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Solubilization and Partial Purification of the Adenosine Triphosphatase from a Corn Root Plasma Membrane Fraction

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ABSTRACT

The K^+-stimulated ATPase was partially purified from a plasma membrane fraction from corn roots (WF9 × Mo 17) by solubilization with 30 millimolar octyl-β-D-glucopyranoside followed by precipitation with dilute ammonium sulfate. The specific activity of the enzyme was increased about five times by this procedure. The molecular weight of the detergent-extracted ATPase complex was estimated to be at least 500,000 daltons by chromatography on a Bio-Gel A-5m column. Negative staining electron microscopy indicated that the detergent-extracted material consisted of amorphous particles, while the ammonium sulfate precipitate was composed of uniform vesicles with an average diameter of 100 nanometers. The protein composition of the ammonium sulfate precipitate was significantly different from that of the plasma membrane fraction when compared by sodium dodecyl sulfate gel electrophoresis. The characteristics of the partially purified ATPase resembled those of the plasma membrane associated enzyme. The ATPase required Mg^{2+}, was further stimulated by K^+, was almost completely inhibited by 0.1 millimolar diethylstilbestrol, and was not affected by 5.0 micrograms per milliliter oligomycin. Although the detergents sodium cholate, deoxycholate, Triton X-100 and Lubrol WX also solubilized some membrane protein, none solubilized the K^+-stimulated ATPase activity. Low concentrations of each detergent, including octyl-β-D-glucopyranoside, activated the ATPase and higher concentrations inactivated the enzyme. These results suggest that the plasma membrane ATPase is a large, integral membrane protein or protein complex that requires lipids to maintain its activity.

Neither the precise transport properties of the plasma membrane ATPase nor the mechanism of K^+ transport into higher plant tissues has been clearly determined (19, 26). A K^+-stimulated ATPase activity is associated with plasma membrane fractions from tissues of higher plants, and correlations between cation stimulation of this ATPase and cation transport into plant tissues have led to the suggestion that the ATPase is a cation pump (16, 19). Measurements of membrane potentials and ion fluxes suggest that the plasma membrane contains an electrogenic, proton-pumping ATPase (26, 28). K^+ transport in higher plants is closely linked to ATP hydrolysis (2, 26), but K^+ may be directly pumped by an ATPase or indirectly driven by the electrical component of the proton motive force. It has been difficult to distinguish between these possibilities. No specific inhibitors of the plasma membrane ATPase or of K^+ transport in plants are known (2, 19) and ATP dependent ion transport has not been demonstrated in plasma membrane vesicles isolated from higher plants (31). It appears that it will be necessary to reconstitute the K^+-stimulated ATPase into impermeable lipid vesicles to determine its transport properties.

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 (15, 18, 27, 35) 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 putative transport ATPase (4, 7, 32). Benson and Tipton (4) report purification of a low mol wt ATPase activity from a membrane fraction from corn roots, but they provide no evidence that this enzyme was associated with the plasma membrane and did not represent the activity of a nonspecific phosphatase. Similarly, Cross *et al.* (7) report that a low mol wt ATP hydrolyzing activity with poor substrate specificity was released from a microsomal membrane fraction from corn coleoptiles by Triton X-100. Tognoli *et al.* (32) used EDTA, trypsin, deoxycholate or perchlorate to release a poorly characterized ATP hydrolyzing activity of extremely low specific activity from a microsomal fraction from corn coleoptiles.

Greater progress has been made with ATPases from fungal plasma membranes. The action of an electrogenic ATPase has been demonstrated in isolated membrane vesicles from *Neurospora* (29) and detergents have been used to purify the plasma membrane ATPase of *Neurospora* (5) and the yeast *Schizosaccharomyces pombe* (10). These reports may be relevant to attempts to determine the structure and function of the plasma membrane ATPase of higher plants, since there are many similarities between the ATPase activity of plasma membrane fractions from higher plants and from fungi (5, 9, 30).

This paper describes our attempts to solubilize and purify the K^+-stimulated ATPase from a plasma membrane fraction from corn roots.

MATERIALS AND METHODS

Plant Material. Corn seeds (*Zea mays* L. WF9 × MO.17) were germinated at 28 C for 4 days in the dark (20). They were then transferred to aeroponics tanks built according to the specifications of Zobel *et al.* (36) and grown in a greenhouse for an additional 8–14 days with the roots suspended in a nutrient mist. The composition of the nutrient solution was slightly modified (the phosphorous concentration was only 1 mM) from Table 3-1 of Epstein (13).

Preparation of Membrane Fractions. Membrane fractions were prepared as previously described (20, 21) with certain modifications. All steps were carried out between 2 and 10 C. Briefly, the fibrous roots were excised, rinsed in ice water, and ground with mortar and pestle in a grinding medium consisting of 0.25 M sucrose, 3 mM EDTA, 2.5 mM DDT, and 25 mM Tris-Mes (pH 7.7). The brei was filtered through cheese cloth and centrifuged at 13,000*g* for 15 min, followed by centrifugation at 80,000*g* for 30

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min. The 13,000 to 80,000*g* microsomal pellet was suspended in a suspension buffer consisting of 0.25 M sucrose, 1 mM DTT and 1 mM Tris-Mes (pH 7.2). The microsomal fraction was used for a number of the preliminary experiments.

A plasma membrane fraction was prepared by the following modification of published procedures (20). The microsomal fraction was adjusted to a final volume of 6 ml and a final concentration of 17% (w/w) sucrose in 1 mM DTT and 1 mM Tris-Mes (pH 7.2), layered over 5 ml of 34% sucrose and centrifuged at 80,000*g* for 1 or 2 h in a Beckman SW 27.1 rotor. The sucrose layers were carefully removed by aspiration and the loose plasma membrane pellet was suspended in suspension buffer. The characteristics of the ATPase activity of this modified plasma membrane fraction were similar to those previously published (12, 20).

Detergent Solubilization. Triton X-100, sodium deoxycholate, sodium cholate and SDS were obtained from Sigma. Lubrol WX was obtained from Supelco (Bellefonte, Pa.). Octyl-glucoside[2] was obtained from Calbiochem. Membrane fractions were adjusted to the indicated concentrations of detergent and of membrane protein in suspension buffer, incubated on ice for the indicated times, and centrifuged at 41,000 rpm (approximately 100,000*g*) for 1 h in a Beckman 50 rotor. The supernatant was carefully decanted and saved. If pellets were to be assayed, they were suspended in suspension buffer.

Molecular Sieve Chromatography. Bio-Gel A-5m and A-1.5m were purchased from Bio-Rad. Columns (2.2 × 45 cm) were eluted by gravity flow, at 10 C. Column effluents were monitored at 280 nm using an ISCO fractionation system. Mol wt standards, purchased from Sigma, were catalase (240,000 daltons) and BSA (68,000 daltons). Blue dextran 200 was purchased from Pharmacia.

Ammonium Sulfate Precipitation. The octyl-glucoside supernatant was immediately diluted with an equal volume of ammonium sulfate in suspension buffer to a final concentration of 15 mM octyl-glucoside and the indicated concentrations of ammonium sulfate, incubated on ice for 30 min and centrifuged at 41,000 rpm for 1 h in a Beckman 50 rotor. The supernatant was carefully decanted and the pellet resuspended in suspension buffer.

Assay Procedures. ATPase activity was measured at 38 C in a 1-ml volume containing the enzyme (20–80 μg protein for the plasma membrane and detergent treated samples; 5–15 μg for the ammonium sulfate precipitate), 3 mM ATP (Tris salt), 30 mM Tris-Mes (pH 6.5), and 3 mM $MgSO_4$, plus 50 mM KCl where indicated (20). K^+-stimulated activity is the difference in ATPase activity in the presence and absence of 50 mM KCl. Any changes in substrate, inorganic ions, or pH are indicated in Figure Legends and Tables.

Inorganic phosphate was determined by a modification of the procedure of Fiske and Subbarow (11). The reaction was stopped by adding 2 ml of a solution of 5% (w/w) SDS and 0.833% (w/v) ammonium molybdate in 1.667 N H_2SO_4, immediately followed by 0.4 ml of the Fiske and Subbarow color reagent. Samples were incubated at 23 C for 35 min before determining A_{660}. SDS eliminated interference due to Triton X-100 and Lubrol WX.

Proteins were estimated by a modification of the Lowry procedure (25). In early experiments, proteins were precipitated with a mixture of deoxycholate and trichloroacetic acid (25). Material solubilized by octyl-glucoside was not quantitatively precipitated by this technique, so the precipitation step was abandoned and the presence of DTT was compensated for by both: (*a*) including equal amounts of DTT in all blanks and standards; and (*b*) letting the protein samples remain for at least 2 h in the alkaline copper reagent before adding the Folin-Ciocalteu reagent, inasmuch as DTT deteriorates under these conditions.

[2] Abbreviations: octyl-glucoside: octyl-β-D-glucopyranoside; DES: diethylstilbestrol; CMC: critical micellar concentration; PNP: *p*-nitrophenylphosphate.

RESULTS

A trial and error procedure was used to search for a method to solubilize the K^+-stimulated ATPase from the plasma membrane fraction. 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. Initially, the criterion for solubilization was simply retention of protein or enzyme activity in the clear supernatant after centrifugation at 100,000*g* for 1 h (24). The microsomal fraction was used for these preliminary experiments, since it could be prepared quickly and in large quantities.

Solubilization of Membrane Proteins. Table I shows a number of detergents and other substances listed in order of their ability to extract protein from the microsomal fraction. There was 6% loss of protein from the control. EDTA, which interferes with cation dependent binding of proteins to membranes, removed 17% of the membrane protein. KI, a chaotropic agent, removed 37% of the protein. Detergents solubilized from 26% (Lubrol WX) to 66% (SDS) of the protein. Of the reagents listed in Table I, the most effective solubilizing agent was the combination of 1% Triton X-100 and 8 M urea. Also, 1% SDS will completely solubilize the plasma membrane fraction for SDS gels (Fig. 7).

Effect of Detergents on ATPase Activity. Figures 1 and 2 illustrate the effect of four different detergents on the ATPase activity of the microsomal fraction. Triton X-100 and octyl-glucoside are mild, nonionic detergents, cholate is a mild bile salt, and SDS a harsh, anionic detergent. The three mild detergents each affected the activity of the enzyme in a similar manner, although with different concentration dependencies. Enzyme activity increased with increasing detergent concentration up to some optimal concentration. Both the basal, Mg^{2+} ATPase activity and the K^+-stimulated ATPase activity were affected by the detergents. Maximal activation of Triton X-100 was at 0.1% (v/v), by octyl-glucoside at 20 mM and by sodium cholate between 2 and 3% (w/w) (Figs. 1 and 2). As detergent concentration was further increased, enzyme activity declined and high concentrations of each detergent eliminated the K^+-stimulated component of the ATPase activity. The "harsh" detergent, SDS, eliminated the K^+-stimulated ATPase activity and greatly reduced all ATPase activity at the lowest concentration tested (0.02%, Fig. 2).

Similar experiments were performed to test the effect of Lubrol WX and deoxycholate on the enzyme activity of the plasma membrane fraction (12). Maximal activation by deoxycholate was at 0.15% (w/v), and 0.5% deoxycholate completely eliminated the

Table I. *Extraction of Protein from the Microsomal Fraction from Corn Roots by Detergents, KI and EDTA*

Aliquots (1.1 mg protein) of the microsomal fraction were adjusted to the indicated concentrations of detergents or other substances in 1.0 ml of suspension buffer, incubated from 20 min at 23 C and centrifuged at 100,000*g* for 1 h at 4 C.

Additions	Protein in Supernatant
	%
None	6
5 mM EDTA	17
5 % (w/w) Lubrol WX	26
1% (w/v) Sodium cholate	33
1 M KI	37
1% (v/v) Triton X-100	42
1% (w/v) Octyl-glucoside	47
1% (w/v) Sodium deoxycholate	61
0.1% (w/w) SDS	66
1% Triton X-100 + 8 M urea	90

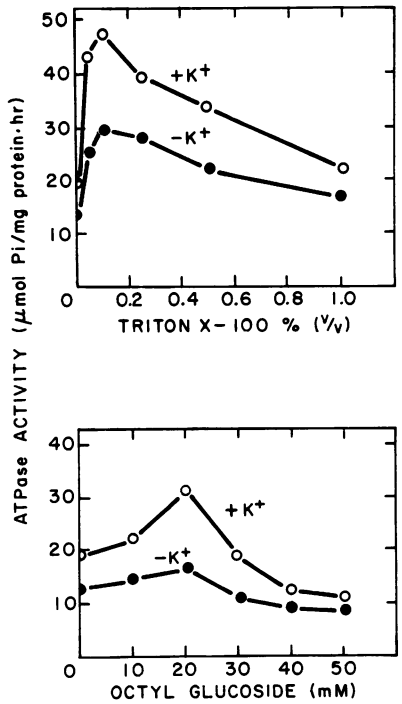

FIG. 1. ATPase activity of the microsomal fraction from corn roots as a function of detergent concentration. Aliquots of the microsomal fraction were adjusted to a protein concentration of 0.82 mg/ml and the indicated concentrations of the detergents Triton X-100 and octyl-glucoside in suspension buffer, incubated for 2 h at 10 C, and assayed for ATPase activity at pH 6.5, with 50 mM KCl (when added).

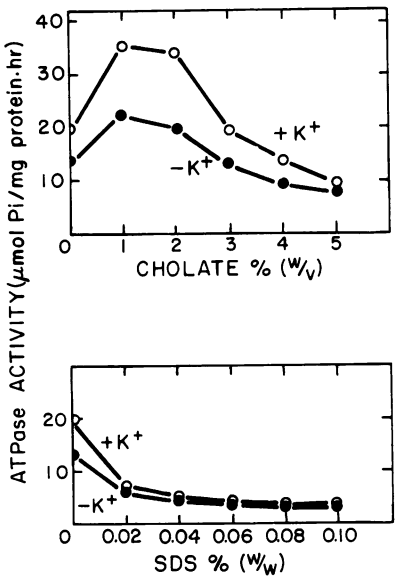

FIG. 2. ATPase activity of the microsomal fractions from corn roots as a function of detergent concentration. See Figure 1 for details. Membranes were adjusted to the indicated concentrations of the detergents sodium cholate and SDS as described in Figure 1 and assayed for ATPase activity with 50 mM KCl (when added).

K+-stimulated activity. Maximal activation by Lubrol WX was at 0.1% (w/w), but activity remained high in the presence of 1.0% Lubrol (the highest concentration tested).

Solubilization of the K+-stimulated ATPase. The abilities of the detergents Triton X-100, sodium cholate, and octyl-glucoside to solubilize the K+-ATPase from the microsomal fraction were compared (Fig. 3). A suitable concentration range for each detergent was chosen, based on the effect of the detergent on ATPase activity (Figs. 1 and 2). All three detergents caused noticeable clearing of the cloudy membrane fractions, and the 100,000*g* supernatants were clear, not cloudy. At the highest concentrations tested, all three detergents released 40 to 50% of the membrane protein to the 100,000*g* supernatant. However, only octyl-glucoside solubilized significant K+-stimulated ATPase activity. The optimal concentration for solubilization was 30 mM octyl-glucoside, as has been reported previously (3). Lubrol WX or sodium deoxycholate were not effective in solubilizing the ATPase (not shown).

Characterization of the Octyl-glucoside Solubilized ATPase. A simplified procedure was developed to prepare a concentrated plasma membrane fraction. The ability of octyl-glucoside to solubilize the plasma membrane fraction was tested, and results of a typical experiment are shown in Table II. Octyl-glucoside solubilized approximately 50% of the protein and 50% of the K+-stimulated ATPase activity. The activity of the solubilized enzyme decayed rapidly and it was necessary to assay the enzyme immediately after solubilization. The K+-stimulated component of the ATPase activity disappeared completely within 24 h if the preparation was stored at 4 C or frozen at −20 C.

The properties of the ATPase of the modified plasma membrane fraction and the octyl-glucoside supernatant were similar to those previously published (12, 20). The K+-stimulated ATPase activity was specific for ATP, although the fractions also had considerable nucleoside diphosphatase activity that was not stimulated by

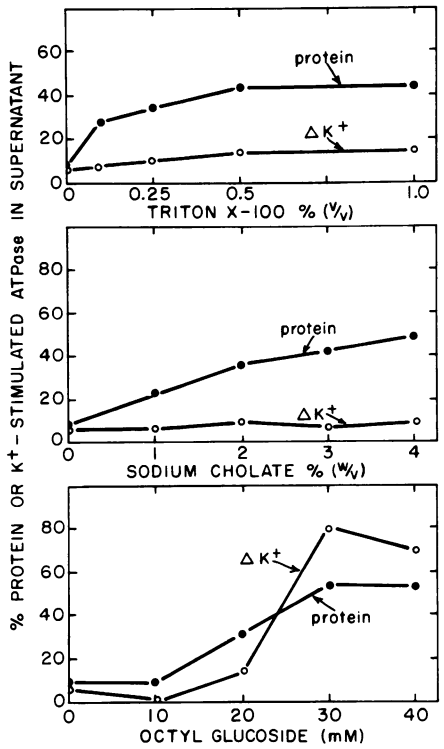

FIG. 3. Solubilization of protein and K+-stimulated ATPase activity from the microsomal fraction from corn roots as a function of detergent concentration. Aliquots (1 mg protein) of the microsomal fraction were adjusted to the indicated concentrations of the detergents Triton X-100, sodium cholate, or octyl-glucoside in 1 ml suspension buffer, incubated on ice for 20 min and centrifuged at 100,000*g* for 1 h. The pellets (resuspended in suspension buffer) and supernatants were assayed for protein and ATPase activity (pH 6.5). Total K+-stimulated ATPase activity of the supernatant is expressed as a percentage of the sum of the total activity in the pellet and supernatant fractions at that detergent concentration.

Table II. *Solubilization of Protein and ATPase Activity from a Plasma Membrane Fraction from Corn Roots by 30 mM Octyl-glucoside*

The plasma membrane fraction was adjusted to a concentration of 1.74 mg/ml protein and 30 mM octyl-glucoside in 6 ml suspension buffer, incubated on ice for 20 min and centrifuged at 100,000*g* for 1 h at 4 C. The plasma membrane fraction, octyl-glucoside pellet, and octyl-glucoside supernatant were assayed (15 min) for ATPase activity at pH 6.5, with 50 mM KCl (when added).

	Total ATPase Activity			Total Protein	ATPase Specific Activity		
	$-K^+$	$+K^+$	K^+-stimulation		$-K^+$	$+K^+$	K^+-stimulation
	μmol Pi/h			*mg*	*μmol Pi/mg·h*		
Plasma membrane	119.7	202.1	82.4	10.5	11.4	19.2	7.8
Octyl-glucoside							
Supernatant	29.5	70.3	40.8	5.4	5.5	13.0	7.5
Pellet	70.5	122.7	52.2	5.0	14.1	24.5	10.4
Total supernatant + pellet	100.0	193.0	93.0	10.3			

monovalent cations (12). The enzyme showed simple Michaelis-Menten kinetics for the substrate ATP:Mg; the K_m for ATP:Mg was between 0.5 and 1 mM in the presence or absence of K^+ and the major effect of K^+ was to increase V_{max}.

Molecular Sieve Chromatography of the Octyl-glucoside Supernatant. Bio-Gel columns were used to estimate the mol wt of the membrane components in the octyl-glucoside supernatant (Fig. 4). The columns were run at 10 C with fairly rapid elution rates to attempt to preserve enzyme activity. The octyl-glucoside supernatant was chromatographed on a Biogel A-5m column (exclusion limit: 5 million daltons) using a low ionic strength buffer (Fig. 4). Similar results were obtained whether the elution buffer contained no detergent (Fig. 4, upper), 25 mM octyl-glucoside (Fig. 4, lower) or 25 mM octyl-glucoside plus 4 M urea (not shown). Only 15% of the total K^+-stimulated ATPase activity that was applied to the column was recovered. The ATPase may have been inactivated when lipids and detergent were removed by the column. PNPase activity was also determined (Fig. 4, upper) and approximately 70% of the original PNPase activity was recovered. Some of the protein, ATPase and PNPase activity were retained on the column, with a minimum mol wt of approximately 500,000 daltons. About half of the ATPase activity emerged in the void volume.

The octyl-glucoside supernatant was also chromatographed on a Bio-Gel A-0.5m column, with an exclusion limit of 500,000 daltons (not shown). All of the protein and enzyme activities emerged in the void volume, indicating a mol wt of at least 500,000 daltons and this result did not depend on the ionic strength of the elution buffer.

Ammonium Sulfate Fractionation. Attempts were made to fractionate the octyl-glucoside supernatant with ammonium sulfate. The detergent was diluted to 15 mM (below its CMC of 25 mM) and samples were centrifuged at 100,000*g* for 1 h. (In preliminary experiments, it was observed that a white flocculent precipitate formed at high concentrations of ammonium sulfate and this floated unless samples were centrifuged at high speeds.) White pellets with some ATPase activity were collected at the higher concentrations of ammonium sulfate (not shown). The greatest enrichment of ATPase activity was obtained when the sample was adjusted to only 10% of saturation (approximately 0.38 M ammonium sulfate). A clear, jelly-like pellet was obtained, and the specific activity of the K^+-stimulated ATPase in this pellet was approximately five times greater than that of the original plasma membrane fraction. Additional experiments indicated that the optimal concentration for precipitation of an enriched pellet of K^+-stimulated ATPase activity was between 0.30 and 0.38 M ammonium sulfate (Fig. 5). A concentration of 0.35 M ammonium sulfate was routinely used. The ATPase activity of the ammonium

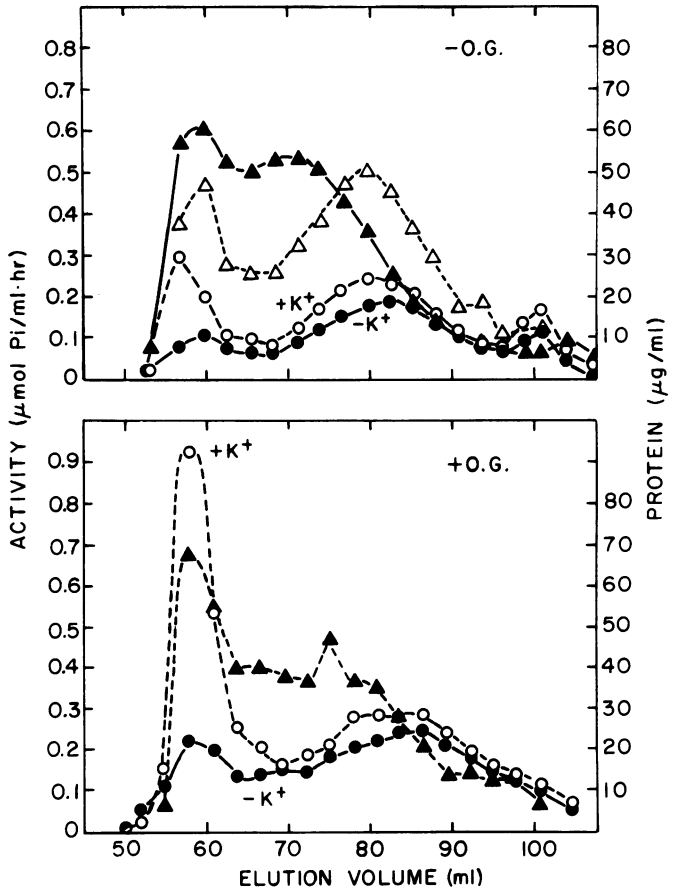

FIG. 4. Chromatography of octyl-glucoside supernatant on a Bio-Gel A-5m column. Plasma membrane fractions were solubilized with 30 mM octyl-glucoside and the 100,000*g* supernatants were chromatographed on a Bio-Gel A-5m column. Fractions (2.9 ml) were collected at a flow rate of 34 ml/h. Fractions were assayed for ATPase activity at pH 6.5 and for *p*-nitrophenylphosphatase activity at pH 5.0. (Upper) Absence of octyl-glucoside. Elution buffer was 10 mM Tris-Mes (pH 7.2) and 1 mM DTT. Sample was 1.84 mg protein and 2.0 ml of 30 mM octyl-glucoside and suspension buffer. Proteins (▲——▲), ATPase, $-K^+$ (●——●), ATPase, $+K^+$ (O---O), and *p*-nitrophenyl phosphatase (Δ---Δ). (Lower) Presence of octyl-glucoside. Elution buffer was 25 mM octylglucoside, 10 mM Tris-Mes (pH 7.2), and 1 mM DDT. Sample was 1.60 mg protein in 2.0 ml of 30 mM octyl-glucoside and suspension buffer. Symbols, same as above.

sulfate precipitate was more stable than that of the octyl-glucoside supernatant and approximately 50% of the activity was retained if the enzyme was stored frozen at −20 C for several days.

Electron Microscopy. The physical appearance of the plasma membrane fraction, octyl-glucoside supernatant, and ammonium sulfate precipitate were compared by negative staining electron microscopy (Fig. 6). The plasma membrane fraction consisted of vesicles of a variety of sizes and an average diameter of approximately 200 nm. The octyl-glucoside supernatant consisted of amorphous particles that were clearly nonmembranous. The smallest particles visible in the micrograph had a diameter of approximately 20 nm. The ammonium sulfate pellet was composed of uniform vesicles with an average diameter of 100 nm. Apparently, when the octyl-glucoside supernatant was diluted to a concentration of 15 mM in the presence of 0.35 M ammonium sulfate, some of the proteins, including the K^+-stimulated ATPase, reformed into membrane vesicles, and these vesicles, enriched in K^+-stimulated ATPase activity, were pelleted by the high speed centrifugation step. The Na^+, K^+-ATPase, and Ca^{2+}-ATPase also form membrane vesicles or other membranous structures when purified (17, 23).

SDS Gels. The protein composition of the ammonium sulfate precipitate was compared with that of the plasma membrane fraction and the octyl-glucoside supernatant by electrophoresis on SDS gels (Fig. 7). The plasma membrane fraction contained at least 20 peptides, which ranged in mol wt from less than 20,000 daltons to greater than 200,000 daltons. The numbered bands

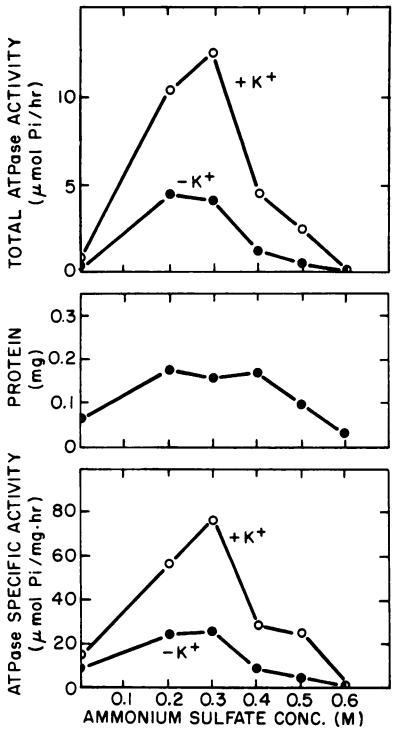

FIG. 5. Precipitation of protein and ATPase activity from the octyl-glucoside supernatant as a function of ammonium sulfate concentration. Aliquots (0.5 mg protein) of the octyl-glucoside supernatant were adjusted to the indicated concentrations of ammonium sulfate in 1.0 ml suspension buffer, incubated on ice for 30 min and centrifuged at 100,000*g* for 1 h. The pellets were resuspended in 1 ml suspension buffer and assayed for ATPase activity at pH 6.5 with 50 mM KCl (when added). Upper: ATPase activity of ammonium sulfate pellets as a function of ammonium sulfate concentration. Middle: protein recovered in ammonium sulfate pellets as a function of ammonium sulfate concentration. Lower: specific activity of ammonium sulfate pellets as a function of ammonium sulfate concentration.

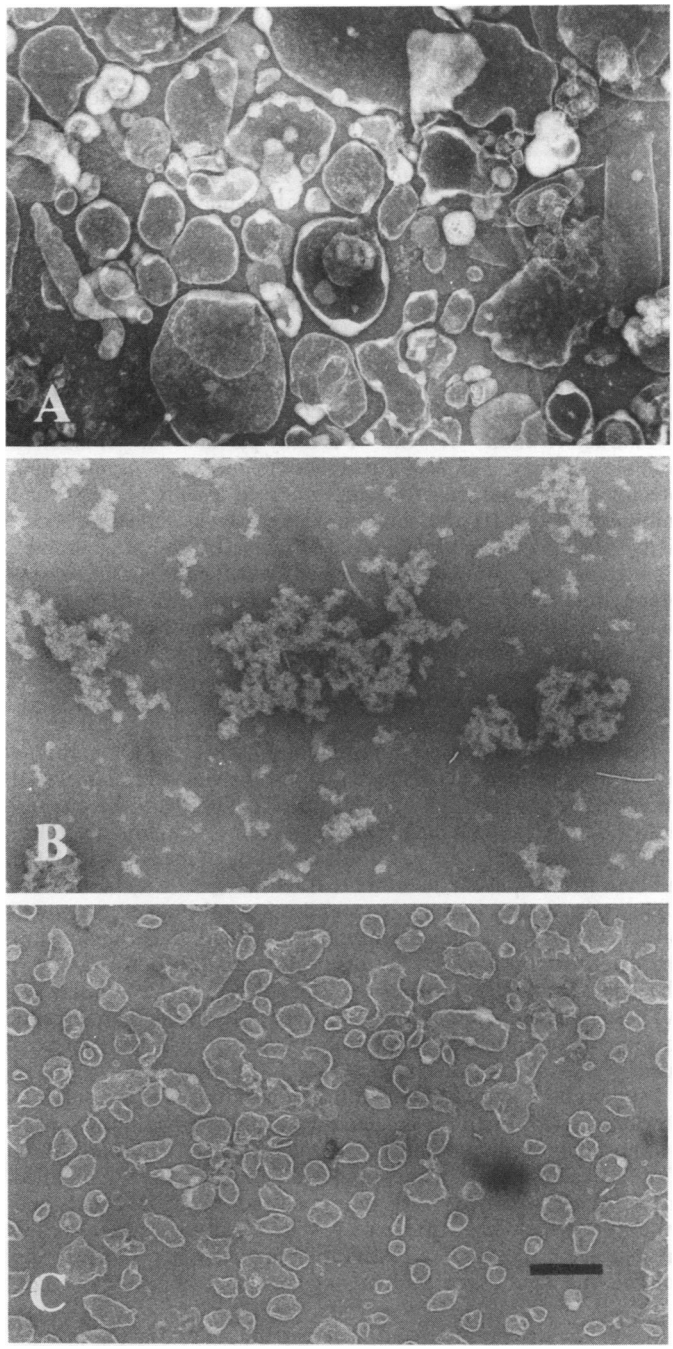

FIG. 6. Electron micrographs of negative stained plasma membrane fraction, octyl-glucoside supernatant, and ammonium sulfate precipitate from corn roots. The plasma membrane fraction, octyl-glucoside supernatant and an 0.35 M ammonium sulfate precipitate were prepared as described. ATPase activity of the ammonium sulfate precipitate was ($-K^+$) 14.7 and ($+K^+$) 37.0 μmol Pi/mg·h. Samples were spread on Formvar grids, stained with a pH 7.0 solution of 1% phosphotungstic acid and viewed in a Phillips 300 electron microscope. Bar represents 0.2 μm. A: plasma membrane fraction; B: octyl-glucoside supernatant; C: ammonium sulfate precipitate.

(Fig. 7) were consistently observed in gels of plasma membrane fractions from corn roots, and a similar banding pattern was observed for plasma membrane fractions from oat roots (not shown). The protein composition of the octyl-glucoside supernatant was similar to that of the plasma membrane fraction (Fig. 7),

suggesting that the solubilized proteins were a representative sample of the original membrane proteins. The protein composition of the ammonium sulfate precipitate (Fig. 7) was clearly different. Bands of approximately 150,000, 100,000, and 20,000 daltons were enriched, while other bands were reduced or eliminated. It was not determined which bands represented the K^+-stimulated ATPase or subunits of the ATPase.

Attempts were made to detect a labile phosphorylated intermediate of the ATPase by incubating the plasma membrane fraction with γ-[^{32}P]ATP, as been done for the Na^+, K^+-ATPase (8), and the Ca^{2+}-ATPase (22). However, ^{32}P formed what appeared to be covalent bonds with proteins in the area of bands 6, 15, and 18–19 (not shown). This labeling resembled the activity of protein kinases (1, 33). It would be difficult to detect a labile intermediate of a transport ATPase in the presence of this apparent protein kinase activity.

Characterization of the Partially-purified K^+-stimulated ATPase Activity. The ATPase activity of the ammonium sulfate precipitate was characterized with respect to sensitivity to ions and inhibitors (Table III), pH optimum (not shown), substrate specificity (not shown), and kinetics of hydrolysis of ATP (not shown). The results were similar to published data for the plasma membrane fraction (12, 20). As shown in Table III, the partially purified ATPase requires Mg^{2+}, is further stimulated by K^+, is insensitive to oligomycin, and is strongly inhibited by DES.

DISCUSSION

Development of a procedure for solubilization of a membrane bound enzyme in an active state is still somewhat a matter of trial and error despite a rapidly growing understanding of the interaction of detergents with membranes and membrane proteins (6, 15, 24). To develop a procedure for solubilization of the K^+-stimulated ATPase from a plasma membrane fraction of corn roots, first the ability of a variety of detergents and other substances to remove proteins from the plasma membrane was tested, then the effect of the detergents on the activity of the K^+-stimulated ATPase was checked, and finally the ability of the detergents to solubilize the ATPase was compared. The alternative strategy, use of detergents to prepare an enriched, membrane bound ATPase, was not pursued.

Membrane fractions obtained from plant tissues may contain a number of ATP hydrolyzing activities, some of which may simply be phosphatase contaminants from the cytoplasm or vacuole (16). The K^+-ATPase was considered to be solubilized only if it retained the substrate specificity, pH optimum, and response to ions and inhibitors which have previously been used to distinguish the enzyme from the other ATP-hydrolyzing activities found in the soluble and membrane fractions of corn root homogenates (20, 21).

All of the detergents tested were capable of extracting protein from the membrane fraction, as was the chaotropic salt, KI. But only octyl-glucoside released the K^+-stimulated ATPase activity from the plasma membrane fraction. The ATPase activity which was released from the membrane by 30 mM octyl-glucoside had a pH optimum of 6.5, simple Michaelis-Menten kinetics with respect to ATP:Mg, and a substrate specificity similar to that of the membrane bound K^+-stimulated ATPase described by Leonard and Hotchkiss (12, 20).

The activity of the solubilized enzyme decayed rapidly, and most of the ATPase activity was lost when the octyl-glucoside supernatant was chromatographed on a Bio-Bel A-5m column. A portion of the remaining activity was retained on the column, with an apparent mol wt of (roughly) 500,000 daltons, and a portion emerged in the void volume (mol wt greater than 5 million daltons). This range of apparent mol wt corresponds with the appearance of the octyl-glucoside supernatant in electron micrographs (Fig. 6). A minimum mol wt of 500,000 daltons is not

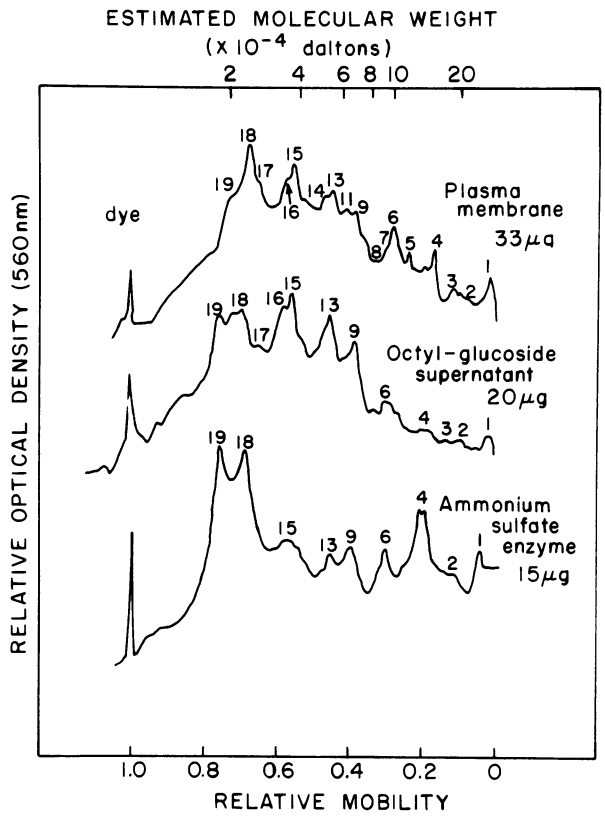

FIG. 7. SDS polyacrylamide gels of a plasma membrane fraction, octyl-glucoside supernatant and ammonium sulfate precipitate from corn roots. The plasma membrane fraction, octyl-glucoside supernatant, and an 0.35 M ammonium sulfate precipitate were prepared as described. The activity of the freshly prepared ammonium sulfate precipitate was ($-K^+$) 22.2 and ($+K^+$) 73.0 μmol Pi/mg·h. Aliquots of the plasma membrane fraction, the octyl-glucoside supernatant and the ammonium sulfate precipitate were solubilized in SDS and electrophoresed on 5.6% SDS gels with a pH 7.4 Tris-acetate buffer system (14). The plasma membrane fraction was adjusted to 4 M urea before electrophoresis. The amount of protein applied to each gel is indicated at the right of each gel scan. Dye bands (Pyronin Y) were marked with an injection of India ink before gels were stained with Coomassie blue and scanned at 560 nm with a Beckman Acta spectrophotometer. Molecular weights were estimated from a plot of log mol wt *versus* relative mobility using gels of the following protein standards (obtained from Sigma): phosphorylase A, 94 kilodaltons; catalase, 60 kilodaltons; and ovalbumin, 43 kilodaltons.

extremely high for a solubilized detergent-lipid-protein complex. The mol wt of the Lubrol WX solubilized Na^+, K^+-ATPase was estimated to be 680,000 daltons by chromatography on a Sepharose column (34); half was estimated to be enzyme protein, and half to be lipids and detergent. The K^+-stimulated ATPase may have also formed a large lipid-protein-detergent complex when solubilized in an active state by octyl-glucoside.

Ammonium sulfate precipitation is used in several procedures for purification of detergent solubilized membrane transport proteins (8, 22). The detergent is usually removed before precipitation with ammonium sulfate. In the procedure described here the octyl-glucoside is simply diluted at the same time as ammonium sulfate is added to the solubilized enzyme preparation. The subsequent formation of pelletable vesicles by the ATPase and other proteins may be an effect of lowering the detergent concentration below the CMC, under the optimal conditions of ionic strength, as in the cholate dilution procedure for reconstitution of membrane proteins described in Racker *et al.* (27).

A minimum of 20 protein bands were observed in SDS gels of

Table III. *Effect of Ions and Inhibitors on the Activity of the Partially Purified ATPase*

A 0.35 M ammonium sulfate precipitate previously frozen in suspension buffer at a protein concentration of 0.17 mg/ml, was assayed for ATPase activity at pH 6.5 with the indicated additions at the following concentrations: 3 mM Mg^{2+}, 50 mM KCl, 5.0 μg/ml oligomycin in ethanol, 0.1 mM DES in ethanol. Final concentration of ethanol was less than 1%. Activity of freshly prepared enzyme was ($-K^+$) 22.2 and ($+K^+$) 73.0 μmol Pi/mg·h.

Additions	ATPase Activity
	μmol Pi/mg·h
No ions	1.5
K^+	1.6
Mg^{2+}	7.2
Mg^{2+}, 1% Ethanol	6.1
Mg^{2+}, Oligomycin	7.2
Mg^{2+}, DES	1.0
Mg^{2+}, K^+	36.0
Mg^{2+}, K^+, Ethanol	35.7
Mg^{2+}, K^+, Oligomycin	33.8
Mg^{2+}, K^+, DES	0.4

the plasma membrane fraction, and these were reduced to a minimum of 10 protein bands in gels of the ammonium sulfate precipitate (Fig. 7). There was enrichment of several high mol wt bands of 100,000 daltons and above, and enrichment of two low mol wt bands of approximately 20,000 daltons each. It is not known which bands represent the K^+-stimulated ATPase or its subunits. Determination of its subunit composition awaits further purification of the enzyme. Chromatography on Bio-Gel A-5m suggests that the active enzyme is of a high mol wt. If the enzyme is an intrinsic membrane protein which spans the membrane in a manner similar to that of the transport proteins and transport ATPases which have been described so far (35) it might be expected to be of a high mol wt and to contain large subunits. The Na^+, K^+-ATPase (8), Ca^{2+}-ATPase (22), anion transporter of the erythrocyte plasma membrane (35), *Neurospora* ATPase (5) and a purified yeast ATPase (10) all contain subunits of approximately 100,000 daltons. It may be significant that several high mol wt bands, including one of approximately 100,000 daltons, are enriched in the ammonium sulfate precipitate.

Negative staining electron microscopy of the octyl-glucoside supernatant showed this fraction to consist of nonmembranous, amorphous particles and aggregates (Fig. 6). The small 20-nm particles might represent the approximately 500,000 dalton species retained on the Bio-Gel A-5m column and the larger aggregates, the material which emerged in the void volume. The ammonium sulfate precipitate was composed of uniform vesicles, as though the solubilized material consisted of lipid-protein complexes kept in suspension by the detergent, which reformed into lipid-protein vesicles when excess detergent was removed in the presence of dilute ammonium sulfate.

The literature contains a number of reports of the purification of an ATPase activity from the plant plasma membrane (4, 7, 32). Benson and Tipton (4) claim to have purified the K^+-stimulated ATPase from a membrane fraction from corn roots, but the enzyme described in their report had a low mol wt, poor substrate specificity and was insensitive to inhibitors of the plasma membrane ATPase. Cross *et al.* (7) report that Triton X-100 failed to solubilize the majority of the K^+-stimulated ATPase activity of a microsomal fraction from corn coleoptiles, although they report separation of a solubilized ATP-hydrolyzing activity with poor substrate specificity and low mol wt on a Bio-Gel A-1.5m column. Triton X-100, Lubrol WX and deoxycholate also did not solubilize the plasma membrane ATPase of *Neurospora* (30) and *S. pombe* (10). Finally, Tognoli *et al.* (32) suggest that the ATPase is a multisubunit complex with an ATP hydrolyzing component that is an extrinsic protein which can be removed with EDTA, trypsin, perchlorate, or deoxycholate. However, the specific activity for ATP hydrolysis reported by Tognoli *et al.* (32) was so low (less than 1 μmol/mg·h) that it is likely that the majority of the ATPase activity was inactivated by their method of preparing the membrane fraction. In each report (4, 7, 32), enzyme activity is retained after treatments that might be expected to remove lipids from a lipid-requiring protein and thus inactivate such a protein. The enzymes studied in these reports are probably nonspecific phosphatase contaminants of the membrane fraction used. The relevance of these studies to the transport ATPase of the plasma membrane should be reconsidered.

Analysis of the structure of the ATPase awaits further purification of the enzyme. However, the results presented in this paper are compatible with the hypothesis that the K^+-stimulated ATPase is an intrinsic membrane protein or protein complex which spans the membrane, is tightly associated with membrane lipids, and retains activity only when it remains associated with lipids. Proof of the involvement of the ATPase in transport of either K^+ ions or H^+ ions awaits the reconstitution of the enzyme into impermeable lipid vesicles. The ability of the ammonium sulfate enzyme to form vesicles (Fig. 6C) suggests that it will be possible to reconstitute the partially purified enzyme in the near future.

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