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Separation of the Mg^{2+}-ATPases from the Ca^{2+}-Phosphatase Activity of Microsomal Membranes Prepared from Barley Roots

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ABSTRACT

Two methods for preparing membrane fractions from barley (*Hordeum vulgare* cv California Mariout 72) roots were compared in order to resolve reported differences between the characteristics of the plasma membrane ATPase of barley and that of other species. When microsomal membranes were prepared by a published procedure and applied to a continuous sucrose gradient, the membranes sedimented as a single broad band with a peak density of 1.16 grams per cubic centimeter (g/cm³). Activities of NADH cytochrome (Cyt) *c* reductase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase were coincident and there was little ATP-dependent proton transport anywhere on the gradient. When the homogenization procedure was modified by increasing the pH of the buffer and the ratio of buffer to roots, the microsomal membranes separated as several components on a continuous sucrose gradient. A Ca^{2+}-phosphatase was at the top of the gradient, NADH Cyt *c* reductase at 1.08 g/cm³, a peak of ATP-dependent proton transport at 1.09 to 1.12 g/cm³, a peak of nitrate-inhibited ATPase at 1.09 to 1.12 g/cm³, and of vanadate-inhibited ATPase at 1.16 g/cm³. The Ca^{2+}-phosphatase had no preference for ATP over other nucleoside di- and tri-phosphates and was separated from the vanadate-inhibited ATPase on a sucrose gradient; approximately 70% of the Ca^{2+}-phosphatase was removed from the microsomes by washing with 150 millimolar KCl. The vanadate-sensitive ATPase required Mg^{2+}, was highly specific for ATP, and was not affected by the KCl wash. These results show that barley roots have a plasma membrane ATPase similar to that of other plant species.

Barley roots have been used so extensively for studies of ion transport and mineral nutrition that they might be called a model system for solute transport by plant roots. However, there are indications that plasma membrane-enriched fractions prepared from barley roots are not similar to those of other plants (3–5, 16). The predominant ATP-hydrolyzing activity in barley is stimulated by millimolar concentrations of Ca^{2+}, prefers Ca^{2+} to Mg^{2+}, shows little substrate specificity, and has a specific activity of 200 to 400 μmol Pi/mg·h. Both the plasma membrane and tonoplast ATPases of many other plant species are highly specific for Mg^{2+} and ATP, are inhibited by millimolar concentrations of Ca^{2+}, and have specific activities of less than 100 μmol Pi/mg·h (2, 6–8, 12, 17). It is important to develop a reliable method to prepare defined membrane fractions from barley roots and to characterize the membrane ATPases that are involved in transport, so that transport properties of the membrane fractions could be correlated with transport by barley roots. In this paper, we demonstrate that the microsomal membranes of barley roots, when separated on continuous sucrose gradients, contain an assortment of Mg^{2+}-ATPases similar to those found in other plants, and that the plasma membrane ATPase of barley has characteristics similar to the plasma membrane ATPase of other plant species. The Mg^{2+}-ATPase activities can be masked by a Ca^{2+}-phosphatase of high specific activity if appropriate methods are not used to prepare the membranes.

MATERIALS AND METHODS

Plant Material. Seeds of barley (*Hordeum vulgare* L. cv California Mariout 72) were sown on moist cheesecloth above aerated 0.5 mM $CaSO_4$ and grown at 22°C in the dark for 7 d.

Membrane Preparations. Roots (25 g) were excised into cold-distilled H_2O, washed, weighed, and immediately ground with a mortar and pestle.

Procedure A. This is a modification of the procedure of Nagahashi *et al.* (16). The homogenization buffer consisted of 3 mM EDTA and 25 mM Tris-Mes (pH 7.2) in 0.25 M sucrose, and was used at a ratio of 4 ml/g fresh weight of roots. The homogenate was filtered through four layers of cheesecloth and centrifuged at 3,000*g* in a Sorval[1] SS34 rotor for 5 min, and the pellet was discarded. The supernatant was centrifuged at 13,000*g* for 15 min and the pellet discarded; the supernatant was again centrifuged at 13,000*g*. The resulting supernatant was centrifuged at 80,000*g* in a Beckman 42.1 rotor for 30 min to obtain the microsomal pellet. The pellet was suspended in a buffer consisting of 1 mM Tris-Mes (pH 7.0) in 0.25 M sucrose. The pellet was suspended with a 2-ml pipette.

Procedure B. The homogenization buffer consisted of 25 mM Tris plus 4 mM EDTA (pH 8.0) and 2 mM DTT in 0.25 M sucrose and was used at a ratio of 8 ml/g fresh weight of roots. The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 3,000*g* for 5 min in a Sorval GSA rotor. The pellet was discarded and the supernatant centrifuged at 10,000*g* for 20 min in a Sorval GSA rotor. The pellet was discarded, and the supernatant was centrifuged at 100,000*g* for 35 min in a Beckman 42.1 rotor to obtain the microsomal pellet. The pellet was suspended in a buffer consisting of 5 mM Pipes-KOH (pH 7.0) in 0.25 M sucrose. The pellet was suspended by gently brushing it into the buffer with a camel's hair brush.

Sucrose Gradients. Two ml of resuspended microsomes were applied to a 15% to 45% linear sucrose gradient of 38 ml. The sucrose contained 1 mM EDTA adjusted to pH 7.2 with Tris and 1 mM DTT, unless otherwise indicated. The gradients were centrifuged for 2 h at 80,000*g*, unless otherwise indicated. Fractions of 2.4 ml each were collected with an ISCO density gradient fractionator and absorbance was monitored at 254 or 280 nm. The fractions were divided into three aliquots, and stored at −70°C. Fractions were assayed within 1 week; results were similar for all assays whether fresh or frozen samples were assayed.

Salt Wash. Membranes (either microsomes or sucrose gradient

[1] 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.

fractions) were suspended in a 6-fold excess of 0.25 M sucrose containing 5 mM Tris-Mes (pH 8.0), 150 mM KCl, and 1 mM DTT and centrifuged at 100,000*g* for 35 min. The pellet was resuspended in 5 mM Pipes-KOH (pH 7.0) in 0.25 M sucrose.

Assays. ATPase activity was assayed at 30°C in a 0.5-ml reaction volume containing 1 mM ATP, 0.25 M sucrose, 5 mM Pipes-KOH (pH 7.0), either 1 mM $MgSO_4$ or 1 mM $CaCl_2$, as indicated, and 50 mM KCl, unless otherwise indicated. Inorganic phosphate was determined by the method of Ames (1). Proton transport was assayed as initial rate of quench of fluorescence of acridine orange (18) at room temperature (approximately 23°C) in a 3.0-ml reaction volume containing 0.25 M sucrose, 5 mM Pipes-KOH (pH 7.0), 1 mM $MgSO_4$, 1 mM ATP, and 1 μM acridine orange. Fluorescence was measured using a Varian spectrophotometer with the fluorescence accessory, excitation was at 493 nm and emission at 510 nm and above. The reaction was started by addition of ATP. NADH Cyt *c* reductase and Cyt *c* oxidase were assayed as previously described (7). Protein was assayed by the method of Lowry (14).

RESULTS

Membrane components were not resolved on continuous sucrose gradients when prepared from barley roots by a method similar to that of Nagahashi *et al.* (16) (Fig. 1, procedure A). The UV absorbance trace indicated that the microsomal membranes sedimented on a continuous sucrose gradient as a single broad band that ranged in density from 1.10 to 1.19 g/cm^3 and peaked at 1.16 g/cm^3. The distribution of ATPase and NADH Cyt *c* reductase activities were nearly coincident with the UV absorbance trace. The ATPase activity was stimulated nearly as much by Ca^{2+} as by Mg^{2+}, and the Ca^{2+} and Mg^{2+} activities coincided. There was little measureable proton transport activity. The distribution of the marker enzymes indicated that the microsomal membranes were aggregated and sedimented together through the gradient. Similar results were obtained in the presence of 1 mM EDTA (not shown). These results were similar to those of Nagahashi *et al.* (16).

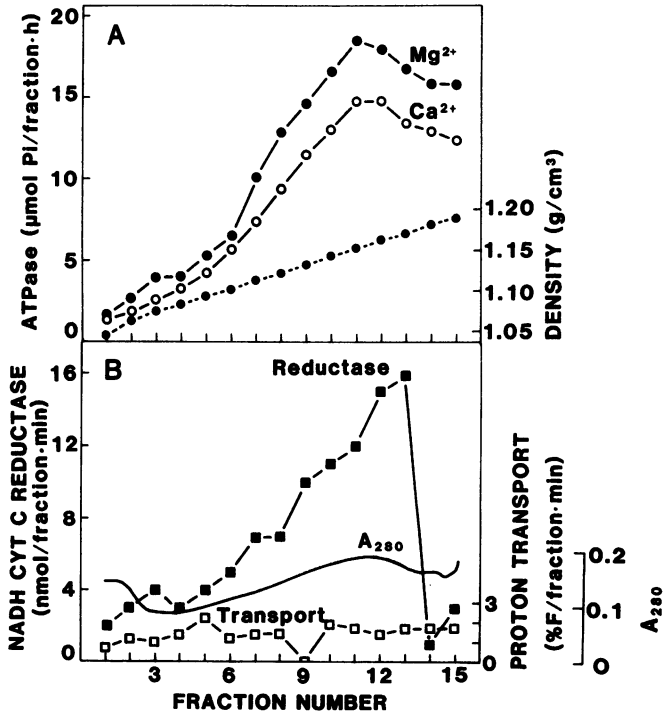

FIG. 1. Distribution of enzyme activities in a continuous sucrose gradient. A 10,000*g* to 80,000*g* membrane pellet prepared by procedure A was layered onto a 15% to 45% sucrose gradient containing 1 mM DTT but no EDTA and centrifuged for 2 h at 80,000*g*. A, Mg^{2+}-ATPase (●), Ca^{2+}-ATPase (○), sucrose density (●—●); B, NADH Cyt *c* reductase (■) and ATP-dependent proton transport (□) were assayed as described in "Materials and Methods."

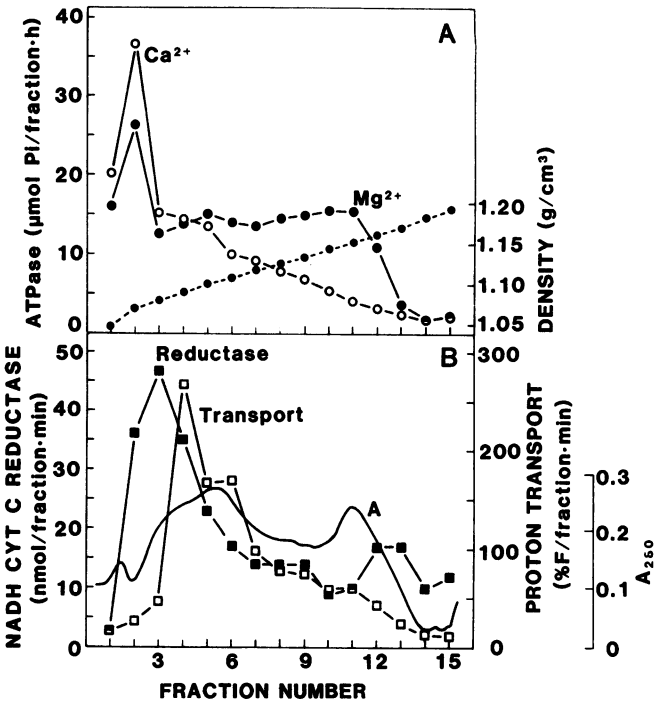

FIG. 2. Distribution of enzyme activities in a continuous sucrose gradient. A 10,000*g* to 100,000*g* membrane pellet prepared by procedure B was layered onto a 15% to 45% sucrose gradient containing 1 mM EDTA and 1 mM DTT and centrifuged for 2 h at 80,000*g*. The symbols for enzyme activities are the same as in Figure 1.

Table I. *Distribution of Ca^{2+}-ATPase and Mg^{2+}-ATPase during Differential Centrifugation of a Barley Root Homogenate Prepared by Procedure B from 30 Grams Fresh Weight of Roots*

Data are from a representative experiment.

Fraction	ATPase Activity			
	Total activity		Specific activity	
	+ Mg^{2+}	+ Ca^{2+}	+ Mg^{2+}	+ Ca^{2+}
	μmol Pi/h		*μmol Pi/mg·h*	
3,000*g* pellet	81 ± 7	104 ± 23	37	48
3,000–10,000*g* pellet	260 ± 18	256 ± 41	46	45
10,000–100,000*g* pellet	337 ± 12	206 ± 26	55	34
100,000*g* supernatant	2,790 ± 150	4,380 ± 570	77	121

A different method (procedure B), used previously to prepare the proton-translocating ATPase from tomato suspension cells (9) gave better separation of the membranes on sucrose gradients (Fig. 2). Barley membranes prepared by procedure B also gave high yields of ATP-dependent proton transport activity. The essential differences between procedures A and B are small, the latter using a greater ratio of homogenization buffer to roots, a higher pH for the homogenization buffer, and a different technique for resuspending the microsomal pellets. The UV absorbance trace of a 2-h gradient revealed four distinct peaks (Fig. 2). One was at the top of the gradient and coincided with a large peak of ATPase activity that was stimulated more by Ca^{2+} than Mg^{2+}. The second peak was at a density of 1.08 g/cm^3 and coincided with the peak of NADH Cyt *c* reductase activity, suggesting that the second peak was primarily ER. The third

Table II. *Substrate Specificity of the 100,000g Supernatant and Various Fractions Obtained by Sucrose Gradient Centrifugation*

Substrate	100,000g Supernatant		Gradient Fractions					
			2–3[a]		6–7[a]		10–13[a]	
	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
				%[b]				
ATP	100	190	100	133	100	100	100	25
UTP	107	160	71	135	40	60	5	15
CTP	53	257	74	123	47	53	10	10
GTP	93	187	61	103	47	60	30	15
ADP	67	140	35	113	13	40	5	10
IDP	60	93	77	84	20	20	10	5
PNP, pH 7.0	13	7	10	0	0	7	0	5
PNP, pH 5.0	47	27	45	35	7	47	—	—

[a] Gradient prepared as in Figure 2; fractions pooled and assayed with 1 mM substrate, 50 mM KCl, and 1 mM $MgSO_4$ or 1 mM $CaCl_2$. [b] Values expressed as per cent of activity with ATP + Mg^{2+}. The specific activities were: fractions 2–3, 66.4 μmol/mg·h; fractions 6–7, 18.1 μmol/mg·h; fractions 10–13, 31.3 μmol/mg·h; and supernatant, 39.8 μmol/mg·h. Data are shown for a representative experiment.

peak was at a density of 1.10 g/cm³ and coincided with proton transport activity. There was a broad plateau, followed by a fourth peak at a density of 1.16 g/cm³ that coincided with a peak of Mg^{2+}-ATPase activity. Although the Ca^{2+}-ATPase activity was predominantly at the top of the gradient, there was also a gradually diminishing Ca^{2+}-ATPase activity that trailed into the gradient. This Ca^{2+}-ATPase activity interfered with measurement of the Mg^{2+}-ATPase activity responsible for the ATP-dependent proton transport.

The results suggested that the predominant Ca^{2+}-ATPase was a soluble enzyme with a tendency to adhere to the microsomes, more so using procedure A than B. This hypothesis is supported by the distribution of Ca^{2+}- and Mg^{2+}-ATPase activities among cellular fractions prepared by differential centrifugation (Table I). A Ca^{2+}-ATPase was distributed among all the membrane fractions. The highest specific activity and total activity was in the 100,000g supernatant, while the lowest specific activity was in the 100,000g microsomal pellet. Nagahashi *et al.* (16) and Caldwell and Haug (3–5) reported a higher ratio of Ca^{2+} to Mg^{2+} activity in the microsomes than we found. Although the former measured the ATPase activity at 38°C, the latter at 16°C, and our measurements were at 30°C, we found that differences in assay temperature did not have a large effect on the ratio of Ca^{2+} to Mg^{2+} activity (DuPont, unpublished results). We suspect that we found a smaller ratio of Ca^{2+} to Mg^{2+} activity than the other authors because more of the Ca^{2+}-ATPase remained in the supernatant, and less adhered to the membranes with our procedures.

Both Caldwell and Haug (3–5) and Nagahashi *et al.* (16) reported that the Ca^{2+}-stimulated 'ATPase' of barley plasma membranes showed only a slight preference for ATP. We compared the substrate specificity of three areas of the gradient and the 100,000g supernatant (Table II). The supernatant contained a phosphatase that is stimulated almost twice as much by Ca^{2+} as by Mg^{2+} and preferred CTP as the substrate; hydrolysis of CTP > ATP > GTP > UTP in the presence of Ca^{2+}. The activity at the top of the gradient (fractions 2–3) was stimulated 30% by Ca^{2+} over Mg^{2+}, and did not discriminate significantly between the nucleoside triphosphates and diphosphates. The Mg^{2+}- and Ca^{2+}-stimulated activities were more specific for ATP in fractions 6–7. The activity of the fraction presumed to be rich in plasma membrane vesicles (fractions 10–13) was highly specific for ATP

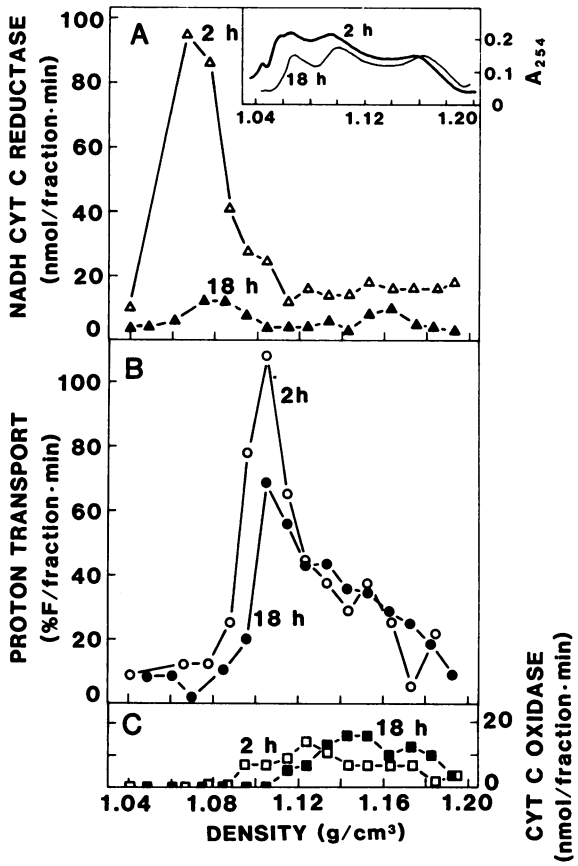

FIG. 3. Comparison of the distribution of enzyme activities in sucrose gradients centrifuged for 2 h and 18 h. A 10,000g to 100,000g membrane pellet prepared by procedure B was divided into two aliquots, and layered onto two 15% to 45% (w/w) sucrose gradients. One was centrifuged for 2 h and the other for 18 h. A, NADH Cyt *c* reductase (△, ▲); B, proton transport (○, ●); C, Cyt *c* oxidase (□, ■). (△, ○, □), 2 h; (▲, ●, ■), 18 h gradients.

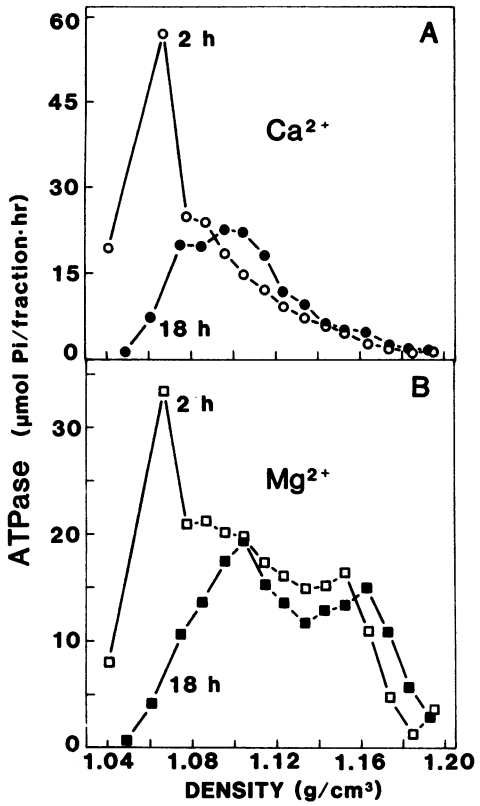

FIG. 4. Comparison of the distribution of ATPase activities in sucrose gradients centrifuged for 2 h and 18 h. Method as in Figure 3. A, Ca^{2+}-stimulated ATPase; B, Mg^{2+}-stimulated ATPase. (○, □), 2 h; (●, ■), 18 h.

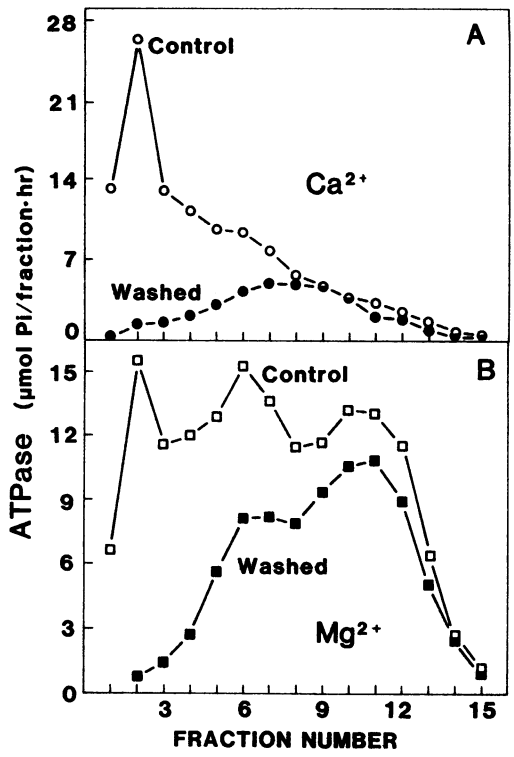

FIG. 5. Comparison of the distribution of ATPase activities of control or salt-washed microsomes in a sucrose gradient. A 10,000*g* to 100,000*g* membrane fraction prepared by procedure B was divided into two aliquots. One was washed with 150 mM KCl, as described in "Materials and Methods," and both were layered onto sucrose gradients and centrifuged for 2 h at 80,000*g*. A, Ca^{2+}-stimulated ATPase; B, Mg^{2+}-stimulated ATPase. (○, □), control; (●, ■), KCl washed.

and Mg^{2+}.

We compared the distribution of marker enzymes in 18-h gradients with the results from 2-h gradients (Figs. 3 and 4). The peaks of optically dense material shifted to slightly greater densities during the longer centrifugation, though the pattern was similar for 2 h and 18 h (Fig. 3A, insert). After 18 h of centrifugation, the NADH Cyt *c* reductase activity had decayed to 10% of the activity measured after 2 h of centrifugation (Fig. 3A). Nagahashi (15) also found that the NADH Cyt *c* reductase activity of barley root membranes decayed rapidly. The trailing edge of the proton transport activity shifted from 1.08 to 1.10 g/cm^3, and the peak of activity decayed by 25%, but the general distribution of proton transport activity was similar at 2 h and 18 h (Fig. 3B). The small amount of Cyt *c* oxidase activity ranged from 1.09 to 1.17 g/cm^3 after 2 h and 1.12 to 1.18 g/cm^3 after 18 h (Fig. 3B). The activity may represent fragments of inner mitochondrial membranes.

The peak of Ca^{2+}- and Mg^{2+}-phosphatase activity that was at the top of the gradient after 2 h moved into the gradient and also decreased after 18 h of centrifugation (Fig. 4). It appears that the Ca^{2+}-phosphatase, like the NADH Cyt *c* reductase, is much more labile than the proton transport, Cyt *c* oxidase, and Mg^{2+}-ATPase activities.

Methods to separate the Ca^{2+}-phosphatase from the microsomal membranes were explored. The membranes were washed with buffers of various pH and ionic strength. A range of pH from pH 5.0 to pH 8.0 was tested with or without EDTA, with KCl concentrations from 0 to 150 mM, or 250 mM KI, and with or without 0.25 M sucrose. The goal was maximum removal of the Ca^{2+}-phosphatase and maximum preservation of the vanadate-sensitive and nitrate-sensitive Mg^{2+}-ATPases. The Ca^{2+}-phosphatase was removed most effectively by 150 mM KCl, irrespective of pH or the presence or absence of 0.25 M sucrose or 4 mM EDTA (data not shown). The best recoveries of vanadate- and nitrate-inhibitable Mg^{2+}-ATPases were at pH 8.0. A wash with 150 mM KCl gave better recovery of the ATPases than 250 mM KI, so a KI wash was not used. A washing procedure was thus devised using 150 mM KCl at pH 8.0, in 0.25 M sucrose and 1 mM DTT that removed approximately 70% of the Ca^{2+}-phosphatase. Two procedures for combining the KCl wash and sucrose gradient fractionation were then compared. When the microsomes were first washed with KCl and then applied to a gradient (Fig. 5), the Ca^{2+}-phosphatase was no longer present at the top of the gradient, although it was still broadly distributed within the gradient, with a peak at 1.10 to 1.12 g/cm^3 (Fig. 5A). Mg^{2+}-ATPase was also removed from the top of the gradient (Fig. 5B). The peak of NADH Cyt *c* reductase was broader than previously observed and coincided with the peak of proton transport activity (not shown); both NADH Cyt *c* reductase and proton transport activity were similar in distribution to the remaining Ca^{2+}-ATPase (Fig. 5A).

We wished to remove the Ca^{2+}-phosphatase, preserving the separation of NADH Cyt *c* reductase from proton transport that we achieved when the microsomes were not washed, as shown in Figure 2. Therefore, we tried an alternative procedure, which was to fractionate the membranes in a gradient and then wash the fractions with KCl. The gradient fractions (1.2 ml each) were pooled in groups of 4, the 4.8-ml pooled fractions each diluted to 25 ml with the wash buffer, and centrifuged at 100,000*g* for 35 min, resuspended in suspension buffer, and assayed (Fig. 6). Again, a broad peak of Ca^{2+}-ATPase remained, with a peak density of 1.08 to 1.10 g/cm^3 (Fig. 6A). The Mg^{2+}-ATPase (Fig. 6A) contained a vanadate-inhibitable component with a peak

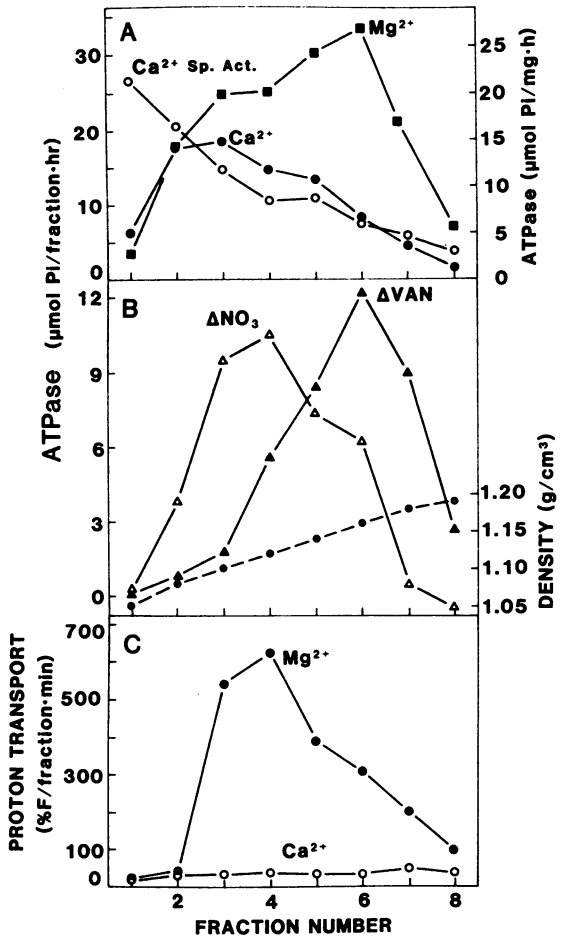

FIG. 6. Distribution of enzyme activities in a continuous sucrose gradient after washing the gradient fractions with 150 mM KCl, as described in "Materials and Methods." A, Distribution of Mg^{2+} (■) and Ca^{2+} (●) -stimulated ATPase activities and of Ca^{2+}-stimulated activity/mg protein (○). B, VAN (▲): the difference in activity between the presence and absence of 100 μM sodium ortho vanadate; NO_3 (△): the reduction in activity when 50 mM KNO_3 was substituted for 50 mM KCl (nitrate-inhibited activity); sucrose density (●—●). C, Distribution of ATP-dependent proton transport in the presence of Ca^{2+} (○) or Mg^{2+} (●).

density of 1.16 g/cm³ (Fig. 6B) and a nitrate-inhibitable component with a peak density of 1.10 to 1.12 g/cm³ (Fig. 6B). The peak of nitrate-inhibitable ATPase coincided with the major peak of proton transport activity (Fig. 6C). The proton-translocating ATPase required Mg^{2+} and no activity was observed if Ca^{2+} was substituted for Mg^{2+} (Fig. 6C). The specific activities of the Mg^{2+}-ATPases were similar in distribution in the gradient to the total activities (not shown), but the distribution of specific activity and total activity of the Ca^{2+}-ATPase gave very different patterns (Fig. 6A).

DISCUSSION

There is a tendency for barley membranes to aggregate (11). Although Nagahashi *et al.* (16) reported partial purification of plasma membrane vesicles from barley roots, our results indicate that aggregation of membrane vesicles probably accounts for the relatively low purity of the fraction. Prepared as described here, barley root microsomes give a pattern of marker enzyme activities in continuous sucrose gradients that is similar to that described for other plants such as red beet (2, 17), rose (10), corn (7, 8, 12), and oats (6). However, barley roots have a large amount of Ca^{2+}-stimulated phosphatase activity that can obscure the Mg^{2+}-ATPases. There are other examples in the literature where large amounts of soluble phosphatase, possibly of vacuolar origin, adhered to membranes and interfered with the detection of the Mg^{2+}-ATPases that are the intrinsic membrane proteins involved in ion transport (13). For example, membranes of red beet storage root were washed with 0.25 M KI to remove phosphatase activity prior to centrifugation on a sucrose gradient (2, 17).

The Ca^{2+}-phosphatase of barley roots remained associated with the microsomal membranes upon passage through a sucrose gradient under conditions that encouraged aggregation, but remained on top of the gradient in the conditions that promoted separation of the membranes. This suggests that the same phenomenon that promoted aggregation promoted adherence of the Ca^{2+}-phosphatase to the membranes. A 'Ca^{2+}-Mg^{2+} plasma membrane ATPase' from barley roots has been described by Caldwell and Haug (3–5). However, we found no evidence for any specific association of the Ca^{2+}-phosphatase with the plasma membrane. Although a third of the Ca^{2+}-stimulated ATP hydrolysis associated with the microsomal membranes was not removed by washes with KCl, EDTA, or buffers ranging from pH 5.0 to pH 8.0, the density distribution of the activity suggests that this remaining activity is associated with tonoplast or ER membranes. The pattern of specific activity shown in Figure 6 suggests that some of the Ca^{2+}-ATPase remaining after a salt wash may be of the same type of activity removed from the top of the gradient; however, we suspect that the majority of the membrane-bound activity is due to a different enzyme than the soluble Ca^{2+}-phosphatase found at the top of the gradients.

There are several ATPases involved in regulating the compartmentation of ions in plant cells. The best characterized enzymes to date are a vanadate-inhibited proton-translocating ATPase associated with the plasma membrane and a nitrate-inhibited proton-translocating ATPase apparently associated with the tonoplast. When the barley membranes were separated on a sucrose gradient and then washed with KCl, it was possible to detect the activities of both ATPases in similar amounts to those reported for other plants. Studies to compare the properties of the transport ATPases of barley root membranes with the transport properties of barley roots thus are feasible. Our results for barley roots are significant because a pattern of ATPase activity that seemed quite different from that of other plants has been shown to be the same. We find an ATPase at the density of plasma membrane that is highly specific for ATP and is inhibited by vanadate. We also find a nitrate-sensitive ATPase and ATP-dependent proton transport, at a lower density that is similar to the nitrate-sensitive tonoplast ATPase described for other plants. The results with barley call for a repetition of the warning (13) that plant membrane preparations are rich in contaminating phosphatase activities that may obscure detection of the transport ATPases, and also a warning that a comparatively small difference in technique may make a large difference in the fractionation of plant membranes.

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