High Purity Preparations of Higher Plant Vacuolar H\(^+\)-ATPase Reveal Additional Subunits

REVISED SUBUNIT COMPOSITION*

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A fast protein liquid chromatography procedure for purification of the V-type H\(^+\)-ATPase from higher plant vacuolar membrane to yield near-homogeneous enzyme with a specific activity of 20–25 \(\mu\)mol/mg·min is described. When precautions are taken to ensure the quantitative recovery of protein before sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the preparation is found to be constituted of seven major polypeptides of 100, 67, 55, 52, 44, 32, and 16 kDa, respectively, and two minor components of 42 and 28 kDa. The 52- and 44-kDa polypeptides do not cross-react with antisera raised to the 67- and 55-kDa subunits of the enzyme, and two independent sample preparation procedures yield the same apparent subunit composition. The additional polypeptides are not breakdown products or aggregates of the previously identified products or aggregates of the previously identified subunits of the ATPase.

The ATPase of tonoplast vesicles is subject to MgATP-dependent cold inactivation, and the conditions for inactivation are identical to those for the bovine chromaffin granule H\(^+\)-ATPase (Moriyama, Y., and Nelson, N. (1989) J. Biol. Chem. 264, 3577–3582). Cold inactivation is accompanied by the detachment of five major polypeptides of 67, 55, 52, 44, and 32 kDa from the membrane, and all five components co-migrate with the corresponding polypeptides of the purified ATPase upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 100- and 16-kDa polypeptides of the ATPase are not removed from the membrane during cold inactivation, but the latter can be purified to homogeneity by chloroform:methanol extraction of the fast protein liquid chromatography-purified enzyme.

It is concluded that the tonoplast H\(^+\)-ATPase is constituted of 6-7 major polypeptides organized into a peripheral sector comprising the 67-, 55-, 52-, 44-, and 32-kDa components and an integral sector consisting of the 100- and 16-kDa polypeptides. The V-type H\(^+\)-ATPases from animal endomembranes and higher plant vacuolar membranes therefore have remarkably similar subunit compositions and gross topographies.

The endomembrane or vacuolar (V-type) H\(^+\)-ATPases of both animal and plant cells constitute a third category of H\(^+\)-translocating phosphohydrolase distinct from the F-type (P,F,-type) H\(^+\)-ATPases of mitochondria, chloroplasts, and eubacterial plasma membranes and the P-type ("plasma membrane-type") H\(^+\)-ATPases of the plasma membranes of plants and fungi (Pederson and Carafoli, 1987; Rea and Sanders, 1987).

V-type H\(^+\)-ATPases have an apparent functional mass of 400,000-600,000 and comprise three to nine different subunits, of which the nucleotide-binding, 67-73- and 55-62-kDa polypeptides and a 16-kDa proteolipid are universal components (e.g. Pederson and Carafoli, 1987; Rea and Sanders, 1987). Immunological cross-reactivity between the nucleotide-binding subunits of the enzymes from plant and fungal vacuolar membranes and (animal) clathrin-coated vesicles and chromaffin granules is demonstrable (Manolson et al., 1987), and all of the enzymes concerned are inhibited by nitrate. Genomic and cDNA clones of the structural genes for the 67-73-kDa subunits of the enzymes from Neurospora and Daucus, respectively, yield deduced amino acid sequences with more than 60% identity (Bowman et al., 1988a; Zimniak et al., 1988), whereas the 55-62 kDa subunits from Arabidopsis (Manolson et al., 1988), Neurospora (Bowman et al. 1988b), and Saccharomyces (Nelson et al., 1989) show greater than 70% sequence identity. Conservation of primary structure within the V-type category is therefore pronounced. A major but as yet unaddressed issue, however, is the extent of conservation of subunit composition within the category.

Most preparations of the V-type enzymes from animal endomembranes comprise six polypeptides of 100–116, 70–73, 57–60, 39–41, 33, and 16 kDa, respectively. Arai et al. (1988) report 9 and Xie and Stone (1986) 8 polypeptides for the enzyme from bovine clathrin-coated vesicles, and Cidon and Nelson (1986) and Percy et al. (1985) report 4–6 and 5–6 polypeptides, respectively, for the bovine chromaffin granule ATPase. By contrast, studies of the vacuolar H\(^+\)-ATPases of plants and fungi have yielded results consistent with a simpler three-subunit heteromultimer consisting of multiples of only 3 polypeptides of 67–72, 55–62, and 16kDa, respectively (Rea and Sanders, 1987).

The reason for the differences between the enzymes from animal endomembranes and plant and fungal vacuolar membranes is not known. On the one hand, it has been suggested that the enzymes in membranes bounding catabolic intracellular compartments, such as lysosomes and plant vacuoles, may differ from those participating in receptor-mediated endocytosis and those present in secretory granules (Nelson, 1988), in that the latter may require "accessory" subunits for intracellular sorting. On the other hand, it should be appreciated that while the H\(^+\)-ATPases from chromaffin granules...
and clathrin-coated vesicles have been purified exhaustively from isolated membranes (Arai et al., 1987; Cidon and Nelson, 1986; Xie and Stone, 1986), the enzymes from plant and fungal vacuolar membranes have only been purified partially, so necessitating affinity labeling in parallel with purification for the ascription of specific polypeptides to the enzyme complex (e.g. Bowman et al., 1986; Mancson et al., 1985; Randall and Sze, 1986). Subunits without reactivity toward the affinity probes tested might consequently have been overlooked.

Thus, in this investigation we present methods for the purification of the H+-ATPase of higher plant vacuolar membrane to near-homogeneity to test the notion of subgroups. The purification data in combination with studies of the mechanism of cold inactivation of the enzyme of native membranes provide the first clear evidence that the V-type ATPases from animal endomembranes and higher plant cells have identical subunit organizations.

**MATERIALS AND METHODS**

**Preparation of Tonoplast Vesicles—**Tonoplast vesicles were isolated from storage root of fresh, greenhouse-grown red beet (Beta vulgaris L.) by differential and sucrose density gradient centrifugation (Rea and Poole, 1985).

**Membrane Solubilization—**Tonoplast vesicles were solubilized with Triton X-100. The tonoplast suspension was adjusted to 3–5 mg/ml membrane protein with suspension medium (1 M glicerol, 1 mM Tris-EDTA, 0.5 mM butylated hydroxytoluene, 0.5 mM dithiothreitol, 5 mM Tris-Mes (pH 8.0)), and an equal volume of 8% (w/v) Triton X-100 (containing 0.8 mg/g butylated hydroxytoluene) dissolved in 20% (v/v) glicerol, 1 mM Tris-EDTA, 4 mM MgSO4, 5 mM dithiothreitol, 5 mM Tris-Mes (pH 8.0) was added dropwise with constant stirring. The mixture was incubated on ice for 30 min before chromatography.

**Chromatography—**Solubilized tonoplast ATPase was purified by two successive chromatographic steps: gel filtration on Sephacryl S-400 and anion-exchange FPLC on Mono-Q. A 100 x 1-cm diameter column packed with Sephacryl S-400 was equilibrated with running buffer (10% (v/v) glicerol, 0.3% (w/v) Triton X-100, 0.05 mg/ml Type IV-S phospholipid, 5 mM dithiothreitol, 1 mM Tris-EDTA, 4 mM MgCl2, 5 mM Tris-Mes (pH 8.0)). Triton X-100-solubilized membrane (3-5 mg of protein) was applied and the column was run at a flow rate of 2–3 ml/h and a temperature of 4°C. Fractions of 1.2 ml were collected and assayed for protein and ATPase activity.

**ATPase Assays—**ATPase fractions from chromatography on Sephacryl S-400 were then subjected to FPLC on an HR 5/5 Mono-Q column packed with Sephacryl s-400 were then subjected to FPLC on an HR 5/5 Mono-Q column equilibrated with running buffer (10% (v/v) glicerol, 0.3% (w/v) Triton X-100, 0.05 mg/ml Type IV-S phospholipid, 5 mM dithiothreitol, 1 mM Tris-EDTA, 4 mM MgCl2, 5 mM Tris-Mes (pH 8.0)). The peak ATPase fractions from chromatography, the reaction media were supplemented with 1.33 mg/ml sonicated Type IV-S phospholipid (Rea and Poole, 1986).

**Protein—**Protein was estimated routinely by the dye-binding method of Lowry et al. (1951) on concave exponential gradient gels (C’Farrel, 1975). The molecular weight markers were: myosin (M, 255,000), 6-galactosidase (M, 116,000), phosphorylase b (M, 97,400), bovine serum albumin (M, 66,000), ovalbumin (M, 45,000), glyceraldehyde-3-phosphate dehydrogenase (M, 36,000), carboxic anhydride (M, 29,000), trypsinogen (M, 24,000), trypsin inhibitor (M, 20,100), and a-lactalbumin (M, 14,200).

The gels were stained with silver (Bio-Rad Laboratories Ltd., Watford, United Kingdom) and for Coomassie Blue. Double-staining was by the method of Dzandu et al. (1984). Quantitative densitometry was performed by scanning the stained gels with an LKB Bromma Ultrascan XL enhanced laser densitometer at 633 nm.

**ATPase Assays—**ATPase activity was determined by measuring the liberation of Pi at 37°C in the reaction medium containing 30 mM Tris-Mes (pH 8.0), 50 mM KC1, 5 mM glicericidin D, 3 mM MgSO4, and 3 mM Tris-ATP. The reaction was initiated by the addition of membrane protein. The reaction was stopped (and, if present, C14Es or Triton X-100 and added phospholipid were precipitated by the addition of ice-cold 4% (v/v) perchloric acid, 4% (v/v) trichloracetic acid, 4% (v/v) acetic acid, and 4% (v/v) perchloric acid. The samples were left on ice for 10 min. The supernatant was collected and assayed for Pi by the method of Ames (1966).

To maximize the activity of the solubilized membranes and the fractions from chromatography, the reaction media were supple-
method of Bradford (1976). Protein in the column fractions was measured by a modification of the Lowry method (Peterson, 1979).

**Chemicals**—Sephacryl S-400 and Mono-Q were from Pharmacia Biotechnology, Inc. (Milton Keynes, United Kingdom). Triton X-100, Type IV-S phospholipid (partially purified soybean L-α-phosphatidylcholine), and protein A-alkaline phosphatase conjugate were purchased from Sigma Chemical Co. Ltd. (Poole, United Kingdom). All of the general laboratory reagents were from Sigma, British Drug House (Poole, United Kingdom), or FSA Laboratory Supplies (Loughborough, United Kingdom).

**RESULTS AND DISCUSSION**

**Solubilization and Chromatography**—Chromatography of Triton X-100-solubilized tonoplast on Sephacryl S-400, equilibrated and eluted with running buffer containing 0.3% (w/v) Triton X-100 and 0.05 mg/ml phospholipid (Fig. 1A), resulted in an approximately 7-fold enrichment of the ATPase relative to native tonoplast and an approximately 9-fold purification relative to solubilized tonoplast (Table I). Subsequent FPLC of the peak ATPase fractions from the Sephacryl S-400 column on Mono-Q (Fig. 1B) enabled a further 8-9-fold enrichment of the ATPase to yield enzyme with a specific activity of 20-22 pmol/mg.min. The overall purifications were 61- and 74-fold relative to native and solubilized membranes, respectively, with an overall recovery of 26% (Table I).

Exogenous phospholipid in the running buffers and additional sonicated phospholipid in the assay media were necessary for the quantitative recovery of activity. Supplementation with phospholipid after chromatography in the absence of added phospholipid did not restore activity, indicating irreversible denaturation of the enzyme. Chromatography in the presence of phospholipid but assay without further supplementation, on the other hand, underestimated ATPase activity by a factor of 6-8. Consequently, phospholipid (0.05 mg/ml) was routinely included in the running buffers and all assays were performed in reaction medium containing 1.33 mg/ml sonicated phospholipid.

**Nitrate Inhibitability**—The identity of the purified ATPase was confirmed by its nitrate inhibitability (Table II). The activity of the purified enzyme was 88% inhibited by 50 mM KNO₃ (in the presence of 50 mM KCl), whereas native tonoplast vesicles and the peak fractions from Sephacryl S-400 chromatography were inhibited by 55 and 69%, respectively.

Contamination by mitochondrial (F-type) ATPase, plasma membrane (P-type) ATPase, and nonspecific phosphatase was negligible: azide (1 mM), orthovanadate (100 μM), and molybdate (200 μM) caused no inhibition of the purified enzyme (data not shown).

**Polypeptide Composition**—Crucial to determining the polypeptide composition of the purified ATPase was the method employed for the preparation of the chromatographic fractions for SDS-PAGE (Fig. 2). Precipitation of the fractions

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**Fig. 1. Chromatographic purification of tonoplast ATPase.** A, Chromatography of Triton X-100-solubilized tonoplast on Sephacryl S-400. Protein (○) and ATPase activity (■). Five mg of solubilized protein was applied. B, FPLC on Mono-Q of fractions 35-41, inclusive, from Sephacryl S-400 chromatography. A₂₆₆nm (○), ATPase activity (■), KCl gradient (Δ). Approximately 8 ml of sample (containing 600-700 μg of protein) was applied to the column with a Superloop. The column was run at 0.5 ml/min and 1-ml fractions were collected. The program parameters were: 0-12 min, 0.00 m KCl; 12-20 min, 0.00-0.15 m KCl; 20-64 min, 0.15-0.26 m KCl; 64-70 min, 0.26-1.00 m KCl; 70-90 min, 1.00 m KCl. ATPase activity was measured on 30-μl aliquots of the fractions in reaction media supplemented with 1.33 mg/ml sonicated phospholipid.
with trichloroacetic acid and sequential extraction of the pellet with 90% (v/v) Triton X-100 (TX-100) (see “Materials and Methods”) and chromatographed on Sephacryl S-400. The peak ATPase fractions (total volume 8 ml) from gel filtration chromatography were then applied to a Mono-Q column and eluted with KCl as described in the legend to Fig. 1B. Native membranes were assayed in the presence of 5 μM gramicidin D to ensure H+/cation equilibration. The solubilized membranes and chromatographic fractions were assayed in the presence of 1.33 mg/ml sonicated phospholipid to ensure maximum phospholipid activation. “Peak ATPase” refers to the column fractions containing the highest ATPase activity. The values shown are the mean from three separate purifications.

### Table I

| Step                  | Specific activity | Purification | Recovery (step) |
|-----------------------|-------------------|--------------|-----------------|
| Tonoplast             | 0.33              | -fold        | %               |
| TX-100 supernatant    | 0.27              | 1.0          | 75.0            |
| Sephacryl S-400       | 2.36              | 8.9          | 32.8            |
| Peak ATPase Mono-Q    | 20.10             | 8.5          | 74.4            |
| Overall               |                   |              | 25.6            |

### Table II

Nitrate inhibition of tonoplast ATPase before and after purification

ATPase activity was measured in a reaction system containing 30 mM Tris-Mes (pH 8.0), 50 mM KCl, 5 μM gramicidin D, 0.3 mM MgSO₄, 1.33 mg/ml sonicated phospholipid, and 50 mM KNO₃ where indicated. The values shown are the mean ± S.E. for n = 6.

| Preparation          | Specific activity | Inhibition |
|----------------------|-------------------|------------|
|                     | −KNO₃             | +KNO₃      |
| Tonoplast            | 0.38 ± 0.01       | 0.17 ± 0.01| 55.3           |
| Sephacryl S-400      | 2.00 ± 0.07       | 0.63 ± 0.01| 68.5           |
| Peak ATPase Mono-Q   | 21.62 ± 0.93      | 2.50 ± 0.19| 88.4           |

### Cold Inactivation

Recent investigations by Moriyama and Nelson (1989) demonstrate that the V-type H⁺-ATPases from a wide range of sources are subject to MgATP-dependent cold inactivation. The conditions for cold inactivation are well defined and the abolition of ATPase activity is associated with detachment of the 72-, 57-, 41-, and 33-kDa subunits of the chromaffin granule H⁺-ATPase from the membrane. Thus, in order to test our deductions concerning the subunit composition of the tonoplast ATPase, experiments were performed to determine if cold inactivation of the tonoplast enzyme has the same requirements as those defined by Moriyama and Nelson (1989) and is associated with the selective detachment of polypeptides identical to those constituting the purified enzyme. The results are summarized in Figs. 4 and 5.

### Incubation of Tonoplast Vesicles

Incubation of tonoplast vesicles at 4°C in the presence of 5 mM Mg²⁺, 5 mM ATP, and 0.15 M NaCl caused a rapid and irreversible abolition of ATPase activity. Inactivation was maximal after 20 min and resulted in 65% loss of activity relative to controls (Fig. 4). ATP, alone, elicited some inactivation but vesicles incubated at 4°C in Mg²⁺, alone, or in the absence of both Mg²⁺ and ATP exhibited negligible loss of activity over the course of the 60-min incubation period. While incubation at 4°C for 5–10 min was sufficient to cause 50% inhibition, more than 60 min were required for similar inhibitions at room temperature. The requirements for cold inactivation of the tonoplast ATPase therefore closely ap-
Fig. 2. SDS-PAGE of purified ATPase. A, successive ATPase-containing fractions from FPLC on Mono-Q after delipidation by the method of Piccioni et al. (1982) (see "Materials and Methods"). The ATPase activities of the fractions (μmol/fraction·h) were: lane 1 (0.6); lane 2 (2.8); lane 3 (8.0); lane 4 (11.1); lane 5 (6.9); lane 6 (4.6); lane 7 (2.6). B, successive ATPase-containing fractions from FPLC on Mono-Q after delipidation with acetone:ethanol (1:1) (see "Materials and Methods"). The ATPase activities of the fractions (μmol/fraction·h) were: lane 1 (0.7); lane 2 (2.7); lane 3 (4.9); lane 4 (8.1); lane 5 (15.3); lane 6 (13.3); lane 7 (10.4); lane 8 (3.3); lane 9 (1.1). C, direct comparison of same peak ATPase fraction from Mono-Q after delipidation by method of Piccioni et al. (1982) (lane 1) versus delipidation with acetone:ethanol (lane 2). The samples were run on 7–12% concave exponential gels in A and B and an 8–14% gel in C. The gels were double-stained with silver stain and Coomassie Blue. Equal volumes of the fractions were electrophoresed in each case to give a maximum load of 6–8 μg/lane.

proximate those elucidated by Moriyama and Nelson (1989) for the chromaffin granule ATPase.

Incubation of tonoplast vesicles with MgATP and NaCl at 4 °C for 60 min before centrifugation at 200,000 × g resulted in the release of 5 prominent polypeptides from the membranes (Fig. 5A), and all 5 co-migrated with the corresponding 67-, 55-, 52-, 44-, and 32-kDa polypeptides of the purified ATPase on SDS gels. Vesicles subjected to the same treatment but in the absence of MgATP, by comparison, liberated negligible protein to the supernatant.

Neither the 100- nor 16-kDa polypeptides of the tonoplast ATPase were released from the membrane during cold inac-
Table III
Proportionality between M, 100,000, 67,000, 55,000, 52,000, 44,000, 32,000, and 16,000 polypeptides in fractions from Mono-Q

The Mono-Q fractions containing ATPase activity were electrophoresed on 7–12% (w/v) or 8–14% (w/v) concave exponential gels and double-stained. Individual bands were quantitated relative to the M, 67,000 band by integrating laser densitometry. The lines of best fit for plots of peak area vs. M were computed using the algorithm of Marquadt (1963). Six fractions from different chromatographic runs were quantitated (n = 30). The values for the correlation coefficient (r) were estimated by the least squares method.

| Polypeptide (M) | r     | Slope (±SEM) |
|----------------|-------|-------------|
| 100,000        | 0.961 | 0.79 ± 0.08 |
| 55,000         | 0.980 | 1.47 ± 0.14 |
| 52,000         | 0.986 | 1.64 ± 0.12 |
| 44,000         | 0.983 | 1.41 ± 0.11 |
| 32,000         | 0.946 | 0.95 ± 0.13 |
| 16,000         | 0.938 | 1.94 ± 0.29 |

Fig. 4. MgATP-dependent cold inactivation of tonoplast ATPase. Tonoplast vesicles were incubated at 4°C in buffer containing 15 mM Tris-Mes (pH 8.0), 0.5 mM dithiothreitol, 0.15 M NaCl (−MgATP), and the same buffer plus 5 mM MgCl₂ (Mg), 5 mM ATP (ATP), or 5 mM MgATP (MgATP). RT denotes incubation with 5 mM MgATP at room temperature. Aliquots of the treated membranes were taken at the times indicated and ATPase activity was assayed as described under "Materials and Methods."

Fig. 5. A, SDS-PAGE analysis of polypeptides released from tonoplast by MgATP-dependent cold inactivation of tonoplast ATPase. Lane 1, tonoplast (20 µg); lane 2, purified ATPase (6 µg); lane 3, polypeptide composition of supernatant from MgATP-dependent cold inactivation of tonoplast (10 µg). B, SDS-PAGE analysis of polypeptides removed from purified ATPase by extraction with chloroform:methanol (2:1). Lane 4, purified ATPase before extraction (6 µg); lane 5, purified ATPase after extraction with chloroform:methanol (6 µg); lane 6, chloroform:methanol extract of purified ATPase (1.5 µg). Cold inactivation of tonoplast vesicles with MgATP and chloroform:methanol extraction of the purified ATPase were performed as described under "Materials and Methods."

CONCLUSIONS

Elucidation of the subunit compositions of the V-type H⁺-ATPases of plant and fungal vacuolar membranes from purification data, alone, has hitherto been impossible because of the limited degrees of purification obtained. Only those subunits with well defined kinetics of interaction with affinity labels have been firmly identified. Thus, the nucleotide-binding, [¹⁴C]N-ethylmaleimide- and [¹⁴C]7-chloro-4-nitrobenzo-2-oxa-1,3-diazole-modifiable, 67–72-kDa subunit (Bowman et al., 1986; Randall and Sze, 1986), [α-³²P]3-O-(4-benzoyl)benzoyl-ATP-binding, 55–62-kDa subunit (Manolson et al., 1985), and [³¹C]dicyclohexylcarbodiimide-binding, 16-kDa proteolipid (Rea et al. 1987a) have been unequivocally demonstrated, but data for or against the coexistence of other subunits have not been forthcoming. As a result, the notion of subclasses has arisen. Nelson (1988), for example, has suggested that although all characterized V-type ATPases are clearly related evolutionarily (see the Introduction), there might nonetheless have been segregation within the category through the acquisition or loss of "accessory" subunits. The elaborate "100–116–70–73–41–38–34–33–16-kDa" subunit composition of the enzymes from clathrin-coated vesicles and chromaffin granules versus the apparently simple "67–72–55–62–16-kDa" pattern of the plant enzyme has, for instance, been attributed to the need for cell-sorting polypeptides for the enzymes of endocytotic and secretory organelles but not lysosomal compartments (Nelson, 1988).

Our findings appear to invalidate this proposal. When precautions are taken to ensure quantitative recovery of all the component polypeptides of the chromatographic fractions, high purity ATPase preparations from higher plant vacuolar membranes have a polypeptide composition which closely corresponds with the enzymes from clathrin-coated vesicles and chromaffin granules (Table IV). Lack of purity, proteolysis, and/or aggregation cannot explain the presence of the 52-, 44-, or 32-kDa polypeptides in the high purity tonoplast ATPase preparation. First, the specific activity of the purified ATPase (20–25 µmol/mg·min) is the highest achieved for any plant V-type enzyme and is closely comparable to the values of 15–18 µmol/mg·min reported for the 100–200-fold purified enzymes from clathrin-coated vesicles (Xie and Stone, 1986) and chromaffin granules (Cidon and Nelson, 1986). Second, the 52-, 44-, and 32-kDa subunits have not been forthcoming. As a result, the notion of subclasses has arisen. Nelson (1988), for example, has suggested that although all characterized V-type ATPases are clearly related evolutionarily (see the Introduction), there might nonetheless have been segregation within the category through the acquisition or loss of "accessory" subunits. The elaborate "100–116–70–73–41–38–34–33–16-kDa" subunit composition of the enzymes from clathrin-coated vesicles and chromaffin granules versus the apparently simple "67–72–55–62–16-kDa" pattern of the plant enzyme has, for instance, been attributed to the need for cell-sorting polypeptides for the enzymes of endocytotic and secretory organelles but not lysosomal compartments (Nelson, 1988).
polypeptides of the tonoplast ATPase are not cross-reactive with polyclonal antibodies raised against the 67- and 55-kDa subunits. Generation of the 52-, 44-, and/or 32-kDa polypeptides by fragmentation of the 67- and 55-kDa subunits does not apply. Third, only the 16-kDa subunit of the purified ATPase freely partitions into chloroform:methanol. If the 52-, 44-, and/or 32-kDa polypeptides were aggregates of the 16-kDa subunit they also should be soluble in chloroform:methanol.

The identity of the 100-kDa band is less clear. Two factors complicate the ascertainment of the 100-kDa band to a discrete polypeptide: (i) It migrates as a diffuse, often bimodal, band on SDS gels, and its intensity of staining is variable between preparations. (ii) Although it does not co-migrate with the 67-kDa subunit, a low intensity, cross-reactive aggregate of the 52-, 44, and 32-kDa subunits (Arai et al., 1987) is observable in all preparations. The identity of two or more of the other subunits of the enzyme to generate a complex of 100 kDa with immunological cross-reactivity to the 67-kDa subunit (e.g. 67 + 32 kDa) cannot be eliminated.

The results of the investigations of cold inactivation of the ATPase of tonoplast vesicles augment the purification data and enable direct comparison between our findings and those of Moriyama and Nelson (1989). Three features of cold inactivation are significant: (i) the exact correspondence between the requirements for cold inactivation of the chromaffin granule ATPase, selective release of the 72-, 57-, 55-, 44-, and 32-kDa polypeptides from the membrane during cold inactivation (Fig. 5A); and (iii) the identity of the dissociated 67-, 55-, 52-, 42-, and 32-kDa polypeptides with the corresponding components of the purified ATPase (Fig. 5A). Since essentially the same results are obtained with the chromaffin granule ATPase, selective release of the 72-, 57-, 51-, 45-, and 33-kDa subunits of the complex from the membrane (Moriyama and Nelson, 1989), the basic subunit organizations of the V-type ATPases from both animal and plant systems are remarkably similar.

To account for detachment of the 67-, 55-, 52-, 44-, and 32-kDa polypeptides of the tonoplast ATPase during cold inactivation they are proposed to be peripherally associated with the membrane. Conversely, the 16-kDa polypeptide and putative 100-kDa polypeptide are inferred to be integral membrane components.

Several independent lines of investigation support this contention: (i) Chotrophic anions preferentially dissociate the 67-72- and 55-62-kDa, but not the 16-kDa, polypeptides of the tonoplast ATPase from native membranes (Rea et al., 1987b), and cDNA clones for the genes encoding the corresponding subunits of Arabidopsis (Manolson et al., 1988) and Dauca (Zimmniak et al., 1988) have deduced amino acid sequences lacking transmembrane α-helices. (ii) The dicyclohexylcarbodimide-binding 16-kDa subunit is a proteolipid (Arai et al., 1987; Rea et al., 1987a; Sun et al., 1987), rich in hydrophobic amino acids and organized into four transmembrane spans (Mandel et al., 1988). (iii) Surface-active reagents (e.g. 1,1′-sulfoasucimidyle-3-(4-hydroxyphenyl)propionate) label the 100-, 73-, 58-, 40-, 38-, and 34/33-kDa polypeptides of the reconstituted clathrin-coated vesicle H+-ATPase (Arai et al., 1988). Agents acting from the phospholipid bilayer, on the other hand, preferentially label the 100- and 16-kDa subunits (Arai et al., 1988).

The significance of the 52-kDa polypeptide of the purified tonoplast ATPase is unknown. While it strictly copurifies with the ATPase (Table III) and undergoes dissociation from the membrane during cold inactivation (Fig. 5B), a similar component has not been reported for any other V-type ATPase (Table IV). Further investigations are required to decide whether it is a contaminant or subunit unique to the V-type ATPases of plant vacuolar membranes, or a ubiquitous subunit which has been overlooked or lost from other preparations.

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