# SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF THE PHOSPHORYLATED COMPONENT(S) OF THE MEMBRANE OF ADRENAL CHROMAFFIN GRANULES

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## 1. Introduction

In the presence of $Mg^{2+}$, ATP produces structural changes of chromaffin granules isolated from the adrenal medulla and there is a simultaneous release of catecholamines, endogenous ATP, and soluble protein [1–6]. When ATP exerts this action on chromaffin granules, it is hydrolyzed by the $Mg^{2+}$-dependent ATPase present in the granule membrane, and part of the $P_i$ liberated from the ATP is transferred to the chromaffin granule membrane [7].

Recent work from our laboratory has shown that the chromaffin granule ATPase can be solubilized and isolated from the granule membrane [8]. The present experiments studied the solubilization, isolation, and partial characterization of the phosphorylated membrane component(s) of the chromaffin granules.

## 2. Materials and methods

Bovine adrenal glands were obtained from a slaughterhouse, kept on ice and the medullae were separated from the cortices. Each medulla was homogenized in 5 vol of ice-cold 0.3 M sucrose pH 7. The homogenates were subjected to differential and density gradient centrifugation and chromaffin granules were isolated as previously described [9]. Chromaffin granule membranes were prepared from the purified granule fractions as described elsewhere [8]. Chromatographic techniques, protein determination, ATPase assay, and membrane phosphorylation procedures have been described in detail in previous publications [7, 8]. The $^{32}P$ radioactivity was measured as previously described [10], and the results were expressed in μmoles or nmoles of $P_i$ per mg of protein, calculated on the basis of the specific activity (1.5 μCi/μmole) of the added [$\gamma$-$^{32}P$] ATP.

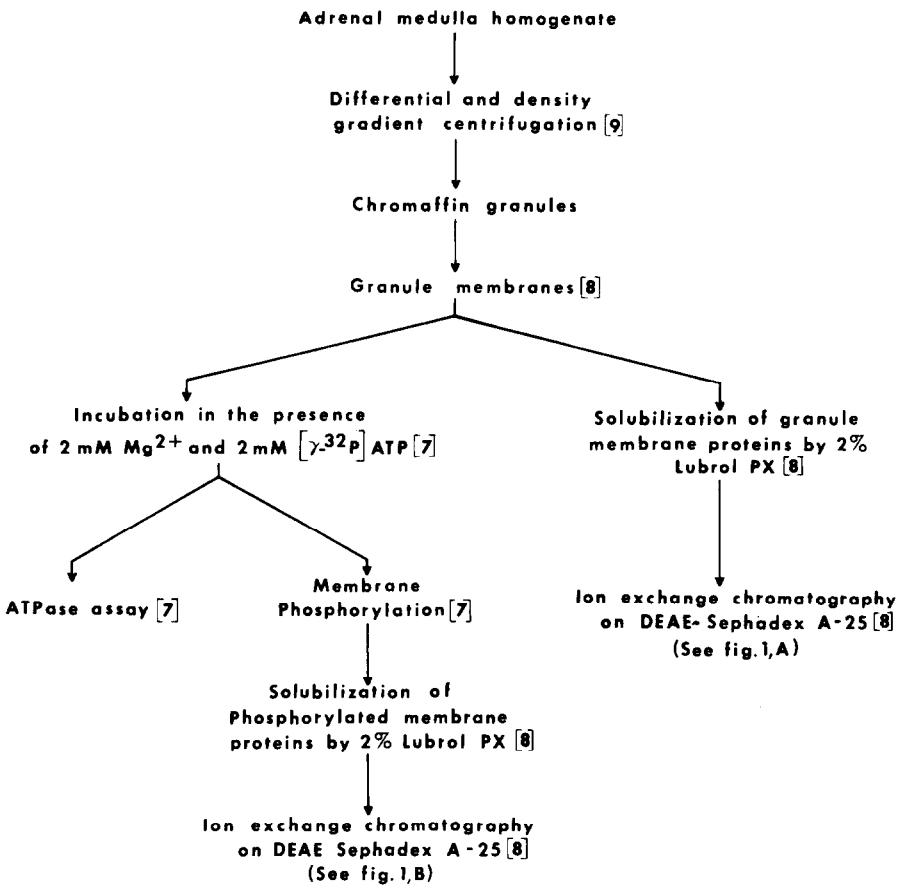
The following protocol was used in the experiments: (see scheme on following page).

## 3. Results and discussion

When chromaffin granules [7], or its membranes, were incubated with ATP in the presence of $Mg^{2+}$, ATP was hydrolyzed and part of the $P_i$ was transferred from ATP to chromaffin granule membranes (table 1). In intact granules, ATP also produces structural changes leading to the release of granule constituents [1–6].

In the present experiments, Lubrol was used as the solubilizing agent, because we have previously demonstrated that of several non-ionic detergents, Lubrol PX most effectively solubilized and preserved the ATPase activity of the granule membrane preparations [8].

The increase in the ATPase specific activity observed in the Lubrol-extracted membrane protein preparation (table 1) is due to the fact that Lubrol solubilized between 40 and 50% of the membrane proteins and all of the membrane ATPase [8]. Similarly, the specific activity of the phosphorylated




component(s) in the Lubrol-extracted membrane proteins was greater than in granule membranes (table 1). In other experiments chromaffin granule membranes were incubated in the presence of 2 mM [$\gamma$-$^{32}$P] ATP and 2 mM $Mg^{2+}$. The phosphorylated membrane components were then solubilized with Lubrol, and chromatographed on DEAE-Sephadex A-25; a fraction (peak II) containing 85–95% of the radioactivity applied to the column was eluted at the ionic strength (I) of 0.2 (fig. 1, B). This is the same ionic strength in which a fraction containing the $Mg^{2+}$-dependent ATPase activity was eluted (fig. 1, A). The value for the transphosphorylation from ATP to granule protein obtained in peak II was 11 times greater than that found in intact granule membranes (table 1). This increase in the specific activity of the phosphorylated component is similar to that found in the specific activity of the membrane ATPase isolated by the same procedure (table 1).

Chromaffin granule membranes have other constituents than ATPase; they contain chromogranin A [11], dopamine $\beta$-hydroxylase [12] and cytochrome $b_{559}$ [13]. It is possible that the other elution peaks (I, III, IV, and V) obtained during ion exchange chromatography contain these proteins.

We have found previously that among the different peaks obtained by ion exchange chromatography only the fraction that corresponds to peak II was phosphorylated when tested in the presence of ATP [8]. The present results differed from those in that the membranes were phosphorylated prior to the solubilization and chromatographic separation.

We have previously demonstrated that this phosphorylation is due to an active transfer of phosphate from ATP to the chromaffin granule membrane components rather than to binding of ATP to the chromaffin granule membranes [7], and that the phosphorylation is possibly related to the labelling of stable sites in the membrane [7].

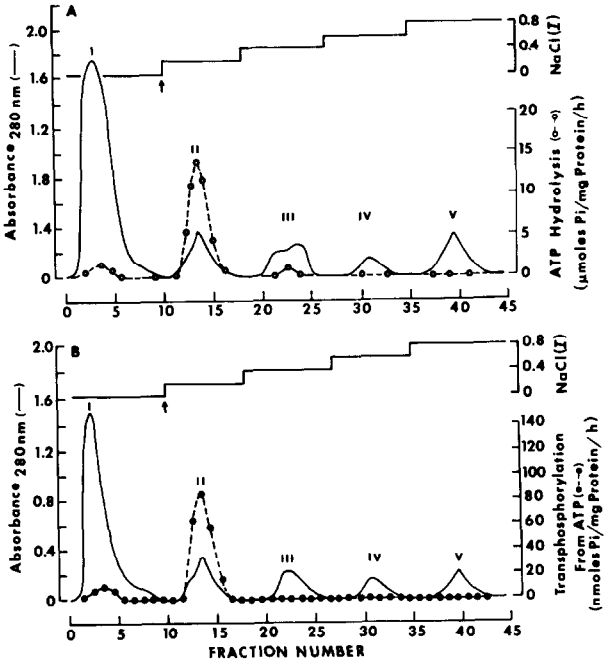



Fig. 1. Ion exchange chromatography of Lubrol-solubilized proteins of chromaffin granule membranes. A) Chromaffin granule membrane proteins were solubilized by 2% Lubrol PX (Lubrol to protein ratio, 10:1). A sample containing 16 mg of protein in 16 ml of starting buffer (0.005 M Tris-HCl buffer pH 6.5 and 0.2% Lubrol) was applied to a DEAE-Sephadex A-25 column. B) Chromaffin granule membranes were incubated for 30 min with 2 mM [$\gamma$-$^{32}$P] ATP (specific activity: 1.5 $\mu$Ci/$\mu$mole) in the presence of 2 mM $MgCl_2$ in the following incubation medium: 160 mM KCl; 5 mM NaCl; and 10 mM Tris-HCl buffer pH 6.5. At the end of the incubation period, the preparation was centrifuged (100,000 $g$ for 60 min) and the phosphorylated membranes were recovered in the sediment. The sediment was treated with a solution containing 2% Lubrol for 30 min. The Lubrol-treated preparation was then centrifuged at 100,000 $g$ for 60 min, and a sample from the supernatant fluid (12 mg of protein in 12 ml of starting buffer) was applied to a DEAE-Sephadex A-25 column.

After the application of the samples, both columns were perfused with 40 ml of starting buffer. At the points indicated by arrows, stepwise ionic strength gradients of NaCl (I from 0.0 to 0.8) in starting buffer were begun. Fractions (4 ml) were collected and assayed for protein content. ATPase activity was measured in A and $^{32}$P radioactivity was determined in B. The results were expressed in $\mu$moles of $P_i$ and nmoles of $P_i$ per mg protein per hr, respectively [7, 8].

The results presented in this paper suggest that the phosphorylated component(s) of the chromaffin granule membrane is part of, or is closely associated with the $Mg^{2+}$-dependent ATPase present in the granule membrane. It is not clear from the present experiments whether the phosphorylated material is a single component or not. Other preliminary results indicate that part of the $^{32}$P radioactivity recovered in peak II (20–40% of the total) can be extracted with chloroform:methanol+[HCl] (2:1+0.25%), and that upon thin layer chromatography [14] of the lipid extract, the radioactivity is recovered with the fractions containing mono-, di-, and triphosphatidyl inositides. Diphosphatidyl inositol had the greatest specific radioactivity among the three inositides.

Although it is not yet known whether these effects of ATP on chromaffin granules occur during the stimulation of the gland *in vivo*, it seems likely that the production of ATP is necessary for maintaining secretion, because the secretory process is blocked by inhibitors of oxidative phosphorylation and glycolysis [15, 16]. It will therefore be of some interest to combine *in vivo* experiments with these isolation and

Table 1
Partial purification of the phosphorylated component(s) of the chromaffin granule membrane.

| Preparation | Additions | | | ATPase activity (μmoles $P_i$/mg protein/hr) | Transphosphorylation from ATP (nmoles $P_i$/mg protein/hr) |
|---|---|---|---|---|---|
| | $Mg^{2+}$ (2 mM) | [$\gamma$-$^{32}$P]ATP (2 mM) | EDTA (2 mM) | | |
| Granule membranes | – | + | + | 0.15 ± 0.01* | 0.09 ± 0.03 |
| Granule membranes | + | + | – | 1.16 ± 0.08 | 7.88 ± 0.14 |
| Lubrol-extracted membrane proteins | + | + | – | 2.25 ± 0.31 | 11.04 ± 1.21 |
| Peak II from DEAE-Sephadex A-25 | + | + | – | 12.5 | 86.3 |

* Mean ± standard error.

Chromaffin granule membranes were suspended in solutions containing 2% Lubrol. Specific ATPase activity and transphosphorylation from ATP were determined [7, 8] before the Lubrol treatment, in the Lubrol-extracted membrane proteins, and in fraction (peak II) obtained by DEAE-Sephadex A-25 chromatography. The Lubrol-extracted membrane protein fraction is the supernatant fluid obtained by centrifuging the Lubrol-treated granule membranes at 100,000 *g* for 60 min. Results are averages of 3–4 different experiments, except for those obtained from peak II, which are averages of 2 separat determinations.

characterization techniques. This approach might provide a better understanding of the cellular and molecular events involved in "stimulus secretion coupling."

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