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Solubilization and Partial Purification of ATPase from a Rose Cell Plasma Membrane Fraction

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ABSTRACT

The K^+-stimulated ATPase was partially purified from a plasma membrane fraction of suspension cultured cells of rose (*Rosa damascena*) by two different solubilization procedures. Solubilization with 30 mM octyl-β-D-glucopyranoside followed by precipitation with ammonium sulfate increased the specific activity of the enzyme about 6-fold. Solubilization with 1% cholate removed all but 1% of the phospholipids and resulted in an almost total loss of ATPase activity. The subsequent addition of polar lipids restored >90% of the ATPase activity with a doubling in specific activity. Fractionation of the cholate-solubilized ATPase activity on a Sephadex G-150 column resulted in 88% of the ATPase activity being recovered in two discrete, approximately equal peaks. Both ATPase activities were similar to plasma membrane ATPase activities in pH optimum, substrate specificity, ion stimulation, and inhibitor sensitivity. Assays of marker enzymes for Golgi apparatus, endoplasmic reticulum, and mitochondria revealed only a low contamination (<7%) from other membranes in the plasma membrane-enriched preparations. Lacking an unequivocal marker for the tonoplast, intact vacuoles were isolated, and their membrane density and ATPase activity were characterized and shown not to correspond to those of the putative plasma membrane preparation. These results suggest that there are two forms of ATPase separable by size in the plasma membrane of rose.

The probable role of ATPases in a variety of energy transducing processes has long been recognized. It is now widely accepted that certain membrane-associated ATPases, such as the Na^+,K^+-stimulated, ouabain-sensitive Mg-ATPase in a variety of animal cells and the Ca^{2+}-dependent Mg-ATPase in the sarcoplasmic reticulum are directly involved in the active transport of cations. In bacteria, membrane-bound ATPases are considered to be biological energy transducers which can utilize the energy of ATP hydrolysis to generate proton gradients and/or membrane potentials by effecting the net separation of H^+ and OH^- across the membrane in which they reside.

Monovalent cation-stimulated ATPases are associated with membranes from many species of higher plants (10), and it is probable that they function in the transport of ions across the plasma membrane (10, 11, 24, 25). However, the precise transport properties of the plasma membrane ATPase have not been clearly determined.

If the ATPase were solubilized with detergents, it might be possible to purify it, reconstitute it, and determine both its structure and its transport properties. Despite rapid advances in purification and characterization of membrane transport proteins from biological membranes (9, 14, 20), there is no example of a purified, well characterized transport protein from the plasma membrane of higher plants. However, there are several reports of the purification of a supposed transport ATPase (1, 23) and a variety of solubilization techniques (5, 6, 22).

Here, we describe the development of a procedure for obtaining two distinct, partially purified ATPase activities from the plasma membrane-enriched fraction and describe the characteristics of the partially purified enzymes.

MATERIALS AND METHODS

Plasma Membrane Vesicle Isolation. Plasma membrane vesicles were prepared as previously described (13) from 4-d-old suspension-cultured cells of rose (*Rosa damascena*). Twelve to 15 g of cells were sonicated in 3- to 4-g aliquots for two 15-s intervals in 3 volumes of grinding medium (25 mM Tris-Mes, pH 6.5, 3 mM EDTA, 25 mM DTE,[2] 250 mM sucrose with 1% PVP, w/v). The sonicator used was BIOSONIK (Bronwill Scientific, Rochester, NY) with a small probe set at an intensity of 60. The homogenate was strained through four layers of cheesecloth and the filtrate was centrifuged for 15 min at 13,000*g*. Membrane vesicles were pelleted from the resulting supernatant by centrifugation at 80,000*g* for 35 min in a Beckman 60 Ti rotor, resuspended in fresh grinding medium, and centrifuged again at 80,000*g* for 35 min. The final pellet was resuspended in 1 ml suspension buffer (1 mM Tris-Mes, pH 6.5, 1 mM $MgSO_4$, 20% sucrose, w/w), layered on a discontinuous sucrose gradient consisting of 3.5 ml of 34% sucrose (containing 0.55 mM Tris-Mes, pH 6.5, and 0.55 mM $MgSO_4$), and 6 ml of 45% sucrose in the same buffer system, and centrifuged for 2 h at 80,000*g*. Plasma membrane vesicles were recovered from the interface between the two sucrose layers.

Detergent Solubilization. Triton X-100, sodium cholate, Lubrol WX, and SDS were obtained from Sigma. Octylglucoside was obtained from Calbiochem. Membrane fractions were adjusted to the indicated concentrations of detergent and membrane protein in suspension buffer, incubated on ice for at least 20 min, and centrifuged at 100,000*g* for 60 min in a Beckman SW 60 Ti rotor. The supernatant was carefully decanted and saved. If pellets were to be assayed, they were resuspended in buffer.

Ammonium Sulfate Precipitation. The octylglucoside supernatant was diluted with an equal volume of saturated $(NH_4)_2SO_4$ in water, incubated on ice for 10 min, and centrifuged at 100,000*g* for 1 h. The supernatant was decanted, incubated with an equal volume of saturated $(NH_4)_2SO_4$ in water on ice for 10 min, and then filtered on a 0.45 μm Millipore filter. The filtrate was collected from the filter in 1 ml of suspension buffer and

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[2] Abbreviations: DTE, dithioerythritol; BHT, butylated hydroxy toluene; octylglucoside; *n*-octyl-β-D-glucopyranoside; PNP, *p*-nitrophenyl phosphate.

was then used as the ATPase preparation.

Gel Filtration. The cleared cholate solution (2.0 ml) was layered on a 50-ml Sephadex G-150 column and eluted by gravity flow at 4°C with a solution of 0.1% cholate, 1 mM Tris-Mes, and 1 mM $MgSO_4$. Thirty 1-ml fractions were collected after the void volume, which was measured using blue dextran 200. Mol wt standards used were the monomer and dimer of BSA (66,000 and 132,000 D).

Polar Lipid Dispersion. Polar lipids were added to the cholate-solubilized fractions following the procedure of Cocucci and Ballarin-Denti (5). A mixture of lipids, approximately 50% L-α-phosphatidylcholine from soybean (Sigma Chemical Co.) was dispersed with a 15-s pulse from the sonicator in a solution containing 2 mM histidine, 0.1 mM EDTA, 2 mM Hepes (pH 6.7) to give a final phosphorus concentration of 10 μM. All phospholipid preparations contained 50 μg BHT/ml. The lipid suspension was added with another 15-s pulse from the sonicator to the fractions from the column to give 10 μmol lipid P/μg protein.

Phospholipid Analyses. Phospholipids were extracted with chloroform:methanol:water (1:1:0.75 v/v/v) according to Wilson and Rinnie (29). To assay for total P, samples of 0.1 ml were evaporated to dryness and digested for 15 min in 0.9 ml $HClO_4$ over a medium flame. Pi standards were carried through the digestion procedure. Samples and standards were diluted with 5 ml deionized H_2O, heated in a boiling water bath for 5 min, and cooled for 20 min. Pi was determined by the Fiske-Subbarow method (7).

Protoplast Isolation. 50 ml of 5-d-old cells were filtered and washed twice with 50 ml of deionized H_2O. The 3 to 4 g of cells were then transferred to 10 ml of an enzyme mixture consisting of 4% Cellulysin (Calbiochem), 0.5% Macerase (Calbiochem), 25 mM Mes, 0.5 M mannitol, 1 mM $CaCl_2$, and 0.1 mM KCl adjusted to pH 5.7 with 1 M KOH (15). The cells were incubated in this mixture at room temperature for 2 to 3 h with constant shaking.

After enzymic treatment, the cells were filtered through two layers of cheesecloth and protoplasts were collected by layering the filtrate over a 45% sucrose pad and centrifuging in a table top centrifuge (International Equipment Co., Needham, MA) for 5 min at setting 5. The supernatant was aspirated off and 1 to 1.5 ml of protoplasts were collected from the top of the sucrose pad with a Pasteur pipette.

Vacuole Isolation. The protoplasts were added to 7 ml of 0.1 M K_2HPO_4 (pH 8.0) with 0.5 mM DTT and gently swirled for 5 min to release intact vacuoles (15). The lysate was filtered through nylon mesh to remove aggregates of cytoplasmic particles, and mixed with 1 ml of 0.75 M mannitol. This solution was divided equally and loaded onto the top of two discontinuous gradients composed of 3.0 ml of 1.5% Ficoll 400 (containing 0.5 M mannitol, 0.5 mM DTT, 0.2 mM $CaCl_2$, and 25 mM Tris-HCl, pH 7.3) and 4 ml of 5% Ficoll in the same buffer system, and centrifuged for 2 h at 100,000*g*. Intact vacuoles collected at the interface of the 1.5% and 5% Ficoll layers. Protoplasts and vacuoles appeared to be intact when examined under the light microscope.

Assays. The enzyme activity of alcohol dehydrogenase was measured according to Scandalios (22). NADPH-Cyt *c* reductase, latent IDPase, and Cyt *c* oxidase were measured using the methods of Hodges *et al.* (11). Acid phosphatase and α-mannosidase activities were assayed by measuring the liberation of *p*-nitrophenol from the substrates *p*-nitrophenyl phosphate and *p*-nitrophenol-α-mannopyranoside, respectively (2). Catalase was assayed spectrophotometrically by monitoring changes in optical density at 240 nm (30). Carboxypeptidase was assayed by measuring hydrolysis of *N*-carbobenzoxy phenylalanylalanine (18). ATPase was assayed by measuring the amount of Pi released at 30°C. This temperature was selected because it is far from the transition temperature of phospholipids and, at this temperature, inactivation of ATPase and oxidation of polar lipids does not take place (5). ATPase activity was measured in a 1-ml volume containing 3 mM ATP (Tris salt), 30 mM Tris-Mes at pH 6.5, 3 mM $MgSO_4$, and 50 mM KCl (when added). The reaction was initiated by introducing 25 to 50 μg of membrane protein. The reaction was terminated after 30 min by adding 1 ml of cold 10% TCA. Inorganic phosphate was determined by the Fiske-Subbarow method (7). ATPase specific activity is expressed as μmol Pi/mg protein·h.

Protein concentration was determined by the method of Lowry *et al.* (16), using BSA standards. Possible interferences were corrected for by including in the standards the same components present in the buffers in which the protein samples were suspended.

RESULTS

First, a variety of detergents and other substances were tested for their ability to remove protein from the membrane fraction; then the effect of the detergents on the activity of the K^+-stimulated ATPase was tested; and finally several detergents were tested for their ability to solubilize the K^+-stimulated ATPase in an active form. The abilities of detergents to extract protein from plasma membrane were similar to those reported by DuPont and Leonard (6). Detergents solubilized from 22% (Lubrol WX, 0.2% w/w) to 69% (SDS, 0.1%, w/w) of the protein. The nonionic detergents Triton X-100, Lubrol WX, and octylglucoside each affected the activity of the enzyme in a similar manner. Both the basal Mg^{2+}-ATPase activity and K^+-simulated ATPase activity increased with increasing detergent concentration up to some optimal concentration. For example, octylglucoside at 30 mM increased ATPase 2.0-fold above the control. As detergent concentration was further increased, enzyme activity declined as high concentrations of each detergent eliminated the K^+-stimulated component of the ATPase activity. Cholate (1%, w/w) and SDS (0.1%, w/w) eliminated the K^+-stimulated ATPase activity. SDS greatly reduced ATPase activity at the lowest concentration tested (0.01%).

In these initial experiments, octylglucoside solubilized the most K^+-stimulated ATPase activity. Fractionation of the octylglucoside supernatant, with 50% to 75% saturated $(NH_4)_2SO_4$, increased the specific activity of the K^+-stimulated ATPase six times above that of the original plasma membrane fraction (134 compared to 23 μmol Pi/mg protein·h, assayed with 50 mM KCl). The properties of the octylglucoside-solubilized ATPase were similar to those previously published (13). The K^+-stimulated ATPase activity was specific for ATP, was stimulated by $K^+ > Na^+ > Li^+$, and was inhibited by vanadate but not by azide or oligomycin (data not shown).

Treatment of the membrane fraction with a high concentration of cholate, followed by centrifugation for 60 min at 100,000*g*, removed all but 1% of the phospholipids (0.0009 of 0.095 μmol/g fresh weight remained) and about 50% of the protein (0.061 of 0.116 mg/g fresh weight remained), and it produced an almost total loss of ATPase activity (Table I). As previously reported (5), the subsequent addition of lipids restored more than 90% of the total ATPase activity and doubled the specific activity of total enzyme measured in the presence of KCl. The addition of lipids to the other detergent preparations had no effect (data not shown).

The cholate-solubilized preparation was fractionated on a Sephadex G-150 column at 4°C. From the column, 66% of the protein was recovered in 30 fractions after the void volume and 88% of the ATPase activity was recovered in two discrete, approximately equal peaks (Fig. 1). Using BSA monomers and dimers as standards, the mol wt of the protein from fractions 5 to 7 was estimated at 127,000 D and that of the protein from

Table I. *Effects of Cholate Treatment on the Specific and Total ATPase Activity*

The plasma membrane fraction from the 34/45% sucrose gradient interface was incubated in 1% Na cholate for 20 min on ice and then centrifuged for 60 min at 100,000*g* to give the cholate-treated preparation; 10 μmol lipid P/μg protein was added to give the final preparation.

	Total Activity		Protein	Specific Activity	
	−KCl	+KCl		−KCl	+KCl
	μmol Pi/h		*mg*	*μmol Pi/mg·h*	
Plasma membrane fraction	814	1490	61.7	13.2	24.2
Cholate-treated preparation	10.4	23.3	25.9	0.4	0.9
Cholate preparation + phospholipids	510	1330	25.9	19.7	51.3

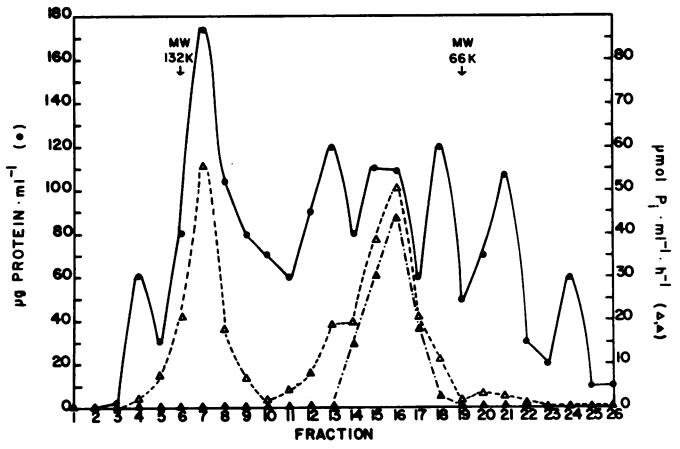

FIG. 1. Gel filtration. The cleared cholate solution was layered on a Sephadex G-150 column and eluted with a solution of 0.1% cholate, 1 mM Tris-Mes, and 1 mM $MgSO_4$ at 4°C. 1-ml fractions were collected and assayed for ATPase activity (△) and protein concentration (●). The octylglucoside preparation was incubated in 1% cholate and then passed through the Sephadex column. The ATPase activity (▲) was recovered in one peak.

fractions 14 to 17 was estimated at 82,000 D. The ATPase specific activity from fractions 5 to 7 represented a 13-fold purification over the initial plasma membrane-associated activity, while that from fractions 14 to 17 represented a 19-fold purification. When the octylglucoside-solubilized preparation was chromatographed on the Sephadex column, all the enzyme activity emerged in the void volume. When the octylglucoside preparation was incubated in 1% cholate and then passed through the Sephadex column, 81% of the ATPase activity was recovered in one peak, fractions 14 to 17, corresponding to the cholate-solubilized ATPase of 82,000 D (Fig. 1).

The ATPase from fractions 5 to 8 and 14 to 17 was characterized. The pH optimum for both enzymes assayed with or without 50 mM KCl was 6.5 (Fig. 2). ATP was the preferred substrate (Table II). The rate of hydrolysis of other di- and triphosphates tested was less than 20% of the rate of ATP hydrolysis. With the phosphatase substrate, *p*-nitrophenyl phosphate, the hydrolysis was less than 5% of that of ATP. The enzymes required Mg^{2+} and were further stimulated by monovalent cations. For both ATPase activities, K^+ gave greater stimulation than the other cations tested: Na^+, Li^+, or NH_4^+. There was no anion-specific stimulation since KCl, K_2SO_4, and KNO_3 had the same effect (Table III).

The inhibitors vanadate, azide, and molybdate were used to distinguish plasma membrane ATPase activity from mitochondrial ATPase activity and phosphatase activity (8). Both fractions of ATPase were inhibited by 15 μM vanadate; fractions 5 to 7 were slightly more vanadate sensitive (Fig. 3). Neither fraction was sensitive to 1 mM Na molybdate, an inhibitor of acid phosphatases or to 5 mM Na azide, a mitochondrial ATPase inhibitor, over a pH range of 5 to 10.

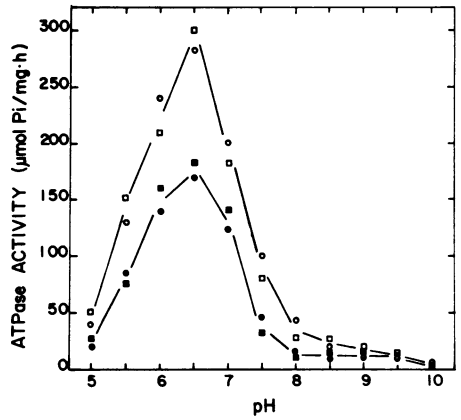

FIG. 2. Activity of the partially purified ATPase as a function of pH. The assay medium contained 3 mM ATP, 3 mM $MgSO_4$, 33 mM Tris titrated with 33 mM Mes to achieve pH, and 50 mM KCl when added. ○, ●: fractions 14 to 17; □, ■: fractions 5 to 7; ○, □: KCl added; ●, ■: no KCl.

Table II. *Activity of Partially Purified Enzyme with Various Substrates*

Assay mixture contained 3 mM substrate (sodium salt), 3 mM $MgSO_4$, 33 mM Tris-Mes (pH 6.5), and 50 mM KCl. Enzyme-containing fractions came from a Sephadex G-150 gel filtration column (Fig. 1).

	Substrate	Activity	
		μmol Pi/mg·h	%
Fractions 5–8	ATP	314	100
	CTP	35.5	11
	GTP	59.6	19
	ITP	28.3	9
	UTP	44.0	14
	ADP	18.8	6
	PNP	9.4	3
Fractions 14–17	ATP	458	100
	CTP	55.0	12
	GTP	96.0	21
	ITP	41.2	9
	UTP	55.0	12
	ADP	22.9	5
	PNP	13.7	3

Marker enzymes for the cytosol, ER, Golgi apparatus, plasma membrane, and mitochondria were assayed to assess the degree to which they contaminated the plasma membrane-enriched preparations. On a continuous 20% to 50% sucrose gradient, we found the expected distributions of marker enzymes (Fig. 4). In the final discontinuous gradient centrifugation in our plasma membrane-enriched preparation, there was no detectable NADPH Cyt *c* reductase (Fig. 5). There was less than 7% of the Golgi membranes and less than 5% of the mitochondria in the fractions routinely collected for use as the plasma membrane-enriched fraction. Less than 2% of the plasma membrane ATPase represented Golgi or mitochondrial ATPase activity.

Using a modified procedure for the gentle osmotic rupture of protoplasts (15), a large number of intact vacuoles were released. The purity of the vacuoles was assessed by following the distribution of marker enzymes. Vacuoles contained 14% of the alcohol dehydrogenase activity, a cytosol enzyme, and 7% or less

Table III. *Effect of Monovalent Ions on Activity of Partially Purified ATPase*

ATPase activity was recovered from the column, lipid suspension added to give 10 μmol P/μg protein, and assayed at 30°C in 30 mM Tris-Mes at pH 6.5, 3 mM ATP (Tris salt), and indicated salts (3 mM $MgSO_4$ + 50 mM KCl, 50 mM NaCl, 50 mM LiCl, 50 mM NH_4Cl, 25 mM K_2SO_4, or 50 mM KNO_3).

Additions	ATPase Activity	Ion Stimulation	Ion Stimulation
	μmol Pi/mg·h		%
Fractions 5–8			
$MgSO_4$	119		
+KCl	316	197	100
+NaCl	260	68	35
+LiCl	200	42	21
+NH_4Cl	292	144	73
+K_2SO_4	315	185	94
+KNO_3	315	196	99
Fractions 14–17			
$MgSO_4$	207		
+KCl	451	244	100
+NaCl	332	95	39
+LiCl	282	70	29
+NH_4Cl	298	154	63
+K_2SO_4	437	209	85
+KNO_3	447	229	94

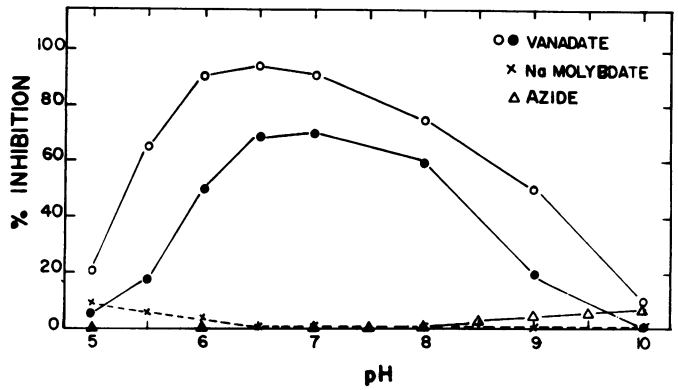

FIG. 3. Inhibition of the two ATPase activities (μmol Pi/mg protein·h), measured in the presence of 3 mM $MgSO_4$ and 50 mM KCl, by vanadate (15 μM) (○, ●), molybdate (1 mM) (×) or azide (5 mM) (△). ○: fractions 5 to 7; ●: fractions 14 to 17.

of the marker enzymes for ER, Golgi, and mitochondria, based on the amount present in the protoplasts from which the vacuoles were prepared (Table IV).

The activities of some hydrolytic enzymes demonstrated to be predominantly, if not exclusively, vacuolar (2, 18) were determined in the protoplast and vacuole preparations (Table IV). Nearly all the acid phosphatase and mannosidase of the protoplasts were located in the vacuoles. The vacuoles also contained high levels of ATPase, carboxypeptidase, and catalase activities.

The characteristics of the ATPase activity associated with vacuoles were different from either plasma membrane ATPase activity in both ion stimulation and inhibitor sensitivity (Table V). The vacuolar ATPase activity was stimulated by the anion Cl^-, insensitive to vanadate, and inhibited by NO_3^-. On a continuous 20% to 50% sucrose gradient, the ATPase of the sonicated vacuoles, used as a marker for tonoplasts (27), was located in the top five fractions (Fig. 4). When centrifuged on a discontinuous 34% to 45% sucrose gradient, 96% of the ATPase activity stayed in the 20% to 34% sucrose interface (Fig. 5). Thus, the plasma membrane-enriched fraction was virtually uncontaminated by tonoplast.

DISCUSSION

Despite a rapidly growing understanding of the interaction of detergents with membranes and membrane proteins (4, 9), developing a procedure for solubilization of a membrane-bound enzyme in an active state is still somewhat a matter of trial and error. The solubilization techniques for plant plasma membrane ATPase reported by other workers, $(NH_4)_2SO_4$ precipitation (6), sonication (23), gel filtration (6, 23), and addition of polar lipids (5), were tested on rose cells with varying degrees of success.

The ATPase activity from the cholate-solubilized preparation fractionated into two distinct, approximately equal peaks when chromatographed on Sephadex G-150. For both ATPase activities recovered from the column, whether solubilized first with octylglucoside or directly with cholate, substrate specificity, pH optimum, and response to ions and inhibitors were very similar. In addition, we have recently reconstituted vesicles from a mixture of each fraction with phospholipids and found that each fraction catalyzes an ATP-dependent acidification of the vesicles' interiors (unpublished data). These multiple similarities may suggest that the two ATPase fractions possess a common catalytic subunit, but we cannot rule out the possibility that the enzymes are quite different without more chemical, enzymological, or immunochemical characterization. The possibility that the two fractions represent a monomer and homodimer seems unlikely from the mol wt estimations, but it is possible that the large enzyme has an additional polypeptide chain not found in the small enzyme, like $(Na^+\text{-}K^+)$-ATPase (4, p. 191). We believe it unlikely that one form of ATPase is formed from the other by our extraction procedures, because (a) between experiments the ratio of forms was fairly constant (fraction of activity in the first peak ranged from 34% to 41% in six representative experiments), (b) the large ATPase form (and no small form) was found in Golgi bodies prepared using the same extraction technique, (c) the small form (and no large form) was found in plasma membrane extracted from 10-d cells using the same extraction technique (12), and (d) UV-inactivation experiments indicate that both forms are present in native plasma membrane vesicles (13).

The mol wt of a phosphorylated polypeptide that presumably represents the catalytic subunit of plant plasma membrane ATPase has been estimated at 100,000 D (3, 21, 26). Vara and Serrano (26) found six major polypeptides ranging in mol wt from 25,000 to 105,000 D with only the heaviest being phosphorylated. The mol wt that we estimated by comparing ATPase peaks to BSA monomer and dimer on Sephadex G-150 do not match the weights in these previous reports. While we believe that one of our peaks corresponds to the phosphorylated protein, confirmation will require that we apply the other workers' techniques to our material. It is possible that cholate-solubilized ATPase binds slightly to Sephadex or that it has a lower axial ratio than BSA. Either could lead us to underestimate its mol wt.

When the octylglucoside preparation was chromatographed, the activity emerged in the void volume, indicating a mol wt of over 150,000 D. A high mol wt was expected, since previous workers identified the solubilized ATPase as a detergent-lipid-protein complex (6). When the octylglucoside-solubilized ATPase activity was incubated with cholate and then chromatographed, the activity was recovered in one peak corresponding very closely to the cholate-solubilized ATPase activity of 82,000 D. This suggests that octylglucoside solubilization resulted in the loss of the larger form of ATPase, perhaps by denaturation.

The purification of a specific membrane-associated ATPase is complicated by the presence of several membrane-associated

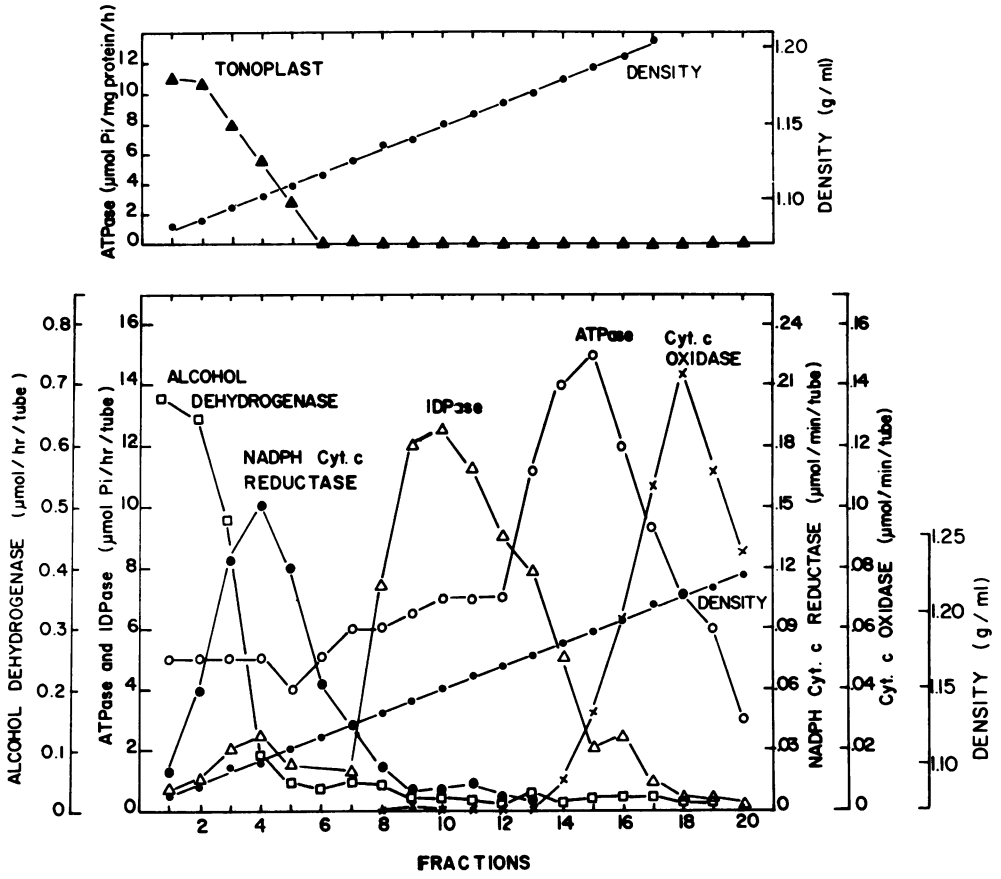

FIG. 4. Results of centrifuging a total cell extract on a continuous sucrose gradient. After sonication, the liquid was strained through four layers of cheesecloth. The filtrate was layered over a continuous 20% to 50% (w/w) sucrose gradient in 1 mM $MgSO_4$ and 1 mM Tris-Mes, pH 7.2, and centrifuged at 28,000 rpm (100,000*g*) for 3 h. The gradient was fractionated into 0.5-ml portions, and the indicated enzymes were assayed in each fraction. Tonoplast distribution was assayed as ATPase activity associated with vacuoles isolated from osmotically shocked protoplasts, and centrifuged on a separate gradient.

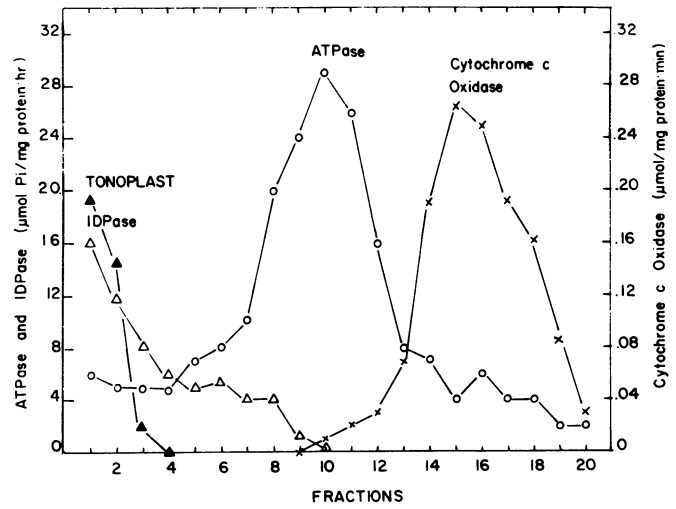

FIG. 5. Results of centrifuging the resuspended 80,000*g* pellet on a discontinuous sucrose gradient (1 ml 20% sucrose, 1.08 g ml^{-1} density; 3.5 ml 34% sucrose, 1.15 g ml^{-1}; 6 ml 45% sucrose, 1.20 g ml^{-1}). The gradient was fractionated into a 0.5-ml portions, and each fraction was assayed for alcohol dehydrogenase (not shown), NADPH Cyt *c* reductase (not shown), latent IDPase (△), Cyt *c* oxidase (×), and K^+-stimulated ATPase activity (○). Shown also is the distribution of intact vacuoles that were sonicated and layered on a separate discontinuous sucrose gradient (▲).

Table IV. *Enzyme Activities and Protein Content in Protoplasts and Vacuoles Isolated from Rose Cell Suspension Cultures*

The activities are reported on a per 0.1-ml protoplast suspension basis with activity in vacuoles related to the amount of protoplast suspension from which the vacuoles came.

	Protoplasts	Vacuoles	Activity in Vacuoles
Alcohol dehydrogenase[a]	4.45	0.62	14
NADPH Cyt *c* reductase[a]	0.67	0.05	7
Latent IDPase[b]	82.2	3.3	4
ATPase (pH 6.5)[b]	147.0	39.2	27
Cyt *c* oxidase[a]	0.54	0.01	2
α-Mannosidase[a]	36.0	34.0	94.4
Acid phosphatase[a]	52.7	46.3	88.0
Catalase[c]	1.98	1.24	62.6
Carboxypeptidase[a]	0.61	0.29	47.5
Protein[d]	207.0	27.0	13.0

[a] μmol/min·0.1 ml protoplasts.
[b] μmol/h·0.1 ml protoplasts.
[c] ΔA_{240}/min·0.1 ml protoplasts.
[d] μg/0.1 ml protoplasts.

ATPases in plant cells (11, 20). *In situ* cytochemical studies have shown ATPase activity to be associated with mitochondria, plastids, plasma membranes, tonoplast, Golgi apparatus, and the ER of plant root cells. Membrane fractions obtained from plant tissues may also contain phosphatases that hydrolyze ATP. The substrate specificity, pH optimum, and response to ions and inhibitors were used to distinguish the solubilized ATPase from the other ATP-hydrolyzing activities found in the soluble and membrane fraction of cell homogenates and to compare them to other ATPase preparations (10, 13). Also, the characteristics of the ATPase activity from the vacuoles were very different from those of either of our plasma membrane ATPase fractions. The vacuolar ATPase activity was stimulated by Cl^- and not by K^+, and was inhibited by NO_3^- but was not inhibited by vana-

Table V. *Effect of Ions and Vanadate on the ATPase Activity of Isolated Vacuoles*

Assayed at 38°C in 30 mM Tris-Mes at pH 6.5, 3 mM ATP (Tris salt), and indicated salts (3 mM $MgSO_4$ + 50 mM KCl, 50 mM NaCl, 50 mM NH_4Cl, 25 mM K_2SO_4, 50 mM KNO_3, 50 mM KCl, and 50 μM vanadate, 50 mM KCl and 1 mM Na molybdate, or 50 mM KCl and 5 mM Na azide).

	ATPase Activity	% of Control
	μmol Pi/mg·h	
$MgSO_4$	2.74	100
+KCl	4.01	146
+NaCl	3.85	141
+NH_4Cl	8.01	292
+K_2SO_4	2.71	99
+KNO_3	1.31	48
+KCl + vanadate	3.87	141
+KCl + Na molybdate	3.29	120
+KCl + Na azide	3.96	144

date. These characteristics are similar to those reported in the literature for vacuolar ATPase activity (17, 19, 27).

Another way of determining the purity of an isolated membrane preparation is to determine the degree of contamination of the preparation by the enzymes associated with other cell membranes: the marker concept (28). From the distribution of the marker enzymes for the cytosol, ER, Golgi apparatus, mitochondria, and plasma membrane, the membranes collected from the 34/45% interface on the discontinuous sucrose gradients were identified as plasma membrane and appeared to be quite pure. There is at present no unequivocal report of a unique tonoplast marker (20). A determination of the extent of contamination by the tonoplast in our plasma membrane preparation was especially important given the recent reports of probable tonoplast ATPase activity in microsomal vesicles (19, 24, 25). Thus, we demonstrated that the intact vacuoles, even after disruption by sonication, were located, after centrifugation on both continuous and discontinuous sucrose gradients, at a fraction corresponding to a much lighter density than that of our plasma membrane preparation. There could have been virtually no contamination of our plasma membrane preparations by the tonoplast.

These results show that there are two forms of K^+-stimulated ATPase associated with the plasma membrane of rose cells. Solubilization with the detergent sodium cholate and fractionation on Sephadex G-150 is an effective method for purifying the two forms. The two forms of ATPase are similar in pH optima, substrate specificity, ion stimulation, and inhibitor sensitivity, but differ in mol wt.

This solubilization procedure has been used in the following paper to address some of the questions dealing with the photoinactivation kinetics of the ATPase activity. Since this procedure strips the enzymes of the phospholipids associated with them, it provides a system to test the effect of specific lipids on the ATPase activities. This enzyme preparation is also being used to test the transport properties of the two ATPases in synthetic lipid vesicles.

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