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# Solubilization and Reconstitution of the Adenosine 5′-Triphosphate Dependent Proton Translocase of Bovine Chromaffin Granule Membrane†

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ABSTRACT: The ATP-dependent proton translocase of chromaffin granule membrane has been solubilized with bile salts and nonionic detergents and reconstituted in vesicular structures by removal of the detergent. Solubilization changed the properties of the ATPase which was no longer inhibited by *N,N′*-dicyclohexylcarbodiimide and trisubstituted tin derivatives. Reconstituted vesicles contained a considerable fraction of the solubilized ATPase, but the $H^+$ pump activity varied with the conditions used for solubilization and reconstitution. Small vesicles (50-nm diameter) possessing an active $H^+$ pump were reconstituted from deoxycholate extracts of the membrane after dilution of the detergent and from cholate extracts after adsorption of the detergent on Biobeads SM-2. Their ATPase was inhibited by *N,N′*-dicyclohexylcarbodiimide and trisubstituted tin derivatives and was resistant to oligomycin. Upon addition of ATP, a transmembrane potential was generated which was monitored with the extrinsic fluorescent probe OX-V [bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethineoxonol]. Imposition of an artificial proton electrochemical gradient to the vesicle interior reversed the ATPase reaction and induced ATP synthesis. Moreover, the vesicles accumulated *l*-noradrenaline in the presence of ATP, suggesting some reconstitution of the complete system of catecholamine uptake. In contrast, when nonionic detergents were used for solubilization, reconstitution of the $H^+$ pump either by dilution or by adsorption of the detergent was unsuccessful.

Chromaffin granules are organelles which are involved in the synthesis, storage, and release of adrenaline and noradrenaline by adrenal medulla. In presence of ATP, they accumulate catecholamines by a reserpine-sensitive process (Kirshner, 1962; Carlsson et al., 1963) which is a property of the membrane since ghosts derived from chromaffin granules are also capable of active amine transport (Phillips, 1974a; Ingebretsen & Flatmark, 1979). The uptake mechanism has been shown to involve inward translocation of protons by an electrogenic ATP-dependent proton pump (Pollard et al., 1976; Casey et al., 1977; Phillips & Allison, 1978; Johnson & Scarpa, 1979). The transmembrane potential $\Delta\Psi$ (interior positive) generated in the presence of ATP can be sensed by the extrinsic probe OX-V,[1] the absorption and fluorescence of which are linearly related to potential changes (Scherman & Henry, 1980).

The $Mg^{2+}$-dependent ATPase of the chromaffin granule membrane has some properties in common with the mitochondrial proton pump but is oligomycin and efrapeptin resistant (Apps & Glover, 1978). It has been solubilized by nonionic detergents such as Lubrol-PX (Trifaro & Warner,

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[1] Abbreviations used: OX-V, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethineoxonol; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; DTT, dithiothreitol; DOC, deoxycholate; DBH, dopamine β-hydroxylase; MAO, monoamine oxidase; DCCD, *N,N′*-dicyclohexylcarbodiimide; cmc, critical micellar concentration; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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1972) or Nonidet P-42 (Apps & Reid, 1977) and by extraction of the membrane phospholipids by methylene chloride (Apps & Glover, 1978). The present study describes solubilization of chromaffin granule membrane by cholate or DOC and reconstitution of vesicles possessing an ATPase activity after removal of the detergent. The reconstituted ATPase is shown to be a functional $H^+$ pump sensitive to inhibitors of $H^+$ translocation in chromaffin granule, capable of generating a $\Delta\Psi$ detected with the probe OX-V and of ATP synthesis when an artificial $\Delta\mu_{H^+}$ composed of a positive $\Delta\Psi$ and a pH gradient (inside acidic) was imposed to the vesicles. Accumulation of catecholamines by reconstituted vesicles having an active $H^+$ pump is also described. A different approach to the same problem has recently been reported (Buckland et al., 1979).

## Experimental Section

*Preparation of Chromaffin Granule Membranes.* Bovine chromaffin granules were prepared by centrifugation on discontinuous sucrose gradients (Smith & Winkler, 1967; Schneider, 1972). The granules were lysed by resuspension in 4–5 volumes/g of initial tissue of a 5 mM Hepes buffer (pH 7.5) containing 0.1 mM DTT, 2 mM $MgSO_4$, and 10 $\mu$M $CaCl_2$ (buffer A) (Ingebretsen & Flatmark, 1979). The suspension was stirred for 15 min at 4 °C and was centrifuged for 20 min at 142000*g*. The pellets were washed with the same volume of buffer A and resuspended in a 10 mM Hepes buffer (pH 7.0) containing 0.3 M sucrose, 0.1 mM DTT, and 2 mM $MgSO_4$ (buffer B) at a concentration of 5–10 mg of protein/mL. The membranes were usually frozen in liquid nitrogen and stored at −80 °C. Alternatively and where indicated, granules have been purified by differential centrifugation (Taugner, 1972; Pollard et al., 1976).

*Solubilization of Chromaffin Granule Membranes.* Membranes were centrifuged (175000*g* for 20 min) and resuspended at ~5 mg of protein/mL in a 0.1 mM DTT–5 mM Hepes buffer (pH 7.3–7.5) containing KCl where specified and the detergent at the indicated concentration. The mixture was stirred for 30 s, sonicated for the same time, and incubated at 4 °C for 30 min before centrifugation at 175000*g* for 60 min. With DOC, the detergent concentration was routinely 0.6 mg/mg of protein (corresponding to 0.3–0.6% DOC) and no KCl was added whereas with cholate, 0.2 M KCl, and 1–1.5% detergent were used. Where specified, the endogenous phospholipids were separated from the proteins in the cholate extract by filtration on Sephacryl S-200 (Pharmacia). The sample was concentrated by ultrafiltration on YM-10 membranes (Amicon) and layered onto a 45 × 2 cm column of Sephacryl S-200 equilibrated in the solubilization buffer. ATPase activity was found in the first protein peak.

*Estimation of ATP.* ATP formation was followed by esterification of $^{32}P_i$ as described (Roisin et al., 1980). The reaction was stopped by addition of 10% trichloroacetic acid (0.2 mL) to the incubation mixture (0.1 mL). After centrifugation at 6000*g* for 10 min, the supernatant (0.25 mL) was mixed with 0.3 mL of 2.5% ammonium molybdate in 2.5 N $H_2SO_4$. Unesterified $^{32}P_i$ was extracted 3 times with 3 mL of 2-methyl-1-propanol–benzene (1:1) saturated with $H_2O$. An aliquot (0.4 mL) of the aqueous phase was counted in 5 mL of 50% ethanol by using Cerenkov emission.

*ATP-Induced Amine Uptake.* The reconstituted vesicle preparation (0.2–2 mg of protein/mL final concentration) was added to a medium containing 5 mM ATP, 2.5 mM $MgSO_4$, 10 mM Hepes (pH 7.0), and either 0.3 M sucrose for noradrenaline uptake or 187.5 mM KCl and 8.5 mM sucrose for methylamine uptake. The final radioactivity of the amines per milliliter was usually *l*-[7-$^3$H]noradrenaline, 4 $\mu$Ci, and methylamine, 7 $\mu$Ci. The complete mixture was incubated for various times at 37 °C. Aliquots (100 $\mu$L) were withdrawn and assayed for membrane-enclosed amine either by filtration through Millipore filters (Phillips, 1974a) or by separation on Sephadex G-50 columns (Toll & Howard, 1978).

*Electron Microscopy of the Preparations.* The pellets were fixed for 1 h in 3.5% glutaraldehyde in 0.1 M Millonig buffer containing glucose (pH 7.4). They were then cut into small blocks which were postfixed for 1 h in 2% buffered osmic acid, dehydrated in ethanol and propylene oxide, and embedded in epon–araldite mixtures. The ultrathin sections were stained with uranyl acetate and lead citrate.

*ATPase Activity.* Membranes (10–25 $\mu$g of protein) were incubated in 25 mM Tris-HCl buffer, pH 7.5 (0.2 mL final volume), for 10 min at 37 °C. The reaction was initiated by addition of 2 mM ATP (sodium salt) and 2 mM $MgSO_4$ (final concentrations). Aliquots (50 $\mu$L) were withdrawn and assayed for inorganic phosphate (Anner & Moosmayer, 1975).

*Analytical Techniques.* DBH activity was assayed at pH 6.5 in 0.1 M phosphate buffer with tyramine (20 mM) as a substrate (Pisano et al., 1960; Wallace et al., 1973). MAO activity was measured with [$^{14}$C]tyramine (50 $\mu$M) as a substrate (Wurtman & Axelrod, 1963). Catecholamines were assayed according to Von Euler & Lishajko (1961). Proteins were estimated by the Lowry procedure with bovine serum albumin as a standard following precipitation in 5% trichloroacetic acid and redissolution in 2% DOC–3% NaOH. Phospholipids estimation was done by assaying inorganic phosphate liberated by perchloric acid hydrolysis.

*Instrumentation.* Fluorescence and absorption changes were measured with a Jobin-Yvon double-monochromator fluorometer and an DW-2 Aminco spectrophotometer.

*Materials.* Bovine adrenals were obtained from a local slaughterhouse. [*side chain*-2-$^{14}$C]Tyramine hydrochloride (50 mCi/mmol) and *l*-[7-$^3$H]noradrenaline hydrochloride (15 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, England); [$^{14}$C]methylamine (38 mCi/mmol) and carrier-free $^{32}P_i$ were from CEA, France. The $^{32}P_i$ was heated at 100 °C for 60 min in 1 N HCl before use. Nonidet P-40 (Fluka) was deionized on Chelex 100 before use. Biobeads SM-2 (Bio-Rad) were washed with methanol and kept in water.

## Results

*Characteristics of the $H^+$ Translocase of Purified Chromaffin Granule Membrane.* Membranes have been derived from chromaffin granules purified by centrifugation through 1.8 M sucrose. The membranes were free of mitochondria as assessed by centrifugation on analytical sucrose gradients and by the resistance of their ATPase activity to oligomycin and efrapeptin (Apps & Reid, 1977). This ATPase activity was sensitive to trisubstituted tin derivatives and DCCD, which are inhibitors of $H^+$ translocation in chromaffin granules [Apps & Glover (1978), Bashford et al. (1976), and Table I) and was enhanced (25–40%) by the uncoupler CCCP at a 10 $\mu$M concentration.

Addition of ATP to a mixture of OX-V and this membrane preparation induced a change of the optical properties of the probe (Scherman & Henry, 1980). An optimal 40% relative fluorescence decrease was obtained with 20 nmol of OX-V/mg of protein at 0.5–2 $\mu$M OX-V (Figure 1A). Under the same conditions a decrease of the differential absorbance ($A_{620} - A_{650}$) was also observed (Figure 1B). These changes were related to the ATPase activity of the membrane since inhibitors of ATPase, DCCD, or triphenyltin affected in a same way the OX-V effect (Table I). The effect was due to the transmem-

Table I: Inhibition of ATPase Activity and OX-V Effect

| prepn used[a] | control[b] act. | inhibition[c] (%) triphenyltin (50 μM) | tributyltin (50 μM) | DCCD (50 μM) | oligomycin (10 μg/mL) |
|---|---|---|---|---|---|
| (1) ATPase activity | (nmol $min^{-1}$ (mg of protein)$^{-1}$) | | | | |
| ghosts | 209 (±23) | 86 (±1) | 90 (±7) | 75 (±12) | −1 (±5) |
| solubilized enzyme | 126 (±10) | 40 (±12) | 26 (±8) | 7 (±10) | 4 (±6) |
| reconstituted vesicles | 414 (±43) | 66 (±13) | 76 (±8) | 70 (±15) | 2 (±3) |
| (2) OX-V effect[d] | (%) | | | | |
| ghosts | 32 (±5) | 96 (±6) | | 100 (±2) | |
| reconstituted vesicles | 57 (±13) | 90 (±5) | | 95 (±3) | |

[a] The solubilized enzyme and the reconstituted vesicles were prepared as described in Table IV. [b] All controls contained ethanol (1% or less), the inhibitors being added in that solvent. [c] Percentages of the appropriate control. Values are means (±SE) of at least three experiments. [d] Relative fluorescence changes as described in the legend of Figure 1A.

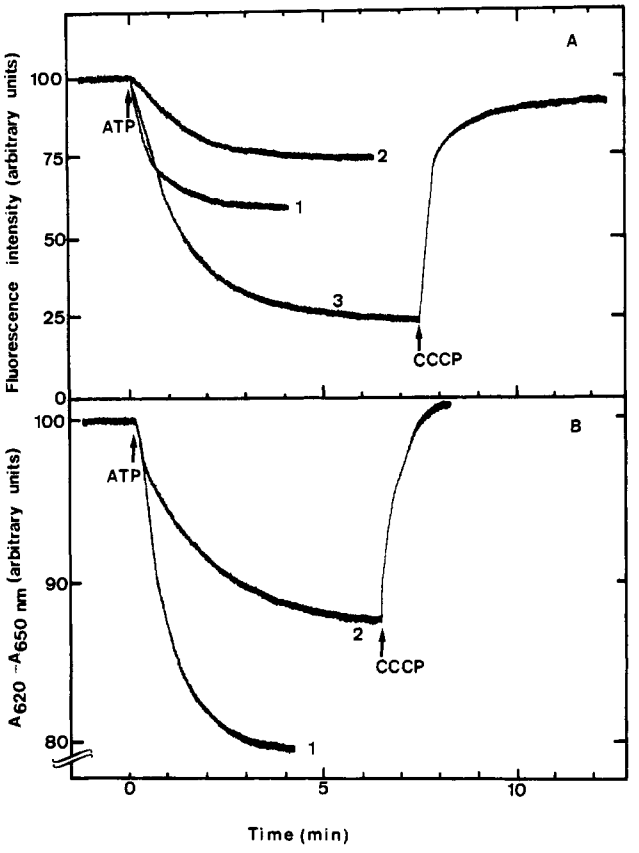


FIGURE 1: OX-V spectral changes associated with transmembrane potential generation induced by ATP–$Mg^{2+}$ addition. ATP and $MgSO_4$ were added to OX-V and sample in 20 mM Mes, pH 6.6 (final volume 2 mL). (A) Relative fluorescence decrease. The concentrations used were as follows: chromaffin granule membrane (curve 1), 63 μg of protein/mL; supernatant of membranes solubilized in 1% cholate (curve 2), 48 μg of protein/mL; vesicles reconstituted as described in Table IV (curve 3), 50 μg of protein/mL; OX-V, 0.5 μM; ATP, 1.25 mM; $MgSO_4$, 0.63 mM; CCCP, 5 μM. Excitation and emission wavelengths were 580 and 650 nm, respectively. Results were corrected for light-scattering effects. (B) Relative absorbance decrease. Concentrations were as follows: chromaffin granule membranes (curve 1), 210 μg of protein/mL; vesicles reconstituted as described in Table III (curve 2), 44 μg of protein/mL; OX-V, 2 μM; ATP, 2.5 mM; $MgSO_4$, 1.25 mM; CCCP, 8 μM. The $A_{620} - A_{650}$ nm differential absorbance was measured with a dual-wavelength spectrophotometer, and the results are expressed as the relative change. The initial $A_{620} - A_{650}$ absorbances were 0.046 and 0.049 for curves 1 and 2, respectively.

brane potential generated by the $H^+$ pump and not to OX-V binding to the ATPase since it was reversed by 5 μM CCCP (Figure 1) or the permeant anion $SCN^-$ (10 mM), which affect the transmembrane potentials and do not inhibit the ATPase activity (Bashford et al., 1975).

Table II: Effect of Detergents on the ATPase Activity of Chromaffin Granule Membrane[a]

| | act. of treated membranes[b] (%) | solubilization yield[c] (%) | sp act. of solubilized ATPase[d] (%) |
|---|---|---|---|
| Lubrol-PX (1%) | 44 | 92 | 87 |
| Triton X-100 (2%) plus KCl, 0.2 M | 100 | 32 | 67 |
| Nonidet P-40 (1%) | 137 (±19) | 45 (±13) | 124 (±13) |
| cholate (1%) plus KCl, 0.2 M | 59 (±8) | 49 (±9) | 55 (±5) |
| DOC (0.48%) plus KCl, 0.2 M | 137 (±16) | 36 (±8) | 68 (±10) |

[a] Membranes from purified chromaffin granules have been treated as described under Experimental Section. Values are means (±SE) of at least three experiments except that of Lubrol-PX and Triton X-100 which are derived from plots of the solubilization yield as a function of detergent concentration. [b] Ratio of the specific activity of treated membranes to that of untreated membranes [230 nmol $min^{-1}$ (mg of protein)$^{-1}$]. [c] Ratio of the solubilized activity to that of the treated membranes. [d] Ratio of the specific activity of the solubilized ATPase to that of the untreated membranes.

*Effects of Detergents on Chromaffin Granule Membrane.* Detergents affected the OX-V effect generated by the ATP-dependent $H^+$ pump at concentrations where solubilization was not observed. A 50% inhibition of the OX-V fluorescence effect was observed at 0.028 mM Nonidet P-40, 0.062 mM Triton X-100, 0.15 mM DOC, and 0.42 mM cholate, these concentrations corresponding respectively to 0.1, 0.26, 0.03, and 0.03 cmc (Helenius & Simons, 1975). Concentrations of respectively 0.084, 0.12, 0.75, and 2.1 mM of Nonidet P-40, Triton X-100, DOC, and cholate completely abolished the fluorescence change but were without effect on the ATPase activity. This result had to be considered when selecting reconstitution conditions.

Increasing the detergents concentration induced solubilization and modifications of the ATPase activity (Table II). Under our experimental conditions the enzymatic activity was inhibited by Lubrol-PX and cholate and was activated by DOC and Nonidet P-40. This result depended largely on the purity of the commercial detergent. With Nonidet P-40 which had not been deionized on Chelex-100, the membranes were not activated but were inhibited (30%). The best solubilization yield was obtained with Lubrol-PX, but there was no increase in the specific activity of the enzyme solubilized. Such an increase was observed only with Nonidet P-40, but this was due to the increase in total ATPase activity and not to selective solubilization. The effect of bile salts, which are widely used

Table III: Solubilization of Chromaffin Granule Membrane by DOC and Reconstitution by Dialysis or Dilution[a]

| | proteins (mg) | ATPase (nmol of $P_i$/min) | DBH (nmol of octopamine/min) | $\Delta I/I$[b] (%) |
|---|---|---|---|---|
| (1) membranes | 34 | 7888 | 15500 | 53 |
| (2) DOC-treated preparation | | | | |
| pellet | 12 | 5740 | 2138 | |
| supernatant | 21 | 2324 | 12740 | |
| (3) reconstitution by dilution | | | | |
| pellet | 4.9 | 2072 | 2332 | 21 |
| (4) reconstitution by dialysis | | | | |
| pellet | 2.9 | 1652 | 2625 | 7.4 |

[a] Membranes from chromaffin granules obtained by differential centrifugation were solubilized by DOC (0.6 mg/mg of protein) as described under Experimental Section. After centrifugation at 140000*g* for 1 h, the supernatant and the pellet were assayed (step 2). The supernatant was divided into two fractions. The first was diluted by slow addition of 60 volumes (v/v) of buffer A, under agitation. The solution was centrifuged at 140000*g* for 90 min; the pellet was resuspended in 1 mL of buffer B and assayed (step 3). The second fraction was dialyzed for 24 h at 4 °C against 1 L of buffer A with two buffer changes. It was then centrifuged at 140000*g* for 1 h; the pellet was resuspended in 1 mL of buffer B and assayed (step 4). The results of step 3 and 4 have been corrected for the division of the preparation. Results of one experiment representative of more than six other different experiments. [b] $\Delta I/I$ is the relative OX-V fluorescence change assayed as in Figure 1A.

Table IV: Solubilization by Cholate and Reconstitution by Adsorption of the Detergent on Biobeads SM-2[a]

| | proteins (mg) | ATPase (nmol of $P_i$/min) | DBH (nmol of octopamine/min) | $\Delta I/I$[b] (%) |
|---|---|---|---|---|
| (1) membranes | 32 | 7440 | 8775 | 40 |
| (2) cholate-treated preparation | 32 | 4080 | 8300 | |
| pellet | 14 | 4200 | 1760 | |
| supernatant | 19 | 2400 | 9500 | |
| (3) reconstituted vesicles | 6 | 2230 | 4460 | 54 |

[a] Washed membranes from purified chromaffin granules were solubilized by 1% cholate–0.2 M KCl as described under Experimental Section (step 2). For reconstitution, Biobeads SM-2 were added to the solubilized membranes (0.5 g/mL), and the mixture was kept under gentle stirring for 18 h at 4 °C. After filtration on glass wool and eightfold dilution in buffer A, the sample was centrifuged for 1 h at 175000*g*. The pellet was resuspended in 0.25 mL of buffer B and assayed (step 3). Results of one experiment are representative of more than 10 other different experiments. [b] $\Delta I/I$ is the relative OX-V fluorescence change assayed as in Figure 1A.

in reconstitution experiments, has been more carefully studied. With DOC, the amount of ATPase solubilized was unaffected by pH in the 6.7–8.0 range and by KCl concentration up to 1.0 M; it increased with DOC concentration, reaching a plateau value of 25–40% of the total activity solubilized at 0.6 mg of DOC/mg of protein. In addition to ATPase, the membrane-bound DBH was also solubilized by these detergents. Thus, when membranes were treated with DOC, 64% of the membrane proteins, 29% of the ATPase activity, and 86% of the membrane-bound DBH activity were solubilized (Table III). Similar results were also obtained when membranes were treated by 1% cholate in the presence of 0.2 M KCl (Table IV).

*Properties of the Solubilized ATPase.* The solubilized ATPase differed from its membrane-bound form by its susceptibility to inhibitors. CCCP (10 $\mu$M), which increased ghost ATPase activity, had no effect on the soluble enzyme. Triphenyltin, tributyltin, and DCCD, which inhibited the membrane-bound activity (Table I), had only a limited effect on the soluble enzyme. On the other hand, both forms of the enzyme were resistant to oligomycin (Table I). The same results were obtained when the detergent-solubilized extract was delipidated by gel filtration on Sephacryl S-200.

Since the ATP-dependent OX-V effect is caused by transmembrane potential generation, it was anticipated to disappear after membrane solubilization. In fact, an OX-V effect was observed when membranes solubilized by cholate or DOC were tested in the usual conditions (Figure 1A, curve 2), but disappeared when a 1% cholate concentration was maintained in the cuvette used for optical measurements. The signal of curve 2, Figure 1A, is attributed to reconstitution of active vesicles induced by the 20–40-fold dilution of the detergent in the cuvette buffer (see below).

*Reconstitution of the Solubilized Membrane.* The solubilized membrane reconstituted upon removal of the detergent.

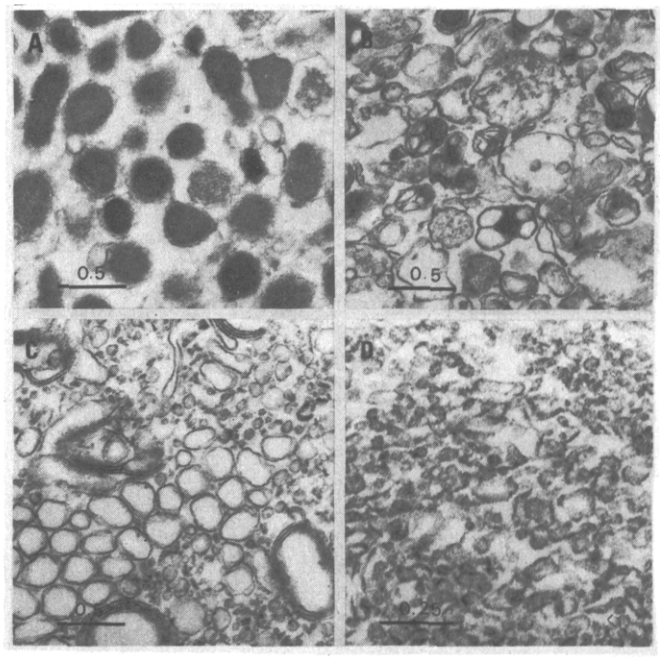


FIGURE 2: Electron micrographs of a typical reconstitution experiment. The procedure described in Table III legend has been followed. (A) Granules isolated by differential centrifugation. (B) Ghosts. (C and D) Vesicules reconstituted respectively by dialysis and dilution. Calibration bars are in micrometers.

A 60-fold dilution of the DOC solubilized membrane which lowered the detergent concentration to 0.02–0.04 cmc, followed by high-speed centrifugation, gave a particulate fraction composed of microvesicles with an average diameter of 50 nm (Figure 2D). Such vesicle formation was also observed after adsorption of the detergent on Biobeads SM-2 resin (Chiesi et al., 1978). This procedure has been applied to membrane solubilized by sodium cholate.

The particulate fractions obtained by either of these two methods contained the bulk of the solubilized ATPase but only 20–30% of the proteins (Tables III and IV). The reconstitution yield for DBH activity was lower than that for ATPase. The phospholipids content of the particles isolated by the Biobeads technique was 4.2–4.7 $\mu$mol/mg of protein, which is higher than that of the initial purified membrane (1.9–2.3 $\mu$mol/mg

of protein), thus indicating a loss of protein.

Reconstitution by the Biobeads technique restored the initial properties of the membrane ATPase. Thus, as in the "ghost" enzyme, the reconstituted ATPase was not inhibited by efrapeptin or oligomycin but was inhibited by DCCD, tributyltin, and triphenyltin (Table I). However, we have not been able to demonstrate a stimulation of the ATPase activity by CCCP similar to that observed with the native granules.

*Generation of Transmembrane Potentials by the $H^+$ Pump of Reconstituted Vesicles.* The ATPase activity of the membranes reconstituted by the above methods was associated with an efficient electrogenic $H^+$ pump. Thus, addition of ATP to these preparations induced a marked decrease in the fluorescence as well as in the absorbance of OX-V (Figure 1; Tables III and IV) generally greater than with the initial "ghosts". As expected, the OX-V effect was blocked by the ATPase inhibitors DCCD and triphenyltin (Table I) and also by thiocyanate (8 mM) as well as by CCCP (Figure 1).

It should be noted that reconstitution of vesicles possessing an active ATPase did not necessarily imply that an active $H^+$ pump was reconstituted. For instance, removal of DOC by dialysis gave rise to vesicles of various shapes, including small vesicles, large vesicles, and multilayered sheaths (Figure 2C) with ATPase and DBH activities similar to those of the vesicles obtained by dilution (Table III). Nevertheless, the $H^+$ pump activity of these preparations was low ($\Delta I/I$, Table III), perhaps because of the long duration of the experiment. Solubilization by nonionic detergents of the Nonidet P-40 type followed either by dilution or by Biobeads adsorption also gave an active membrane-bound ATPase but an inactive $H^+$ pump as judged from the resistance to DCCD of the ATPase activity and the absence of an OX-V effect.

*ATP Synthesis and $^{32}P_i$–ATP Exchange by Reconstituted Vesicles.* Since $H^+$ translocation by chromaffin granule membrane has recently been shown to be reversible (Roisin et al., 1980), it was of interest to investigate the reversibility of the $H^+$ pump of reconstituted vesicles. ATP synthesis by vesicles to which an artificial $\Delta\mu_{H^+}$ was imposed was first tested. For generation of a large $\Delta\mu_{H^+}$ the vesicles were first incubated with valinomycin at pH 5.0 in $K^+$-free malonate buffer (acid stage) and to this medium was then added a basic buffer containing $K^+$ ions associated with the impermeant sulfate anion, to give a final pH of 8.3 and a $K^+$ external concentration of 80 mM (base stage). This generated a transient pH gradient (inside acidic) and $\Delta\Psi$ (inside positive) which, when $Mg^{2+}$, ADP, and $^{32}P_i$ were added to the base stage solution, induced an esterification of the $^{32}P_i$ (Table V). Omission of valinomycin in the acid stage solution (which suppressed the $\Delta\Psi$) or of the acid stage (which suppressed the $\Delta$pH) resulted in complete inhibition of the reaction. Addition to the acid stage solution of the ionophore nigericin, which catalyzes the electroneutral $H^+$–$K^+$ exchange and thus decreases both the $\Delta\Psi$ and the $\Delta$pH, or of tributyltin also blocked the reaction. The reversibility of the $H^+$ translocase was also demonstrated by the $^{32}P_i$–ATP exchange reaction (Table V). This reaction was associated with the $\Delta\mu_{H^+}$ generated by the ATPase since it was inhibited by CCCP or by nigericin and $K^+$ ions. It was also sensitive to tributyltin.

*Transport of Amines by Reconstituted Vesicles.* It has been shown that methylamine permeates chromaffin granule membrane by a nonspecific diffusion process (Johnson & Scarpa, 1976) whereas *l*-noradrenaline is transported through a specific saturable carrier (Phillips, 1974b; Pletscher, 1976). In presence of ATP, reconstituted vesicles accumulated [$^{14}$C]methylamine when resuspended in KCl-containing media (23–38 pmol of methylamine transported/mg of protein at 20 $\mu$M initial concentration). They also accumulated [$^3$H]noradrenaline in sucrose-containing media. The latter reaction was inhibited by reserpine (1 $\mu$M) and by tetrabenazine (10 $\mu$M). Typical kinetics are presented in Figure 3: ~20 min was required to reach a plateau value which was somewhat variable from one preparation to another. Representative figures were in the 500–1000 pmol of noradrenaline/mg of protein range at 100 $\mu$M initial noradrenaline concentration which should be compared with 14000 pmol/mg of protein for the initial membrane preparation. At 10 $\mu$M noradrenaline, the reconstituted vesicles accumulated from 40 to 160 pmol of amine/mg of protein whereas the initial membrane preparation took up 500–3000 pmol/mg of protein. Similar values have been obtained by using a gel filtration method, thus showing that retention of the reconstituted vesicles on the filters is not the limiting factor.

Table V: Reversibility of the Reconstituted Vesicles $H^+$ Pump

| | ATP synthesis (nmol of $^{32}P_i$ esterified/mg of protein) |
|---|---|
| complete system[a] | 0.38 (±0.11) |
| acid stage omitted | 0.04 (±0.01) |
| valinomycin omitted | 0.03 (±0.04) |
| plus nigericin (5 $\mu$g/mL)[b] | 0.11 (±0.03) |
| plus tributyltin (50 $\mu$M)[b] | 0.01 (±0.01) |
| | $^{32}P_i$–ATP exchange [nmol of $^{32}P_i$ esterified min$^{-1}$ (mg of protein)$^{-1}$] |
| complete system[c] | 0.39 (±0.13) |
| plus CCCP (2 $\mu$M) | 0.05 (±0.03) |
| plus nigericin (5 $\mu$g/mL) plus $K_2SO_4$ (25 mM) | 0.16 (±0.06) |
| plus tributyltin (50 $\mu$M) | 0.03 (±0.02) |

[a] Vesicles (100–110 $\mu$g of protein) prepared as described in Table IV and washed twice in $K^+$-free media were first incubated at 37 °C for 10 min with $Na^+$–malonate (2.5 $\mu$mol), pH 5.0, and with valinomycin (0.4 nmol) in a final volume of 0.05 mL. A base stage solution (0.05 mL) containing $K_2SO_4$ (4.0 $\mu$mol), Na-ADP (0.2 $\mu$mol), $MgCl_2$ (0.2 $\mu$mol), sucrose (5 $\mu$mol), Na-EPPS (5 $\mu$mol), and $^{32}P_i$, $Na^+$ salt (0.5 $\mu$mol, $10^6$ cpm), was then added. The final pH of the reaction mixture was 8.3. The reaction was terminated after 10 s of base stage and was processed as described under Experimental Section. Values are mean (±SE) of four experiments. A figure of 2.0 ± 0.9 nmol/mg of protein was obtained when ghosts were assayed under the same conditions. [b] Added to the acid stage solution. [c] Reconstituted vesicles (85–110 $\mu$g of protein) were incubated at 37 °C for 30 min in a reaction mixture containing ATP (1.5 $\mu$mol), $MgSO_4$ (1.5 $\mu$mol), $^{32}P_i$, $Na^+$ salt (1.0 $\mu$mol, $10^6$ cpm), sucrose (30 $\mu$mol), and Hepes, pH 7.0 (1.0 $\mu$mol), in a final volume of 0.1 mL. Esterified $^{32}P_i$ was measured as described under Experimental Section. Values are mean (±SE) of two experiments. The exchange activity of ghosts assayed under the same condition was 1.6 ± 0.5 nmol (mg of protein)$^{-1}$ min$^{-1}$.

## Discussion

Reconstitution of biological membranes is widely used to gain information on the functions of their different components. Complete reconstitution requires purification of the membrane proteins and reassembly of these proteins with phospholipids of known composition. Nevertheless, before attempting purification of the solubilized proteins, solubilization and reconstitution conditions should be determined. The present communication describes solubilization of the chromaffin granule ATP-dependent $H^+$ translocase and its reconstitution from unfractionated extracts. Solubilization of the ATPase was a major problem since with most of the detergents tried, only partial solubilization was observed and, moreover, the results were often obscured by changes in the

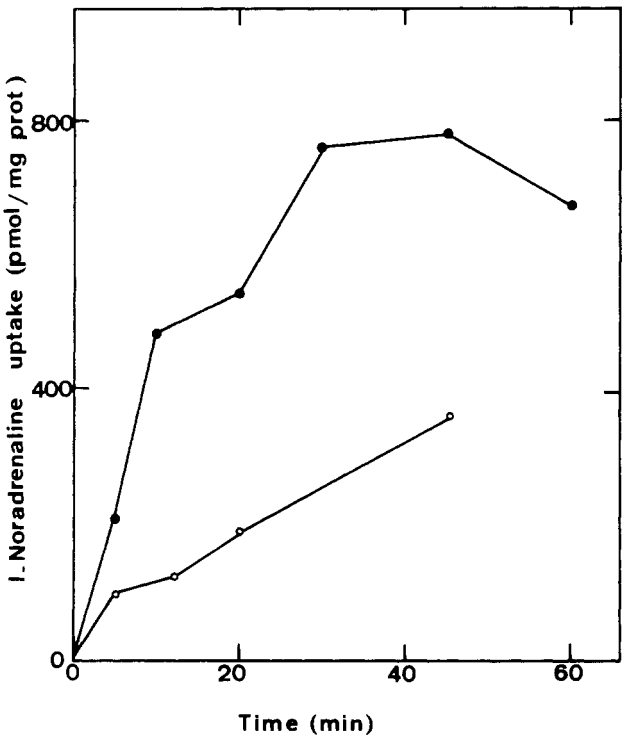


FIGURE 3: Uptake of *l*-[7-$^3$H]noradrenaline by reconstituted vesicles. Reconstituted vesicles (69 $\mu$g of protein) prepared by the DOC dilution procedure were incubated under standard conditions with 100 $\mu$M *l*-[7-$^3$H]noradrenaline, without (●) or with (○) 10 $\mu$M reserpine. After incubation the vesicles were isolated by filtration on Millipore filters. The results have been corrected for adsorption of noradrenaline to the filters.

total enzyme activity (activation or inhibition). Lubrol-PX inhibited ATPase activity and solubilization by this detergent did not increase the enzyme specific activity, as previously reported (Trifaro & Warner, 1972). Nonidet P-40 slightly increased the ATPase activity (Apps & Reid, 1977) but solubilization was not complete. With bile salts, solubilization conditions were similar to those utilized for sarcoplasmic $Ca^{2+}$-dependent ATPase (Meissner & Fleischer, 1974) or mitochondrial ATPase (Kagawa & Racker, 1966), except that with DOC high salt concentration and high pH were not required.

The solubilized ATPase reconstituted easily. Removal of cholate or DOC by dialysis, dilution or adsorption on Biobeads SM-2 led to formation of active particles. Nonidet P-40 or Triton X-100 could also be removed by Biobeads with the same result. Since the chromaffin granule ATPase has some properties in common with the mitochondrial enzyme (Apps & Glover, 1978), the question arises as to the origin of the reconstituted ATPase (Laduron et al., 1976). A mitochondrial origin of this enzyme is excluded since, as the ghost enzyme, it is oligomycin and efrapeptin resistant. When membranes derived from granules purified only by differential centrifugation were used (Table III), they were solubilized under conditions (DOC at low ionic strength) which resulted in selective solubilization of the granule ATPase. The efficiency of this approach is shown by the fact that solubilization by DOC under these conditions and reconstitution by dilution of a mitochondria-rich fraction (with a MAO/DBH ratio of 20.6 in arbitrary units) and a granule-rich fraction (with a MAO/DBH ratio of 1.8 in the same units) gave reconstituted vesicles having 6.5 and 21% of their initial ATPase activity and characterized by OX-V effects $\Delta A_{620-650}/A_{620-650}$ of respectively 5.7 and 29.8%.

The contention that vesicles reconstituted as described in Tables III and IV contain an active $H^+$ pump is supported by three lines of evidence: effect of ATPase inhibitors, generation of transmembrane potentials, and reversibility of ATP-dependent $H^+$ translocation. Inhibitors such as DCCD or trisubstituted tin derivatives which did not affect the lipid-free ATPase inhibited the reconstituted enzyme. Chromaffin granule ATPase thus resembles the other ATP-dependent $H^+$ translocase (Kagawa & Racker, 1966; Hare, 1975; Serrano et al., 1976). The ATPase of these reconstituted vesicles is capable of generating transmembrane potentials as shown by the electrogenic effects monitored with the probe OX-V. The OX-V fluorescence and absorbance changes were associated with the ATPase activity since they were blocked by triphenyltin and DCCD (Table I), and they indicated potential generation since they were reversed by $SCN^-$ and CCCP. It should be pointed out that the fluorescence changes of the reconstituted vesicles were often greater than those of the initial membranes (Figure 1A and Table IV). Reconstituted vesicles were smaller than ghosts but a priori the decrease in size cannot explain higher potentials since both the number of charges transferred per vesicle and the electric capacity of the membrane should be proportional to the vesicle area. An alternate hypothesis would be the generation of more pronounced fluorescence effects by the same potential difference in reconstituted vesicles than in ghosts. In spite of its qualitative aspect, the OX-V technique gave invaluable results since the [$^{14}$C]SCN partition technique which has been used to measure transmembrane potentials in granules or ghosts is time consuming and requires too large amounts of material, especially with vesicles having a small internal volume.

As noted above, reconstitution of vesicles possessing an ATPase activity did not imply reconstitution of an active $H^+$ pump. The difficulties encountered in the case of nonionic detergents might be due to their low cmc which hampered complete removal of the detergent and to the inhibitory effect of the low amounts remaining.

ATP synthesis by reconstituted vesicles to which an artificial $\Delta\mu_{H^+}$ was imposed and $^{32}P_i$–ATP exchange are further evidence of the reconstitution of an active $H^+$ pump (Table V). These reactions are essentially similar to those described in chloroplasts (Jagendorf & Uribe, 1966), submitochondrial particles (Thayer & Hinkle, 1975; Conover et al., 1963), reconstituted bacterial vesicles (Sone et al., 1977), and chromaffin granule ghosts (Roisin et al., 1980). The low amount of ATP synthetized by the reconstituted vesicles can be explained by the small size of these structures since the amount of esterified $^{32}P_i$ is related to the amount of $H^+$ stored.

Reconstituted vesicles capable of generating a $\Delta\Psi$ accumulated noradrenaline (Figure 3). Since noradrenaline uptake requires not only an $H^+$ pump but also a specific transporter, our results suggest reconstitution of a functional transporter. Recently, solubilization and reconstitution of the 5-HT transport of chromaffin granule membranes have been described (Maron et al., 1979). The optimal conditions used to reconstitute the transporter (0.9% cholate) resulted in an almost completely ineffective ATP-dependent uptake of 5-HT. On the other hand, the concentration of cholate found by us to allow optimal reconstitution of the $H^+$ pump [1.5% cholate, M.F. Isambert and J.-P. Henry (unpublished observation)] is not optimal for transporter reconstitution. Reconstitution of the complete system from independently solubilized components is now under investigation in this laboratory.

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