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Separation and Immunological Characterization of Membrane Fractions from Barley Roots[1]

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ABSTRACT

Tonoplast and plasma membranes (PM) were isolated from barley roots (*Hordeum vulgare* L. cv California Mariout 72) using sucrose step gradients. The isolation procedure yielded sufficient quantities of PM and tonoplast vesicles that were sealed and of the right orientation to measure ATP-dependent proton transport *in vitro*. The proteins of the endoplasmic reticulum, tonoplast-plus-Golgi membrane (TG) and PM fractions were separated on sodium dodecyl sulfate gels, and immunoblots were used to test for cross-contamination between the fractions. Proteins that cross-reacted with antibodies to the PM ATPase from corn roots and *Neurospora* were greatly enriched in the PM fraction, as were proteins that cross-reacted with monoclonal antibodies to an arabinogalactan protein from the PM of tobacco cells. Proteins that cross-reacted with antibodies to the 58- and 72-kilodalton subunits of the tonoplast ATPase of red beet storage tissue were greatly enriched in the TG fraction. The results with immunoblots and enzyme assays indicated that there was little cross-contamination between the tonoplast and PM vesicles. The molecular weights and isoelectric points of the PM ATPase and the tonoplast ATPase subunits were also determined using immunoblots of two-dimensional gels of the PM and TG proteins.

For studies of the relationship between environmental stress and ion transport in barley roots we needed to obtain both PM[2] and tonoplast membranes, capable of *in vitro* transport, from the same roots. In a previous paper we demonstrated the separation of the PM and tonoplast H^+-ATPases on continuous sucrose gradients (7). Tonoplast vesicles were collected on a step gradient and the properties of the tonoplast H^+-ATPase were characterized in some detail (7, 12). However, it was not certain that the PM ATPase could be adequately separated from the tonoplast ATPase and obtained in sufficient quantities on a step gradient so that detailed studies of transport by the PM ATPase could also be performed.

There are few studies where sufficient quantities of sealed, inside-out PM and sealed, right-side-out tonoplast vesicles were obtained from the same tissue, so that ion transport by both membranes could be measured and compared. There are a variety of reasons why this has not been accomplished routinely. The choice of tissue used for membrane isolation may determine whether higher rates of transport by PM vesicles (29) or tonoplast vesicles (2, 5, 6) are observed. The method of preparing the membranes also influences the type of vesicle that is obtained. For example Giannini *et al.* (14) reported that one grinding medium gave predominantly sealed, right-side-out tonoplast vesicles while another method gave predominately sealed, inside-out PM vesicles.

Two-phase partitioning of membranes with PEG and dextran resulted in a highly purified PM fraction. The procedure was particularly valuable for green tissue because it was possible to obtain PM without heavy contamination with chloroplast membranes. However, the two-phase procedure was used only to prepare PM and not tonoplast, and the procedure selected for right-side-out PM vesicles (4, 21) that were not suitable for measurements of ATP-driven transport. Good measurements of *in vitro* transport were obtained by using the purified, reconstituted PM ATPase (1, 28). However, for many studies it would be desirable to start with intact membrane vesicles, in which the ATPase, antiports, and symports function as an integrated system. Continuous sucrose or dextran gradients are commonly used to separate intact tonoplast, PM and other membrane vesicles, although the degree of purity of the membrane fractions obtained has been questioned, and the yield of sealed, inside-out PM vesicles is generally much lower than the yield of sealed, right-side out tonoplast vesicles (2, 5, 7, 31).

In this paper ER, tonoplast, and PM vesicles were collected from the same homogenate, under identical conditions. We utilized SDS-polyacrylamide gels, antibodies to membrane proteins, and immunoblots to demonstrate that the PM ATPase was separated from the tonoplast ATPase on sucrose step gradients. We used immunoblots to locate the ATPase subunits on 2D gels, and we demonstrated that transport by both H^+-ATPases could be measured *in vitro* with little cross-contamination between the two activities.

MATERIALS AND METHODS

Plant Material. Seeds of barley (*Hordeum vulgare* L. cv California Mariout 72) were sown above an aerated solution containing full strength nutrients plus 100 mM NaCl (9, 20). Seedlings were grown in the dark at 22°C and 100% humidity for 7 d.

Membrane Preparations. The roots were excised, rinsed in ice cold water and immediately homogenized using a chilled mortar and pestle. The grinding mix consisted of 0.25 M sucrose, 4 mM DTT, 7.2 $\mu g \cdot ml^{-1}$ PMSF and 50 mM Tris plus 8 mM EDTA, which together give a buffered solution of pH 8.0. The PMSF was added to the grinding mix from a concentrated stock solution (12 mg/ml in ethanol) immediately before grinding the roots. A total volume of 400 ml grinding mix was used for 60 g fresh weight of roots. The roots were ground four or five times, using 75 to 100 ml of buffer at a time. The homogenate was pooled,

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[2] Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenyl hydrazone; PM, plasma membrane; PMSF, phenylmethylsulfonyl fluoride; TG, tonoplast and Golgi membranes; 1D and 2D, one- and two-dimensional, respectively; DCCD, dicyclohexylcarbodiimide; pI, isoelectric point.

filtered and centrifuged. The 10,000 to 100,000*g* pellet (7, 8) was resuspended in 6 ml of suspension buffer consisting of 0.25 M sucrose and 2 mM DTT buffered with 5 mM Pipes adjusted to pH 7.2 with KOH. This was divided into two aliquots and layered over two sucrose gradients, consisting of 12 ml of 40% (w/w) sucrose and 8 ml each of 34, 30, and 22% (w/w) sucrose and containing 1 mM DTT, 1 mM EDTA, and 1 mM Tris-HCl (pH 7.2). The step gradients were centrifuged for 3 h at 80,000g_{av} in a Beckman[3] SW28 rotor and the interfaces were collected using a syringe by piercing the sides of the centrifuge tubes. The interfaces were diluted with a KCl solution to a final concentration of 150 mM KCl and 2 mM DTT in 25 mM Tris-HCl (pH 8.0), in a volume of 28 ml and then centrifuged at 100,000g_{av} for 35 min in a Beckman 42.1 rotor. The pellets were resuspended in 6 ml of suspension buffer, divided into 1 ml aliquots and frozen at −70°C.

Assays. Proton transport was assayed as the quenching of acridine orange fluorescence as previously described (7, 12). The standard assay solution for proton transport and ATPase assays consisted of 0.25 M sucrose in 5 mM Pipes KOH (pH 7.0), unless otherwise indicated, plus 2 μM acridine orange for the transport assays. The concentrations of ATP, $MgCl_2$, salts and inhibitors that were used are indicated in the figure legends. A stock solution of 5 mM Na metavanadate was adjusted to pH 7.2 with Tris and it was boiled prior to use. Similar results were obtained with metavanadate or orthovanadate. Inorganic phosphate was determined by a modification of the method of LeBel *et al.* (1, 22).

Immunoblotting. Proteins were solubilized, separated on SDS-polyacrylamide gels, blotted to nitrocellulose, and reaction products detected essentially as described by Hurkman and Tanaka (20). Proteins were solubilized by suspension of the membrane pellets in a buffer containing 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol, and 2 mM PMSF in 100 mM Tris-HCl (pH 8.5), to a final dilution of approximately 4 μg protein/μl buffer. The sample was then heated for 3 min at 80°C. Proteins were separated on 10% SDS-polyacrylamide gels 1.5 mm thick. Approximately 55 μg of protein were loaded per lane. Apparent mol wt were calculated by reference to the mobilities of marker proteins (19, 32). The values obtained are approximations that were used to facilitate the identification of the various membrane proteins, rather than to assign precise mol wt.

Polyclonal antibodies produced against a 100-kD protein cut from a 1D SDS gel of the partially purified PM ATPase from corn roots (10) were a gift from R. T. Leonard, University of California, Riverside. Polyclonal antibodies raised against the purified PM ATPase from *Neurospora crassa* (17) were a gift from C. W. Slayman, Yale University School of Medicine. Polyclonal antibodies to the 72- and 58-kD subunits of the partially purified tonoplast ATPase of red beet storage tissue were a gift from A. B. Bennett, University of California, Davis. The antibodies were prepared against proteins cut from 1D gels of the partially purified tonoplast ATPase. A monoclonal antibody to a surface protein of tobacco protoplasts (AB No. 164B4) (18, 25) was a gift from C. Lamb, Salk Institute, San Diego, CA.

Immunodetection of the PM ATPase and the tonoplast ATPase subunits was carried out using the goat anti-rabbit, horseradish peroxidase Immun-Blot kit from Bio-Rad. Immunodetection of the tobacco surface protein was carried out using the protocol for the goat anti-mouse, alkaline phosphatase method in the ProtoBlot Immunoblotting System from Promega except that 3% BSA was used in the blocking solution and 1% BSA in the primary antibody solution. The goat anti-mouse-IgM (μ-chain specific alkaline phosphatase conjugate) and the color development substrates, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, were obtained from Sigma. Immunostained blots were stained with Ponceau S (30) to correlate the immunostained spots with specific proteins on the 2D gels.

2D Gel Electrophoresis. Proteins were extracted from the membrane fractions, partitioned into phenol and separated by 2D PAGE as previously described (19). Approximately 60 μg of protein were loaded onto the focusing gel. The second dimension SDS gel was silver stained by the method of Morrissey (24).

RESULTS

Immunological Characterization of Step Gradient Fractions. A sucrose step gradient was used to separate barley microsomes into fractions enriched in PM, ER, and tonoplast. The choice of sucrose concentrations was based on previous results with continuous and step gradients (7, 8). The sample/22% sucrose interface (ER fraction) was enriched in NADH Cyt *c* reductase. The 22/30% sucrose interface (TG fraction) was enriched in NO_3^--inhibited ATPase, a marker enzyme for tonoplast membranes, and in glucan synthase I, a marker enzyme for Golgi membranes. A 30/40% interface (PM fraction) was enriched in vanadate inhibited ATPase (7). We improved the degree of purity of the PM fraction by collecting it from a 34/40% interface instead of a 30/40% interface as was done previously (7, 19). The 30/34% interface was discarded.

SDS-PAGE was used to compare the protein patterns of the ER, TG, and PM fractions (Fig. 1). To assist comparisons between the fractions, the calculated mol wt of the proteins that were enriched in each fraction are indicated on Figure 1. The protein patterns of the ER (lane 1) and TG (lane 2) fractions were qualitatively very similar, but there were notable quantitative differences and a greater number of proteins were enriched in the ER fraction than in the TG fraction. The ER fraction had prominent bands at 143, 105, 96, 80, 75, 67, 56.5, 53, 43, 41, 40, 35.5, 33.5, and 32.5 kD. The TG fraction had prominent bands

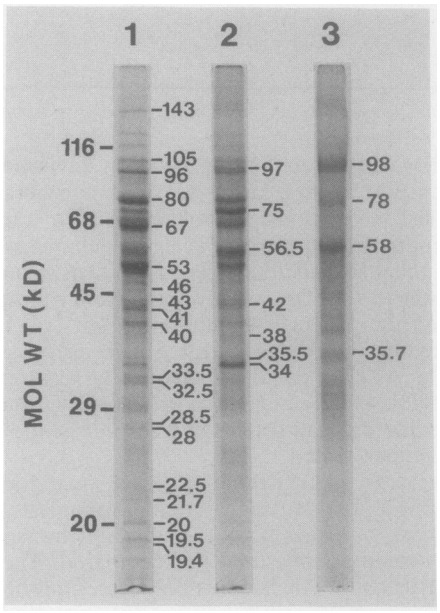

FIG. 1. SDS-polyacrylamide gel of proteins of ER, TG, and PM fractions. Proteins were detected using Coomassie blue. Lane 1, membranes from the sample/22% interface (ER), lane 2, from the 22/30% interface (TG) and lane 3, from the 34/40% interface (PM) of a sucrose step gradient of the 10,000 to 100,000*g* membrane fraction. Calculated mol wt are indicated to the right of lanes 1 to 3 and identify proteins enriched in each fraction. Mol wt of marker proteins are indicated to the left of lane 1.

[3] Mention of a specific product name by the United States Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

at 97, 96, 80, 75, 67, 56.5, 53, and 34 kD. The protein pattern of the PM fraction was qualitatively different from those of the ER and TG fractions. Overall, the proteins in the PM fraction were not as clearly resolved as the proteins in the ER and the TG fractions, and the PM fraction contained significantly fewer prominent bands than the ER and TG fractions. The PM fraction (lane 3) contained two prominent protein bands at 98 and 58 kD and two other major bands at 78 and 35.7 kD.

Antibodies that were prepared against several membrane proteins were used to verify the cellular origin of the barley membrane fractions and to evaluate the degree of cross-contamination of the different membrane fractions (Figs. 2 and 3). A polyclonal antibody that was prepared against the PM ATPase from corn roots (10) showed cross-reactivity with proteins in the PM fraction from barley roots (Fig. 2B, lane 3). The antibody reacted most strongly with a protein in the 100-kD region (98-kD) on the blot, a M_r characteristic of the catalytic subunit of the PM ATPase (1, 11, 27). The antibody also cross-reacted with several higher M_r proteins. Intense immunostaining was observed for two broad protein bands at approximately 150 and 200 kD and also for material that was retained at the top of the resolving gel. These three bands were only slightly stained by Coomassie blue (Fig. 2A, lane 3). Additionally, there was faint immunostaining of two broad bands of approximately 28 and 48 kD. A small amount of cross-reactivity of the antibody with proteins at 97, 150, and 200 kD was also detected in the TG fraction (Fig. 2B, lane 2). No cross-reactivity of the antibody with proteins in the ER fraction was detected (Fig. 2B, lane 1). The antibodies to the corn ATPase cross-reacted with a similar set of protein bands whether the proteins were extracted with SDS, as shown, or in phenol (not shown). Antibodies to the PM ATPase of *Neurospora* also cross-reacted with a protein band at 100 kD in the PM fraction, although the reaction was faint compared to that of the corn antibody (not shown), in agreement with the results of Oleski and Bennett (27).

The monoclonal antibody No. 164B4, that was prepared against proteins from the surface of tobacco protoplasts (18, 25), cross-reacted with a number of PM proteins in the 95- to 200-kD range, but cross-reacted most strongly with two protein bands of 100- and 114-kD (Fig. 2C, lane 3). Proteins that were immunostained on blots incubated with the antibody AB No. 164B4 were not stained with Coomassie blue on SDS gels (Fig. 2A, lane 3). Faint immunostaining of proteins in the ER and TG fractions (Fig. 2C, lanes 1 and 2) occurred in the 95- to 200-kD region of the blot. Faint immunostaining of proteins that had lower M_r (approximately 50–70 kD) was observed only in the ER and TG fractions and not in the PM fraction. If the membrane proteins were extracted with phenol instead of SDS, the antibody cross-reacted with only a single protein band on a blot of the PM proteins, at a M_r of approximately 200 kD (not shown).

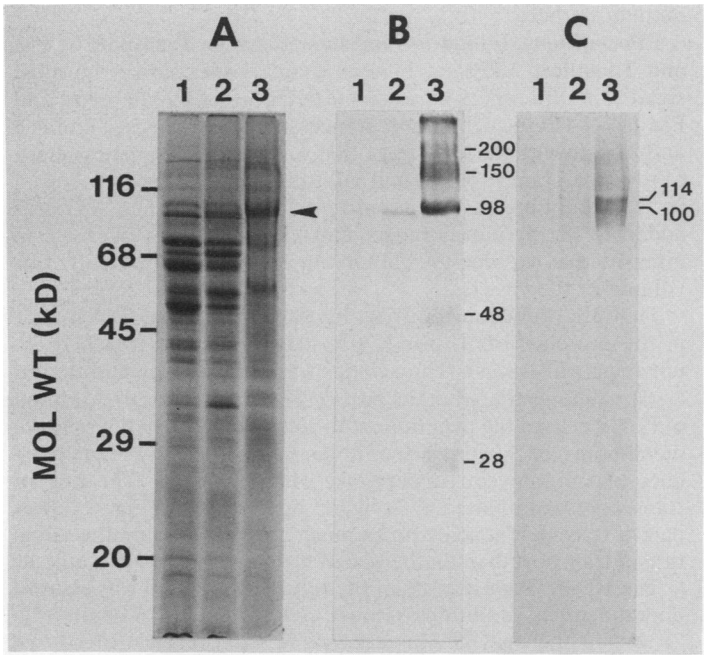

FIG. 2. Immunodetection of barley PM proteins. Lanes 1 to 3 as in Figure 1. A, SDS-polyacrylamide gel stained with Coomassie blue. B, Nitrocellulose blot immunostained for the PM ATPase of corn roots. C, Nitrocellulose blot immunostained for a surface protein of cultured tobacco cells.

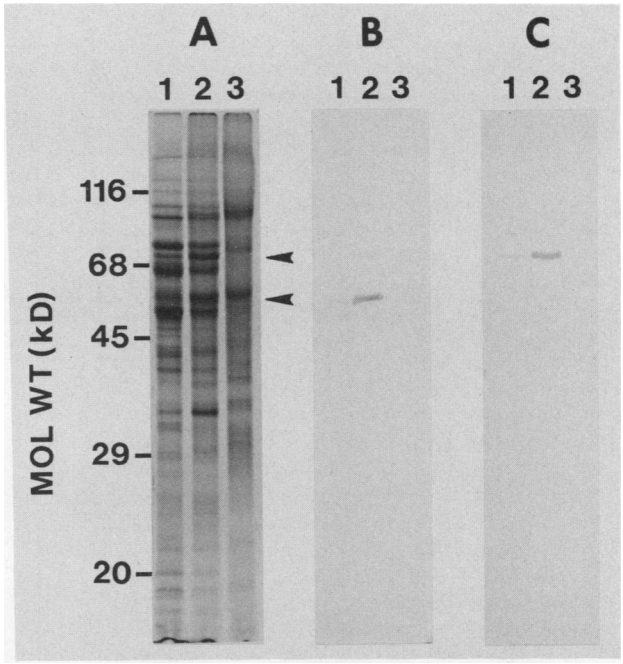

FIG. 3. Immunodetection of barley tonoplast proteins. Lanes 1 to 3 as in Figure 1. A, SDS-polyacrylamide gel stained with Coomassie blue. B, Nitrocellulose blot immunostained for the M_r = 58,000 tonoplast ATPase subunit of red beet. C, Nitrocellulose blot immunostained for the M_r = 72,000 tonoplast ATPase subunit.

Antibodies prepared against subunits of the tonoplast ATPase from the storage tissue of red beet cross-reacted strongly with proteins in the TG fraction (Fig. 3, B and C). The antibodies prepared against the 72-kD subunit cross-reacted with a 75-kD protein in the barley TG fraction (Fig. 3C, lane 2). The antibodies prepared against the 58-kD subunit cross-reacted with a 56.5-kD protein, as well as showing a slight cross-reaction with a protein of 75 kD (Fig. 3B, lane 2). The differences in the estimates of M_r for the ATPase subunits may simply be due to differences in the electrophoresis systems used in the various laboratories. For example, Mandala and Taiz (23) obtained an estimate of 72 and 62 kD for the subunits of the corn and barley ATPases with their electrophoresis system. Some immunostaining of the 75- and 56.5-kD proteins occurred on the blot of the ER fraction (Fig. 3, B and C, lane 3), but no cross-reaction was detected for proteins in the PM fraction.

The proteins in the PM and the TG fractions were separated by 2D PAGE in order to identify the specific proteins that cross-reacted with the antibodies on 1D gels (Figs. 4 and 5). Single bands in 1D gels, stained with Coomassie blue (Figs. 1–3) were resolved into as many as 20 spots on 2D gels stained with silver (Figs. 4A and 5A). When a blot of a 2D gel of proteins of the PM fraction was reacted with antibodies to the PM ATPase from *Neurospora*, two proteins were immunostained (Fig. 4B). The

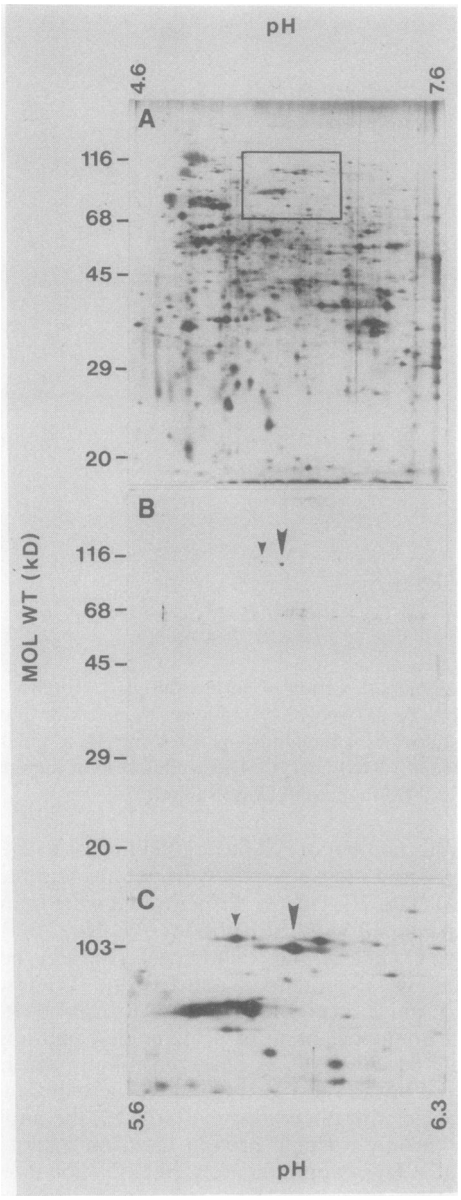

FIG. 4. Identification of the plasma membrane ATPase on a 2D gel of proteins of the PM fraction. A, Silver-stained 2D gel of proteins of the PM fraction. B, Nitrocellulose blot of a 2D gel immunostained for the plasma membrane ATPase. The large arrow indicates the major protein and the small arrow the minor protein that cross-reacted with the antibodies. C, Enlargement of the area indicated on the 2D gel in A that contains the immunostained proteins. The membranes were from roots of plants grown in full nutrients without NaCl.

major one had a mol wt of approximately 103,000 and pI of 6.0 and the minor one had a slightly greater mol wt and a pI of 5.9 (Fig. 4C). A band running across the gel at a mol wt of 65,000 to 68,000 was also immunostained. It may correspond to an artifact that is commonly observed on silver stained gels (26). The membranes used for the gel in Figure 4 were obtained from roots grown in full nutrients without NaCl, unlike the membranes used for the other gels shown in this paper. However, the protein patterns of the gels from roots grown with or without NaCl were very similar and there was no obvious effect of NaCl on the ATPase proteins (WJ Hurkman, FM DuPont, unpublished data).

Results were unsatisfactory when blots of 2D gels of the PM proteins were reacted with antibodies to the PM ATPase from corn roots or with AB No. 164B4. The antibody to the corn ATPase did not cross-react with the two proteins that reacted with the *Neurospora* antibody (not shown), nor with any other distinct protein spots of 100 kD. Several experiments to explain the lack of cross-reactivity of the antigen on a 2D gel were tried. To rule out the possibility that the phenol extraction method changed the antigenicity of the ATPase, the PM fraction was solubilized instead by the SDS procedure of Hurkman and Tanaka (19). However, no cross-reaction with a protein that corresponded to the ATPase was detected on the blot of the 2D gel. Two additional solubilization methods were tried. In one, the PM fraction was solubilized with a urea buffer that also contained SDS (13) and in the other, the PM fraction was solubilized with an SDS buffer to which NP-40 was later added (27). No cross-reaction with a protein that corresponded to the ATPase was detected on the blots of 2D gels of proteins solubilized by either method. No satisfactory method was found for 2D gel electrophoresis of the proteins that cross-reacted with the AB No. 164B4. When the PM proteins were extracted with SDS the cross-reactive material remained at the top of the isoelectric focusing gel (not shown).

Antibodies to the subunits of the tonoplast ATPase cross-reacted with distinct protein spots on 2D gels of the tonoplast fraction (Fig. 5). One prominent feature in 2D gels of the TG fraction was a large cluster of protein spots that had approximate mol wt of 78,000 and pIs near 5.2. The enlargement of this region of the gel (Fig. 5B) shows that this cluster consisted of eight protein spots. A second smaller cluster of four spots with approximate mol wt of 58,000 and pIs near 5.2 was observed below the 78-kD cluster. On a blot of a 2D gel incubated with antibodies to the 72-kD subunit of the red beet tonoplast ATPase (Fig. 5C) two 78-kD protein spots were immunostained. On the silver stained gel, these two proteins were located in the lower row of the 78-kD cluster of proteins (Fig. 5B, large arrows). On a blot of a 2D gel incubated with antibodies to the 58-kD subunit of the red beet tonoplast ATPase (Fig. 5D), two 58-kD spots were immunostained. On the silver stained gel, the two 58-kD cross-reactive proteins were located in the 58-kD cluster of proteins (Fig. 5B, small arrows). The two 78-kD spots were also faintly immunostained.

Effect of Ions, Inhibitors, and Ionophores on Transport by PM and Tonoplast ATPases. Figures 6 and 7 summarize the most significant differences between the activities of the tonoplast and PM H^+-ATPases. The fluorescence intensity traces in Figures 6 and 7 begin with the level trace that was obtained in the presence of the membranes, buffer and indicated ions. This was followed by the abrupt increase in intensity that occurred when ATP was added to the acridine orange, and the subsequent decrease in intensity that was due to ATP-driven acidification of the interior volume of the vesicles.

The rate of proton transport by the PM ATPase was greater in the presence of 50 mM KNO_3 than in 50 mM KCl (Fig. 6, upper pair of traces). The activity was increased by addition of 1 μM valinomycin, whether KCl or KNO_3 were used. Addition of FCCP caused the pH gradient to collapse slowly in the absence of valinomycin (lower trace of upper pair) or rapidly in the presence of valinomycin (upper trace of upper pair). The rate of transport was inhibited 87% by 0.5 mM vanadate (Fig. 6, lower pair of traces). Vanadate had a much greater effect on the initial rate of transport than on the size of the steady state pH gradient.

The H^+-ATPase activity of the tonoplast fraction was assayed under identical conditions to those used for the PM ATPase (Fig. 7). When the enzyme was assayed in the presence of 50 mM KNO_3, a characteristic two-phase time course for fluorescence was observed in which there was a small initial quench of fluorescence followed by a return of fluorescence to the original level. This two phase time course was attributed to a short initial period during which NO_3^- stimulated transport, because of its action

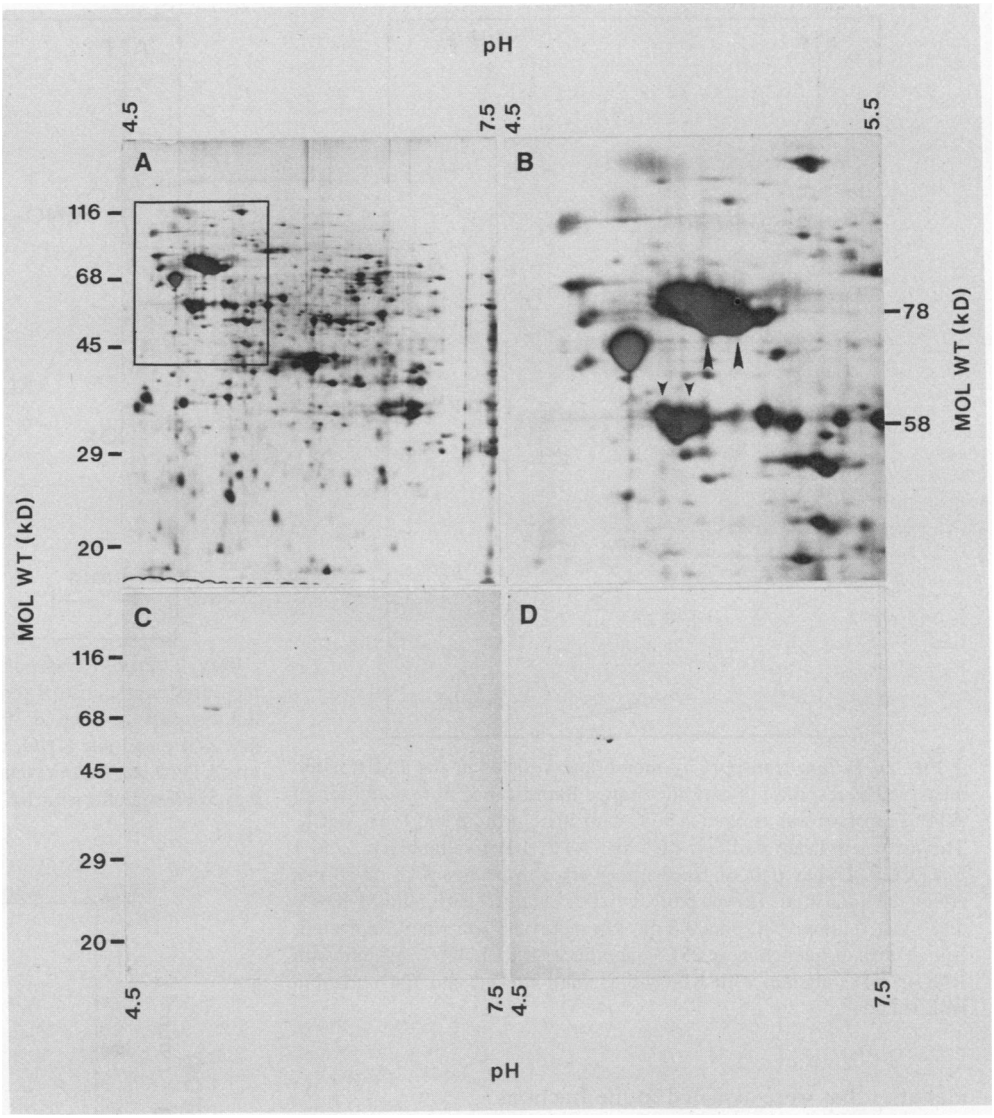

FIG. 5. Identification of tonoplast ATPase subunits on a 2D gel of the TG fraction. A, Silver-stained 2D gel of proteins of the TG fraction. B, Enlargement of the area indicated on the 2D gel in A that contains the 72- and 58-kD subunits of the tonoplast ATPase. The large arrows indicate proteins that cross-reacted with antibodies to the 72-kD subunit and the small arrows those that cross-reacted with antibodies to the 58-kD subunit. C, Nitrocellulose blot of a 2D gel immunostained for the M_r = 72,000 tonoplast ATPase subunit. D, Nitrocellulose blot of a 2D gel immunostained for the M_r = 58,000 tonoplast ATPase subunit.

as a permeant anion, before it subsequently inhibited the ATPase (7). A similar effect was observed when the enzyme was assayed with quinacrine (not shown), so the two phase time course observed with KNO_3 was not an artefact of the use of acridine orange. The enzyme was stimulated by KCl, but addition of 1 μM valinomycin caused a partial collapse of the pH gradient. As with the PM ATPase, addition of FCCP caused the pH gradient to collapse slowly, in the absence of valinomycin, or rapidly, in the presence of valinomycin.

The effect of pH on transport by the PM and tonoplast ATPases was compared (Fig. 8). The enzymes were assayed with KCl and without valinomycin, so the assay conditions were identical, except that the tonoplast fraction was assayed at 20°C and the PM fraction was assayed at 30° C. The tonoplast ATPase was assayed at a lower temperature because rates of quench were highest at assay temperatures between 15 and 20°C, whereas the PM ATPase had higher rates at 30°C than at 20°C (FM DuPont, unpublished data). The PM ATPase had an optimum transport rate between pH 6.5 and 7.0 and the tonoplast ATPase had an optimum between pH 7.5 and 8.0.

The effect of inhibitors on ATP hydrolysis by the plasma membrane ATPase was evaluated (Table I). Azide and oligomycin were included to test for contamination of this fraction by the mitochrondria F_1F_0-ATPase, and ammonium molybdate to test for contamination by molybdate-inhibited phosphatases. Vanadate and DCCD were included to estimate the percent of the Mg^{2+}-stimulated ATPase that was sensitive to those inhibitors. Mg^{2+}-stimulated ATPase accounted for 87% of the total activity (Table I). Of the Mg^{2+}-stimulated ATPase, 80% was inhibited by 100 μM DCCD and 47% was inhibited by 200 μM vanadate. A minor amount of the activity was inhibited by oligomycin (5%), azide (6%) or ammonium molybdate (4%). There was little Ca^{2+}-stimulated ATPase activity in the PM fraction (7, 8). Previously, it was demonstrated that the ATPase activity in the tonoplast fraction was not inhibited by vanadate, only 50% was inhibited by NO_3^-, and the remaining activity was stimulated by Ca^{2+} as well as Mg^{2+} (7, 8).

DISCUSSION

The distribution of membranes from barley roots on sucrose gradients was different from typical membrane distributions reported in the literature. The ER equilibrated at a lighter density than the tonoplast and Golgi membranes, and the PM was widely separated from the endomembranes (7, 8). This difference in distribution was an advantage in separating the PM from the other membranes. Initially, the different fractions were identified by the associated enzyme activities (7, 8). In this paper, identification of protein patterns on SDS gels, along with immunological techniques, were used as additional methods to assess the degree of purity of the membrane fractions and to confirm the

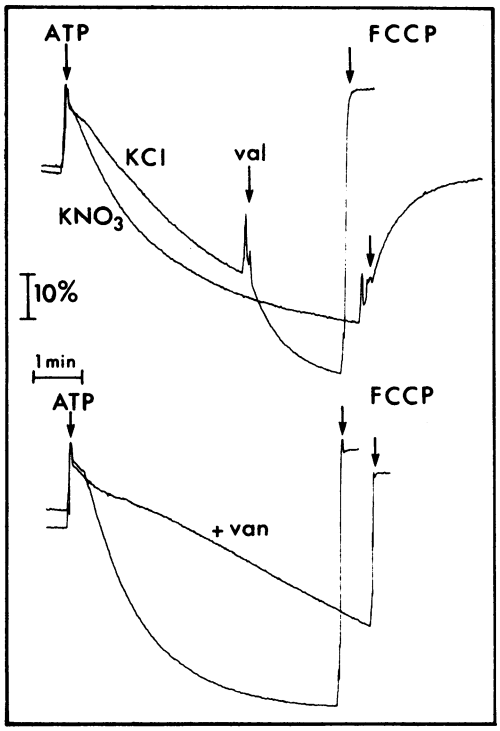

FIG. 6. Proton transport by membrane vesicles in the PM fraction measured as response of acridine orange fluorescence to the addition of ATP. Transport was assayed at 30°C with 50 μg protein and 1 mM $MgCl_2$. The arrows indicate addition of 1 mM ATP, 1 μM valinomycin, and 1 μM FCCP. Upper pair of traces: assayed with 50 mM KCl or 50 mM KNO_3, as indicated. Lower pair of traces: assayed with 50 mM KNO_3 and 1 μM valinomycin, plus 0.5 mM Na metavanadate where indicated. Initial rates of quench were 281%/mg/min with KCl, 425%/mg/min with KNO_3, 757%/mg/min with KNO_3 plus valinomycin, and 104%/mg/min with vanadate.

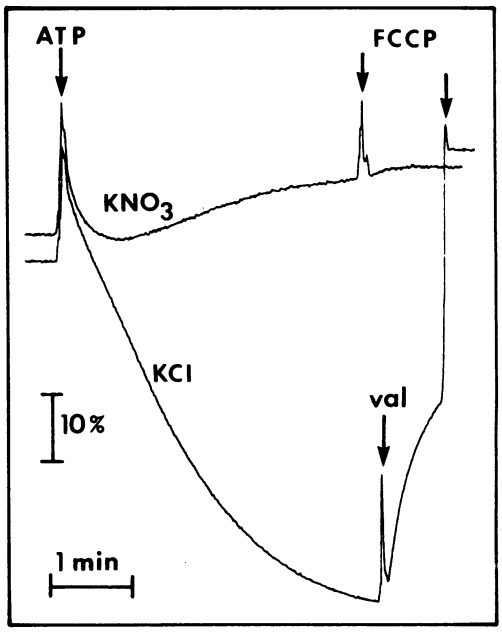

FIG. 7. Proton transport by membrane vesicles in the TG fraction measured as response of acridine orange fluorescence to addition of ATP. All assays were done at 30°C with 50 μg protein, 1 mM $MgCl_2$ and 50 mM KCl or 50 mM KNO_3 as indicated. Arrows indicate additions of 1 mM ATP, 1 μM valinomycin, and 1 μM FCCP. The initial rate of quench was 574%/mg/min with KCl.

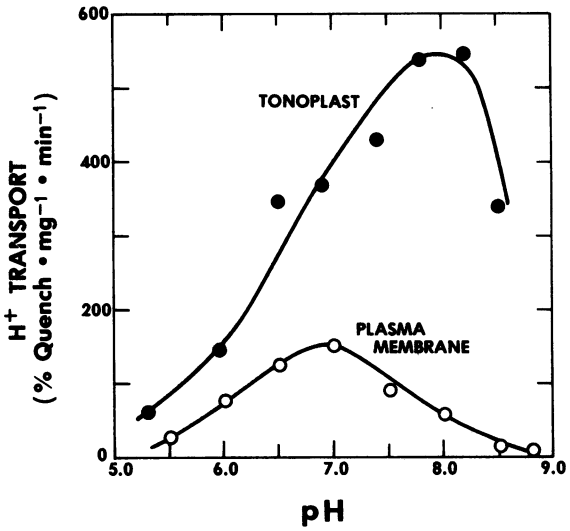

FIG. 8. Initial rate of transport as a function of assay pH for the PM and TG fractions. Transport was assayed with 50 mM KCl, 1 mM $MgCl_2$, 1 mM Tris-ATP, and 10 mM Tris-Mes at the indicated pH values. The PM fraction was assayed at 30°C, using 30 μg protein per assay and the TG fraction was assayed at 20°C, using 42 μg protein per assay.

identities that were assigned to the fractions.

The protein patterns of the Coomassie blue stained gels of the different membrane fractions demonstrated the extent of separation of the membranes. The protein patterns of the TG and PM fractions were qualitatively different, whereas the protein patterns for the TG and ER fractions were only quantitatively different. This corresponds to the distribution of the activities on sucrose gradients, where the peaks of tonoplast and PM ATPase activity were widely separated and the peaks of tonoplast ATPase and NADH Cyt *c* reductase activity were adjacent to one another (7, 8). Antigens to plasma membrane and tonoplast proteins were also well separated, as indicated by the immunoblot results. For example, when the monoclonal antibody No. 164B4 was used, immunostaining of the barley membrane proteins was much more intense for proteins in the PM fraction than in the TG or ER fractions. This is different from the results of Lamb *et al.* (18, 25), who reported that the antibody labeled two membrane peaks on a linear sucrose gradient of tobacco membranes.

The SDS gels of the PM fraction appeared diffuse and poorly resolved in contrast to the more clearly resolved protein bands in the ER and TG fractions. Gallagher and Leonard (11) pointed out that boiling the membrane samples in SDS caused proteins to aggregate, but the barley samples were not boiled. When the PM proteins were extracted with phenol, as for the 2D gels, they were clearly resolved on the SDS gels (not shown); the phenol extraction apparently removed substances that contributed to the poor resolution of the proteins. It is possible that the phenol extraction removed large glycoproteins. For instance, the monoclonal antibody No. 164B4 that was prepared against tobacco membranes was shown to react with arabinogalactan proteins of 130 to 230 kD on the outer surface of tobacco protoplasts (C Lamb, personal communication). The antibody reacted with a number of proteins in the mol wt range of 100,000 to 200,000 in the blot of the SDS extracted barley PM fraction, but it did not cross-react with a blot of a phenol extracted PM fraction.

The polyclonal antibody against the PM ATPase from corn

Table I. *Effect of Inhibitors on ATP Hydrolysis by PM-Enriched Fraction*

The membranes were assayed with 50 mM KNO_3, 3 mM Na ATP, 1 μM valinomycin, and 25 mM Tris Mes (pH 6.5) in 0.25 M sucrose, in the presence or absence of 3 mM $MgCl_2$. Assays were at 30°C for 30 min, with 14 μg protein per assay. Inhibitor concentrations were 100 μM DCCD, 200 μM vanadate, 2 μg/ml oligomycin, 100 μM sodium azide, and 100 μM ammonium molybdate. Numbers are average of 3 replicates ± SD.

Additions	ATPase Activity			Inhibition
	$-Mg^{2+}$	$+Mg^{2+}$	Mg^{2+} stimulation	
	μmol Pi/mg · min			%
None	0.15 ± 0.04	1.19 ± 0.02	1.04	0
DCCD	0.10 ± 0.01	0.29 ± 0.01	0.19	82
Vanadate	0.09 ± 0.002	0.64 ± 0.03	0.55	47
Oligomycin	0.11 ± 0.0	1.09 ± 0.05	0.98	6
Azide	0.08 ± 0.006	1.06 ± 0.03	0.98	6
Molybdate	0.09 ± 0.01	1.09 ± 0.03	0.99	5

roots cross-reacted strongly with a protein band of approximately 100 kD on SDS gels. A 100-kD protein band was a major band in the Coomassie blue stained gels of the barley PM fraction. The 100-kD subunit of the PM ATPase was also a major band in SDS gels of highly purified plasma membranes from the fungi *Schizosaccharomyces pombe* and *Neurospora crassa* (15), but this has not been observed generally for gels of PM fractions prepared from higher plants (11 [and references therein], 27). Because the antibody for the PM ATPase of corn also cross-reacted with several protein bands, and since it was prepared against a band cut fom a 1D gel, it is possible that the antibody was cross-reactive to components other than the PM ATPase. It is clear, however, that all of the antigens were largely restricted to the PM fraction.

The antibodies raised against the PM ATPase of *Neurospora* cross-reacted with two proteins on blots of 2D gels of the PM fraction. The protein that reacted most strongly with the antibody had a mol wt of 103,000 and a pI of 6.0. Using the same antibodies, Oleski and Bennett (27) demonstrated that the antibodies cross-reacted with a 97-kD protein that had a pI of 6.5 on a blot of a 2D gel of red beet PM proteins. The differences in estimates for mol wt and pI may be due to differences in the electrophoresis systems used in the two laboratories. The reasons the antibodies to the PM ATPase of corn did not cross-react with a protein of similar mol wt and pI on blots of the 2D gels are not known. One possibility is that the *Neurospora* and corn antibodies were cross-reactive with different proteins. For example, the corn antibody could have been raised to a 100-kD protein that was copurified with the ATPase, instead of to the ATPase itself. If so, this other 100-kD antigen was not present in the 2D gel. Another possibility is that the *Neurospora* and corn antibodies recognized different epitopes of the ATPase. The ATPase might have assumed sufficiently different conformations in the blots of the 1D gel and the 2D gel so that the antibody to the corn ATPase did not cross-react with the protein in the blot of the 2D gel.

The antibodies prepared against the subunits of the tonoplast ATPase of red beet cross-reacted with bands of 58 and 72 kD in the TG and ER fractions, but the antigens were greatly enriched in the TG fraction. The immunoblot results are in agreement with the results of enzyme assays that showed a sixfold enrichment of the proton translocating ATPase in the TG fraction, compared with a fivefold enrichment of NADH Cyt *c* reductase, an ER marker, in the ER fraction (7). Silver stained 2D gels of the TG fraction demonstrated that both the 72-kD and 58-kD subunits of the tonoplast ATPase had a pI of approximately 5.2. The antibodies to the tonoplast subunits each reacted with only two proteins in larger clusters of proteins of a similar mol wt and pI. The identity of the other proteins in the clusters is unknown.

The antibodies to the PM ATPase and to the tonoplast ATPase subunits each detected a pair of protein spots on the blots of the 2D gels. The proteins in each pair had slightly different mol wt and pIs. There are several possible explanations for the presence of the pairs, including the existence of isozymes, incompletely processed subunits, minor proteolysis, protein phosphorylation or artefacts of the 2D gel procedure. There is insufficient information to decide between any of the alternatives at present.

The SDS gels, immunoblots and enzyme assays demonstrated that the PM and tonoplast ATPases were present in the PM and TG fractions, respectively, with little cross-contamination between them. In contrast to the two-phase procedure (4, 22), the membranes obtained from barley roots by the step gradient procedure contained sealed, inside-out PM and sealed, right-side-out tonoplast vesicles that were useful for measuring proton transport by both the PM and tonoplast H⁺-ATPases. Under the assay conditions used in Figures 6 and 7 the rates of transport by the two ATPases were approximately equal.

There were several distinct differences in proton transport by the ATPases, as has been previously described for other plants (1–3, 5, 6, 16, 29, 31 and references therein). The tonoplast ATPase was inhibited by NO_3^-, stimulated by Cl^-, and not affected by vanadate. Transport by the PM ATPase was stimulated more by NO_3^- than Cl^-, in agreement with the results of Giannini *et al.* (14) and Rasi-Caldogno *et al.* (29). Transport was inhibited by vanadate and there was a lower pH optimum for transport for the PM ATPase than for the tonoplast ATPase.

The data given in this paper are insufficient for comparing the yields of properly oriented, sealed vesicles containing the PM ATPase and the tonoplast ATPase, because temperature, anions and pH had very different effects on the two enzymes. In Figure 6, for example, the transport rates for the PM ATPase were stimulated greatly by assaying with KNO_3 plus valinomycin, but those conditions eliminated transport by the tonoplast ATPase. In Figure 8, the activity of both enzymes was assayed in the presence of KCl, but the PM ATPase was assayed at 30°C whereas the tonoplast ATPase was assayed at 20°C. Under those conditions, the rates of transport by the tonoplast ATPase were much greater than those for the PM ATPase. A preliminary comparison of the activities of the two H⁺-ATPases under a variety of conditions indicated that there were probably 4 to 10 times as many sealed, properly oriented tonoplast vesicles as PM vesicles in a typical preparation (FM Dupont, unpublished data), but both were present in ample quantities for detailed transport assays.

There was one outstanding difference between the two ATPases from barley roots. Although valinomycin stimulated transport of protons by the PM ATPase, it collapsed the pH gradient formed by the tonoplast ATPase. This characteristic was used to distinguish between the activity of the two enzymes on a continuous sucrose gradient (7). Presumably, valinomycin stim-

ulated the inward transport of protons by the PM ATPase because it acted as a K^+ carrier, relieved the membrane potential and allowed the build up of a greater pH gradient. However, valinomycin directly or indirectly promoted the efflux of protons from the tonoplast vesicles. The rate of transport by the tonoplast ATPase may have been limited by a membrane potential, however, since FCCP did not give a rapid collapse of the pH gradient unless valinomycin was also present.

The results with the SDS gels, immunoblots and H^+-ATPase assays demonstrated that a PM fraction could be obtained from a sucrose gradient with minimal contamination from tonoplast or ER. The properties of the tonoplast and PM H^+-ATPases could be compared directly using membranes obtained from a single root homogenate. The ATP-generated pH gradients can therefore be used to study the antiports and symports that are associated with the two membranes. For example, we have demonstrated that a Na^+/H^+ antiport activity in the tonoplast membranes appears to be induced when the roots are grown in NaCl (12).

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