Determination of the proportion of sealed vesicles in a preparation of chromaffin granule membrane 'ghosts'

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Received 1 June 1982

Chromaffin *Membrane* *Lectins* *Antibodies* *Complement* *Glycoproteins*

1. INTRODUCTION

The secretory granules of the adrenal medulla, known as chromaffin granules, accumulate and store high concentrations of catecholamines, nucleotides, Ca^{2+} and other components [1]. Because of their ready availability and similarity to other types of secretory vesicles, chromaffin granules are a favoured model system for the study of active transport [2]. However, the intact granules have certain disadvantages for transport studies: when purified by differential centrifugation they are contaminated by mitochondria and other organelles, but further purification by isopycnic centrifugation tends to inactivate the transport system; lysis of some granules during incubation may significantly alter the concentration of substrates in the bathing medium; the activities of solutes within the granule matrix cannot be measured; and because of the high concentration of matrix components, any transport observed may be the result of exchange across the granule membrane, rather than net accumulation.

These difficulties are largely overcome by studying resealed chromaffin granule membrane vesicles (ghosts), and a number of groups have used such preparations [3–8]. Although the 'ghosts' appear in the electron microscope to be extensively resealed [3], it is not known what fraction of them is leaky or everted. Since such incorrectly sealed or broken membranes would be inactive in ATP- driven membrane transport, the measured specific activities of transport procecces in 'ghosts' may be underestimates of the true rates.

The asymmetry of biological membranes allows discrimination between correctly sealed vesicles and inside-out or broken membranes, on the basis of exposure of different proteins. We now report experiments in which we used specific antisera to measure the fraction of sealed vesicles in our 'ghost' preparations, and affinity chromatography on columns of lectin–Sepharose and IgG–Sepharose to remove broken and everted vesicles, thereby increasing the specific activity of ATP-dependent amine uptake by the 'ghosts'.

2. MATERIALS AND METHODS

Rabbit antiserum to chromaffin granule membrane glycoprotein III [9] was the gift of Professor H. Winkler (University of Innsbruck). Cytochrome b_{561} and soluble dopamine β-hydroxylase (DBH) were prepared as in [10,11] and antisera raised in rabbits by conventional means. Wheat-germ agglutinin (WGA) and *Ricinis communis* agglutinin 1 (RCA-1) were obtained from BDH, and coupled directly to Sepharose 4B [12]. Immunoglobulins were purified on DEAE–Affigel Blue (Bio-Rad) before coupling to Sepharose. Coupling yields (mg protein/ml Sepharose) were: serum albumin, 1.5; pre-immune IgG, 1.3; anti-DBH, 2.0; RCA-1, 0.8; WGA, 0.8. Affinity columns (2.5 ml bed vol.) were run in 0.3 M sucrose/10 mM Hepes (pH 7.2)/0.05% NaN_3, at 4°C and were loaded with 0.5–1.0 mg 'ghosts'.

Complement fixation [13] was performed at 37°C in micro-titre plates, each well containing sensitized sheep erythrocytes (diluted 1:60), guinea pig serum

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

(1:80), heat-treated antiserum (1:100) and various dilutions of antigen, in 0.3 M sucrose, 0.1% serum albumin, buffered with Oxoid buffer tablets.

Chromaffin granule membranes and 'ghosts' were prepared and assayed for uptake of 5-hydroxytryptamine as in [14]. ATPase activity was measured in a coupled assay system containing 2 mM ATP, 10 mM $MgSO_4$, 1 mM phosphoenol-pyruvate, 0.2 mM NADH, 3 units lactate dehydro-genase/ml, 3 units pyruvate kinase/ml, 50 mM Hepes–KOH (pH 7.2), 0.3 M sucrose. Ghosts (5 mg/ml) were radiolabelled with ^{32}P by incubation with 55 μM [γ-^{32}P]ATP (spec. radioact. 1.7×10^{13} Bq/mol), 10 mM $MgSO_4$, 1 mM $MnSO_4$, 0.3 M sucrose, 10 mM Hepes–NaOH (pH 7.2), for 5 min. at 30°C. This procedure had no effect on the specific activities of amine uptake or ATP hydrolysis.

3. RESULTS AND DISCUSSION

3.1. *Topical specificity of antisera*

By the sensitive immune replica technique [15] we showed that each antiserum was specific for a single membrane protein, while pre-immune serum did not react with any membrane component. In micro-complement fixation, anti-cytochrome *b*-561 reacted strongly with intact chromaffin granules, granule membrane fragments and resealed 'ghosts'. This supports the finding [16,17] that cytochrome *b*-561, previously known as chromomembrin B, is exposed on the cytoplasmic face of the granule. Cytochrome *b*-561 appears on SDS–polyacryl-amide gels as a characteristic doublet, M_r 26 000–28 000 [10]; treatment of intact granules with pronase ([17] fig.1a) results in the disappearance

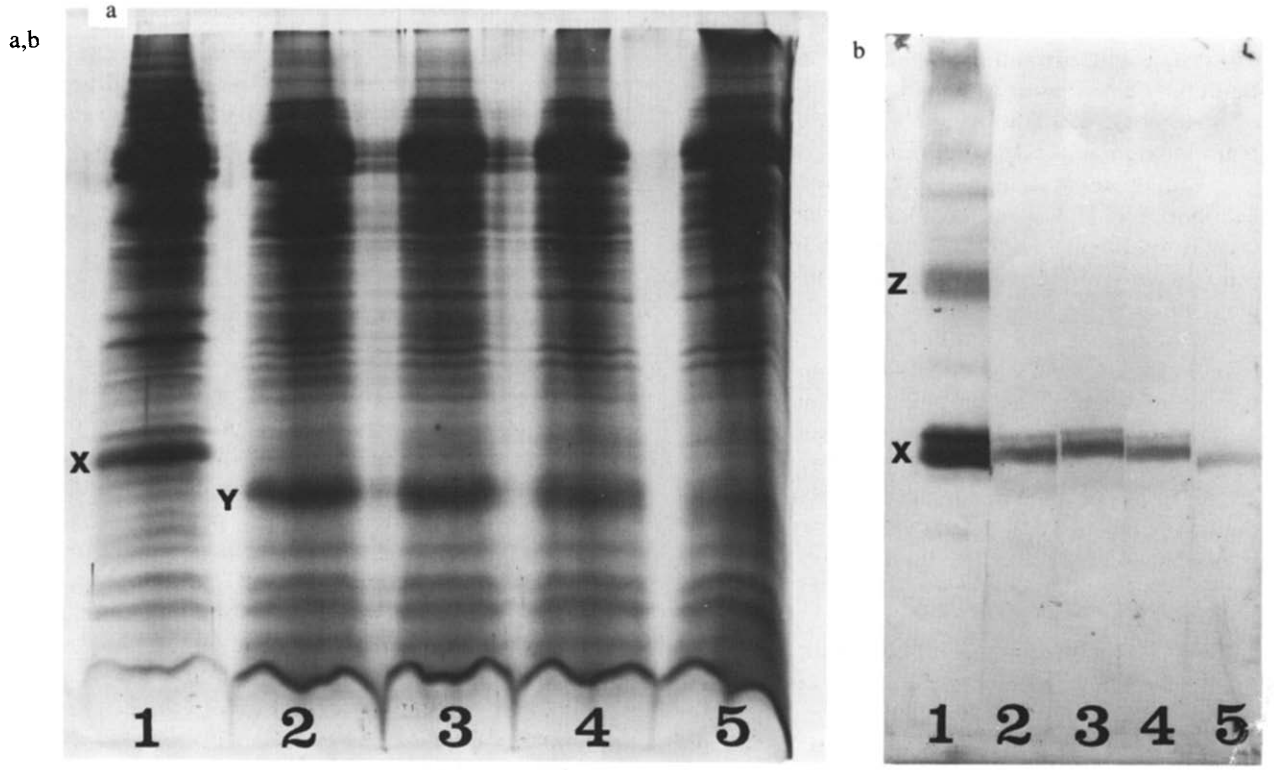

Fig.1. Proteolytic removal of antigenic determinants from cytochrome *b*-561 in intact chromaffin granules. Chromaffin granules were subjected to proteolytic digestion [17], purified by isopynic centrifugation, and the membranes isolated and subjected to SDS–polyacrylamide gel electrophoresis: (a) stained gel; (b) autoradiograph of an immune replica [15] of tracks cut from a similar gel. After electrophoretic transfer of proteins to cellulose nitrate, the replca was washed with anti-cytochrome *b*-561 serum and ^{125}I-protein A. Control incubations: (1) room temperature, 4 h. Protease incubation (0.1 mg pronase/ml, room temperature): (2) 10 min (3) 1 h; (4) 2 h; (5) 4 h. Band X is cytochrome *b*-561; Y its putative digestion product; Z is thought to be undissociated cytochrome oligomer.

of this band, and the abolition of reactivity with anti-cytochrome *b*-561 serum (fig.1b). A new band (M_r 22 000–25 000) appears during pronase digestion, and is probably a degradation product of the cytochrome; it shows very weak reactivity with the antiserum, and is further degraded during prolonged incubation. The fact that the immune reactivity of intact granules is susceptible to protease suggests that our antiserum against cytochrome *b*-561 reacts only with determinants exposed on the outer (cytoplasmic) face, even though cytochrome *b*-561 is a transmembrane protein [17]. Similar results were obtained on digestion with papain, trypsin or *Staphylococcus aureus* V8 protease (not shown).

Complement fixation experiments with anti-DBH and anti-glycoprotein III showed that these antisera reacted strongly with broken membranes and 'ghosts', but only very weakly with preparations of intact granule. DBH and the carbohydrate of glycoprotein III have been shown to be exposed only on the inner (extracytoplasmic) face of the granule membrane [9,17] and our antibodies also recognize determinants on this face only, since the reactivity of chromaffin granules with this serum is completely resistant to protease digestion.

Lysis of granules releases soluble DBH which may contaminate 'ghosts', so only the antisera against glycoprotein III and cytochrome *b*-561 were used in quantitative complement fixation experiments.

3.2. *Determination of the proportion of sealed 'ghosts' by quantitative complement fixation*

A single concentration of each antiserum was titrated with a range of concentrations of chromaffin granule membranes and of resealed 'ghosts' (fig.2). With anti-glycoprotein III, a higher concentration of 'ghosts' than of membrane fragments was required to achieve 50% complement fixation, as would be expected if a significant fraction of the 'ghosts' is correctly sealed, with the antigenic determinants buried, while in the membranes these determinants were all available for reaction. The ratio of titres is 2.20, indicating that 55% of the 'ghosts' are correctly resealed. This antiserum cannot distinguish be-

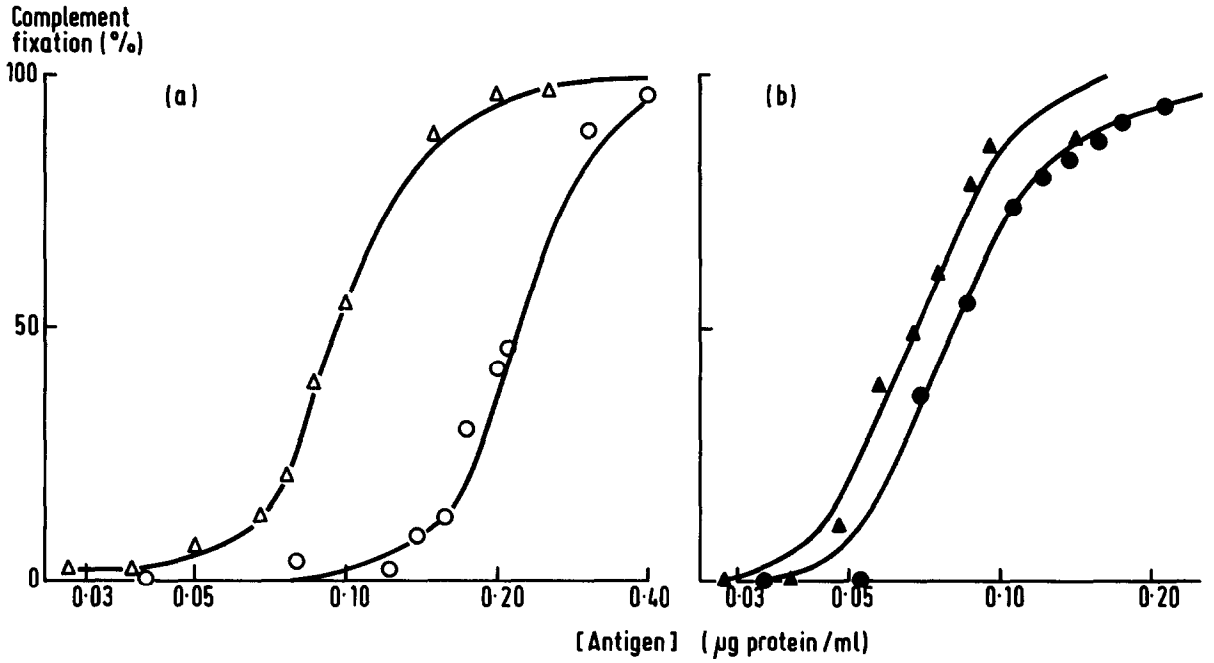

Fig.2. Complement fixation titration of chromaffin granule membranes and 'ghosts'. Antisera against (a) glycoprotein III and (b) cytochrome *b*-561 were titrated with chromaffin granule membrane fragments (△, ▲) and 'ghosts' (○, ●).

Table 1

Activities of chromaffin-granule membrane 'ghosts' eluted from lectin immunoglobulin–Sepharose columns

	Uptake of 5-hydroxytryptamine (nmol . min^{-1} . mg protein^{-1})			Uncoupler-stimulation of ATPase (%)	
Experiment:	1	2	3	1	2
(Untreated ghosts)	7.9	7.0	7.0	21	24
Albumin–Sepharose	5.9			31	
Pre-immune-IgG–Sepharose		3.8	5.1		29
Anti-DBH–Sepharose		8.0	7.5		48
Ricin (RCA–1)–Sepharose	9.8			36	
WGA–Sepharose	10.9			33	

In expt. 1 and 2 the affinity columns were run at 4°C; in expt. 3, at 20°C. The unstimulated rate of ATP hydrolysis was 140 nmol . min^{-1} . mg protein^{-1}: uncoupler stimulation is the fractional increase in rate produced by the addition of 1 μM FCCP

tween everted and broken membranes, but such a distinction is possible using anti-cytochrome *b*-561 serum, since this would react with correctly resealed 'ghosts' and with membrane fragments, but not with everted vesicles. Here, the ratio of titres is 1.13, suggesting that 11% of the ghosts are everted. This result could, however, derive from a weak reaction of the antiserum with cytochrome determinants on the inner face of the membrane, which are exposed in membrane fragments but not in resealed 'ghosts'.

3.3. *Resolution of sealed and unsealed 'ghosts' by affinity chromatography*

Since a significant fraction of the ghosts appeared to be broken or everted, as indicated by complement fixation with anti-glycoprotein III serum, we attempted to resolve the 'ghost' preparation with affinity columns which would remove only those membranes exposing proteins from the extracytoplasmic granule face. Since the carbohydrates of chromaffin-granule membranes are not exposed to the cytoplasm [9], lectin columns are suitable for this purpose. Concanavalin A–Sepharose reacts with numerous chromaffin granule membrane protein [9,18], but its binding in inhibited by sucrose, and we have been unable to prepare active 'ghosts' in other media. We therefore used WGA and RCA-1, attached to Sepharose; both lectins react with a number of proteins [18], although it has been reported that RCA-1 fails to bind SDS-solubilized glycoproteins [9], and that WGA reacts even with intact granules [19]. Sepharose-bound anti-DBH globulin was also used.

Two criteria were employed in assessing intactness of the eluted 'ghosts': the specific activity of ATP-driven amine uptake, using 5-hydroxytryptamine as a substrate, and the sensitivity of ATPase activity to protonophore uncouplers, such as FCCP. 5-Hydroxytryptamine is accumulated by the same mechanism as catecholamines, and would not be taken up by membrane fragments, nor by everted ghosts, in which the ATPase active site is occluded. The ATPase activity of chromaffin granules and 'ghosts' is stimulated by uncouplers, which collapse the membrane potential developed as a result of H^+-translocation; such stimulation should not occur unless the ghosts are sealed.

The results of these experiments are shown in table 1. Specific activity determinations could be complicated by leakage of lectins or IgG from the columns, or by binding of contaminating soluble proteins (such as DBH) in the 'ghost' preparation;

we therefore labelled the 'ghosts' before fractionation, using [γ-^{32}P]ATP and the endogenous phosphatidylinositol kinase [20], and assayed uptake of 5-hydroxy[^{14}C]tryptamine into the eluted ghosts by measuring $^{14}C/^{32}P$ ratios.

The specific activity of amine uptake is significantly increased by passage of the 'ghosts' through lectin or anti-DBH columns, but quantitative interpretation of the results is complicated by the fact that control columns, of serum albumin or pre-immune serum, reduced the specific activity of uptake. This means that, quite apart from the time and expense involved, affinity chromatography is unlikely to be of value in preparing 'ghosts' with high transport activities.

4. CONCLUSION

Complement fixation titration suggests that 55% of our 'ghosts' membranes are correctly resealed, 11% sealed inside out, and the remaining 34% broken. This conclusion is critically dependent on the specificity of the antibodies, and it is a surprising and fortunate finding that the very strongly reacting anti-cytochrome *b*-561 serum is directed only against the cytoplasmic, protease-sensitive region of this antigen.

In reasonable agreement with this result, lectin–Sepharose and anti-DBH–Sepharose columns produce a 1.5–2.0-fold increase in the specific activity of amine uptake, relative to the control columns. Since the phosphatidyl inositol kinase used to label the ghosts is external [17,20], everted ghosts would presumably not become labelled on incubation with [γ,^{32}P]ATP; but the specific radioactivity of the 'ghosts' was unchanged after passage through the columns, which again suggests that everted ghosts are only a small fraction of the total, and that the increase in the activity of amine uptake is a result of the removal of labelled membrane fragments.

In contrast, stimulation of ATPase activity by 1 μM FCCP is only marginally increased by the columns. This stimulation is in any case small, suggesting that ATPase activity is largely uncoupled from inhibition by the membrane potential, even in correctly sealed 'ghosts'.

Our results indicate that the specific activities of amine uptake determined with 'ghosts' preparations of this type [14,21] underestimate the true rate, which should be increased by a factor of ~ 2, to 15–25 nmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1}, at 37°C. Although this value is among the highest reported, it is not large in absolute terms, and suggests that there may be rather few molecules of catecholamine transporter in each granule. The fraction of 55% 'ghosts' sealed appears constant; presumably this depends on the conditions under which the membranes are resealed, rather than an equilibrium between membranes in different configurations. 'Ghosts' prepared by other procedures may be correctly resealed to greater or lesser extents, which would partly account for variations in the reported specific activities of uptake.

ACKNOWLEDGEMENTS

This work was supported by grants from The Medical Research Council and the Scottish Home and Health Department. We are indebted to Dr John Phillips and Professor Hans Winkler for discussions of this work and for gifts of materials.

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