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Characterization of a Partially Purified Adenosine Triphosphatase from a Corn Root Plasma Membrane Fraction[1]

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ABSTRACT

The (K^+,Mg^{2+})-ATPase was partially purified from a plasma membrane fraction from corn roots (WF9 × Mol7) and stored in liquid N_2 without loss of activity. Specific activity was increased 4-fold over that of the plasma membrane fraction. ATPase activity resembled that of the plasma membrane fraction with certain alterations in cation sensitivity. The enzyme required a divalent cation for activity ($Co^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+} > Ca^{2+}$) when assayed at 3 millimolar ATP and 3 millimolar divalent cation at pH 6.3. When assayed in the presence of 3 millimolar Mg^{2+}, the enzyme was further activated by monovalent cations (K^+, NH_4^+, $Rb^+ \gg Na^+$, Cs^+, Li^+). The pH optima were 6.5 and 6.3 in the absence and presence of 50 millimolar KCl, respectively. The enzyme showed simple Michaelis-Menten kinetics for the substrate ATP-Mg, with a K_m of 1.3 millimolar in the absence and 0.7 millimolar in the presence of 50 millimolar KCl. Stimulation by K^+ approached simple Michaelis-Menten kinetics, with a K_m of approximately 4 millimolar KCl. ATPase activity was inhibited by sodium orthovanadate. Half-maximal inhibition was at 150 and 35 micromolar in the absence and presence of 50 millimolar KCl. The enzyme required the substrate ATP. The rate of hydrolysis of other substrates, except UDP, IDP, and GDP, was less than 20% of ATP hydrolysis. Nucleoside diphosphatase activity was less than 30% of ATPase activity, was not inhibited by vanadate, was not stimulated by K^+, and preferred Mn^{2+} to Mg^{2+}. The results demonstrate that the (K^+,Mg^{2+})-ATPase can be clearly distinguished from nonspecific phosphohydrolase and nucleoside diphosphatase activities of plasma membrane fractions prepared from corn roots.

A Mg^{2+}-requiring, K^+-stimulated ATPase activity is associated with microsomal membranes obtained from higher plants. There is considerable evidence that the ATPase is located on the plasma membrane (18, 24 and references therein); it appears to be an intrinsic membrane protein (9) and it is a logical candidate for an ion transport pump, although its transport capabilities have not been demonstrated. The multiplicity of ATP-hydrolyzing activities in plant tissues and membrane preparations, including plasma membrane fractions prepared from corn roots (10, 14), makes careful characterization of any proposed transport ATPase a necessity. A previous paper (9) described a method to obtain 5-fold enrichment of the K^+-stimulated ATPase activity from a plasma membrane fraction from corn roots. Here, we describe a modified procedure for obtaining milligram amounts of the partially purified enzyme and storing it without loss of activity and describe the characteristics of the partially purified enzyme.

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MATERIALS AND METHODS

Preparation of Membrane Fractions. Membrane fractions were prepared as previously described (9, 14) with the following modifications. Corn seeds (*Zea mays* L. WF9 × Mol7) were soaked in aerated tap H_2O for 1 day and germinated 5 days on damp paper towels in deep plastic trays covered with aluminum foil. Fifty g roots were excised, washed, and ground with mortar and pestle in 175 ml grinding medium consisting of 0.25 M sucrose, 3 mM EDTA, 2.5 mM DTT, 10 to 25 mg BSA, and 25 mM Tris adjusted to pH 8.0 with Mes. A 14,000- to 80,000*g* microsomal pellet (9, 14) was prepared, suspended in 3.8 ml suspension buffer consisting of 0.25 M sucrose and 1 mM DTT in 5 mM Tris-Mes (pH 6.5), stored in liquid N_2 for 1 to 4 days, or used immediately. The microsomal suspension was adjusted to 20% (w/w) sucrose, 1 mM DTT, and 5 mM Tris-Mes (pH 6.5) in a volume of approximately 10 ml, layered over 5 ml 34% (w/w) sucrose in the same buffer, and centrifuged at 80,000*g* for 1.5 h. Sucrose layers were removed by aspiration, the plasma membrane pellet (9) was suspended in suspension buffer, and protein concentration was determined immediately. Total protein recovered varied from 2 to 10 mg/50 g fresh weight of roots.

Partial Purification of ATPase. The ATPase was purified further using the method of DuPont and Leonard (9). The plasma membrane fraction was diluted to a final concentration of 1.5 to 2.0 mg protein/ml and 30 mM octyl-glucoside[2] (Calbiochem-Behring) in suspension buffer and then was incubated on ice for 20 min and centrifuged at 100,000*g* for 30 min. Immediately following centrifugation, the 100,000*g* detergent supernatant was diluted with an equal volume of 0.7 M ammonium sulfate in H_2O, incubated on ice for 20 min, and centrifuged at 100,000*g* for 1 h. The ammonium sulfate pellet was suspended in suspension buffer at a concentration of from 1 to 3 mg protein/ml and stored in liquid N_2 for up to 1 month without loss of activity. Results of a typical experiment are shown in Table I.

Assays. Proteins were determined using a Coomassie blue dye-binding procedure (Bio-Rad protein assay) to avoid interference from DTT and to allow quick estimates of protein concentration before solubilization. Results were comparable to those obtained with the Lowry procedure. ATPase activity was determined by the following modifications of previously described methods (9). Except where indicated, the reaction mixture consisted of 3 mM ATP (Boehringer) converted to the Tris salt, 3 mM $MgSO_4$, and 30 mM Tris-Mes (pH 6.3) plus 50 mM KCl (when added). K^+ stimulation was calculated as the difference in activity in the presence and absence of K^+. pH was determined at the reaction temperature of 38 C. The ammonium sulfate enzyme (3 to 10 μg protein) was added to the reaction mixture on ice; final volume was 0.5 ml and

[2] Abbreviations: octyl-glucoside, octyl-β-D-glucopyranoside; DES, diethylstilbestrol; NDPase, nucleoside diphosphatase.

Table I. *Partial Purification of Plasma Membrane ATPase of Corn Roots*

	ATPase Activity		Total Protein
	$-K^+$	$+K^+$	
	μmol Pi/mg·h		*mg*
Plasma membrane	18 (135)[a]	21 (158)	7.5
$(NH_4)_2SO_4$ enzyme	66 (73)	88 (97)	1.1
% total recovered	(54)	(61)	15

[a] Numbers in parentheses indicate total ATPase activity.

residual ammonium sulfate was less than 1 mM. The reaction was started by placing the tubes in a 38 C water bath and stopped by returning the tubes to ice. Assay time was 20 min, the reaction was approximately linear over that time period and substrate concentration, and less than 20% of substrate was used. For analysis of the kinetics of Mg-ATP hydrolysis (Fig. 2), assay time was reduced to 10 min to keep hydrolysis of ATP below 20%. Pi was determined as follows: 1.0 ml 0.822 M ammonium molybdate in 1.33 M HCl and 0.2 ml Fiske and Subbarow reagent was added to each tube, tubes were incubated at room temperature 35 min, and A_{660} was measured. Substitution of HCl for H_2SO_4 eliminated the unpleasant odor associated with the Fiske and Subbarow procedure (19).

RESULTS

Purification. There was approximately a 4-fold enrichment of Mg^{2+}-ATPase specific activity, measured in the presence or absence of 50 mM KCl (Table I). The experiment depicted in Table I shows recovery of about 60% of the plasma membrane ATPase activity in the ammonium sulfate pellet. Estimates of total ATPase activity recovered are affected by the ability of octyl-glucoside either to activate or inactivate the ATPase, depending on experimental conditions (9), but it is clear that the 4-fold increase in specific activity was not achieved by enrichment of a minor component of the ATPase activity of the plasma membrane fraction. The enzyme described here appears to be the principal ATP-hydrolyzing enzyme of the plasma membrane fraction.

General Characteristics. The pH optimum of the enzyme was approximately 6.5 in the presence of 3 mM $MgSO_4$ and decreased to 6.2 or 6.3 with the addition of 50 mM KCl (Fig. 1). Considerable K^+ stimulation was observed below pH 6.0. The pH curve was qualitatively similar to that reported for the plasma membrane fraction (14) but was shifted downward approximately 0.5 pH units at the higher pH values, possibly because previous reports did not consider the sensitivity of Tris buffer to temperature. The enzyme was assayed at pH 6.3 for all other experiments.

ATP was the preferred substrate (Table II). Hydrolysis of ADP, CDP, AMP, and *p*-nitrophenyl phosphate was less than 10%, hydrolysis of other nucleoside triphosphates was less than 20%, and hydrolysis of GDP, UDP, and IDP was less than 30% of the rate of ATP hydrolysis. The activity of the ammonium sulfate enzyme showed good specificity for ATP in the presence or absence of K^+, whereas only the K^+-stimulated activity of the plasma membrane fraction was clearly specific for ATP (14).

Divalent cation specificity was similar but not identical to that reported for the plasma membrane fraction. The order of stimulation was $Co^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+} > Ca^{2+}$ for the ammonium sulfate enzyme (Table III) and $Mg^{2+} > Mn^{2+} > Co^{2+}$ for the plasma membrane fraction (14). There was little difference between the activity of the sulfate and chloride salts. Presence of a divalent cation (Co^{2+}, Mg^{2+}, or Mn^{2+}) was required for additional stimulation by a monovalent cation (Table III). The monovalent cations fell into two distinct groups, with good stimulation by K^+, NH_4^+, and Rb^+ and lesser stimulation by Na^+, Cs^+, and Li^+ (Table IV).

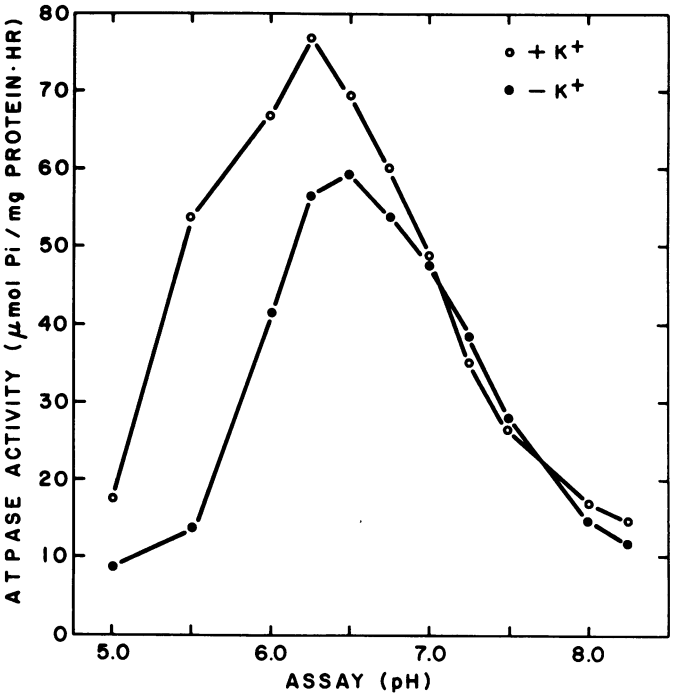

FIG. 1. Activity of the partially purified ATPase as a function of pH. The assay medium contained 3 mM ATP, 3 mM $MgSO_4$, 30 mM Tris titrated with 30 mM Mes to achieve pH, and 50 mM KCl (when added). pH was measured at 38 C.

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

Assay contained 3 mM substrate (sodium salts), 3 mM $MgSO_4$, 30 mM Tris-Mes (pH 6.3), and 50 mM KCl.

Substrate	Activity	
	μmol Pi/mg·h	%
ATP	57.4	100
CTP	5.8	10
GTP	9.7	17
ITP	7.6	13
UTP	9.4	16
ADP	4.7	8
CDP	2.5	4
GDP	16.2	28
IDP	15.2	26
UDP	14.4	25
AMP	1.9	3
PNP[a]	2.8	5

[a] PNP, *p*-nitrophenyl phosphate.

Kinetics. The ammonium sulfate enzyme showed simple Michaelis-Menten kinetics for hydrolysis of the substrate ATP-Mg (Fig. 2). The apparent K_m for ATP-Mg was 1.3 mM in the absence of K^+ and 0.7 mM in the presence of 50 mM KCl, nearly identical to the values reported for the plasma membrane fraction (14) and similar to the estimated ATP concentration of the cytoplasm of beet root cells (20). Hill plots of the data give a slope of 1.0 in the presence or absence of K^+ (not shown).

K^+ stimulation approached simple Michaelis-Menten kinetics (Fig. 3) with a K_m of 4 mM and saturation of K^+ stimulation above 30 mM KCl. A Hill plot of the data (not shown) gave a slope of 1.2. These results differ from those reported for the plasma membrane fraction, where K^+ stimulation gave negative cooperative kinetics resembling the kinetics of ^{86}Rb transport into roots (10, 14). The effect of monovalent cations on ATPase activity was

Table III. *Effect of Divalent Cations on Activity of Partially Purified ATPase*

Assay contained 3 mM Tris ATP, 30 mM Tris-Mes (pH 6.3), 50 mM KCl (when added).

Additions	ATPase Activity		
	$-K^+$	$+K^+$	K^+ stimulation
	μmole Pi/mg·h		
None	1 6	1.7	0.1
$CoSO_4$	43.2	68.9	25.7
$MgSO_4$	32.5	53.1	20.6
$MnSO_4$	32.5	44.9	12.4
$ZnSO_4$	9.5	10.5	1.0
$CaSO_4$	3.8	3.8	0
$CoCl_2$	30.4	68.7	38.3
$MgCl_2$	27.3	51.8	24.5
$MnCl_2$	30.9	41.5	10.6
$ZnCl_2$	7.8	8.8	1.0
$CaCl_2$	3.3	4.8	1.6

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

Assay contained 3 mM Tris-ATP, 3 mM $MgSO_4$, 30 mM Tris-Mes (pH 6.3), and 50 mM chloride salts of the indicated monovalent cations.

Additions	ATPase Activity	Ion Stimulation	
	μmol Pi/mg·h		%
None	63.5		
KCl	86.4	22.9	100
NH_4Cl	85.3	21.9	96
RbCl	84.5	21.0	92
LiCl	72.7	9.3	41
CsCl	71.1	7.7	34
NaCl	71.0	7.5	33

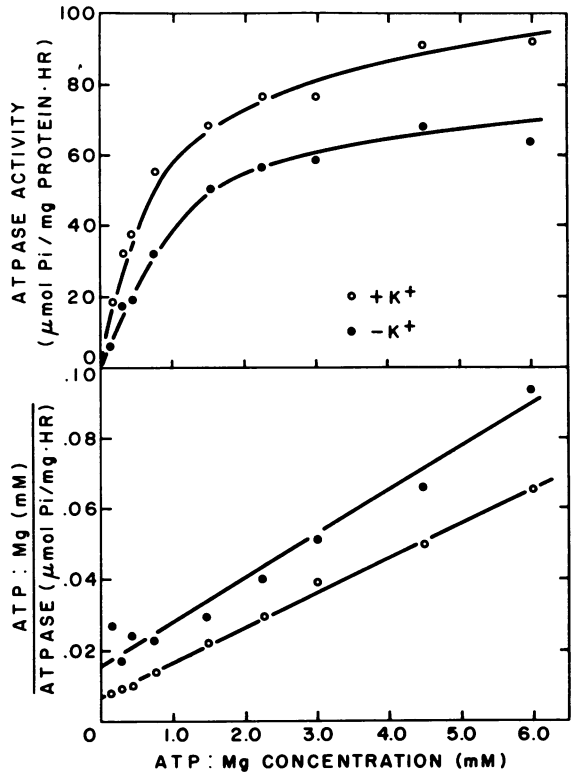

FIG. 2. Top, ATPase activity as a function of increasing ATP-Mg concentration. The enzyme was assayed at pH 6.3 with 50 mM KCl (when added) and equal concentrations of ATP and Mg^{2+} as indicated. Bottom, Woolf plot. Kinetic constants were calculated by linear regression analysis. K_m was 1.3 mM and V_{max} was 81 μmol Pi/mg·h in the absence of K^+. K_m was 0.7 mM and V_{max} was 103 μmol Pi/mg·h in the presence of 50 mM KCl.

examined carefully because similarities between the transport of monovalent cations into roots and stimulation of the ATPase by monovalent cations support the proposal that the enzyme is a cation pump (10, 14, 22). Preliminary results suggested that K^+ stimulation was enhanced and basal Mg^{2+}-ATPase activity was reduced during purification of the ATPase (9). The results here show about a 4-fold enrichment of both the activity in the presence of K^+ and that with Mg^{2+} alone (Table I), when assayed at pH 6.3. Co-purification of the Mg^{2+}-ATPase and the (Mg^{2+},K^+)-stimulated activity and their similar kinetics (Fig. 2) and pH optima (Fig. 1) suggest, but do not prove, that the activities are from a single enzyme.

Inhibitors. The effect of vanadate on enzyme activity was tested, as vanadate inhibits a number of transport ATPases (3, 4, 5, 16, 25). Sodium orthovanadate also inhibited the ammonium sulfate enzyme (Fig. 4). At pH 6.3, half-maximal inhibition was at 150 μM in the presence of Mg^{2+} alone, decreased to 35 μM with the addition of 50 mM KCl, and was only 5 μM for K^+ stimulation.

Sensitivity to sodium azide and sodium vanadate have been used to distinguish between mitochondrial and plasma membrane ATPase activity of *Neurospora* (3). The mitochondrial ATPase of *Neurospora* was inhibited by azide and not vanadate while the plasma membrane ATPase was inhibited by vanadate and not azide. Azide inhibited both the intact F_1F_0 ATPase and the solubilized F_1 ATPase, whereas oligomycin inhibited only the intact mitochondrial ATPase (3). A previous report (9) showed only slight inhibition of the ATPase activity of the ammonium sulfate enzyme by 5 μg/ml oligomycin, when assayed at pH 6.5, and we concluded that there was little contamination by the mitochondrial ATPase. Sodium azide (10 mM) inhibited up to 10% of the ATPase activity when assayed at pH 6.3 (not shown), suggesting a greater contamination by mitochondrial ATPase than was initially assumed. Additional tests for mitochondrial contamination were made. Oligomycin (20 μg/ml) inhibited up to 25% of the ATPase activity and sodium azide inhibited up to 70% of the ATPase activity when assayed at pH 8.0 (not shown). The mitochondrial ATPase contributes up to 10% of the pH 6.3 ATPase activity shown here. As suggested by Bowman *et al.* (3), azide may be more useful than oligomycin for detecting mitochondrial contamination in membrane preparations.

NDPase Activity. NDPase activity is primarily associated with fractions rich in golgi vesicles (11, 15). It is also associated with plasma membrane fractions prepared from corn roots (14, 15) but not from oat roots (10) or from corn-leaf mesophyll protoplasts (18). NDPase activity was reduced, but not eliminated, by the purification procedure (Table II). The effect of ions and inhibitors on ATPase and IDPase activity was compared (Table V). Both ATP and IDP hydrolysis required a divalent cation. In the absence of K^+, ATPase activity did not discriminate between Mg^{2+} and Mn^{2+} (Table III and Table V), whereas IDPase activity preferred Mn^{2+} (Table V). Only ATPase activity was stimulated by K^+ and inhibited by 0.5 mM vanadate, and ATPase activity was more sensitive than IDPase activity to 0.1 mM DES and to 0.2% cholate. Both IDPase and ATPase activities (not shown) were greatly inhibited by 1% cholate. The NDPase activity resembled that of onion root tips, which was preferentially stimulated by Mn^{2+}, slightly inhibited by K^+, and utilized the substrates GDP, IDP, and UDP (11). Reduction of NDPase activity during purification of the ATPase and differences in sensitivity to cations and inhibitors suggest that nucleoside diphosphate and ATP hydrolysis are

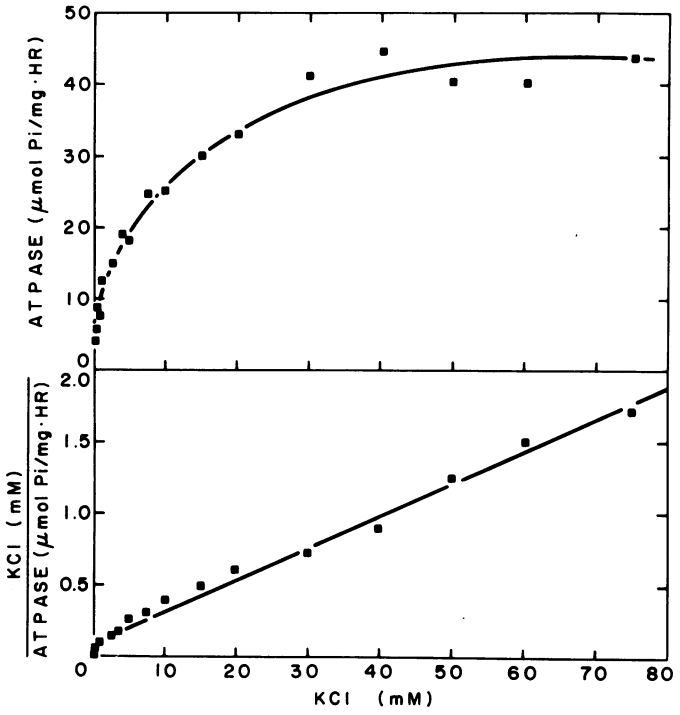

FIG. 3. Top, K^+-stimulated ATPase activity as a function of increasing K^+ concentration. The assay medium contained 3 mM ATP, 3 mM $MgSO_4$, 30 mM Tris-Mes (pH 6.3), and the indicated concentrations of KCl. Bottom, Woolf plot. Kinetic constants were calculated by linear regression analysis. K_m was 4 mM and V_{max} was 45 μmol Pi/mg·h. A Hill plot (not shown) gave a slope of 1.2.

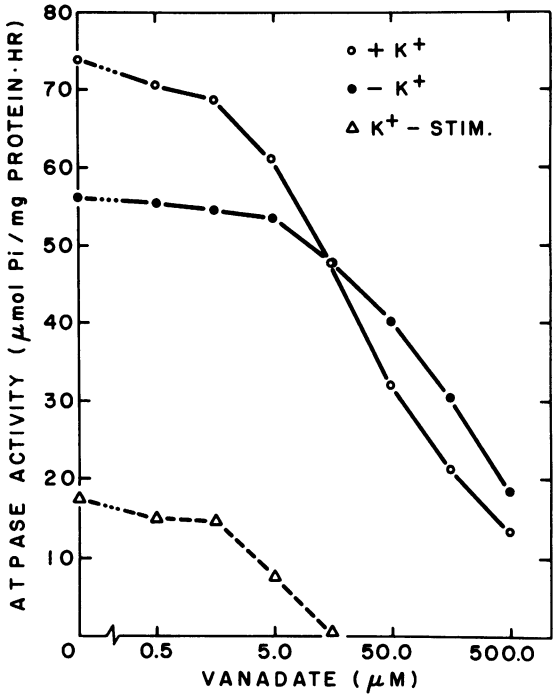

FIG. 4. ATPase activity as a function of increasing sodium orthovanadate concentration. Assayed as in Table I. The concentration of vanadate giving half-maximal inhibition was 150 μM in the absence of K^+, 34 μM in the presence of K^+, and 5 μM for K^+ stimulation.

functions of two separate enzymes.

In a previous report (9) ATPase activity of the ammonium sulfate enzyme was more sensitive to DES than is shown here; also, K^+ stimulation was greater. Differences between the results here and those previously reported (9) may reflect small changes in methods or a real difference in the ATPase activity of the younger, tray-grown roots used here and the older, aeroponics-grown roots used earlier.

DISCUSSION

Partial purification of the (Mg^{2+},K^+)-ATPase from a plasma membrane fraction from corn roots by solubilization with the detergent octyl-glucoside and precipitation with dilute ammonium sulfate increased the specific activity 4-fold (Table I). Enzyme activity remained constant for at least 1 month when the enzyme was stored in liquid N_2. Microsomal and plasma membrane fractions were stored in liquid N_2 before use without reduction of enzyme activity.

The characteristics of the ammonium sulfate enzyme were examined for comparison with the plasma membrane-bound enzyme (14) and with several published reports where solubilization and/or purification of the plasma membrane ATPase has been claimed (1, 7, 23). The characteristics of the ammonium sulfate enzyme were similar but not identical to those described for the plasma membrane-bound enzyme (14) and markedly different from those of solubilized and/or purified ATPases described in other reports (1, 7, 23) where substrate specificity was either poor, or not reported, and the enzymes were insensitive to procedures that would strip them of lipids.

The plasma membrane ATPases both from higher plants and from *Neurospora* are stimulated by monovalent cations (2, 4, 10, 14, 18, 22). The *Neurospora* ATPase is postulated to be a proton pump that is, incidentally, stimulated by K^+ (2). The results here confirm the sensitivity of the higher plant ATPase to monovalent cations but leave open the question of whether there is a relationship between K^+ stimulation of the enzyme and K^+ transport by the enzyme. K^+ lowered the K_m for hydrolysis of the substrate ATP-Mg and increased the sensitivity to vanadate. K^+, NH_4^+, and Rb^+ gave much greater stimulation than other monovalent cations (Table IV) and K^+ stimulation showed simple saturation kinetics (Fig. 3) rather than the negative cooperative kinetics found for both the plasma membrane fraction and for K^+ transport into roots (10, 14). Little is known about the sidedness of the ATPase with respect to substrate, inhibitors, and cations but, if the enzyme is involved in transport, it is likely that it is an intrinsic membrane protein, spans the membrane, and hydrolyzes ATP at the cytoplasmic side. The ammonium sulfate enzyme preparation consists of vesicles (9). If the vesicles are impermeable to ATP, then only the activity of ATPase molecules oriented in an "inside-out" manner was measured. The ATPase assay may have measured the

Table V. *Comparison of Effect of Salts and Inhibitors on Hydrolysis of Substrates ATP and IDP*

Assay contained 30 mM Tris-Mes (pH 6.3) and, where indicated, 3 mM ATP or IDP (Tris salts), 3 mM $MgSO_4$ or $MnSO_4$, 50 mM KCl, 0.5 mM Na_3VO_4, 0.1 mM DES in ethanol (final concentration of ethanol, 0.1%), and 0.2% sodium cholate.

Additions	Activity		Inhibition	
	ATP	IDP	ATP	IDP
	μmol Pi/mg·h		%	
None	3.2	3.5		
$MgSO_4$	43.6	14.4		
$MnSO_4$	44.6	21.4		
$MgSO_4$ + KCl	72.4	13.6	0	0
$MgSO_4$ + KCl + cholate	32.5	11.1	55	18
$MgSO_4$ + KCl + DES	24.7	9.2	66	32
$MgSO_4$ + KCl + Na_3VO_4	21.2	13.6	71	0

effects of monovalent cations at the cytoplasmic side of the ATPase and also at the extracellular side if the vesicles are freely permeable to cations. Differences between the pattern of cation stimulation here and that in previous reports might be explained in part by differences in permeability of the ammonium sulfate vesicles and the plasma membrane vesicles.

The ATPase was inhibited by vanadate, which also inhibits the (Na^+,K^+)-ATPase, three other plasma membrane ATPases of animal cells, the K^+-ATPase of gastric mucosa, the *Escherichia coli* K^+-ATPase, the Ca^{2+}-ATPase of sarcoplasmic reticulum, and the Mg^{2+}-ATPase of fungal plasma membranes (3–5, 16, 17, 25). All of the above are half-maximally inhibited by concentrations of vanadate less than 20 μM. K^+ increased the sensitivity of the partially purified ATPase to vanadate (Fig. 3) and only K^+ stimulation was as sensitive to vanadate as the animal and fungal ATPases. Vanadate is also reported to inhibit the ATPase activity of a microsomal fraction from radish seedlings (6). It has been suggested that vanadate inhibits transport ATPases which form phosphorylated intermediates by acting at the intracellular side of the enzyme and interfering with formation of the phosphorylated intermediate (5, 25). Its use as an inhibitor of transport is limited since vanadate must enter the cell to inhibit transport *in vivo* and, once inside the cell, it may be reduced to a noninhibitory form by compounds such as ascorbate and glutathione (17). However, it is taken up by phosphate-starved *Neurospora* and subsequently appears to inhibit the electrogenic pump (2, 12). There are several reports of *in vivo* inhibition of plant functions by vanadate that may indicate inhibition of transport (6, 13, 21). Lado *et al.* (13) report inhibition of fusicoccin-induced H^+ secretion and K^+ uptake in corn roots. Cocucci *et al.* (6) report inhibition of K^+ reabsorption and a decreased membrane potential in germinating radish seedlings. Saxe and Satter (21) report inhibition of leaf closure by *Albizzia*. Vanadate may be a useful tool for further investigations of ATPase activity and transport in higher plants.

Our results demonstrate that a distinct ATPase activity can be separated from contaminating nonspecific phosphohydrolase activities found in the plasma membrane fraction. The ATPase is highly specific for ATP, has a pH optimum near 6.3, requires a divalent cation (Co^{2+}, Mg^{2+}, or Mn^{2+}), is further stimulated by K^+, is inhibited by DES and vanadate, and is sensitive to mild detergents such as cholate. Similar characteristics were described for the plasma membrane ATPases of *Neurospora* and *Schizosaccharomyces*, putative electrogenic proton pumps that have each been purified to a single subunit (2–4, 8). There is now a need to purify the higher plant ATPase, determine its subunit composition, determine whether it forms a phosphorylated intermediate, and, most importantly, provide proof that the ATPase is involved in the transport of protons and/or K^+ ions.

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