieved by density-gradient centrifugation in either sucrose or Percoll-sucrose. In either case, the isolated granule fractions were contaminated with mitochondria to about the same degree.

3. Chromaffin granule ghosts, obtained by hypoosmotic lysis of granules isolated by sucrose density-gradient centrifugation, were subjected to centrifugation on a discontinuous density gradient (buffer/0.9 M sucrose). By this procedure a substantial purification of the ghosts was achieved as determined from measurements of protein and various marker enzymes.

4. In contrast to preparations of chromaffin granule ghosts prepared by previous standard procedures, those purified by gradient centrifugation (on 0.9 M sucrose) did not reveal any NADH-linked cytochrome *b*-561 reductase activity. However, experimental evidence is presented for the existence of an intrinsic NADH-oxidizing enzyme system in the granule membrane.

5. No significant difference was observed in the specific content of cytochrome *b*-561 and NADH:(acceptor) oxidoreductase activities between ghost preparations obtained from populations of heavy and light chromaffin granules.

6. The functional significance of cytochrome *b*-561 and the NADH:(acceptor) oxidoreductase activities of the granule membrane remains to be determined.

Introduction

It is generally accepted that the membranes of chromaffin granules contain at least two redox active enzymes, i.e., dopamine β-monooxygenase (EC 1.14.17.1) [1] and cytochrome *b*-561 [2—4]. In addition to these membrane-bound enzymes, we have repeatedly observed that the chromaffin granule ghost preparations contain an NADH:(acceptor) oxidoreductase activity [3,5], and this finding has later been confirmed by others [6—8]. A number of compounds have been found to function as electron acceptors in this reaction in vitro, e.g. ferricyanide, 2,6-dichlorophenolindophenol, mammalian ferricytochrome *c* as well as endogenous acceptors including the membrane-bound ferricytochrome *b*-561 [3]. The NADH-dependent reduction of cytochrome *b*-561 [3], which has later been confirmed by others [6,9], suggested the possible presence of an electron transfer chain, including this cytochrome, in the chromaffin granule membrane [5]. However, the fact that we have never been able to obtain a rapid and extensive (i.e. >15%) reduction of cytochrome *b*-561 by NADH [3], which is considerably lower than reported later from another laboratory [6], have prompted a reinvestigation of the reason for this discrepancy as well as of the possible function of cytochrome *b*-561 in the chromaffin granules. Our recent findings of a high degree of polydispersity of these organelles [10,11] has further increased the importance of such an investigation. In the present study a procedure is presented for the isolation of the total population of chromaffin granules as well as of their ghosts in order to clarify some of the problems inherent in previously applied isolation procedures.

Materials and Methods

Preparation of homogenate and crude populations of chromaffin granules

A homogenate of bovine adrenal medulla was prepared as previously described [10].

Based on our recent estimates of the sedimentation coefficients of the two main subpopulations of chromaffin granules ($s_H \simeq 12\,400$ S and $s_L \simeq 3900$ S) [10], the following conditions were selected for the preparation of a crude population of these organelles by differential centrifugation containing a maximal amount of both subpopulations. The homogenate was first centrifuged (in Sorvall RC-5 refrigerated centrifuge, HB-4 rotor with r_{min} = 6.2 cm and r_{max} = 14.4 cm) at a centrifugal effect of $\int_0^t (\text{rev./min})^2\, dt = 11.0 \cdot 10^7\ \text{min}^{-1}$ (t = 10 min) to remove unbroken cells and large particles. The pellet (nuclear fraction) thus obtained was resuspended and resedimented twice as described above. The combined supernatants (post nuclear fraction) were centrifuged at a centrifugal effect $\int_0^t (\text{rev./min})^2\, dt = 328 \cdot 10^7\ \text{min}^{-1}$ (t = 17 min) to sediment both populations of chromaffin granules [10]. The sediment was resuspended and resedimented twice in order to reduce the microsomal contamination. By this procedure, approx. 97% of the 3900 S population of chromaffin granules was recovered (calculated as in Ref. 12). The final pellet, representing a crude preparation of the total population of chromaffin granules, was carefully resuspended in 20 ml (total volume) of 0.25 M sucrose medium.

The heavy ($s_H \simeq 12\,400$ S) and light ($s_L \simeq 3900$ S) populations were isolated as recently described [11] using a centrifugal effect $\int_0^t (\text{rev./min})^2\, dt = 328 \cdot 10^7\ \text{min}^{-1}$ ($t = 17$ min) to sediment the 3900 S population.

Isolation of chromaffin granules

In order to separate the chromaffin granules from other contaminating subcellular particles, the crude populations of chromaffin granules were subjected to either: (1) rate zonal centrifugation in 1.6 M sucrose [13] as the medium, or (2) isopycnic density-gradient centrifugation in Percoll-sucrose [11].

Preparation of chromaffin granule ghosts

The purified populations of chromaffin granules (from rate zonal or isopycnic density-gradient centrifugation) was subjected to hypoosmotic lysis in large volumes of 50 mM potassium citrate buffer, pH 6.5 [6], and the granule ghosts thus obtained were washed twice in the same medium. The final pellet of granule ghosts was resuspended in 2—10 ml 50 mM potassium citrate buffer, pH 6.5, and layered over 4 ml of 0.9 M sucrose in a 15 ml Corex tube and centrifuged (in Sorvall RC-5 refrigerated centrifuge, HB-4 rotor at a centrifugal effect of $\int_0^t (\text{rev./min})^2\, dt = 14.1 \cdot 10^9\ \text{min}^{-1}$; $t = 90$ min). The highly purified chromaffin granule ghosts were collected as a band at the interface of 0.9 M sucrose/buffer separated from a small brown pellet, with a slightly turbid sucrose medium between these two fractions (see Fig. 1 and Results). The collected chromaffin granule ghosts were diluted with standard citrate buffer (see above) and recentrifuged ($\int_0^t (\text{rev./min})^2\, dt = 328 \cdot 10^7\ \text{min}^{-1}$). The final pellet was resuspended in buffer at a concentration of 2—6 mg protein $\cdot$ ml^{-1}.

Isolation of mitochondrial and microsomal fractions from bovine adrenal medulla homogenates

The mitochondria, which band at the interphase of 1.6 M/0.25 M sucrose by rate zonal centrifugation of the crude population of chromaffin granules [13], were collected and washed twice in 50 mM potassium citrate buffer, pH 6.5, using a centrifugal effect of $\int_0^t (\text{rev./min})^2\, dt = 2 \cdot 10^9\ \text{min}^{-1}$. The mitochondrial pellet was resuspended and layered over 0.9 M sucrose as described above for the isolation of chromaffin granule ghosts. The mitochondrial pellet thus obtained was washed once at $\int_0^t (\text{rev./min})^2\, dt = 2 \cdot 10^9\ \text{min}^{-1}$.

A microsomal fraction was obtained from the post chromaffin granule supernatant using a centrifugal effect of $\int_0^t (\text{rev./min})^2\, dt = 8.9 \cdot 10^{10}\ \text{min}^{-1}$ ($t = 60$ min) in the Beckman 40 rotor.

Assay of marker enzymes

Dopamine β-monooxygenase and cytochrome *b*-561 were used as marker enzymes of chromaffin granule membranes. Dopamine β-monooxygenase was assayed by the hydroxylation of tyramine to octopamine determined by high-performance liquid chromatography and a fluorimetric detector [14]. Since dopamine β-monooxygenase is a latent enzyme in chromaffin granule ghosts [15], the activity was assayed in the presence of 0.2% (w/v) Triton X-100. The assumption was made that the enzymic activity was inhibited [16] to the same extent by the detergent in all fractions measured. Cytochrome *b*-561 was

determined by dual wavelength spectrophotometry as described [3] except that 0.2% (w/v) Triton X-100 was used to solubilize the membrane. The reduced minus oxidized difference spectrum of the membranes were obtained on a Cary Model 219 spectrophotometer as described [3], except that 1% Triton X-100 was present.

Malate dehydrogenase (EC 1.1.1.37) activity (mitochondrial matrix) was assayed spectrophotometrically by measuring the rate of oxidation of NADH [17]. Amine oxidase (EC 1.4.3.4) activity (mitochondrial outer membrane) was assayed by the method of Wurtman and Axelrod [18]. Cytochrome *c* oxidase (EC 1.9.3.1) activity was measured polarographically [19] in a medium containing: 50 mM potassium citrate buffer of pH 6.5, 1% (w/v) Brij 58, 2.8 μM cytochrome *c*, 3.3 mM ascorbate, 0.16 mM *N,N,N',N'*-tetramethyl *p*-phenylenediamine and about 0.3 mg of protein in a total volume of 3.0 ml at 25°C. The low pH of 6.5 was selected to reduce the background utilization of oxygen due to autoxidation of endogenous catecholamines, and the cytochrome *c* oxidase activity was defined as the cyanide-sensitive oxygen consumption; the final concentration of KCN was 3.3 mM.

Glucose-6-phosphatase (EC 3.1.3.9) activity (microsomal vesicles) was assayed as described [20]. The concentration of glucose 6-phosphate was 30 mM, and the liberated phosphate was measured as described [21].

Other enzymic assays

NAD(P)H:ferricyanide oxidoreductase activities were measured as previously described [3]. NAD(P)H-dependent aldehyde reductase activity was assayed by a standard procedure [22] using glyceraldehyde as the substrate. NAD(P)H: (acceptor) oxidoreductase activities were also assayed using dehydroascorbate or catecholamine quinones as acceptors. The incubation mixture contained 50 mM potassium citrate buffer (pH 6.5), 0.05 mM NAD(P)H, and 1.0 mM dehydroascorbate or 0.1—1.0 mM catecholamine quinones generated from dopamine, noradrenaline and adrenaline by monophenol monooxygenase (EC 1.14.18.1). The rate of NAD(P)H oxidation was measured as described [3].

Other analytical procedures

Protein was determined by the Coomassie brilliant blue method of Bradford [23]; the endogenous catecholamines in the chromaffin granule ghosts do not interfere with the assay of protein by this method [24]. Bovine serum albumin was used as standard [25].

Chemicals

Percoll was supplied by Pharmacia (Uppsala, Sweden). NADH, NADPH, Brij 58, and bovine heart cytochrome *c* (Type IV) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monophenol monooxygenase was obtained from Worthington Biochemical Co. (NJ, U.S.A.) and dehydroascorbate from Fluka AG (Switzerland). Coomassie brilliant blue solution for protein assay was obtained from the Bio Rad Laboratories (CA, U.S.A.). Triton X-100 was the product of Serva Feinbiochemia (Heidelberg, F.R.G.). *N,N,N',N'*-tetramethyl *p*-phenylenediamine was obtained from Eastman (NY, U.S.A.), and ascorbate from Merck (Darmstadt, F.R.G.).

Results

Evaluation of the purity of various populations of chromaffin granules isolated by rate zonal centrifugation in 1.6 M sucrose

In the standard preparations [13] of the total population of chromaffin granule ghosts, obtained by the standard lysis procedure and no further purification, significant activities of cytochrome *c* oxidase, malate dehydrogenase and amine oxidase were always measured (Table I).

After centrifugation of the granule ghost preparations on a discontinuous density gradient (Fig. 1), four fractions were recovered. Fraction I represented the suspending medium only, without any measureable amount of protein or enzyme activity. Fraction II, which accounted for approx. 80% of the applied protein ($n = 4$), was a narrow, pink coloured band of chromaffin granule ghosts formed at the interphase between the suspending medium and 0.9 M sucrose. Fraction III was slightly turbid, and accounted for less than 5% of the protein applied. Fraction IV, which accounted for approx. 15% of the applied protein ($n = 4$), was a firm, brownish pellet of mitochondria and probably other organelles including some incompletely lysed chromaffin granules.

Measurements of marker enzymes revealed that the specific activity of dopamine β-monooxygenase and the specific content of cytochrome *b*-561 in the ghost preparations increased by approx. 20%, whereas that of cytochrome *c* oxidase, malate dehydrogenase (latent) and amine oxidase was lowered by approx. 92%, 65% and 50%, respectively, by this centrifugation step (Table I). The specific activity of the microsomal marker enzyme glucose-6-phosphatase did not change at all (Table I), and was found to be about 10% of that present in the isolated microsomes. The data given in Table I, therefore, clearly show that the chromaffin granule ghost preparations, isolated essentially by the classical procedure of Smith and Winkler [13], is contaminated by mitochondria, although it is difficult to estimate exactly the degree of this contamination based on marker enzymes. It is evident, however, that an appreciable purification with respect to mitochondria was obtained by the centrifugation step on 0.9 M sucrose (Fig. 1). First, the specific activity of cytochrome *c* oxidase was reduced to less than 8% (Table I) of that present in the standard

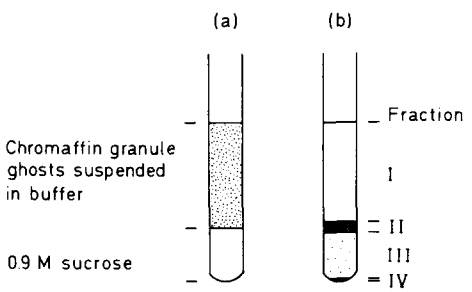


Fig. 1. Outline of the procedure to fractionate chromaffin granule ghosts on 0.9 M sucrose (for experimental details, see Methods). (a) Initial state: Chromaffin granule ghosts suspended in 10 ml of 50 mM potassium citrate buffer, pH 6.5, layered over 4.0 ml of 0.9 M sucrose. (b) After centrifugation at a time integral of $\int_0^t (\text{rev./min})^2\, dt = 1.4 \cdot 10^{10}$ min^{-1} ($t = 90$ min). Fraction I, suspending medium; fraction II, chromaffin granule ghosts; fraction III, 0.9 M sucrose (slightly turbid); fraction IV, pellet.

TABLE I

MARKER ENZYME ACTIVITIES IN THE TOTAL POPULATION OF CHROMAFFIN GRANULE GHOSTS, THE MITOCHONDRIAL AND THE MICROSOMAL FRACTIONS

Data were obtained from randomly selected preparations; the numbers are given in parentheses. Specific activities given as (a) nmol/min per mg protein, i.e. octopamine formed (dopamine β-monooxygenase), NADH oxidized (NADH: ferricyanide oxidoreductase and malate dehydrogenase), oxygen consumed (cytochrome *c* oxidase) and phosphate liberated (glucose-6-phosphatase) or as (b) c/20 min per mg protein (amine oxidase); the content of cytochrome *b*-561 is given in nmol per mg protein (c).

Marker enzyme	Cell fraction			
	Chromaffin granule ghosts		Mitochondrial fraction	Microsomal fraction
	As obtained from granules isolated in 1.6 M sucrose	Gradient-purified		
Dopamine β-monooxygenase (a)	1800 ± 200 (6)	2200 ± 200 (6)	N.D. *	N.D.
Cytochrome *b*-561 (c)	6.1 ± 1.6 (7)	7.4 ± 1.2 (7)	N.D.	N.D.
NADH: ferricyanide oxidoreductase (a)	524 ± 70 (9)	366 ± 63 (8)	3193 ± 900 (5)	2250
Malate dehydrogenase (a)				
—Triton X-100	36	N.D.	700	N.D.
+Triton X-100	188 ± 86 (15)	67 ± 16 (7)	1062 ± 450 (7)	N.D.
Amine oxidase (b)	4762 ± 762 (5)	2431 ± 424 (4)	35 700 ± 2000 (3)	N.D.
Cytochrome *c* oxidase (a)	5.70 ± 2.0 (7)	0.55 ± 0.44 (7)	235 ± 62 (5)	N.D.
Glucose-6-phosphatase	1.8 ± 0.2 (16)	1.8 ± 0.2 (7)	N.D.	18.6

* N.D., not determined.

TABLE II

MARKER ENZYME ACTIVITIES IN GHOST PREPARATIONS FROM THE LIGHT AND HEAVY POPULATION OF CHROMAFFIN GRANULES

Data were obtained from randomly selected preparations; the numbers are given in parentheses. Specific activities are in nmol/min per mg protein (a) (see text in Table I); the content of cytochrome *b*-561 is given in nmol per mg protein (b).

Marker enzyme	Chromaffin granule ghosts			
	As obtained from granules isolated in 1.6 M sucrose		Gradient-purified	
	Light granules	Heavy granules	Light granules	Heavy granules
Dopamine β-monooxygenase (a)	N.D. *	N.D.	1200 ± 220 (7)	1700 ± 100 (9)
Cytochrome *b*-561 (b)	N.D.	N.D.	6.4 ± 0.9 (5)	7.2 ± 1.1 (5)
NADH: ferricyanide oxidoreductase (a)	501 ± 90 (5)	844 ± 200 (6)	513 ± 107 (5)	432 ± 92 (6)
Malate dehydrogenase (a)	91 ± 25 (5)	281 ± 70 (6)	60 ± 10 (5)	70 ± 11 (6)
Glucose-6-phosphatase (a)	1.8 ± 0.3 (5)	4.6 ± 0.5 (5)	1.8 ± 0.3 (5)	3.5 ± 0.4 (6)

* N.D., not determined.

preparation, indicating that intact mitochondria were effectively removed. Secondly, the specific activity of malate dehydrogenase was reduced to 35% of that in the original standard membrane preparations, but this figure may well be too high since the latency factor of malate dehydrogenase was 2 in the mitochondria recovered in the pellet ($n = 4$) and about 5 when measured in the granule ghost preparations (Table I). Thus, malate dehydrogenase activity is in some way enhanced by the presence of chromaffin granule ghosts when measured in the presence of Triton X-100. A decrease in the specific activity of amine oxidase, on the other hand, was only about 50%.

In the ghost preparations of the heavy and light subpopulations, of chromaffin granules, the specific activity of malate dehydrogenase (latent) was found to be significantly higher in the ghosts derived from the heavy granules as compared to those obtained from the light granules (Table II). Following purification on 0.9 M sucrose (Fig. 1), however, the membranes derived from both types of granules revealed the same latent malate dehydrogenase activity (Table II), also very similar to that found for the purified membranes of the total population (Table I). On the other hand, the microsomal marker enzyme glucose-6-phosphatase was found to be higher in the membranes of the heavy as compared to the light granules both before and after the purification step on 0.9 M sucrose (Table II). From the data in Tables I and II, it is evident that the purified membranes of the light granules are the most pure ones of the various preparations obtained. The reduced minus oxidized difference spectrum of the

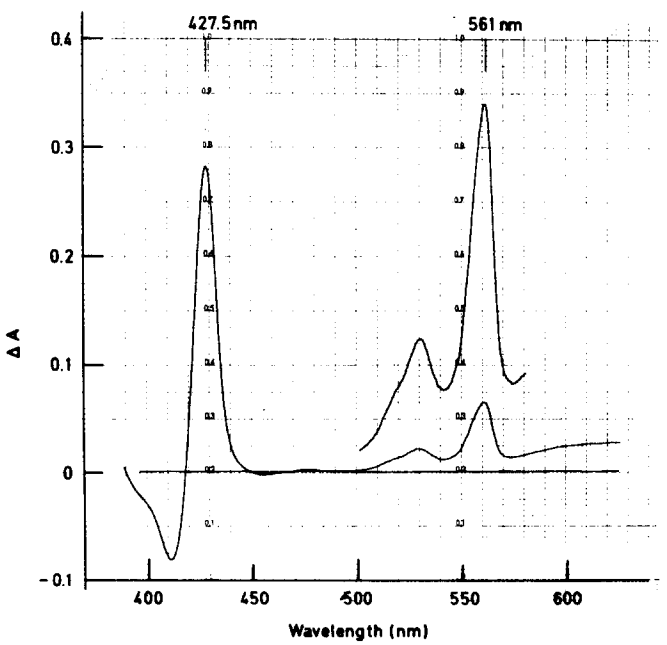


Fig. 2. The reduced minus oxidized difference spectrum of highly purified chromaffin granule ghosts isolated from light chromaffin granules. The membranes were solubilized by 1% (w/v) Triton X-100, and the baseline recorded following the addition of 10 μM ferricyanide to both cuvettes. Solid dithionite was then added to the sample cuvette and the reduced minus oxidized spectrum was recorded. The inset represents the spectrum at scale expansion X5. The lightpath was 1 cm.

highly purified ghost preparation of the light granules (Fig. 2) revealed the characteristic absorption bands at 561 nm and 427.5 nm [3], but very little absorbance was observed in the 450 nm region characteristic of flavoproteins.

When the chromaffin granules were isolated by isopycnic gradient centrifugation on Percoll-sucrose [11], the specific activity of malate dehydrogenase in the granule ghost preparation was on an average (n = 6) the same as that found for granules isolated by the method of Smith and Winkler.

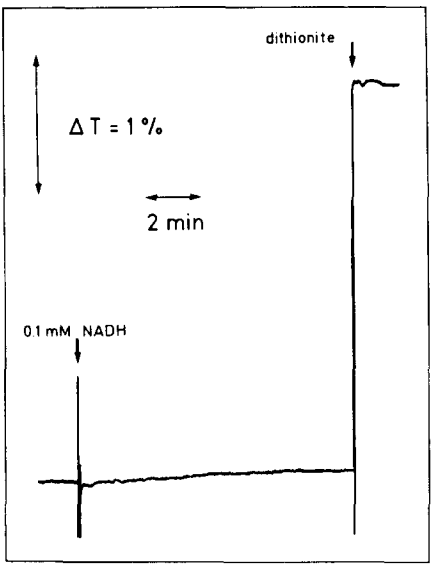
NADH:(acceptor) oxidoreductase activities in preparations of chromaffin granule ghosts

From Table I it is seen that the preparations of total chromaffin granule ghosts have a high NADH:ferricyanide oxidoreductase activity (524 nmol/min per mg protein; n = 9) in accordance with previous findings [5,6]. Following the purification step on 0.9 M sucrose (Fig. 1), the specific activity was reduced to 366 nmol/min per mg protein, i.e. to approx. 70% of the original value, and the relative specific activity of NADH:ferricyanide oxidoreductase to dopamine β-monooxygenase or cytochrome *b*-561 was lowered by 43%. From this finding it may be concluded that about 40% of the NADH:ferricyanide oxidoreductase activity previously measured in granule ghost preparations [3,5—7] is contributed by contaminating organelles and membrane fragments, notably mitochondria. Furthermore, it is seen that following the purification step on 0.9 M sucrose (Fig. 1), the specific activity of the NADH:ferricyanide oxidoreductase changed in a different manner when the granule ghosts obtained from heavy and light granules were compared (Table II). In the ghosts of the light granules, in which the specific activity of malate dehydrogenase was lowered by 32% (n = 5), the specific activity of NADH:ferricyanide oxidoreductase revealed a slight increase (approx. 3%). In the ghosts prepared from heavy granules, with a higher degree of mitochondrial contamination, the specific activity of malate dehydrogenase and NADH:ferricyanide oxidoreductase were both markedly lowered by the final purification step (Table II). However, in the highly purified membrane preparations of both populations of granules almost the same NADH:ferricyanide oxidoreductase activity and cytochrome *b*-561 content was observed (Table II).

We have not been able to detect any NAD(P)H:(acceptor) oxidoreductase activity with glyceraldehyde, dehydroascorbate [26] and catecholamine quinones as potential acceptors in any preparation of chromaffin granule membranes tested (i.e., no increase in NADH oxidase activity above the background due to endogenous acceptors [5]).

Reduction of the endogeneous cytochrome b-561 by NADH

From Fig. 3 it is seen that 0.1 mM NADH induces only a very slow reduction of cytochrome *b*-561 in preparations of chromaffin granule ghosts purified on 0.9 M sucrose, and that less than 1% of the cytochrome is reduced within a period of 2 min. This finding is at variance with previous observations [3,6] using membranes of lower purity. On the other hand, when preparations of purified mitochondria are added to the highly purified ghost preparations, a NADH-dependent reduction of the endogenous cytochrome was again observed (Fig. 4). The rate as well as the degree of reduction at equilibrium, increased in



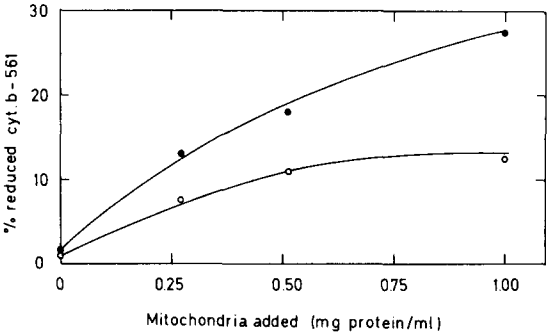


Fig. 3. The reduction of cytochrome *b*-561 in highly purified chromaffin granule ghosts by NADH. Highly purified chromaffin granule ghosts were suspended in 50 mM potassium citrate buffer, pH 6.5 (0.3 mg protein · ml^{-1}), and NADH was added to a final concentration of 0.1 mM. The reduction of cytochrome *b*-561 was followed by the wavelength-pair 561 nm/575 nm; solid dithionite was added to obtain the cytochrome in the fully reduced form.

Fig. 4. The effect of added mitochondria on the reduction of cytochrome *b*-561 in highly purified chromaffin granule ghosts by NADH. Chromaffin granule ghosts of low latent malate dehydrogenase activity was suspended in 50 mM potassium citrate buffer, pH 6.5 (0.75 mg protein · ml^{-1}), and increasing amounts of purified mitochondria was added. The reduction of cytochrome *b*-561 was initiated by 0.1 mM NADH, and the degree of reduction measured at t = 2 min (○) and t = 6 min (●); for experimental details, see legend to Fig. 3.

proportion to the amount of mitochondrial protein added (Fig. 4), and the maximal degree of reduction obtained (approx. 30%) compares well with that reported by Hollenbeck et al. [6] using a partly purified preparation of chromaffin granule ghosts.

Discussion

In the characterization of the biochemical properties of chromaffin granules isolated from bovine adrenal medulla, the purity of the granule preparations obtained is of critical significance, and particularly important in studies of NADH:(acceptor) oxidoreductase activities since such activities are widely distributed throughout the membrane systems of mammalian cells [27]. Of particular interest and significance is the possibility of a mitochondrial contamination since adrenal medulla mitochondria revealed an s value in 0.25 M sucrose (i.e., 6190 S) intermediate between the heavy ($s_H \simeq$ 12 400 S) and the light ($s_L \simeq$ 3900 S) population of chromaffin granules [10]. Although the classical sucrose gradient centrifugation procedure of Smith and Winkler [13], e.g. using 1.6 M sucrose, separated the chromaffin granules (as a pellet) from

the bulk of mitochondria (at the interphase of 0.25 M and 1.6 M sucrose), several workers have reported some activity of mitochondrial marker enzymes in the chromaffin granule pellet [28] whereas it has been denied by others [29] in agreement with electron microscopic studies [30].

The data reported in this paper, using biochemical methods, show that the chromaffin granules obtained by the procedure of Smith and Winkler [13] is always contaminated to a certain extent by mitochondria or mitochondrial fragments (Fig. 1 and Table I). It is difficult to estimate exactly the degree of such contamination, but it is markedly lowered by centrifugation of the granule ghost preparations on a discontinuous density gradient (Fig. 1 and Tables I and II). The recovery of protein in the pellet obtained by this procedure was on an average $\simeq$15% of the total amount applied. Since 98% of the dopamine β-monooxygenase activity was recovered at the interphase (the specific activity increased by approx. 20%), it is likely that most of the material removed in the pellet represents non-chromaffin granule protein, partly of mitochondrial origin. It should be noted, however, that the same degree of purity (based on assay of marker enzymes) was obtained whether crude preparations of the total, heavy or light populations of the granules were selected as the starting material (Table I and II). Based on measurements of cytochrome *c* oxidase activity, less than 0.2% of the protein of the purified granule ghosts may be derived from mitochondria.

From Tables I and II it is seen that the highly purified preparations of chromaffin granule ghosts have a significant and constant specific activity of NADH:ferricyanide oxidoreductase. Several lines of evidence support the notion that this residual enzymic activity has a true localization to the chromaffin granule membrane. First, on purification of the granule ghosts by centrifugation on 0.9 M sucrose, there is no proportional decrease in the activities of the mitochondrial marker enzymes and the NADH:ferricyanide activity (Table I). In particular, when the crude light population of granules was used as the starting material, the final purification step on a discontinuous density gradient resulted in a slight increase in the specific activity of NADH: ferricyanide oxidoreductase in contrast to that of malate dehydrogenase (Table II). It is therefore unlikely that this activity can be accounted for by mitochondrial contamination. Secondly, a contribution of microsomal vesicles is unlikely due to the efficient removal of microsomes by sucrose gradient centrifugation [29] and the low specific activity of glucose-6-phosphatase measured in the granule ghost preparations (Tables I and II). No correlation was observed between the NADH:ferricyanide oxidoreductase activity and the glucose-6-phosphatase activity (Table II), and no cytochrome *P*-450 has been detected in such preparations [31]. It should also be mentioned that glucose-6-phosphatase activity is not a specific microsomal marker [32]. Thirdly, it has been reported [7] that the H^+-ATPase (which is an integral protein of the granule membrane) and NADH:(acceptor) oxidoreductase activities of granule membrane preparations both have been found to reveal discontinuous Arrhenius temperature versus activity relationships with 'transitions' at 33°C, which is removed by treatment with the non-ionic detergent Triton X-100. Finally, it is unlikely that the amine oxidase activity is largely derived from the mitochondrial outer membrane. Thus, the highly purified ghost preparations

revealed almost no NADH:ferricytochrome *b*-561 activity (Fig. 3) characteristics of partly purified chromaffin granule ghosts [3,6] and that induced by adding mitochondria to highly purified granule ghosts (Fig. 4). The possibility that amine oxidase is an enzyme not exclusively confined to the mitochondrial outer membrane has also been considered in subfractionation studies of rat liver microsomes [33].

Thus, all experimental evidence obtained so far point to the NADH:(acceptor) oxidoreductase as a true activity of the chromaffin granule membrane, and the question is now the nature of the physiological electron acceptor. Although previous studies have pointed to the membrane-bound cytochrome *b*-561 as a likely candidate [3,6], this possibility is almost excluded by the present study. In the highly purified granule ghost preparations, having high NADH:ferricyanide activities, even high concentrations of NADH were unable to induce any significant reduction (>1%) of cytochrome *b*-561 (Fig. 3). Since the addition of purified mitochondrial fractions resulted in a substantial, although slow, reduction of cytochrome *b*-561 in the granule ghosts, it is likely that the reduction reported previously in chromaffin granule ghost preparations [3,6,9] is due to mitochondrial contamination. Furthermore, no NADH:dehydroacorbate, NADH:catecholamine quinone, and NADH:aldehyde oxidoreductase activities were detected in the preparations. Thus, the question of the physiological acceptor of this NADH dehydrogenase remains to be answered. Finally, it should be mentioned that no vectorial transport of H^+, linked to oxidation of NADH, has been detected in the ghost preparations (Flatmark, T., unpublished data). In general, the problems faced with in studies on the chromaffin granule NADH:(acceptor) oxidoreductase are very similar to those reported for the corresponding activity in plasma membranes [34], including the very low flavin content (Fig. 2).

The present study gives no indication as to the possible functional significance of cytochrome *b*-561 in the granule membrane, but confirms our previous suggestion [5] that it at least faces the cytosolic (outer) side of the membrane. Thus, cytochrome *b*-561 establishes a redox equilibrium with exogenously added bovine heart cytochrome *c* [5] and is reduced by NADH when mitochondria are present (Fig. 4). It should be noted that cytochrome *b*-561 is rapidly reduced by ascorbate in a concentration-dependent manner [25]. This finding, together with the observation that ascorbate is present at high concentrations both in the cytosol [35] and in the granule matrix compartment [36], points to the possibility that the cytochrome in some way functions as a redox buffer in the membrane. Finally, it should be noted that the measured specific content of cytochrome *b*-561, being the same in ghost preparations of light and heavy granules (Table II), was higher than previously reported [31]. This difference is explained partly by the use of more efficient lysis procedure in the present study and partly by the use of a new assay of protein in which there is no interference by catecholamines [24]. Finally, the protein assay of Bradford [23] may underestimate the amount of protein when compared to the Lowry method using bovine serum albumin as standard [37].

Acknowledgements

This work was supported by the Norwegian Council for Research on Mental Retardation and Nordisk Insulin Foundation. O.T. is a Fellow of the Norwegian Research Council for Science and the Humanities.

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