Vacuolar H⁺-ATPase of adrenal secretory granules

Rapid partial purification and reconstitution into proteoliposomes

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A procedure has been developed for the rapid purification and reconstitution into phospholipid vesicles of the proton-translocating ATPase of bovine adrenal chromaffin-granule membranes. It involves fractionation of the membranes with Triton X-114, resolubilization of the ATPase with n-octyl glucoside, addition of purified lipids and removal of detergent by gel filtration. The entire process can be completed within 2 h. H⁺ translocation was detected by the ATP-dependent quenching of the fluorescence of a permeant weak base. The effect of varying the lipid composition of the vesicles on ATP hydrolysis and H⁺ translocation by the reconstituted enzyme was examined. ATPase activity was maximally increased about 4-fold by added lipid, but was relatively insensitive to its composition, whereas vesicle acidification was absolutely dependent on the addition of phospholipids and cholesterol.

INTRODUCTION

H⁺-translocating ATPases fall into three classes, called F, P and V (Pederson & Carafoli, 1987). Those of the V (vacuolar) type occur in the tonoplasts of higher plants, in fungal vacuolar membranes and in endomembranes bounding the acidic compartments of animal cells (Forgac, 1989). Animal V-type ATPases have been isolated from a number of sources, including secretory granules (Percy *et al.*, 1985; Cidon & Nelson, 1986), clathrin-coated vesicles (Xie & Stone, 1986) and kidney medulla microsome (Gluck & Caldwell, 1988) and also identified in the Golgi (Young *et al.*, 1988; Moriyama & Nelson, 1989b). They contain at least six (and possibly more) types of subunit (Forgac, 1989) and have a structure that is generally similar to that of F-type ATPases (Arai *et al.*, 1988; Apps *et al.*, 1989; Moriyama & Nelson, 1989a). Immunological cross-reactivity has been demonstrated among V-type ATPases from various sources, the F-type ATPases from eubacteria and the H⁺-ATPases from archaeobacteria (Manolson *et al.*, 1989), and comparison of the amino acid sequences of the two nucleotide-binding subunits confirms this similarity, suggesting that V- and F-type ATPases have a common evolutionary origin (Gogarten *et al.*, 1989; Sudhof *et al.*, 1989).

Published procedures for the isolation of animal V-type ATPases involve several steps and yield a product of specific activity 3–5 µmol/min per mg of protein (Moriyama & Nelson, 1987, 1989a). The fractionation of chromaffin-granule membranes by phase separation in Triton X-114 (Bordier, 1981; Pryde & Phillips, 1986) affords extensive purification of the H⁺-ATPase complex in a single step, but the product is of low specific activity (Percy *et al.*, 1985). In order to investigate the properties of this ATPase we required a reconstituted enzyme capable of H⁺ translocation. We now report an adaptation of the Triton X-114 procedure that yields H⁺-pumping vesicles of high specific ATPase activity and can be completed within 2 h.

Chromaffin-granule membranes contain a P-type ATPase of unknown function, termed 'ATPase II' (Percy *et al.*, 1985; Moriyama & Nelson, 1988), as well as the H⁺-translocating V-type ATPase (ATPase I). The former ATPase is separated during the first stage of purification, appearing in the detergent-rich phase of Triton X-114 fractionation (Pryde & Phillips, 1986).

MATERIALS AND METHODS

Materials

Triton X-114 was obtained from Fluka AG, Buchs, Switzerland, and purified by the method of Bordier (1981). n-Octyl β-glucoside was purchased from Sigma. Bio-Gel P6-DG was from Bio-Rad. Cholesterol was supplied by Boehringer, Lewes, East Sussex, U.K. Bovine spinal-cord phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were obtained from Lipid Products, Nutfield, Redhill, Surrey, U.K. Soybean phosphatidylcholine was from Karlshamns AB, Stockholm, Sweden. 9-Amino-6-chloro-2-methoxyacridine (ACMA) and bafilomycin A₁ were generously given by Dr. R. Kraayenhof, Free University, Amsterdam, The Netherlands, and Dr. K. Altendorf, University of Osnabrück, Osnabrück, Germany.

Analytical methods

ATP hydrolysis was measured at 34 °C (see Figs. 1–6 below) or 37 °C (see Table 1 below), by a coupled spectrophotometric assay described previously (Percy *et al.*, 1985), with the addition of carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) (2 µM). H⁺ translocation by reconstituted ATPase proteoliposomes was measured at 30 °C by recording the quenching of ACMA fluorescence in a Perkin–Elmer 3000 fluorimeter, with excitation and emission wavelengths of 420 and 480 nm respectively. The assay medium (0.5 ml) contained 0.3 M-sucrose, 10 mM-Hepes/NaOH buffer, pH 7.4, 3 mM-ATP, 3 mM-MgSO₄, ACMA (0.2 µg/ml), valinomycin (0.4 µg/ml) and reconstituted ATPase vesicles (4 µg of protein/ml). Protein concentrations were measured by an adaptation of the Folin–Lowry method (Peterson, 1977). Concentrations of cholesterol and of phospholipids were measured by spectrophotometric assays, using assay kits purchased from Boehringer. Triton X-114 was assayed by the method of Garewal (1973). Concentrations of bafilomycin A₁ were determined spectrophotometrically, using the published extinction coefficient of 25 mm⁻¹ cm⁻¹ (Werner *et al.*, 1984).

Purification of the H⁺-ATPase

Chromaffin-granule membranes were purified from fresh bovine adrenal medullae as described elsewhere (Apps *et al.*,

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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1980) and stored at -20°C in 10 mM-Hepes/NaOH (pH 7.4)/0.1 mM-EDTA/1 mM-dithiothreitol. After thawing, membranes were sedimented (Beckman TL-100.3 rotor; 100 000 rev./min, 10 min; $412\,000\,g_{av}$). The membrane pellet was resuspended in 0.15 M-KCl/10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/1 mM-dithiothreitol, with gentle homogenization in a Teflon/glass homogenizer. Triton X-114 was added so that the final concentrations were: detergent, 20 mg/ml; membranes, 4 mg of protein/ml. After 5 min at 0°C , the white pellet was removed by centrifugation as described above and washed by homogenization in the same buffer, containing Triton X-114 (20 mg/ml), followed by centrifugation. It was then resuspended in half the original volume of buffer, containing n-octyl β -glucoside (18 mg/ml); after 10 min at 0°C it was centrifuged and the clear supernatant carefully removed. This was the solubilized partially purified ATPase.

Reconstitution into phospholipid vesicles

Pure lipids [solutions in chloroform/methanol (2:1, v/v)] were dispensed into glass tubes in the required amounts and dried under a stream of N_2 . (Typically, for 0.2 ml of resolubilized ATPase, 350 μg of phosphatidylcholine, 350 μg of phosphatidylethanolamine, 115 μg of phosphatidylserine and 215 μg of cholesterol gave near-optimal activities; see the Results section.) The solubilized ATPase (0.2 ml) was added and the lipids dissolved by vortex-mixing, followed by gentle homogenization. The solution was incubated at room temperature (about 20°C) for 10 min and loaded on to a 1 ml column of Bio-Gel P6-DG that had been equilibrated with 0.15 M-KCl/10 mM-Tris/HCl (pH 7.4)/1 mM-dithiothreitol/1 mM-EDTA/10% (w/v) methanol, and packed by centrifugation in a bench centrifuge (1 min; 1400 rev./min, $150\,g_{av}$). The sample was passed through the column by centrifugation under the same conditions, and stored on ice after collection.

RESULTS

Purification of H^+ -ATPase

Table 1 summarizes the purification of the H^+ -ATPase. Although there is a considerable loss of activity in the second step, washing of the pellet is essential to minimize contamination with ATPase II and endogenous lipids. Some ATPase activity is found in the supernatant, but it is partially vanadate-sensitive and therefore represents ATPase II (Percy *et al.*, 1985). The final specific activity reported in Table 1 is that obtained on reconstitution with a mixture of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol, in the proportion defined in the Materials and methods section; this is the optimal lipid composition that we have found, but it may be possible to achieve higher activities with other combinations of lipids.

Fig. 1. SDS/PAGE of ATPase fractions

Track A, chromaffin-granule membranes; track B, protein precipitated with Triton X-114; track C, washed precipitate; track D, reconstituted ATPase; outer tracks, molecular-mass standards [from top to bottom: β -galactosidase (116 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)]. Tracks A–D contained 50 μg of protein each. Staining was with Coomassie Blue. The numbers to the right of track D indicate the ATPase subunits (120, 72, 57, 41, 33 and 16 kDa).

Fig. 1 shows an SDS/polyacrylamide gel of the ATPase through the various stages of purification. The reconstituted enzyme contains the six major polypeptides of 120, 72, 57, 41, 33 and 16 kDa that have been assigned to the ATPase (Percy *et al.*, 1985; Moriyama & Nelson, 1987) and contaminants of 78, 38 and 36 kDa. It is noteworthy that reconstitution is selective, the proportion of contaminant proteins decreasing during the last step.

The procedure was usually applied to about 6 ml of purified chromaffin-granule membranes (24 mg of protein); on a smaller scale, mechanical losses became significant and the overall recovery was reduced. The reconstituted ATPase had good stability and lost only 4–10% of its ATPase activity in 24 h at 4°C . However, at the penultimate stage (solution in octyl glucoside) activity was rapidly lost, and it was important to proceed at once with the reconstitution.

Table 1. Reconstitution of chromaffin-granule ATPase 1

The data shown are for a typical preparation; values in parentheses are the means $\pm$ S.D. of specific activities and recoveries obtained in four separate preparations.

Stage	ATPase activity (nmol/min per ml)	[Protein] (mg/ml)	Specific activity (nmol/min per mg)	Recovery (%)
Membranes	2815	7.6	370 (348 $\pm$ 26)	100
Precipitate	1546	1.4	1104 (1263 $\pm$ 222)	90.0 (87.7 $\pm$ 6.9)
Washed precipitate	711	1.0	711 (1120 $\pm$ 355)	19.7 (26.1 $\pm$ 4.3)
Solubilized ATPase	200	0.21	952 (894 $\pm$ 195)	6.8 (7.0 $\pm$ 1.8)
Reconstituted ATPase	500	0.21	2380 (2453 $\pm$ 139)	15.1 (16.6 $\pm$ 1.9)

In the final step, 0.15 M-KCl could be replaced by 0.3 M-sucrose. The resulting vesicles initially had similar ATPase and H⁺-translocating activity (provided KCl was added to the assay medium; see below), but were less stable than those containing KCl. Similarly, if methanol was omitted from the reconstitution buffer, the resulting vesicles were less active in both assays, and also lost activity rapidly on storage.

Assay of H⁺ translocation

The quenching of ACMA fluorescence provides a sensitive and convenient assay for the development of a transmembrane pH difference. A typical trace of fluorescence quenching by reconstituted ATPase vesicles is shown in Fig. 2. Initial rates of fluorescence quenching were directly proportional to the amount of ATPase added, at least up to 4 μ g of protein/ml (results not shown) and were absolutely dependent on added ATP and Mg²⁺, and also on valinomycin (see below). Quenching was reversed by the addition of the uncoupler FCCP. ATPase activity and H⁺ translocation were inhibited in parallel by bafilomycin A₁ (Bowman *et al.*, 1988), 50% inhibition occurring at 1 nmol/mg of protein (results not shown).

Conditions for optimal activity

Both the ATPase and the H⁺-translocation activities were dependent on the lipid/protein ratio in the reconstituted vesicles (Fig. 3). These results were obtained with the mixture of lipids that gave optimal activities (see below), but the optimal lipid/

Fig. 2. Typical time course of ACMA fluorescence quenching by reconstituted ATPase vesicles (4 μ g of protein/ml)

Additions of ATP (3 mM) and FCCP (4 μ M) were made at the points shown. The vesicles contained phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol in the proportions 9:9:3:5 (by wt.), and the lipid/protein ratio was 26:1. Valinomycin (0.4 μ g/ml) was present throughout.

Fig. 3. Dependence of ATPase activity (■) and ACMA fluorescence quenching rate (▲) on the lipid/protein ratio

The lipid composition was the same as in Fig. 2, and valinomycin (0.4 μ g/ml) was present.

Fig. 4. Dependence of initial rate of quenching of ACMA fluorescence on valinomycin concentration

The vesicle concentration was 4 μ g of protein/ml and vesicle composition was the same as in Fig. 2.

protein ratio was not dependent on the lipid composition; with each lipid mixture tried, vesicles containing 26 μ g of total lipid/ μ g of protein had maximal H⁺-translocating activity, and almost maximal ATPase activity. Similar activities were obtained using phospholipids from soybean and from bovine spinal cord.

Since H⁺ translocation is electrogenic, the maximal activity is found when the membrane potential induced by H⁺ translocation is overcome by compensating ion movements. In these experiments this was achieved by the addition of valinomycin, which permits exit of K⁺ from the vesicles in exchange for H⁺. At very low concentrations of valinomycin, H⁺ translocation was apparently limited by the rate of K⁺ movement (Fig. 4); in all other experiments we used approx. 100 ng of valinomycin/ μ g of protein. ATPase activity is also stimulated by valinomycin and by FCCP; the stimulation observed depended on the lipid/protein ratio in the vesicles (results not shown), but was up to 50%. ATPase activity was therefore routinely assayed in the presence of FCCP.

Effect of lipid composition on ATPase and H⁺-translocating activities

In order to examine the effect on the reconstituted ATPase of the lipid composition of the vesicles, we made vesicles containing

Fig. 5. Dependence of ATPase activity (a) and ACMA fluorescence quenching rate (b) on the phosphatidylcholine/phosphatidylserine ratio

The total phospholipid content was 30 μg/μg of protein and the cholesterol/protein ratio was 0 (♦), 3 (▲) or 6 (■) μg/μg of protein. The valinomycin concentration was 0.4 μg/ml.

a constant amount of phospholipid, but varied the ratio of phosphatidylcholine to phosphatidylserine, and we examined the effect of incorporating cholesterol into these vesicles. These results are shown in Fig. 5; ATPase activity is relatively insensitive to lipid composition, but is highest with a 2:1 ratio of phosphatidylcholine to phosphatidylserine; the inclusion of cholesterol has little effect. In contrast, H⁺ translocation is sharply dependent on the phospholipid ratio and on the cholesterol content of the vesicles. Phosphatidylethanolamine is able to substitute for phosphatidylcholine in reconstitution of the enzyme; Fig. 6 shows the effect of replacing one lipid by the other, the amount of phosphatidylserine and cholesterol being fixed. Independent variation of the amount of phosphatidylserine (results not shown) showed that the optimum was 3 μg/μg of protein. From these results we could define a standard vesicle composition that would give optimal reconstituted ATP-dependent H⁺-translocation activity: this was phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol at 9, 9, 3 and 5 μg/μg of protein respectively.

DISCUSSION

The initial aim of this work was to develop the previously reported procedure of fractionating chromaffin-granule membranes with Triton X-114 to give a product of specific activity comparable with that of the ATPase purified chromatographically. The new procedure can be completed within 2 h,

Fig. 6. Effect of replacing phosphatidylcholine by phosphatidylethanolamine

The phosphatidylserine content was constant at 10 μg/μg of protein, and other conditions were as in Fig. 5.

giving a product that is remarkably stable. It is completely inhibited by bafilomycin A₁ and insensitive to vanadate, so is free of ATPase II (Percy *et al.*, 1985). In the studies by Moriyama & Nelson (1987), reconstitution of H⁺-pumping activity was achieved using endogenous lipids; the ATPase activity of their preparation was slightly higher than that reported here, but the lipid composition of the vesicles was undefined. Our procedure can be modified so that enough endogenous lipid remains to reconstitute ATPase activity, by decreasing the concentration of Triton X-114 to 1.15%, and omitting the second step. ATPase activity is then reconstituted, to a specific activity of about 1.7 μmol/min per mg, on solubilization with octyl glucoside and removal of this detergent by gel filtration; but the vesicles so formed were unable to support a measurable transmembrane pH gradient, so this approach was not pursued. The ATPase pellet has a phospholipid content of < 1 μg/μg of protein, so that the composition of the vesicles was defined by the lipids added.

Vesicular acidification was monitored by the quenching of ACMA fluorescence. A number of observations suggest that the quenching rate is proportional to the rate of H⁺ translocation: the proportionality of quenching rate to the protein concentration, its dependence on the addition of valinomycin, its instantaneous reversal by FCCP, and the parallel inhibition by bafilomycin A₁ of fluorescence quenching and ATP hydrolysis.

The observed fractional fluorescence change (*Q*) of self-quenching permeant monoamines such as ACMA is given by:

$$\Delta pH = \log \left(\frac{[ACMA]_{in}}{[ACMA]_{out}} \right) = \log \left(\frac{Q}{1-Q} \right) - \log V$$

(Schuldiner *et al.*, 1972). In this expression, V is the 'osmotic volume', that is, the fraction of the volume in the assay that is within the vesicles. Because it is so small, this term dominates the expression for ΔpH . It has not been measured, so the intravesicular pH cannot be calculated. Variation in vesicle size with changing lipid concentration will obviously have a profound effect on the observed fractional quench. The size of the vesicles can, in principle, be measured by photon correlation spectroscopy, and preliminary studies with vesicles containing the optimal lipid/protein ratio of 26:1 suggest a mean diameter of about 180 nm (J. R. Perez-Castineira & D. K. Apps, unpublished work). Although these considerations show that the rate of ACMA fluorescence quenching depends on the size of the vesicles, the response of the probe is not intrinsically dependent on their lipid composition, as results very similar to those presented in Fig. 5(b) have been obtained with two other probes, 9-amino-acridine and quinacrine (J. R. Perez-Castineira & D. K. Apps, unpublished work).

Although optimization of the reconstitution conditions does not depend on a quantitative interpretation of the rate of quenching, the fractional quench can be related to the ΔpH in vesicles of a given lipid composition. Haigh *et al.* (1989) showed that the equation of Schuldiner *et al.* (1972) can be applied to the behaviour of ACMA in intact chromaffin granules, and we have confirmed this using ATPase proteoliposomes of the optimal lipid composition and lipid/protein ratio. In these, the slope of a plot of $\log[(Q/1) - Q]$ against ΔpH was close to its ideal value of 1.0 (results not shown).

Variation of the lipid composition of the vesicles affected both the rate and the final extent of quenching (results not shown). The quench is the resultant of several variables, each of which may be affected by the lipid composition: the activity of the H^+ -translocating ATPase, the size of the vesicles and their H^+ permeability. In the experiments reported here, H^+ permeability of the reconstituted vesicles may be the most important determinant. At low lipid/protein ratios, maximal ATPase activity is achieved without measurable ΔpH (Fig. 4), presumably because sealed vesicles are not formed, and, similarly, omission of cholesterol inhibits the development of ΔpH without much effect on the ATPase activity (Figs. 5 and 6).

The optimal phospholipid composition for vesicle H^+ -translocating activity is quite similar to that found in the membranes of chromaffin granules (Winkler & Westhead, 1980), though the latter contain lysophosphatidylcholine (not tested here). Granule membranes contain a larger fraction of cholesterol than was required for ATPase or H^+ -translocating activity in our vesicles, but greater molar fractions of cholesterol were not inhibitory. Our results can also be compared with those of Xie *et al.* (1986), who investigated the lipid-dependence of H^+ translocation by the ATPase of clathrin-coated vesicles. They found that a much larger ratio of lipid to protein (200:1) was required for optimal H^+ translocation, but their optimal lipid composition was remarkably similar to that found in the present work. The difference may arise in the different method of vesicle preparation used, rather than in the intrinsic lipid requirement of the enzymes.

Furthermore, they optimized the maximal extent of accumulation of their pH probe, Acridine Orange, rather than its initial rate.

The purification procedure reported here provides a rapid and convenient method for preparing vesicles containing purified chromaffin-granule ATPase 1, active in H^+ translocation. Because of the difficulties of interpreting rates of fluorescence quenching, it is not possible to make detailed inferences about the lipid requirement of the enzyme, but these results have established the conditions for making a preparation that can be used for further studies of its catalytic properties, and may be applicable to other V-type ATPases.

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