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# Properties of a Novel ATPase Enzyme in Chromaffin Granules[1]

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## Abstract

Membranes were isolated from mitochondria and chromaffin granules of bovine adrenal medullae. The cross-contamination between the two membranes was examined by comparing the radioactive bands on autoradiograms of gels after phosphorylation of the membranes with [$\gamma$-$^{32}$P]-ATP and decoration with [$^{125}$I]concanavalin A and [$^{125}$I]protein A with antibody that was raised against chromaffin-granule membranes. It was found that the membranes cross-contaminated each other by less than 10%. The technique of immunodecoration with antibodies against $\beta$ subunits of proton-ATPases from yeast mitochondria, spinach chloroplasts, and *E. coli* membranes was used for quantitative estimation of proton-ATPase complexes in chromaffin granules and mitochondrial membranes. It was found that chromaffin-granule membranes contain less than 10% of the amount of proton-ATPase complex in mitochondrial membranes. The specific ATPase activity of chromaffin-granule membranes was on the order of 30 to 50% of the mitochondrial membranes. The ATPase activity of the chromaffin-granule membranes was more sensitive to 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene and 4-chloro-7-nitrobenzofurazan. It was much less sensitive than the mitochondrial membranes to antibody against $\beta$ subunit of proton-ATPase from *E. coli* membranes. After solubilization of chromaffin-granule membranes by octyglucoside and cholate and subsequent centrifugation on sucrose gradient, two different ATPase enzymes were separated. The heavier enzyme was identical to the mitochondrial-ATPase complex, while the lighter enzyme was identified as a novel ATPase, which might be responsible for the special properties of the ATPase activity of chromaffin-granule membranes.

**Key Words:** ATPase; chromaffin granules; membranes; immunodecoration; polypeptides.

[1]Abbreviations: DCCD, dicyclohoxylcarbodiimide: NBD-Cl, 4-chloro-7-nitrobenzofurazan; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethane sulfonic acid; FITC, fluorescein isothiocyanate.

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## Introduction

Neurotransmitters are stored in storage vesicles that upon stimulation deliver their content into the extracellular space. Chromaffin granules are such storage vesicles that function in the adrenal medulla cells. These granules store catecholamines at high concentration, and protonmotive force, which is generated by ATPase enzyme, is the driving force for this process (Njus *et al.*, 1981). Several lines of evidence led to the conclusion that a proton-ATPase enzyme might function in storage vesicles. Uncouplers of the weak acid type and energy-transfer inhibitors like DCCD affected the catecholamine uptake and the ATPase activity of the chromaffin granules in a fashion resembling their effect on mitochondrial membranes (Bashford *et al.*, 1976; Apps *et al.*, 1980). Recent studies on the chromaffin-granule membranes showed the presence of over 60 polypeptides, 13 of which were identified as glycoproteins (Abbs and Phillips, 1980). In these membranes a mitochondrial-type proton-ATPase complex was positively identified (Apps and Glover, 1978; Apps and Schatz, 1979). Upon chloroform treatment, the catalytic sector of the enzyme was released from the membrane, and following purification it was found to be identical to the mitochondrial enzyme in the molecular weights and one-dimensional proteolytic fingerprinting of its subunits (Apps and Schatz, 1979). Therefore, it was proposed that a mitochondrial-type proton-ATPase is generating the protonmotive force for catecholamine uptake into the granules (Apps and Glover, 1978; Apps *et al.*, 1980).

It is the purpose of this communication to describe the properties of a novel ATPase enzyme of the chromaffin-granule membranes, which might play a key role in the function of the storage vesicles.

## Experimental

Published procedures were used for protein determination (Lowry *et al.*, 1951), preparation of [$\gamma$-$^{32}$P]–ATP (Nelson, 1980), and gel electrophoresis in slabs containing an exponential gradient of 10 to 15% acrylamide (Douglas and Butow, 1976). ATPase activity was measured as previously described (Nelson, 1980). Twofold reaction mixture contained 10 mM Tricine-NaOH (pH 8), 2 mM ATP, 2 mM $MgCl_2$ and about $10^5$ cpm/ml of [$\gamma$-$^{32}$P]-ATP. The reaction mixture contained, in a final volume of 1 ml, 40 $\mu$mol of MES-Tricine (brought to pH 5 or 8 by NaOH) and 0.5 ml of the reaction mixture. The reaction was started by the addition of the enzyme preparation and terminated by the addition of 50 $\mu$l of 100% trichloroacetic acid. After centrifugation 0.5 ml of the supernatant was assayed for the hydrolysis of ATP as previously described (Nelson, 1980). Electrotransfer from acrylamide gel to

mitocellulose paper and immunodecoration by antibodies and [$^{125}$I]protein A were performed as previously described (Towbin *et al.*, 1979; Rott and Nelson, 1981; Nelson, 1982). Antibodies against individual subunits of ATPases from spinach chloroplast and *E. coli* membranes were obtained as previously described (Nelson *et al.*, 1973; Nelson, 1982). The antibody against $\beta$ subunit of yeast mitochondria ATPase was a generous gift from Dr. G. Schatz, Biocenter, Basel. Membranes of chromaffin granules and mitochondria from bovine adrenal medullae were prepared as previously described (Cidon and Nelson, 1982).

## Results

The purity of membranes and the degree of cross-contaminations can be assessed by the use of chemical markers. Figure 1 shows that [$^{125}$I]concanavalin A interacted with both chromaffin granules and mitochondrial membranes that were electrophoresed on SDS-gels and electrotransfered to mitrocellulose paper. The radioactive pattern is remarkably different for each of the membrane preparations and, therefore, it enables assessment of possible cross-contamination. Quantitative determination of the radioactive band in the autoradiographs revealed that the degree of cross-contamination does not exceed 5%. Similar conclusion could be obtained from the assessment of the phosphorylation of polypeptides in the chromaffin granules and mitochondrial membranes (Fig. 2). This experiment shows that both membranes contain protein kinase activity and that different polypeptides are phosphorylated in the various membranes. The purity of the mitochondrial membranes was checked by antibody that was raised against purified chromaffin-granule membranes (Fig. 3). The antibody interacted mainly with two peptides of the chromaffin-granule membranes, while the purified mitochondrial membranes only slightly interacted with the antibody, and it is quite safe to conclude that the cross-contamination is less than 5%.

ATPase activity can be readily detected in chromaffin-granule membranes (Kirshner, 1962). It was proposed that this activity could be attributed to a proton-ATPase enzyme that is similar to the mitochondrial one (Apps and Glover, 1978; Apps and Schatz, 1979). Recently it was shown in our laboratory that antibodies raised against $\beta$ subunits of proton-ATPases from chloroplasts, yeast mitochondria, and *E. coli* membranes cross-reacted with every $\beta$ subunit of similar enzymes tested so far (Rott and Nelson, 1981). This observation, together with the technique of electrotransfer and immunodecoration, made it possible to determine the relative amounts of proton-ATPase complexes that are present in chromaffin granules and mitochondrial membranes from the bovine adrenal medulla. Figure 4 shows that antibodies

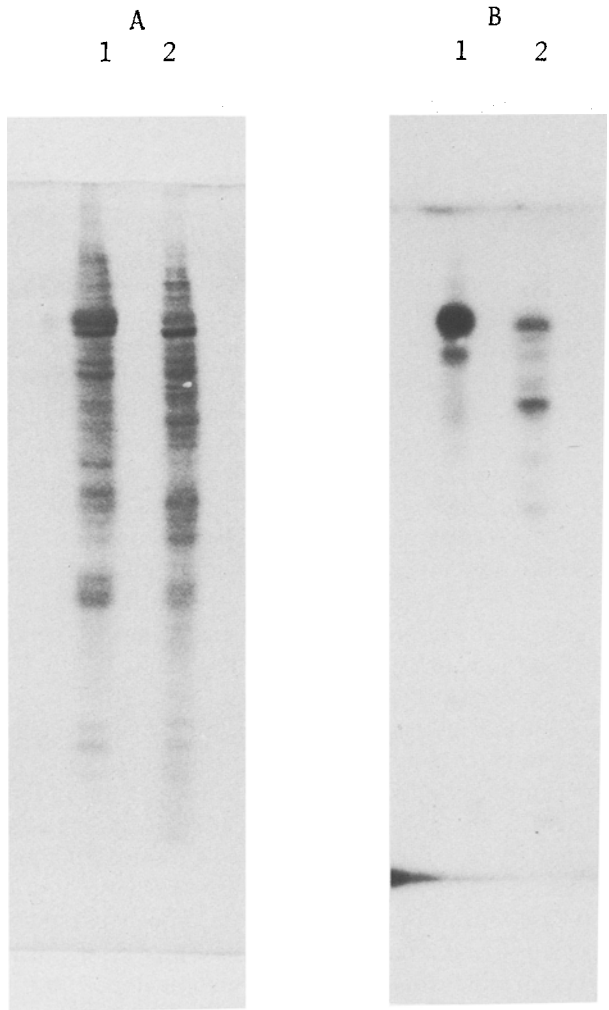


**Fig. 1.** Binding of concanavalin A to polypeptides from chromaffin granules and mitochondrial membranes. About 50 $\mu$g of membranes were electrophoresed on two identical exponential gels of 10 to 15% acrylamide in the presence of SDS. The peptides of one of the gels were electrotransferred onto nitrocellulose paper. The paper was decorated with [$^{125}$I]concanavalin A in a procedure similar to immunodecoration (Nelson, 1982). (A) Stained gel; (B) autoradiogram after decoration with [$^{125}$I]concanavalin A. (1) Chromaffin-granule membranes; (2) mitochondrial membranes.

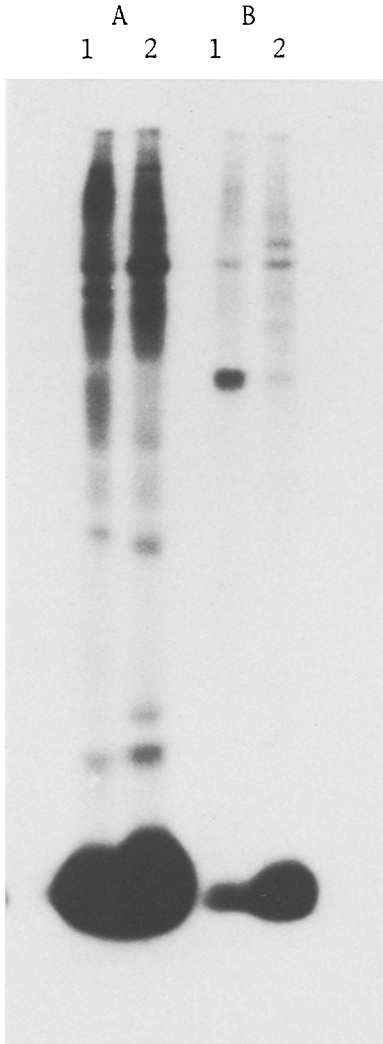


**Fig. 2.** Phosphorylation of chromaffin granules and mitochondrial membranes by ATP. The reaction mixture contained the following in a total volume of 0.1 ml: 60 $\mu$mol sorbitol, 0.4 $\mu$mol $MgCl_2$, 4 $\mu$mol MES-Tricine (pH 7), 20 nmol ATP, 20 $\mu$Ci [$\gamma$-$^{32}$P]-ATP, and membranes containing about 60 $\mu$g of protein. The reaction was carried on at room temperature for 15 min. It was terminated by the addition of protease inhibitors and SDS-dissociation buffer. Aliquots of 25 $\mu$l were electrophoresed on SDS gels and after drying the gels were exposed to X-ray film. (A) Chromaffin-granule membranes; (B) mitochondrial membranes. (1) Control; (2) the reaction mixture contained 0.5% octylglucoside.

against $\beta$ subunits of proton-ATPases from yeast mitochondria, spinach chloroplasts, and *E. coli* membranes reacted with mitochondrial and chromaffin-granule membranes. The relative amounts of radioactivity in the two kinds of membranes was determined both by densitometry and by a newly developed technique (M. Swissa and G. Schatz, manuscript in preparation). The measurements revealed that the amounts of proton-ATPase complex in chromaffin-granule membranes is less than 10% in comparison with the mitochondrial membranes. On the other hand, the specific ATPase activity of

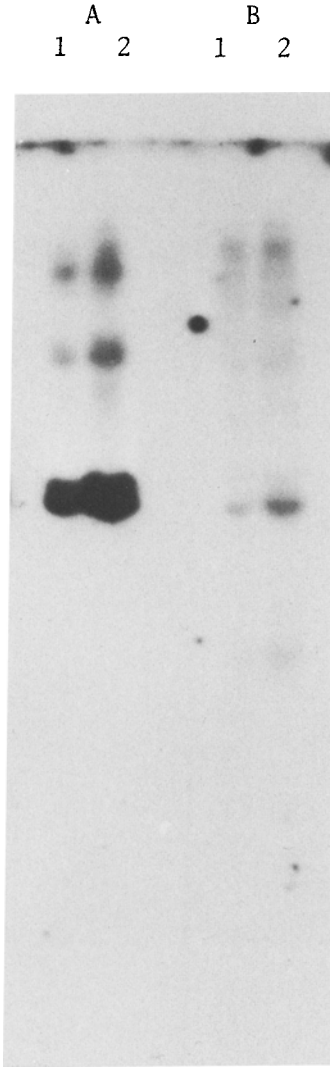


**Fig. 3.** Immunodecoration of chromaffin granules and mitochondrial membranes with antibody against purified chromaffin-granule membranes. Chromaffin granules and mitochondrial membranes were electrophoresed on gel with exponential gradient of 10 to 15% acrylamide in the presence of SDS. The polypeptides were electrotransfered onto nitrocellulose paper and decorated by 25 $\mu$l of antibody against chromaffin-granule membranes and [$^{125}$I]protein A. (A) Chromaffin-granule membranes; (B) mitochondrial membranes. (1) 10 $\mu$g of protein; (2) 40 $\mu$g of protein.

both membranes is quite similar and that of the mitochondrial membranes does not exceed threefold of the chromaffin-granule membranes (Apps and Schatz, 1979). Therefore, it is quite likely that a second ATPase enzyme functions in chromaffin granules.

Figure 5 shows that the ATPase activity of the granule membrane is much less sensitive to antibody against $\beta$ subunit of *E. coli* ATPase than the mitochondrial membranes. In order to prevent interference by membranes, the experiment was performed in the presence of octylglucoside. The ATPase

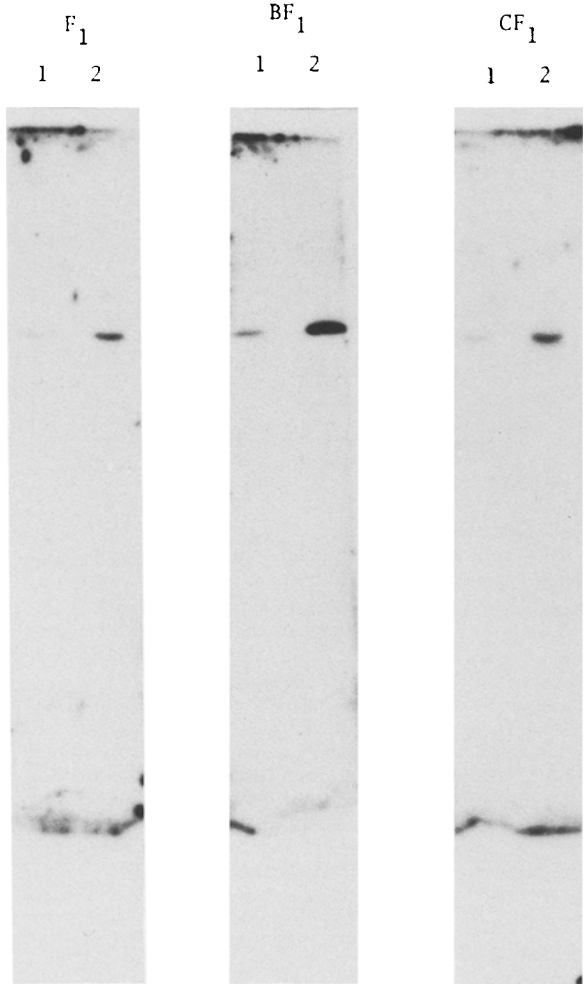


**Fig. 4.** Antibodies against $\beta$ subunits of ATPase from yeast mitochondria, *E. coli* membranes, and spinach chloroplasts cross-reacted with chromaffin granules and mitochondrial membranes. Aliquots of about 50 $\mu$g of chromaffin granules or mitochondrial membranes were electrophoresed and electrotransferred as was described in Fig. 3. The nitrocellulose papers were decorated with 25 $\mu$l antibodies against $\beta$ subunits of ATPases from yeast mitochondria ($F_1$), *E. coli* membranes ($BF_1$), and spinach chloroplasts ($CF_1$) Chromaffin-granule membranes; (2) mitochondrial membranes.

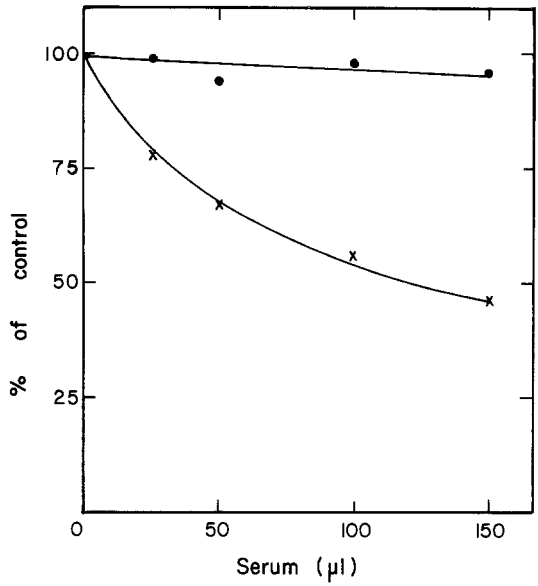


**Fig. 5.** Effect of antibody against $\beta$ subunit of *E. coli* ATPase on the ATPase activity of chromaffin granules and mitochondrial membranes. The reaction mixture contained the following in a final volume of 0.5 ml: 40 $\mu$mole MES-Tricine (pH8), 1% octylglucoside, and the specified amounts of antibody and membranes equivalent to 100 $\mu$g of protein. After 10-min incubation at room temperature, 0.5 ml of a mixture containing 1 $\mu$mol of ATP, 1 $\mu$mol of $MgCl_2$, and about $10^5$ cpm of [$\gamma$-$^{32}$P]-ATP was added. After incubation at 37°C for 10 min the reaction was terminated by the addition of 5% trichloroacetic acid. Following centrifugation the supernatant was assayed for the hydrolysis of ATP (Nelson, 1980). The control values of specific activity were 239 and 738 for chromaffin granules and mitochondrial membranes, respectively. (●) Chromaffin; (X) mitochondria.

activity of proton-ATPases is sensitive to 4-chloro-7-nitrobenzofurazan (NBD-Cl) (Nelson *et al.*, 1974; Deters *et al.*, 1975; Ferguson *et al.*, 1975). However, other ATPases like $Na^+$, $K^+$-ATPase are also sensitive to this chemical (Cantley, 1981). Figure 6 shows that the ATPase activity of chromaffin-granule membranes is more sensitive to NBD-Cl. The presence of octylglucoside in the assay medium enhanced the sensitivity of the mitochondrial membranes, but it did not reach the extent of inhibition of chromaffin-granule membranes.

Pazoles *et al.* (1980) have demonstrated the possible involvement of a $Cl^-$ channel in chromaffin-granule membranes. They showed that, at low pH, $Cl^-$ ions increase the ATPase activity of the membranes about twofold and

4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS) abolished the inclination. Figure 7 shows that at pH 5 NaCl increased the ATPase activity of the purified membranes about fourfold and SITS inhibited most of the enhanced activity. At pH 8 NaCl did not increase the ATPase activity but it was sensitive to SITS (Fig. 8). The concentration dependence of the inhibition revealed two effects. At low concentrations up to 10 $\mu$M the inhibition was probably due to a blockage in the chloride channel, while higher concentrations inhibited the solubilized enzyme as well. The ATPase activity of the mitochondrial membranes was not sensitive up to 100 $\mu$M SITS; however, in the presence of 0.5% octylglucoside the ATPase activity inhibited about 50% (not shown). The data presented so far strongly indicate that a novel ATPase enzyme having specific properties is functioning in the chromaffin-granule membranes. Yet there is very convincing evidence that a mitochondrial type proton-ATPase is present in the granules (Apps and Schatz, 1979). After solubilization by octylglucoside and cholate and subsequent sucrose gradient centrifugation two different ATPase enzymes could be

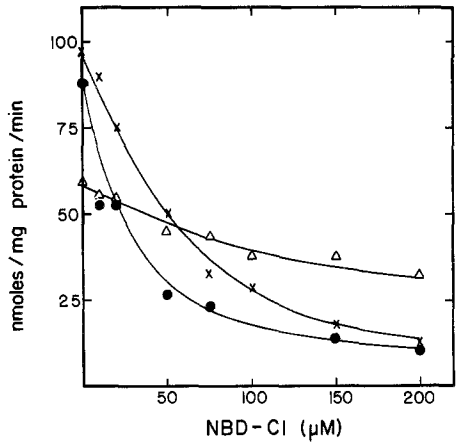


**Fig. 6.** Effect of NBD-Cl on the ATPase activity of chromaffin granules and mitochondrial membranes. The reaction mixture contained the following in a final volume of 1 ml: 40 $\mu$mol MES-Tricine (pH 8), 1 $\mu$mol ATP, 1 $\mu$mol $MgCl_2$, about $10^5$ cpm [$\gamma$-$^{32}$P-ATP, and membranes equivalent to 200 $\mu$g of protein. After incubation for 10 min at 37°C the ATPase activity was assayed as in Fig. 1. A fresh solution of NBD-Cl in dimethyl sulfoxide was added to the reaction mixture prior to the addition of the membranes. (△) Mitochondrial membranes; (X) mitochondrial membranes and 0.5% octylglucoside in the reaction mixture; (●) chromaffin-granule membranes.

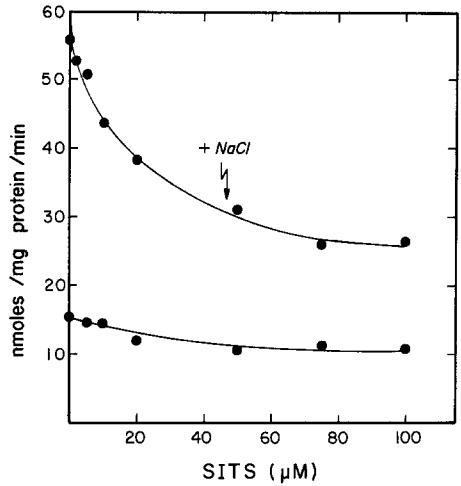


**Fig. 7.** Effect of SITS on the ATPase activity of chromaffin granules at pH 5. The reaction was performed as described in Fig. 6 except that MES-Tricine at pH 5 was used and, when specified, 80 mM NaCl was added.

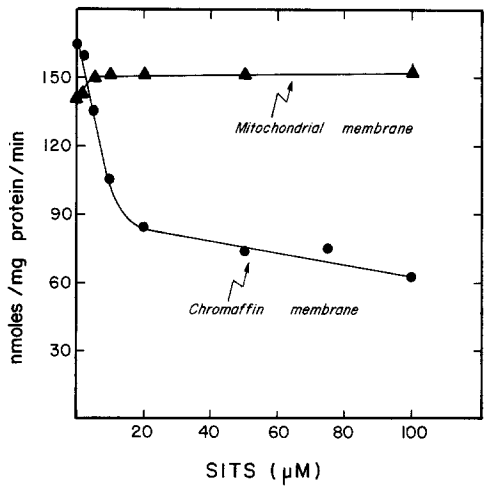


**Fig. 8.** Effect of SITS on the ATPase activity of chromaffin granules and mitochondrial membranes at pH 8. The reaction was performed as was described in Fig. 6 except that 63 $\mu$g of chromaffin granules and 60 $\mu$g of mitochondrial membranes were used.

separated from the chromaffin-granule membranes. The purification procedure was identical to that developed for the purification of proton-ATPase complex from rat liver mitochondria (Rott and Nelson, 1981). Figure 9 shows fractions 7 and 8 of the sucrose gradients from solubilized mitochondria and chromaffin-granule membranes, respectively. A similar polypeptide pattern to fraction 7 from mitochondria was obtained from solubilized chromaffin-granule membranes, except that the staining intensity of the bands was less than 10% of the corresponding fraction from mitochondria. The specific activity of those two fractions from chromaffin-granules was quite similar, while the polypeptide composition was remarkably different. Four main

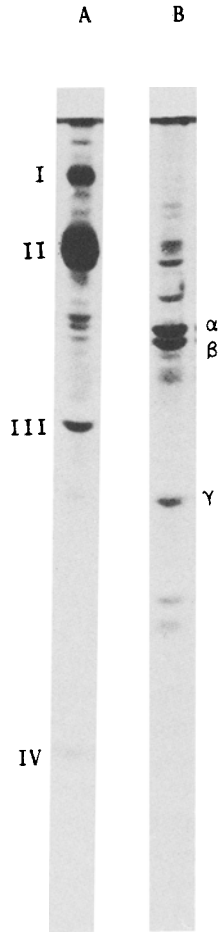


**Fig. 9.** Sodium dodecyl sulfate gels of chromaffin-granule ATPase and mitochondrial proton-ATPase complex. Aliquots of 1 ml of chromaffin granules and mitochondrial membranes containing 10 mg of protein were treated with 1% octylglucoside and 0.5% sodium cholate. After incubation at 0°C for 20 min the suspension was centrifuged at 200,000 × *g* for 1 hr. 0.5-ml portions of the supernatants were applied on sucrose gradients under conditions similar to those described for the purification of the rat liver mitochondrial ATPase complex (Rott and Nelson, 1981), except that 1 mM ATP was present in the gradient solution. Eleven fractions were collected from each tube. (A) 50 μl of fraction 8 from the chromaffin-granule membranes was disassociated and electrophoresed on SDS gel; (B) 50 μl of fraction 7 from the mitochondrial membranes was disassociated and electrophoresed on SDS gel.

protein bands were identified in the novel ATPase enzyme from chromaffin granules. The polypeptides were tentatively denoted as subunits I, II, III, and IV with molecular weights of about 125, 80, 40, and 20 kD, respectively. Subunits II, which is the main protein in the preparation, interacted with concanivalin A and, therefore, it seems to be a glycoprotein. FITC was covalently bound to subunit II upon treatment of membranes. Studies with specific inhibitors and various cations ruled out the possibility that this ATPase might be $Na^+$, $K^+$-ATPase or a $Ca^{++}$-ATPase.

## Discussion

The matrix of chromaffin granules contains about 0.6 M of catecholamines together with 0.12 M ATP, 20 mM calcium, and 20 mM ascorbate. The catecholamines are translocated across the chromaffin-granule membrane by a protonmotive force which should be generated by a proton-ATPase enzyme. Since the discovery of ATPase activity in chromaffin granules (Kirshner, 1962), the reaction was extensively investigated and found to be sensitive to inhibitors that are supposed to be characteristic for the mitochondrial type proton-ATPase complexes (Bashford *et al.*, 1976, Apps *et al.*, 1980). Moreover, a catalytic sector of proton-ATPase, which resembled the mitochondrial enzyme, was isolated from chromaffin-granule membranes (Apps and Schatz, 1979). These findings have led to the conclusion that a proton-ATPase is functioning in the chromaffin granules, and it is suggested that this enzyme is generating the protonmotive force for catecholamine uptake.

We demonstrated remarkable differences in the sensitivity of the ATPase activity of chromaffin granules and mitochondrial membranes toward DCCD (Cidon and Nelson, 1982), NBD-Cl, and SITS. The amount of proton-ATPase complex in chromaffin-granule membranes was found to be 5 to 10% of its amount in mitochondrial membranes (see Fig. 4). A contamination of about 5% mitochondrial membranes in the chromaffin-granule preparation could not be ruled out (Figs. 1–3). Therefore, the presence of mitochondrial type ATPase in the chromaffin-granule preparations might be due to contamination by mitochondrial membranes. However, this work could not eliminate the possibility that a mitochondrial type proton-ATPase complex functions in chromaffin-granule membranes. If this is the case, a few mechanistic obstacles should be overcome by the adrenal medulla cells. At least one of the mitochondrial proton-ATPase subunits is a product of the organelle genome, and the rest of the subunits are synthesized on cytoplasmic ribosomes as larger precursors and transported across the organelle membranes (Nelson and Schatz, 1979, Schatz, 1979). The chromaffin granules lack DNA and their ATPase is facing the cytoplasmic side of the membrane.

At least three solutions can be visualized for these problems. One is that a special set of genes coding for the chromaffin-granule ATPase exists alongside the set of genes coding for the mitochondrial enzyme. The former set should code for all of the ATPase subunits, and the gene products should be of the size of the mature polypeptides. A spontaneous assembly without vectorial processing should take place in the chromaffin-granule membranes (Nelson and Schatz, 1979). A second possibility is that in the adrenal medulla cells all of the mitochondrial ATPase subunits are synthesized on cytoplasmic ribosomes. A special device for processing is present in the vicinity of the chromaffin granules. Consequently, part of the precursors for the mitochondrial proton-ATPase is processed and assembled on the chromaffin-granule membranes. A third possibility is that in the course of biogenesis of the chromaffin-granule membranes these are fused with the mitochondrial membranes. During this short period of time the granules are loaded with ATP and a few protein complexes including the proton-ATPase are laterally diffused into the chromaffin-granule membranes.

Among the properties of the ATPase activity that were found to be different in chromaffin granules and mitochondrial membranes was the sensitivity to detergent treatment. While the ATPase activity of chromaffin granules was inhibited by octylglucoside, the ATPase activity of mitochondrial membranes was enhanced by similar treatment (Cidon and Nelson, 1982). After detergent treatment, followed by sucrose gradient centrifugation, two kinds of ATPase enzymes could be separated from the chromaffin-granule membranes (Cidon and Nelson, 1982). The heavier enzyme was detected in similar sucrose density to the mitochondrial enzyme, and it was found to be identical to the mitochondrial proton-ATPase complex. The lighter enzyme contained four major protein bands on SDS gels (Fig. 9) and had enzymatic properties that are not common to $Na^+$, $K^+$-ATPase, $Ca^{++}$-ATPase, proton-ATPase complex or the vanadate-sensitive enzyme class. After solubilization the total ATPase activity of the two enzymes was about equal. Since the activity of the novel enzyme is inhibited by the detergents, it would be fair to assume that this enzyme comprised over 70% of the ATPase activity of the membranes. We propose that this novel enzyme is the main ATPase enzyme which functions in the building up of the protonmotive force for the uptake of neurotransmitters.

It was reported that uncouplers, DCCD, and NBD-Cl inhibit ATP-dependent norepinephrine transport in synaptic vesicles from rat brain (Toll and Howard, 1978). Similarly, ATP-dependent dopamine transport into storage granules of PC12 clonal cell line was sensitive to uncouplers and DCCD (Rebois *et al.*, 1980; Toll and Howard, 1980). It is quite likely that an ATPase enzyme, similar to the one described in this paper, is functioning in storage vesicles for biogenic amines in cells with embryonic origin of the neural crest.

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