Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6922–6926, November 1982
Botany

Partial characterization of a phosphorylated intermediate associated with the plasma membrane ATPase of corn roots

(ion transport/energy coupling/transport reaction mechanism)

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Communicated by Emanuel Epstein, August 19, 1982

ABSTRACT **The phosphorylated protein associated with a deoxycholate-extracted plasma membrane fraction from corn (*Zea mays* L. var WF9 × Mol7) roots was characterized in order to correlate its properties with those of plasma membrane ATPase. Its phosphorylation, like that of plasma membrane ATPase, was dependent on Mg^{2+}, substrate specific for ATP, insensitive to azide, oligomycin, or molybdate, and sensitive to *N,N'*-dicyclohexylcarbodiimide, diethylstilbestrol, or vanadate. Monovalent cations affected the phosphorylation of the protein in a manner consistent with their stimulatory effects on ATPase. For K^+, this was shown to occur through an increase in the turnover of the phosphoenzyme. Analysis of the phosphorylated protein by $NaDodSO_4$/polyacrylamide gel electrophoresis revealed the presence of a single labeled polypeptide with a molecular weight of about 100,000. Phosphorylation of this polypeptide was dependent on Mg^{2+}, sensitive to K^+, and inhibited by vanadate. It is concluded that this polypeptide represents the catalytic subunit of the plasma membrane ATPase. These results are discussed in terms of a model for the coupling of metabolic energy to H^+ and K^+ transport in higher plants.**

An important primary transport event in the cells of higher plants is the efflux of protons from the cytoplasm to the exterior of the cell (1). This process is believed to be mediated by an ATP phosphohydrolase (ATPase) localized on the plasma membrane (2, 3), and the electrochemical gradient of protons produced could be linked to the transport of other substances by cotransport and countertransport mechanisms (1, 4). It is unclear whether monovalent cations are transported directly by the enzyme or transported in response to the membrane potential (3). A plasma membrane ATPase from higher plants has been solubilized and partially purified (5), but complete purification has not been achieved.

Several transport ATPases from animal (6–8) and fungal (9–11) cells have been shown to form a covalent phosphorylated reaction intermediate during the course of ATP hydrolysis. For these enzymes, the formation and subsequent breakdown of the phosphorylated intermediate are thought to represent key events in the mechanism of energy coupling to ion transport with phosphorylation-induced conformational changes effecting the transport process (7, 8, 12).

We have presented preliminary evidence for a phosphorylated reaction intermediate that might be associated with the catalytic subunit of a plasma membrane ATPase from corn roots (13). In this paper, we further characterize this phosphorylated intermediate in order to compare its properties with those of plasma membrane ATPase. Based on the results of this study, a model is presented to explain ATPase-mediated coupling of metabolic energy to H^+ and K^+ transport in higher plants.

MATERIALS AND METHODS

Plant Culture. Corn (*Zea mays* L. var WF9 × Mol7) plants were grown in aeroponic tanks for 18–21 days as described (5, 13).

Isolation of Plasma Membrane Vesicles. A plasma membrane fraction was prepared according to the method of DuPont and Leonard (5) modified as described (13). Briefly, roots were homogenized at ice temperature with a precooled mortar and pestle in 250 mM sucrose/3 mM EDTA/25 mM Tris MES, pH 7.7/2.5 mM dithiothreitol. The filtered homogenate was centrifuged at 13,000 × *g* (9,000 rpm) for 15 min in a Sorval GSA rotor. The 13,000 × *g* supernatant was centrifuged at 80,000 × *g* (32,000 rpm) for 30 min in a Beckman type 35 rotor to obtain a microsomal pellet. The plasma membrane fraction was isolated from the microsomal pellet by using the discontinuous sucrose gradient of DuPont and Leonard (5). The gradient was centrifuged at 82,500 × *g* (25,000 rpm) for 2 hr in a Beckman SW 27.1 rotor. The resultant plasma membrane pellet was suspended in 250 mM sucrose/1 mM Tris MES, pH 7.2/1 mM dithiothreitol (suspension buffer), and 0.5-ml aliquots were immediately frozen and stored under liquid nitrogen. (There was no significant loss in ATPase activity for up to 5 days.)

Treatment with Deoxycholate. The plasma membrane fraction was treated with 0.1% sodium deoxycholate as described by Briskin and Leonard (13) to remove endogenous protein kinase activity. The frozen plasma membrane fraction was thawed and transferred to a 7-ml polycarbonate centrifuge tube, and the protein concentration was adjusted to 2–3 mg/ml with suspension buffer. One volume of 0.2% sodium deoxycholate/4 mM EDTA/10 mM ATP (disodium salt)/200 mM KCl/1 mM dithiothreitol/50 mM Tris·HCl, pH 7.5, was added dropwise at ice temperature with constant stirring. After incubation at ice temperature for 20 min, the preparation was centrifuged at 100,000 × *g* (41,000 rpm) for 1 hr in a Beckman type 50 rotor. The supernatant was decanted, and the 100,000 × *g* pellet was suspended in suspension buffer.

Phosphorylation. Phosphorylation was performed essentially by the method of Post and Sen (14). The assay was carried out at ice temperature in a 1.0-ml volume containing 40 μM [γ-^{32}P]ATP (45–70 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels), 40 μM $MgSO_4$, 30 mM Tris MES (pH 6.5), 2 mg of carrier bovine serum albumin (Sigma, fraction V), and 100–200 μg of membrane protein. The reactions were started by the addition of

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol.

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[γ-^{32}P]ATP and quenched at the appropriate time by the addition of 25 ml of ice-cold 10% trichloroacetic acid containing 40 mM NaH_2PO_4, 5 mM $Na_2P_2O_7$, and 1 mM ATP (disodium salt). Rapid mixing was provided by a small magnetic stirring bar. The quenched reaction mixture was centrifuged at 27,000 × g (15,000 rpm) for 15 min in a Sorval SS-34 rotor at 0°C. The supernatant was aspirated and the pellet was suspended with a chilled glass rod in 0.5 ml of the quenching solution. An additional 0.5 ml of quenching solution was added to the sample to rinse the glass rod. Aliquots (0.5 ml) were applied to two separate 0.45-μm Millipore (cellulose acetate) filters, and each was washed three times with 2.5 ml of the quenching solution. The filters were transferred to scintillation vials, and radioactivity was determined by liquid scintillation spectroscopy in 10 ml of dioxane/Cellusolve scintillation cocktail (15). In some experiments, a 0.3-ml reaction volume (mixed manually) containing the components listed above without albumin but with 25–100 μg of membrane protein was quenched with 3 ml of 10% trichloroacetic acid/40 mM NaH_2PO_4/10 mM ATP. The precipitated proteins were collected and washed (five times with 3 ml of quench solution per wash) on 0.45-μm Millipore filters.

Phosphorylated samples for $NaDodSO_4$ gel electrophoresis were prepared as described for the 1-ml reaction volume except that the samples were washed by a second centrifugation in 8 ml of 30 mM HCl instead of by Millipore filtration.

$NaDodSO_4$ Gel Electrophoresis. This was carried out by the method of Fairbanks and Avruch (16) on 5.6% acrylamide slab gels containing 0.1% $NaDodSO_4$ and buffered to pH 2.4 with 50 mM NaP_i. Membrane samples were suspended in 1.0% $NaDodSO_4$/50 mM NaP_i, pH 2.4/2% 2-mercaptoethanol/4 M urea/20% (vol/vol) glycerol containing 10 μg of pyronin Y per ml and incubated at room temperature for 10 min. Generally, 10–20 μl of sample (about 100 μg of protein) was applied per slab gel lane.

Slab gels were electrophoresed at 50 mA per gel constant current for 3–4 hr at 15°C in a Bio-Rad electrophoresis system. The tank buffer was 50 mM NaP_i, pH 2.4/0.1% $NaDodSO_4$. After electrophoresis, the gels were immediately dried onto blotter paper and subjected to autoradiography (36 hr to 2 weeks) against Kodak AR x-ray film with Cronex Lightning Plus intensifying screens at −80°C.

Protein Assay. Protein was determined by the method of Peterson (17) after a trichloroacetic acid precipitation to eliminate interference by dithiothreitol.

The data shown are for a representative experiment which was repeated one or more times.

RESULTS AND DISCUSSION

Phosphoenzyme Turnover and Substrate Specificity. Corn root plasma membranes that have been extracted with 0.1% deoxycholate contain a rapidly turning over enzyme phosphorylated intermediate possibly associated with a catalytic subunit of a transport ATPase (13). This detergent treatment was required to remove endogenous protein kinase activity that was present in the membrane fraction. Turnover was clearly demonstrated by the rapid decrease of the steady-state phosphorylation level in response to the addition of an excess of unlabeled ATP. This type of pulse–chase experiment was carried out to assess the effect of various unlabeled nucleoside phosphates on the level of steady-state phosphorylation (Table 1). ATP was the most effective nucleoside phosphate for decreasing the level of steady-state phosphorylation. This implies that the phosphorylation reaction is fairly substrate specific for ATP. This preference is a characteristic of the plasma membrane ATPase activity that has been correlated with ion transport in plants (3, 18). In addition, recent studies on electrogenic ATPase activity in sealed membrane vesicle preparations from plant cells have shown that ATP is the preferred substrate (2, 19).

Table 1. Effect of unlabeled nucleoside phosphates on steady-state level of ^{32}P-labeled phosphorylated intermediate in a deoxycholate-treated plasma membrane fraction from corn roots

Addition	Incorporation of ^{32}P	
	pmol P/mg protein	% of control
None (control)	48.9	100
ATP	8.0	16
ADP	26.5	54
IDP	53.9	110
CTP	47.2	97
GTP	37.5	77
UTP	36.0	74

Phosphorylation was assayed by using the 1-ml reaction described in *Materials and Methods*. After 20 sec, 1.5 μmol of the indicated nucleoside phosphate was added. The reaction was quenched 40 sec after the addition of the unlabeled nucleoside phosphate. ATP was added as the Tris salt; all other nucleoside phosphates were added as sodium salts.

The reduction in the phosphorylation level by ADP (Table 1) appears to be greater than can be accounted for simply by its hydrolysis as a substrate (13). This may represent the effect of an ATPase-catalyzed ATP/ADP exchange reaction during which protein-bound phosphate is lost to the resynthesis of ATP (20). The phosphorylated intermediates of a number of the transport ATPases are sensitive to ADP, and ATP/ADP exchange reactions have been demonstrated for the Na^+,K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase of animal cells (20, 21).

Effect of Various Substances on the Phosphorylation Reaction. Steady-state phosphorylation was dependent on the presence of Mg^{2+} (Table 2) with maximal activity at equimolar Mg·ATP (not shown). Among various divalent ions, Mg^{2+} was the most effective in supporting the phosphorylation reaction; Mn^{2+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, and Zn^{2+} gave 86%, 53%, 32%, 21%,

Table 2. Effect of additions on the level of steady-state phosphorylation in a 0.1% deoxycholate-treated plasma membrane fraction from corn roots

Addition	Steady-state phosphorylation, % of control
None (control)	100
No Mg^{2+}	18
KCl	19
KBr	17
KNO_3	13
K_2SO_4	20
K malate	20
Sodium azide	108
Oligomycin	98
Sodium molybdate	92
DCCD	8
DES	19
Sodium vanadate	39

Phosphorylation was assayed for 10 sec with the 0.3-ml assay; the indicated additions were present throughout the assay. All assays contained 40 μM $MgSO_4$, except as indicated. K salts were added at 50 mM except for K_2SO_4 which was at 25 mM. Final concentrations: azide, 1 mM; oligomycin, 5 μg/ml; molybdate, 1 mM; DCCD, 10 μM; DES, 100 μM; and vanadate, 100 μM. Oligomycin, DCCD, and DES were added in ethyl alcohol to a final alcohol concentration of <1%.

and 16%, respectively, of the phosphorylation level observed with Mg^{2+}. Addition of K^+ salts resulted in a lower level of steady-state phosphorylation, and this effect was independent of the accompanying anion (Table 2). Phosphorylation was maximal at about pH 6 and decreased in a linear fashion to near zero at pH 8 (not shown). Hence, like ATPase activity in the plasma membrane fraction (13), the phosphorylation reaction required Mg^{2+}, was influenced by the presence of K^+, and was maximal at slightly acidic pH.

Phosphorylation was insensitive to azide and oligomycin, inhibitors of mitochondrial ATPase. Molybdate, an inhibitor of acid phosphatase (22), had little effect on phosphorylation (Table 2). In contrast, DCCD, DES, and vanadate, inhibitors of ATPase activity in the plasma membrane fraction (18, 22, 23), were potent inhibitors of the phosphorylation reaction.

Effect of Monovalent Cations on the Steady-State Level of Phosphorylation. The effect of monovalent cations on phosphorylation was examined in greater detail because cation-stimulated ATPase activity may represent an important property of the enzyme linking it to cation transport (3, 18). The addition of certain monovalent cations (after phosphorylation reached steady-state) decreased the level of ^{32}P labeled phosphoprotein (Table 3). NH_4^+, K^+, and Rb^+ were about equally effective in this; Cs^+ was less so. Na^+ and Li^+ were ineffective. The relative order of effectiveness of the various monovalent ions in decreasing steady-state phosphorylation is similar to the relative order of effectiveness in stimulating plasma membrane ATPase activity (18, 23). Therefore, the decrease in the steady-state level of phosphorylation by monovalent cations might be accounted for by increased turnover of the phosphoenzyme intermediate. This proposal was examined in more detail for K^+ by investigating the effect of K^+ on the dephosphorylation reaction.

Dephosphorylation Reaction. The dephosphorylation reaction was specifically studied by adding an excess of unlabeled ATP at steady-state and then quenching the reactions at various times after the addition of the chase.

The time course of dephosphorylation (Fig. 1) followed a first order kinetic relationship which would be expected for the unimolecular breakdown of an enzyme reaction intermediate. The slope of the plot can be related to the first-order rate constant for the decay of the phosphoenzyme and this was found to be 0.028 sec^{-1} at 2°C.

When the dephosphorylation reaction was studied in the presence of 50 mM KCl (i.e., the chase contained both ATP and KCl), the slope of the plot increased, indicating increased turnover of the phosphoenzyme. This increase in turnover was reflected in a nearly doubling of the rate constant for dephosphorylation, to 0.051 sec^{-1}. Although the net effect of KCl on the turnover of the phosphoenzyme was much less than that observed for Na^+,K^+-ATPase (24), these results are consistent with the enzymatic properties of the corn root plasma membrane ATPase (13, 18) and suggest that this enzyme has a sub-

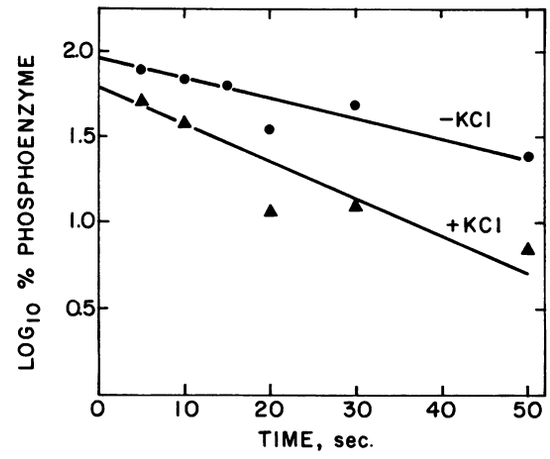

FIG. 1. Effect of K^+ on the turnover of the phosphorylated intermediate associated with a deoxycholate-treated plasma membrane fraction from corn roots. Phosphorylation was assayed by using the 1-ml reaction. After 20 sec, 1.5 μmol of ATP (Tris salt, pH 6.5) and 50 μmol of KCl (if indicated) were added; this is taken as time 0. The reaction was quenched at the times indicated thereafter. Best fit lines were calculated by linear regression, and the first-order rate constant was calculated to be $k = 2.303$ (− slope). The rate constants for dephosphorylation were 0.028 sec^{-1} and 0.051 sec^{-1} in the absence and presence of K^+, respectively.

Table 3. Effect of various monovalent cations on the level of steady-state phosphorylation in a deoxycholate-treated plasma membrane fraction from corn roots

Addition	Steady-state phosphorylation	
	pmol/mg protein	% of control
None (control)	71	100
KCl	47	66
NH_4Cl	48	68
RbCl	51	72
CsCl	62	88
LiCl	70	99
NaCl	80	113

Phosphorylation was assayed by using the 1-ml reaction. After 20 sec, 50 μmol of the indicated monovalent salt was added. The reaction was quenched 40 sec after the addition of the monovalent salt.

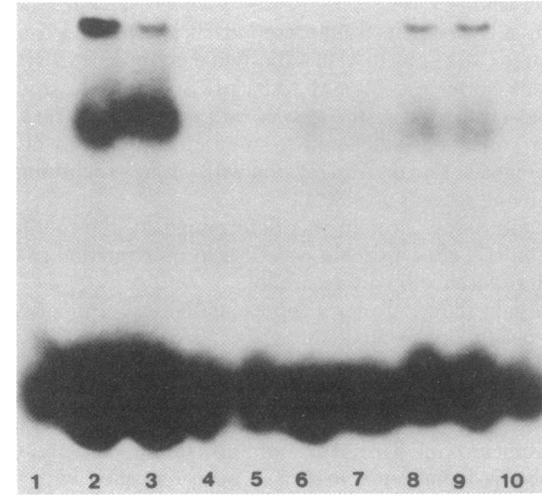

FIG. 2. Autoradiograph demonstrating various characteristics of the phosphorylated intermediate associated with a deoxycholate-treated plasma membrane fraction from corn roots. Phosphorylation was assayed by using about 723 μg of membrane protein in the 1-ml reaction. The dried gel was placed against x-ray film for 36 hr at −80°C with a Cronex Lightning Plus intensifying screen. The radioactivity that ran with the dye front (bottom of the gel) was associated with low molecular weight phosphorus compounds. The major band of radioactivity near the top of the gel corresponds to a molecular weight of about 100,000 (see figure 6 in ref. 13). Lanes: 1 and 10, sample boiled 5 min prior to a 20-sec phosphorylation assay; 2 and 3, control, 20-sec phosphorylation; 4 and 5, phosphorylated for 20 sec followed by a 40-sec chase with 1.5 μmol of unlabeled ATP; 6 and 7, phosphorylated for 20 sec followed by a 40-sec chase with 1 μmol of Tris/EDTA; 8 and 9, phosphorylated for 20 sec followed by a 40-sec chase with 50 μmol of KCl.

FIG. 3. Gel autoradiograph demonstrating vanadate sensitivity of the phosphorylated intermediate associated with a deoxycholate-treated plasma membrane fraction from corn roots. See Fig. 2 for details. Lanes: 1 and 2, control, phosphorylated for 10 sec; 3 and 4, phosphorylated for 10 sec in the presence of 500 μM Na_3VO_4.

stantial velocity in the presence of Mg^{2+} alone and that 50 mM K^+ acts to double this velocity.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. The phosphorylated polypeptide associated with corn root plasma membranes was analyzed by $NaDodSO_4$ gel electrophoresis. Electrophoresis was carried out under acidic conditions (pH 2.4) because preliminary studies indicated that the phosphorylated protein was most stable in acid (data not shown). In this respect, the phosphorylated protein resembles the phosphoenzymes of other transport ATPases, perhaps as the result of the presence of an acyl phosphate bond (6–8, 12, 21).

The autoradiograph of the gel showed that the phosphorylated intermediate appeared as a single radioactive band with a molecular weight of about 100,000 (Fig. 2). The radioactivity that migrated with the tracking dye at the base of the gel was nonenzymatic in nature because it was present when a sample was boiled (5 min) before phosphorylation. This radioactivity most likely represents residual [γ-^{32}P]ATP or [$^{32}P_i$] not removed by the washing steps during sample preparation.

The molecular weight 100,000 phosphorylated polypeptide showed rapid turnover because it was decreased by a chase with unlabeled ATP (Fig. 2). Phosphorylation of this polypeptide was Mg^{2+}-dependent because a 40-sec chase with 1 mM Tris/EDTA (pH 6.5) decreased the radioactivity in the band. This also indicated that the dephosphorylation reaction did not require free Mg^{2+} because substantial turnover occurred in the presence of the chelating agent. The plasma membrane ATPase from both oat and corn roots requires Mg^{2+} for activity, and it is believed that the true substrate for the enzyme may be the MgATP complex (3, 25). Potassium partially decreased the labeling of the band in keeping with its effects on phosphoenzyme turnover.

Phosphorylation was sensitive to vanadate present at 500 μM in the reaction solution (Fig. 3). This was not unexpected because orthovanadate inhibits enzymes that form a covalent phosphoenzyme intermediate (26), and the plasma membrane ATPase of corn roots has been shown to be sensitive to orthovanadate (22, 23). This result is similar to what has been observed for the phosphorylated intermediate associated with the plasma membrane ATPase from *Schizosaccharomyces pombe* (9).

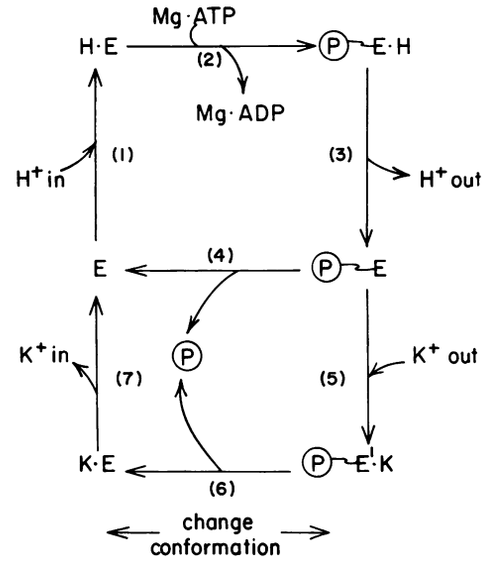

FIG. 4. Possible enzymatic sequence for a H^+,K^+-ATPase on the plasma membrane of plant cells. The reaction sequence might occur as follows: (1) H^+ binding to cytoplasmic surface of the ATPase (E); (2) phosphorylation by ATP to produce a phosphorylated intermediate (P ~ E·H) and a conformation change; (3) H^+ release to cell exterior; (4) spontaneous breakdown of the phosphoenzyme; or (5) K^+ binding with increased lability of the phosphoenzyme bond; (6) breakdown of the phosphoenzyme with bound K^+ and a return to the original conformation; (7) release of K^+ to the cell interior and regeneration of the free enzyme. The pump would be electrogenic if dephosphorylation by reaction 4 exceeded that occurring by reaction 6.

GENERAL DISCUSSION

In this study, the phosphorylated intermediate associated with detergent extracted plasma membranes from corn roots was characterized further. The results suggest that the phosphorylation observed most likely represents a reaction intermediate of the plasma membrane ATPase. Both plasma membrane ATPase and phosphorylation were Mg^{2+}-dependent and relatively substrate-specific for ATP. Furthermore, the effects of monovalent cations on phosphorylation were consistent with their action in stimulating ATPase activity. Finally, both phosphorylation and ATPase showed similar inhibitor sensitivities.

The phosphorylation occurring for the plasma membrane fraction is associated with a polypeptide of molecular weight 100,000. Presumably, this polypeptide represents the catalytic subunit of the ATPase. This result is significant because it is now known that a number of transport ATPases from animal and fungal cells are phosphorylated on polypeptides with molecular weights of about 100,000 (6–11, 21). In addition, a K^+-transport ATPase in *Escherichia coli*, the KdP ATPase, has been shown to be phosphorylated on a polypeptide subunit that has a molecular weight of 90,000 (27). Therefore, the plasma membrane ATPase of higher plants most likely represents an enzyme that is similar mechanistically and structurally to the transport enzymes associated with other organisms.

Although the plasma membrane ATPase from higher plants has not been purified and its transport capabilities have not been demonstrated *in vitro*, much indirect evidence suggests that it functions in the coupling of metabolic energy directly to H^+ transport and indirectly, through a protonmotive force, to the transport of other solutes. Cellular ATP levels have been correlated with the rates of ion uptake (28), and recent studies with isolated membrane vesicles have demonstrated ATP-dependent, electrogenic proton transport (2, 19). Similarities between the kinetics of K^+ stimulation of ATPase and K^+ uptake

into roots (18) and an analysis of K^+ uptake data with respect to membrane potential (29) are consistent with a direct role for this enzyme in driving K^+ transport.

On the basis of the results of this study and of what is currently known about the transport ATPases of other organisms, we propose a simple model to explain the coupling of metabolic energy to H^+ and K^+ transport in higher plants (Fig. 4). Central to this model is the formation and breakdown of a covalent phosphorylated intermediate. The resultant protein conformational changes are visualized as effecting the transport process in a manner similar to that believed to occur for enzymes such as Na^+,K^+-ATPase (12) and sarcoplasmic reticulum Ca^{2+}-ATPase (21). H^+ transport may occur with phosphorylation of the enzyme while K^+ transport may occur in the dephosphorylation reaction. The reaction scheme allows the enzyme to act both as a primary H^+ pump (with Mg^{2+}-ATPase) and as a H^+/K^+ exchange pump (with Mg^{2+},K^+-ATPase) by allowing dephosphorylation to occur in the absence of K^+ binding (reaction 4). The pump can act in an electrogenic manner if dephosphorylation in the absence of K^+ binding exceeds that induced by K^+. This concept of a transport ATPase with multiple modes of action with regard to H^+ and K^+ transport is similar to that proposed by Mercier and Poole (29) for transport in red beet.

We thank Dr. R. L. Post and M. O. Walderhaug for valuable discussions during portions of this research. This research was supported by Grant PCM 8025004 from the National Science Foundation awarded to R.T.L.

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