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Potassium Stimulation of Corn Root Plasmalemma ATPase[1]

I. Hydrolytic Activity of Native Vesicles and Purified Enzyme

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ABSTRACT

Potassium stimulation of the plasmalemma (*Zea mays* L. var Mona) was studied by using a constant ionic strength to prevent indirect stimulation by the electrostatic effect of K^+ salts. The transmembrane electrochemical H^+ gradient was eliminated by using gramicidin. In these conditions, K^+ stimulation was attributable to a direct effect of the cation on plasmalemma proteins. We used both native vesicles isolated on a sucrose cushion, and solubilized and purified ATPase from phase-partitioned plasmalemma, according to the method of T. Nagao, W. Sasakawa, and T. Sugiyama ([1987] Plant Cell Physiol 28: 1181-1186). The purified enzyme had a high specific activity (15 micromoles per minute per milligram protein), but was only about 20% stimulated by K^+. In both preparations, potassium (in the range around 1 millimolar) specifically decreased two-fold the vanadate inhibition constant, and increased the maximum rate of ATP hydrolysis. In plasmalemma vesicles, the Eadie-Scatchard graph of the K^+-dependent ATPase activity as a function of K^+ concentration was linear only at constant ionic strength. The purified ATPase preparation appeared as two closely spaced bands in the 100 kilodalton region with isoelectric point about 6.5. Nevertheless, this biochemical heterogeneity seems unlikely to be related to K^+ stimulation, since K^+ modified neither the pH optimum of the activity (pH 6.5) nor the monophasic kinetics of the vanadate inhibition, in both native plasmalemma and purified enzyme preparation.

The vanadate-sensitive ATPase of plant plasma membrane is stimulated by potassium to a variable extent according to the species, the tissue, and the experimental conditions (23). Potassium stimulates the rates of ATP hydrolysis and phosphoenzyme turnover (4). This could correspond to a true activation of a potassium transport (possibly, a H^+/K^+ exchange catalyzed by ATPase), or only to a stimulation of the ATPase. Presently, no direct evidence of K^+ transport by the ATPase has been obtained. Various studies have shown that the purified ATPase is capable of ATP hydrolysis and H^+ transport even in the absence of K^+ (28). The existence of a plasmalemma ATPase which only transports H^+ is thus widely postulated (23). Nevertheless, two recent findings argue in favor of the existence of a K^+-transporting ATPase on the plasmalemma. First, an absolute potassium requirement for ATP hydrolysis has been reported for ATPase purified from corn root plasmalemma (16). Second, two phosphorylated intermediates of the beet root plasmalemma ATPase have been demonstrated, the relative amounts of which were shifted in the presence of potassium (5). These two findings are reminiscent of the properties of the two main K^+-transporting ATPase in animal cells, the (Na^+/K^+)ATPase and (H^+/K^+)ATPase (25).

On the other hand, Gallagher and Leonard (8) have reported that in SDS-PAGE of sucrose gradient plasmalemma, the ATPase appears as two closely spaced bands in the 100 kD region, even in the presence of antiproteasic agents. According to these authors, "it is likely that this region of the one-dimension gel contains several polypeptides of similar size, but from different proteins." Furthermore, it should be remembered that complex kinetics for K^+ stimulation are generally observed, as well as a shift in the pH optimum upon K^+ addition (23, 24). The absolute K^+ requirement has been observed with a purified ATPase from phase-partitioned plasmalemma (16), whereas the H^+-pumping in the absence of K^+ have been demonstrated on ATPase purified from plasmalemma isolated on sucrose gradient (28). Such considerations led to the proposal that there are two distinct ATPases, a H^+ and/or a H^+-K^+ transporting species, possibly related to the two bands observed on SDS-PAGE gel (7, 8), the phase-partitioned plasmalemma being enriched in the H^+/K^+ pump.

One difficulty in studying ATPase stimulation by K^+ is to distinguish between the so-called direct and indirect effect of the cation (4). First, indirect potassium stimulation of the proton pump may result from the dissipation of both membrane potential and pH gradient by potassium channels and H^+/K^+ carriers (15). A second indirect stimulating effect comes from a diminution of the electrostatic repulsion of the anion Mg-ATP by the negative charge of the membrane, due to the screening effect of the cation (11).

In this paper, we examine the direct effect of K^+ on ATP hydrolysis, *i.e.* in the absence of a transmembrane electrochemical H^+ gradient and of electrostatic effects. We have used both plasmalemma vesicles from corn roots isolated on a sucrose cushion, and a solubilized and purified ATPase from phase-partitioned plasmalemma, according to Nagao *et al.* (16).

MATERIALS AND METHODS

Plant Material

Corn seeds (*Zea mays* L. var Mona) were surface-sterilized for 15 min with 3% calcium hypochlorite, rinsed in running

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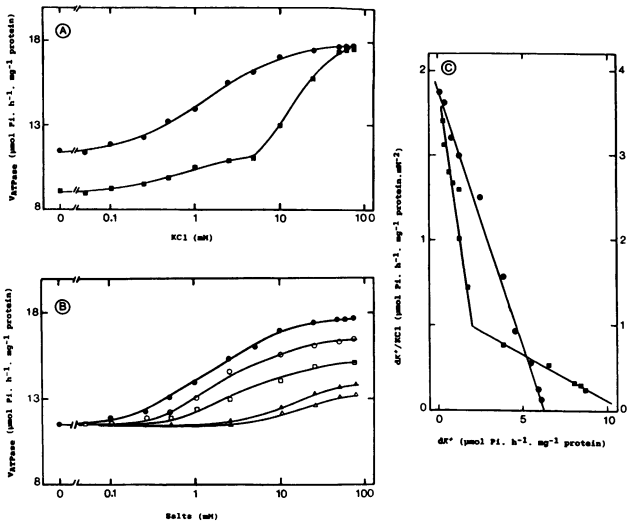

Figure 1. ATPase activity of plasmalemma vesicles as a function of cation concentration. At variable ionic strength, various KCl concentrations were added to the standard assay medium indicated in "Materials and Methods"; constant ionic strength was obtained by supplementing KCl containing medium with Tris-Cl (Tris is a univalent cation at pH 6.5) to obtain a constant ionic strength (110 mM). (A) Effect of K^+ at variable (■), or constant (●) ionic strength; (B) effect of various chloride salts at constant ionic strength (110 mM); Li^+ (△), Na^+ (▲), NH_4^+ (□), Rb^+ (○), K^+ (●); (C) Eadie-Scatchard plot of the K^+-dependent component of the activity from data in (A).

tap water, and grown hydroponically on a plastic grid over an aerated solution containing 0.1 mM $CaSO_4$ for 5 d in the dark. The solution was changed once.

Membrane Preparation

Two methods were used to prepare plasma membrane vesicles. Tightly sealed vesicles were prepared according to De Michelis and Spanswick (6) with minor modifications: BTP-Mes was replaced by BTP[2]-Cl, and KI was replaced by NaI for washing membranes in order to avoid contamination by K^+. Roots were gently chopped with razor blades and homogenized with mortar and pestle in 4 mL per g fresh weight of a grinding medium containing 25 mM BTP-Cl buffer (pH 7.6), 250 mM sucrose, 2 mM DTT, 2 mM $MgSO_4$, 2 mM ATP, 10% glycerol, 2 mM EGTA, 1 mM PMSF, and 0.5% BSA. The homogenate was filtered and centrifuged for 10 min at 13,000*g*. The supernatant was centrifuged for 30 min at 80,000*g* and the pellet was suspended in the grinding medium containing 250 mM NaI, incubated on ice for 15 min, and then sedimented for 30 min at 80,000*g*. The pellet was gently suspended in a buffer containing 2 mM BTP-Cl (pH 7.0), 250 mM sucrose, and 1 mM DTT. This suspension was then layered over a cushion of 30% sucrose (w/w) and centrifuged for 90 min at 90,000*g*. The plasmalemma vesicles passed through the sucrose layer and were pelleted. The supernatant was discarded, and the plasma membrane-enriched pellet was suspended in 2 mM BTP-Cl (pH 7.0), 250 mM sucrose, 10% glycerol, 1 mM DTT, 0.2% BSA, and stored in liquid N_2.

Phase-partitioned plasma membrane was used for ATPase purification. Phase partition was performed according to the batch procedure of Widell *et al.* (29), from a crude microsome in the 50,000*g* pellet of root homogenates (16). Roots were homogenized with a Waring Blendor for 1 min in 2 mL per g fresh weight of grinding medium containing 25 mM Tris-Mes (pH 7.8), 250 mM sucrose, 3 mM EDTA, 0.2 mM PMSF, and 2.5 mM DTT. The brei was strained through two layers of cheesecloth and the filtrate was centrifuged twice for 10 min at 8,000*g*. The supernatant was centrifuged for 30 min

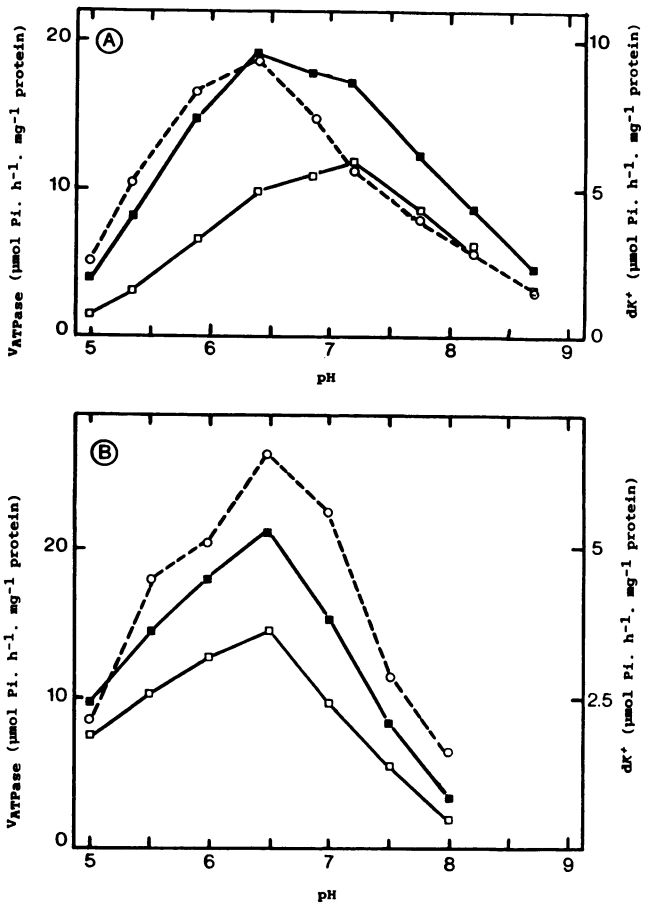

Figure 2. pH optimum of the ATPase activity of plasmalemma vesicles at variable ionic strength (A) and constant ionic strength (B). The standard assay medium indicated in "Materials and Methods" was used, except that at variable ionic strength, the pH was adjusted by varying the ratio of Tris/Mes (25 mM total concentration). ATPase activity in the presence (■) or not (□) of 50 mM KCl, and K^+-dependent component of the ATPase activity (○). In (B) total Tris concentrations were calculated as to obtain a constant ionic strength (100 mM) with the ionized $Tris^+$ species (100 mM $Tris^+$ in the absence of K^+, 50 mM $Tris^+$ in the presence of 50 mM K^+). The pK_a of Tris was taken as 7.8 at 38°C. In the absence of K^+, 75 mM HCl was first added to the Tris containing medium, and thereafter the assay pH was adjusted with Mes. In 50 mM KCl medium, only 25 mM HCl was added. Furthermore, 6 mM free Mg^{2+} was added in B to minimize the variation of the electrostatic surface charge as a function of pH (see text).

[2] Abbreviations: BTP, 1,3-bis(tris(hydroxymethyl)amino)propane; lysoPC, lysophosphatidylcholine.

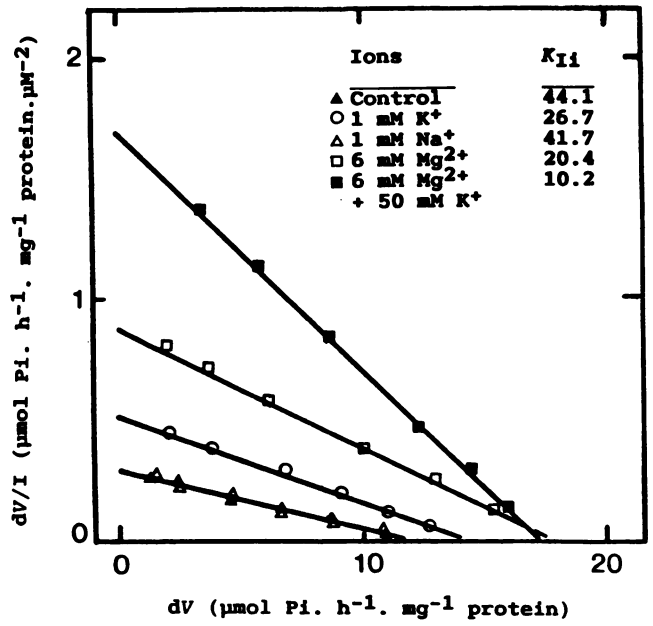

Figure 3. Eadie-Scatchard plot of the decrease (*dV*) of the ATPase activity of plasmalemma vesicles as a function of vanadate concentration. Standard assay medium was used except that the ionic strength was adjusted at 100 mM with the ionized Tris species, as indicated in the legend of Figure 2. (▲) control; (□) 6 mM free Mg^{2+}; (△) 1 mM Na^+; (○) 1 mM K^+; (■) 6 mM free Mg^{2+}, and 50 mM K^+. The values of the inhibition constant are given by the reciprocal of the slopes of the regression lines.

at 50,000*g* and the pellet once suspended in grinding medium was sedimented again for 30 min at 80,000*g*. Finally, the pellet was suspended in 15 mL of 5 mM potassium phosphate buffer (pH 7.8) containing 250 mM sucrose. Crude microsomes were purified using a two-phase system composed of 6.3% (w/w) Dextran T500, 6.3% (w/w) PEG 3350, 250 mM sucrose, and 5 mM K-phosphate buffer. The purified plasma membrane fraction was collected in the upper phase (PEG phase), diluted four-fold in 5 mM Tris-Mes buffer (pH 6.5) containing 250 mM sucrose and 1 mM DTT, and pelleted for 1 h at 80,000*g*. Pellets were suspended in 1 mM Tris-Mes (pH 6.5), 20% glycerol (v/v), and 1 mM DTT and then stored at −80 °C until use.

Solubilization and Purification of the ATPase

The phase-partitioned plasma membrane fraction (2 mg/mL) was treated with 2 mg/mL Triton X-100 for 10 min at 4°C and centrifuged for 45 min at 100,000*g*. The resulting pellet was suspended in 1 mM Tris-Mes buffer (pH 6.5) containing 20% glycerol (v/v), 1 mM DTT, and treated with lysoPC at a lysoPC-to-protein concentration ratio of 5 (mg/mg). Following incubation for 10 min at room temperature, the mixture was centrifuged at 100,000*g* for 1 h at 20°C. The supernatant (4 mL of solubilized ATPase) was applied to a linear glycerol gradient (34 mL, 25% to 50% v/v, in 1 mM Tris-Mes [pH 6.5], 1 mM DTT), and centrifuged 16 h at 150,000*g* in a vertical rotor (Beckman VAC 50) according to Serrano (23). Fractions of 1.5 mL were collected from the bottom and assayed for ATPase activity and protein. Enriched fractions (fractions 8–12) were pooled and stored at −80°C with no loss of activity for at least 2 months.

ATPase Assay

Plasmalemma ATPase activity was operationally defined as the Mg-dependent ATP hydrolysis (10). The standard incubation medium (0.5 mL final volume) contained (unless otherwise indicated) 1 mM NaN_3, 100 µM Na-molybdate, 2 µM gramicidin to dissipate pH gradient, Tris-Mes (pH 6.5) at the indicated concentrations, 3 mM Tris-ATP, and 3 mM $MgSO_4$. Additions and modifications to the ATPase reaction mixture, including various pH and concentrations of salts, vanadate are given in the figures and legends. The ATPase enrichment at the different purification steps was estimated by measuring the activity of both inside-out and right side-out vesicles in the presence of 100 µM lysoPC (13). The reaction was started by addition of 10–15 µg membrane proteins, or 0.4 µg purified ATPase and allowed to proceed for 15 min at 38°C. The released inorganic phosphate was determined by the method of Ames (1). The control was run without $MgSO_4$ and substracted from the assay in the presence of $MgSO_4$ to calculate ATPase activity. Triplicate samples were run for each assay.

SDS-Gel Electrophoresis

The purified ATPase was analysed in one-dimensional SDS-Disc PAGE performed according to Laemmli (14), except for the sample buffer which contained 2 M urea, 1% SDS, 1 mM EDTA, 50 mM DTT, 10 mM Tris-Cl (pH 6.8), and 0.2 $mg \cdot mL^{-1}$ bromophenol blue. The concentration of acrylamide in the separation gel was 10%. Two µg of the fraction 10 were diluted with one volume of twofold concentrated sample buffer, and heated for 20 min at 30°C prior to electrophoresis. Proteins were silver stained according to Oakley *et al.* (17) without the glutaraldehyde step.

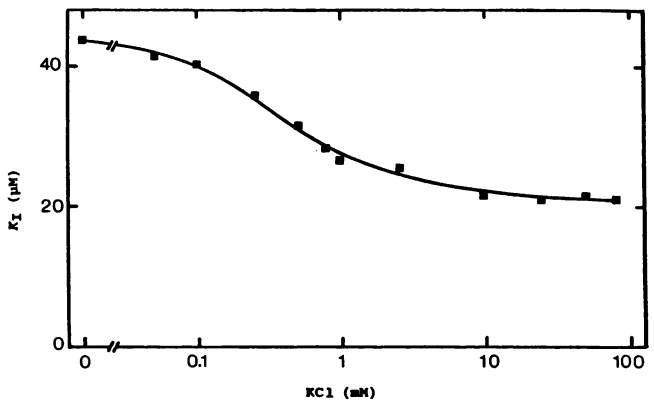

Figure 4. Effect of K^+ on the vanadate inhibition constant of ATPase activity of plasmalemma vesicles. The inhibition constant (K_I) was determined as indicated in the legend of Figure 3 (constant ionic strength).

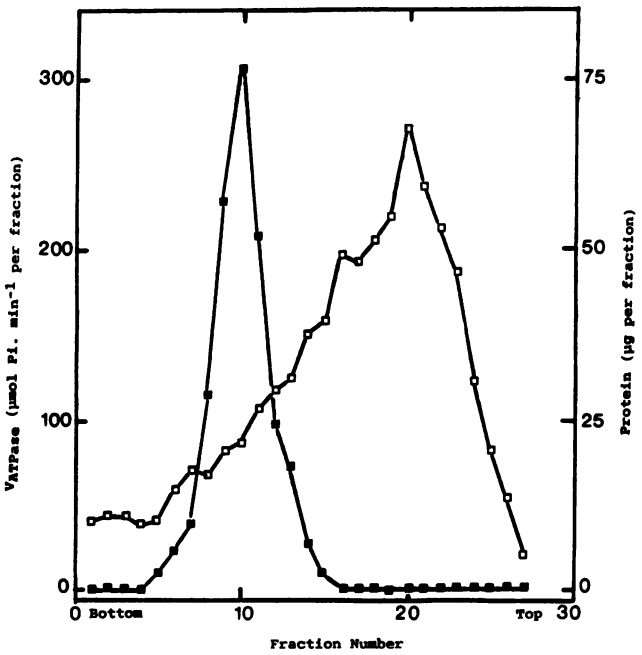

Figure 5. Distribution of ATPase and proteins in the glycerol gradient after lysoPC solubilization. ATPase activity (■) and proteins (□) were assayed as described in "Materials and Methods," except that inhibitors were omitted in the ATPase assay.

Isoelectric Focusing

The purified ATPase fraction 10 was concentrated five-fold (70 $\mu g \cdot mL^{-1}$) and solubilized in an equal volume of twofold concentrated lysis buffer giving a final concentration of 8 M urea, 5% 2-β-mercaptoethanol (v/v), 2% Nonidet P-40 (w/v), and 2% (v/v) Resolyte (pH 4–8 stock solution, BDH electran). The sample was incubated for 1 h at room temperature and insoluble material was removed by a 15 min centrifugation (Beckman Microfuge). Isoelectric focusing tube gels (2 × 14 mm) were made according to O'Farrell (19) with minor modifications. Samples containing 1.8 μg protein were loaded at the acidic end of the focusing gels. The upper (anode) buffer was 0.2% (v/v) H_2SO_4 and the lower (cathode) buffer was 0.5% (v/v) ethanolamine. After electrofocusing, the gels were placed in a SDS-equilibration buffer for 30 min. The second dimensional SDS slab gels (16 × 16 × 1.5 mm) contained 10% acrylamide and electrophoresis was performed at 30 mA according to Laemmli (14). The 2-D gels were fixed overnight in 40% methanol and 10% acetic acid. Proteins were silver stained as indicated above.

Table I. *Purification of Plasmalemma ATPase*

Activity was assayed as described in "Materials and Methods," in the presence of 50 $\mu g \cdot mL^{-1}$ lysoPC, 25 mM KCl, and 25 mM KNO_3 with native vesicles, or 50 mM KCl with purified ATPase.

Fraction	Protein	ATPase Activity	
		Total	Specific
	mg	$\mu mol \cdot min^{-1}$	$\mu mol \cdot min^{-1} \cdot mg^{-1}$
Phase partition	18.40	13.4	0.73
Triton X-100 treated	10.70	12.0	1.12
Solubilized ATPase	2.10	2.6	1.25
Glycerol gradient			
All Fractions	2.30	ND[a]	ND
Fraction 10	0.08	1.2	14.80
Fractions 8–12	0.25	2.8	11.30
			6.80[b]

[a] Not determined. [b] Assayed in the absence of lysoPC.

Protein Assay

Protein was determined according to Schaffner and Weissman (21). This method avoids interferences with the lysoPC used for ATPase solubilization and purification.

RESULTS

Ionic Strength Effects on K^+ Stimulation of the Plasmalemma ATPase

The Mg-dependent ATP hydrolysis activity of plasmalemma isolated on sucrose cushion was measured as a function of KCl concentration (Fig. 1A). In this experiment, the ionic strength varied from 15 mM to 90 mM. A first slight stimulating effect of K^+ was observed in the range around 1 mM, and a second higher one around 10 to 50 mM. When a constant ionic strength (110 mM) was maintained by replacing Tris (which is totally ionized as a cation at pH 6.5) by K^+, the stimulating effect was only observed in the range around 1 mM. The maximum activities were nearly the same in both experiments, but the activity in the absence of K^+ was higher at 110 mM ionic strength. Changes in Tris buffer concentration had no effect on the V_{max} of the hydrolysis activity. Similar results were obtained when BTP was used to adjust ionic strength (data not shown). Eadie-Scatchard plot of the K^+ component of the activity (*i.e.* the increase of the activity due to K^+ addition) was biphasic at variable ionic strength but linear at a constant one (Fig. 1C). The cationic selectivity of the stimulation was studied at a constant ionic strength (110 mM) using 10 mM chloride salts. A clear selectivity was observed, with the following sequence of decreasing efficiency to stimulate Mg-ATP hydrolysis: $K^+ > Rb^+ > NH_4^+ > Na^+ > Li^+$ (Fig. 1B). The pH optimum of the activity was studied by varying the ratio of Tris to Mes (final Tris plus Mes concentration: 25 mM), which corresponded to variable ionic strength. The pH optimum in the absence of K^+ was 7.2, and was shifted to 6.5 upon addition of 50 mM K^+ (Fig. 2A). The addition of 25 mM KNO_3 plus 25 mM KCl gave the same result, indicating that contamination by the tonoplastic ATPase could be neglected. The pH optimum of the K^+ component of the activity was also 6.5. When the pH was adjusted at a constant ionic strength, as indicated in the legend of Figure 2B, and in the presence of 6 mM free Mg^{2+}, a unique pH optimum at 6.5 was observed in the presence and in the absence of K^+.

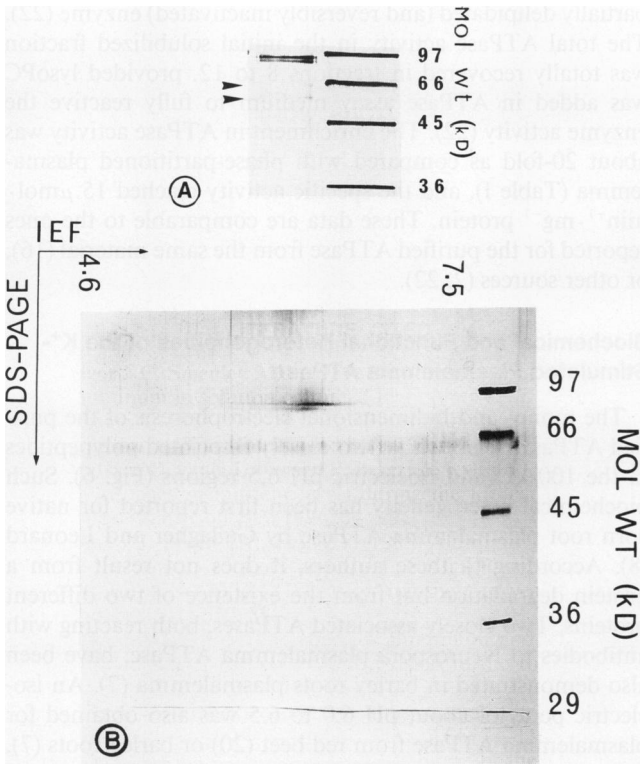

Figure 6. Gel electrophoresis of purified plasmalemma ATPase. Electrophoresis were performed as described in "Materials and Methods." (A) SDS-disc electrophoresis; (B) two-dimensional gel electrophoresis; measured pH and molecular masses are indicated at the top and left side of the tracings, respectively. Molecular mass standards (SIGMA) were: phosphorylase *b* (97.4 kD), BSA (67.6 kD), ovalbumin (43 kD), lactic dehydrogenase (36 kD), and carbonic anhydrase (29 kD).

Effect of K^+ on Vanadate Inhibition Constant of Plasmalemma ATPase

The inhibition constant (K_I) for vanadate, which uncompetitively inhibits the ATPase of corn roots plasmalemma, was determined from Eadie-Scatchard plot from the decrease of the ATPase activity (dV) at saturating ATP concentration, as a function of vanadate concentration (I) (10). When dV/I was plotted as a function of dV, the points lay on a straight line which reciprocal slope gives K_I value (Fig. 3). The plot of K_I as a function of K^+ concentration shows that the cation only acts in the range around 1 mM (Fig. 4). Comparing the effects of 50 mM chloride salts showed that the selectivity of the direct effect of univalent cations on K_I is similar to that of their effect on Mg-ATP hydrolysis. The following K_I values were obtained for Tris (control), K^+, Rb^+, NH_4^+, Na^+, and Li^+, respectively (μM): 44, 20, 23, 22, 42, and 35.

As previously reported (10), the value obtained in the presence of 6 mM free Mg^{2+} (9 mM Mg^{2+} and 3 mM ATP) is the intrinsic vanadate inhibition constant (K_{Ii}, *i.e.* in the absence of electrostatic repulsion of vanadate ion by the negative surface charge of the membrane). Approximately, a two-fold decrease of K_I was observed upon 1 mM or 50 mM K^+ addition in the presence or absence of 6 mM free Mg^{2+}, while Na^+ had no effect (Fig. 3).

Purification of the ATPase

One major peak of ATPase activity occurred at about 35% glycerol after centrifugation on a continuous gradient of the phase-partitioned, Triton X-100 washed and lysoPC solubilized plasmalemma (Fig. 5). The maximum specific activity was routinely 15 μmol $Pi \cdot min^{-1} \cdot mg^{-1}$ protein, when the ATPase assay medium was supplemented with 50 $\mu g \cdot mL^{-1}$ lysoPC (Table I). The ATPase activity of the lysoPC solubilized plasmalemma was totally recovered in fractions 8 to 12, indicating that there was no significant loss of activity during the glycerol gradient step. Only 12% of the proteins layered on the gradient were recovered in fractions 8 to 12, in agreement with the mean 10-fold increase of the specific activity. A maximum 15-fold increase was obtained in fraction 10. Similar ATPase distribution and activity in the glycerol gradient were obtained when the enzyme was purified from plasmalemma isolated on sucrose gradient (data not shown). The one-dimensional SDS-PAGE gel appeared essentially as two closely spaced bands in the 100 kD region, with slight contaminating bands of lower molecular weights (Fig. 6A). The two-dimensional gels revealed only two spots in the 100 kD region corresponding to isoelectric points of approximately 6.5 (Fig. 6B). Since the increased sensitivity of the silver stain technique results in artefactual bands (55–68 kD) in two-dimensional gels, several studies have attributed these

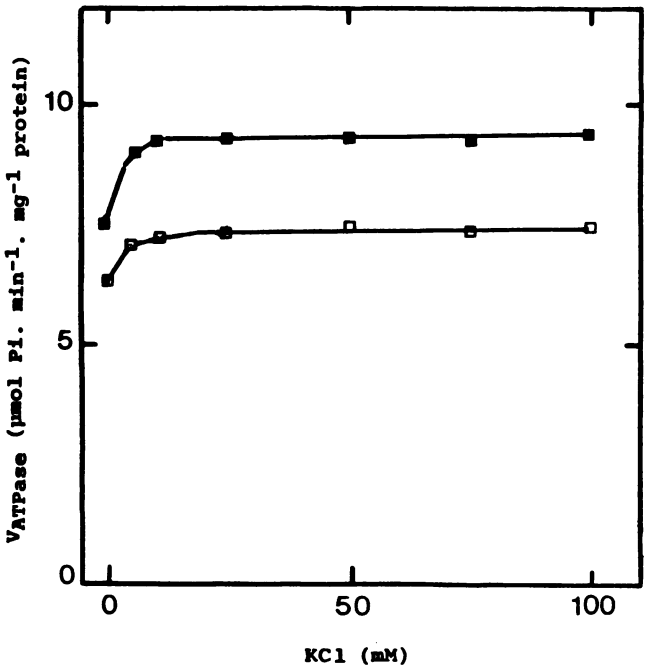

Figure 7. Effect of K^+ on the activity of the purified ATPase. The activity was assayed as described in Materials and Methods (except that inhibitors were omitted), in the absence (□), and in the presence of 50 $\mu \cdot mL^{-1}$ lyso PC (■).

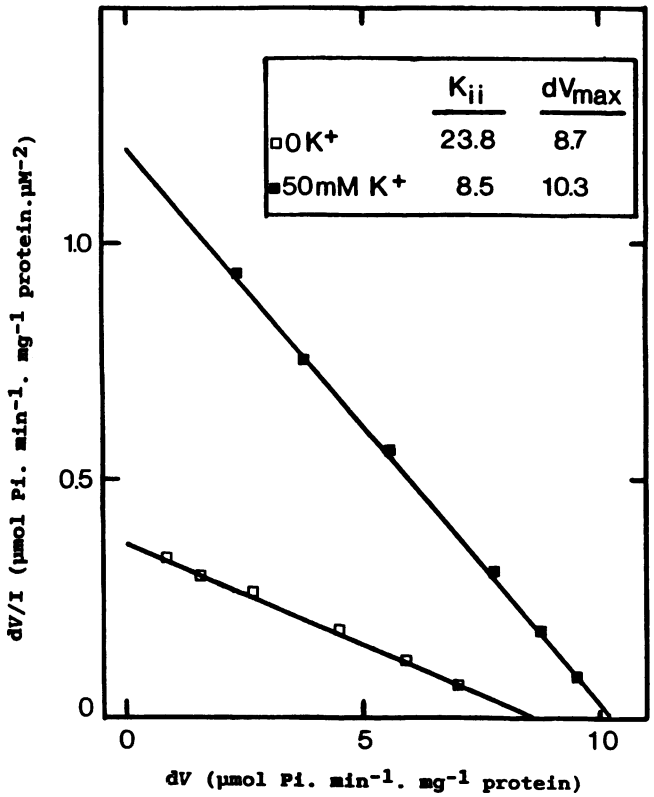

Figure 8. Effect of K^+ on the vanadate inhibition constant of ATPase activity of solubilized and purified enzyme. The intrinsic inhibition constant (K_{ii}) was determined as indicated in the legend of Figure 3, in the presence of 6 mM free Mg^{2+} with (■) and without 50 mM KCl (□).

bands to the presence of 2-mercaptoethanol in the sample (18).

Potassium Effects on the Activity and Vanadate Inhibition of the Purified Enzyme

The activity of the purified enzyme was assayed in the same conditions as those previously used by Nagao *et al.* (16). The activity remained high in the absence of K^+, and was only slightly stimulated (about 20%) by this cation in the range around 1 mM (Fig. 7). Similar results were obtained when ATPase was purified from plasmalemma isolated on sucrose gradient (data not shown). Vanadate inhibition in the presence of 6 mM free Mg^{2+} gave linear plots of dV/I as a function of dV (Fig. 8). A twofold decrease of K_{ii} was observed upon addition of 50 mM K^+.

DISCUSSION

Purification of the ATPase

After glycerol gradient centrifugation of lysoPC solubilized ATPase, the peak of purified enzyme (fractions 8–12) occurred at about 35% glycerol, as classically reported (2, 16, 22). Only 12% of the proteins layered were recovered in these high density fractions, as a result of the aggregation of the partially delipidated (and reversibly inactivated) enzyme (22). The total ATPase activity in the initial solubilized fraction was totally recovered in fractions 8 to 12, provided lysoPC was added in ATPase assay medium to fully reactive the enzyme activity (22). The enrichment in ATPase activity was about 20-fold as compared with phase-partitioned plasmalemma (Table I), and the specific activity reached 15 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. These data are comparable to the ones reported for the purified ATPase from the same material (16), or other sources (2, 22).

Biochemical and Functional Heterogeneities of the K^+-Stimulated Plasmalemma ATPase

The mono- and bidimensional electrophoresis of the purified ATPase clearly show two closely associated polypeptides in the 100 kD and isoelectric pH 6.5 regions (Fig. 6). Such biochemical heterogeneity has been first reported for native corn root plasmalemma ATPase by Gallagher and Leonard (8). According to these authors, it does not result from a protein degradation but from the existence of two different proteins. Two closely associated ATPases, both reacting with antibodies to Neurospora plasmalemma ATPase, have been also demonstrated in barley roots plasmalemma (7). An isoelectric point of about pH 6.0 to 6.5 was also obtained for plasmalemma ATPase from red beet (20) or barley roots (7), and of pH 6.5 for purified (H^+/K^+)ATPase from gastric mucosa plasma membrane (26). Our working hypothesis was to consider these polypeptides as two types of ATPase with different K^+ sensitivity (possibly at H^+-ATPase and a H^+/K^+-ATPase). In animal K^+-transporting ATPase, Mg^{2+} and K^+ enhance both vanadate binding and inhibition of the ATPase activity (3). Potassium addition also enhanced the vanadate inhibition of corn roots ATPase (Fig. 4). If the response of the vanadate inhibition of K^+ was due to only one of the two peptides, a biphasic inhibition curve should be expected. This hypothesis was ruled out, since the vanadate inhibition curves remained monophasic both in the presence and in the absence of K^+ at constant ionic strength in native and purified preparations (Figs. 3 and 8). Similar results were obtained at variable ionic strength (10).

The shift of the optimum pH toward lower pH values, upon K^+ addition at variable ionic strength (Fig. 2A), should not be related to the existence of two peptides, but to electrostatic effect of K^+ salts. As previously reported (9), increasing salt concentration diminishes the electrostatic attraction of cation H^+ by the negative surface charge of the membrane. This induces a shift of the pH in the vicinity of the membrane toward higher values, for the same pH in the bulk. A high constant ionic strength and 6 mM free Mg^{2+} (11) were used to eliminate electrostatic interactions at the membrane interface (the negative surface charges are screened and masked due to Mg^{2+}-binding). We have previously shown that the intrinsic values (*i.e.* corrected for the electrostatic repulsion of ionic substrates by the negative surface charge) of K_M for MgATP (11) and vanadate inhibition constant (12) were the same in the absence or in the presence of 6 mM free Mg^{2+}. In this condition, K^+ was no longer able to shift the optimum pH of the ATPase activity (Fig. 2B).

Similarly, the biphasic Eadie-Scatchard plot of the K^+-dependent ATPase activity at variable ionic strength (Fig. 1C) should not be related to two different sites for K^+, but to electrostatic effect of K^+ salts. Indeed, the screening effect of K^+ salts decreases the electrostatic repulsion of the anion Mg-ATP by the negative surface charge (11), and thus induces an additional and aspecific increase of the ATPase activity at about 50 mM (Fig. 1A). When the screening effect of K^+ salts was eliminated by using a constant ionic strength, K^+ stimulation of the ATPase activity was only observed in the range around 1 mM, in native plasmalemma (Fig. 1, A and C). The same result was obtained at constant ionic strength for the H^+-pumping stimulation by K^+ in both native vesicles and reconstituted vesicles with purified ATPase (12).

In conclusion, the electrophoresis experiments demonstrate a biochemical heterogeneity which seems not related to some heterogeneity of the ATPase response to pH and vanadate. Consequently, the K^+-dependent ATPase activity cannot be attributed to only one of the two polypeptides revealed in the 100 kD region. As already pointed out, the significance of this biochemical heterogeneity of plasmalemma ATPase remains unclear and deserves further experiments (7).

Potassium Stimulation of ATPase Activity of Native Plasmalemma and Purified Enzyme

In Figure 1B, K^+ stimulation cannot be related to electrostatic effects (since the ionic strength was constant) (11), nor to the relaxation by K^+ salts of an electrochemical H^+ gradient created by the pump (because gramicidin was present). In these conditions, K^+ stimulation can be attributed to a direct effect of the cation on plasmalemma proteins. Since this effect also was observed in purified ATPase preparation, it probably corresponds to a direct effect of K^+ on the enzyme. Two distinct direct effects of K^+ were revealed, namely an increase of the specific activity (Figs. 1B and 7), and a decrease of the vanadate inhibition constant (Figs. 3, 4, and 8). One major point is that we have failed, by using the isolation and purification scheme of Nagao *et al.* (16), to obtain the absolute K^+ requirement for the catalytic function of the solubilized and purified ATPase from phase-partitioned corn root plasmalemma, as reported by these authors. Furthermore, the K^+ stimulation of the specific activity decreased after purification of the solubilized enzyme (Fig. 7). The origin of this discrepancy remains unclear. No significant differences were observed either in the specific activities, or in the K^+ stimulation, when ATPase was purified from plasmalemma isolated by phase partition or by sucrose gradient centrifugation (data not shown). Generally, K^+ stimulation of the ATPase does not seem to be strongly affected by enzyme purification on glycerol gradient from crude or sucrose gradient plasmalemma from oat (22), tomato (2), and red beet (24). On the other hand, the K^+ effect on the vanadate inhibition constant is not affected by the solubilization-purification steps (Figs. 3, 4, and 8). Nevertheless, the effects of K^+ on the vanadate inhibition constant and on the specific ATPase activity are both observed in the range around 1 mM, and exhibit the same cationic selectivity ($K^+ > Rb^+ > NH_4^+ > Na^+ > Li^+$). This suggests that the same K^+ site is involved in both aspects, but with different sensitivity to the solubilization-purification step.

The biochemical and physiological significance of these K^+ effects depends greatly on the affinity and the orientation of K^+ site. In our experiments, the two direct effects of K^+ are observed in the range around 1 mM. This is markedly below the K^+ concentration in the cytoplasm, and above the concentration known to trigger *in vivo* the active K^+-H^+ exchange (27). Such an active K^+ transport requires that K^+ binds to its transport system at the extracellular face of the membrane. Thus, it would be important to determine the orientation of the ATPase site responsible for the direct effect of K^+. Study of the H^+-pumping stimulation by K^+, in both native plasmalemma and reconstituted vesicles with the purified ATPase, will give further insight into the orientation of K^+ sites, and thus into their biochemical significance. Such a study will be presented in a following paper.

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