Phytochemistry, Vol. 24, No. 12, pp. 2797–2802, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

PLASMA MEMBRANE ATPase OF SUGARBEET

DONALD P. BRISKIN*† and W. ROBERT THORNLEY*

*Plant Biochemistry and Bioregulation Laboratory, United States Department of Agriculture, Agricultural Research Service and †Department of Chemistry and Biochemistry, Utah State University, UMC 63, Logan, UT 84322, U.S.A.

(*Revised received* 28 *May* 1985)

Key Word Index—*Beta vulgaris*; Chenopodiaceae; sugarbeet; storage taproot; plasma membrane ATPase; phosphoenzyme.

Abstract—A membrane fraction enriched with magnesium-dependent ATPase activity was isolated from sugarbeet (*Beta vulgaris* L.) taproot by a combination of differential centrifugation, extraction with KI and sucrose density gradient centrifugation. This activity was inhibited by vanadate, *N*,*N*′-dicyclohexylcarbodiimide and diethylstilbestrol, but was insensitive to molybdate, azide, oligomycin, ouabain, and nitrate, suggesting enrichment in plasma membrane ATPase. The enzyme was substrate specific for ATP, had a pH optimum of 7.0, but showed little stimulation by 50 mM KCl. The sugarbeet ATPase preparation contained endogenous protein kinase activity which could be reduced by extraction of the membranes with 0.1% (w/v) sodium deoxycholate. Reduction of protein kinase activity allowed the demonstration of a rapidly turning over phosphorylated intermediate on a M_r 105000 polypeptide, most likely representing the catalytic subunit of the ATPase. Phosphorylation was magnesium dependent, sensitive to diethylstilbestrol and vanadate but insensitive to oligomycin and azide. Neither the ATPase activity nor phosphoenzyme level were affected by combinations of sodium and potassium in the assay. These results argue against the presence of a synergistically stimulated NaK-ATPase at the plasma membrane of sugarbeet.

INTRODUCTION

Plasma membrane associated ATP phosphohydrolase (ATPase) activity that is Mg^{2+}-dependent, stimulated by K^+ and inhibited by vanadate has been commonly found in membrane fractions isolated from glycophytic plants [1–3 and refs therein]. It is widely believed that this enzyme may be responsible for driving transport across the plasma membrane through the production of an inwardly directed H^+-gradient [1, 3, 4]. In contrast, ATPase activity that is synergistically stimulated by combinations of Na^+ and K^+ has been reported to occur in membrane fractions isolated from mangrove [5], *Halocnemum strobilaceum* [6] and sugarbeet [7 and refs therein] which are halophytic plants. In the case of sugarbeet, several kinetic studies by Lindberg and co-workers [7–12] have suggested that the ATPase associated with sugarbeet membranes may have many similarities to the Na^+K^+-ATPase present in animal cells ([13] for review). Although the membrane preparations used in these studies were fairly crude (12000–25000 *g* pellet from homogenate), it has been proposed that this Na^+K^+-ATPase is associated with the plasma membrane of sugarbeet and is responsible for ATP-dependent, Na^+-efflux coupled to K^+ influx [7, 10]. This Na^+ extrusion process would presumably play an important role in reducing the cytoplasmic Na^+ concentration and promote survival under conditions of salt stress [10, 14].

In this communication, the properties of ATPase activity associated with sugarbeet membrane fractions purified to a greater degree than previous studies [7–12] were examined. The observed differential sensitivity to phosphohydrolase inhibitors and substrate specificity for ATP support the proposal that the fractions are enriched with plasma membrane ATPase. The response of the enzyme to monovalent cations, however, strongly questions the proposal for a Na^+K^+-ATPase at the plasma membrane of sugarbeet taproot.

RESULTS AND DISCUSSION

Distribution of inhibitor sensitive ATPase activity

Previous studies on red beet by Briskin and Poole [15] demonstrated the feasibility of using plant storage tissue for the large scale isolation of plasma membrane enriched fractions. With minor modifications (see Experimental), this methodology could be used with the storage tissue of sugarbeet. All of the fractions examined during the isolation of a plasma membrane-enriched fraction from sugarbeet contained substantial levels of ATP hydrolytic activity (Table 1). Since many different types of enzymes are capable of hydrolysing ATP, it was useful to examine the effects of phosphohydrolase inhibitors on the activity in each sub-fraction. The data shown in Table 1 represent the difference in activity observed in the absence and presence of each inhibitor (inhibitor-sensitive ATPase). It is apparent that the proportion of the control ATPase activity that is inhibited by vanadate is increased during the fractionation procedure. Although vanadate sensitivity is characteristic of the plant plasma membrane ATPase [1, 3, 16, 17], this compound can also inhibit non-specific phosphatase activity [16]. To test for the presence of non-specific phosphatase in each sub-fraction, the sensitivity to molybdate, an inhibitor of phosphatase [16 and refs therein], was examined. Molybdate-sensitive phosphohydrolase activity was progressively decreased during the fractionation procedure, indicating that the vanadate sensitive component of the control ATPase

Table 1. Distribution of inhibitor-sensitive ATPase and protein during the isolation of a plasma membrane fraction from sugarbeet

	Fraction			
	Homogenate	Microsomal	KI-extracted	Plasma membrane
ATPase activity (μmol Pi/hr·mg)				
Control	39.7 (100)	27.8 (100)	32.8 (100)	47.4 (100)
ΔNa_3VO_4	25.2 (64.5)	19.8 (71.2)	24.3 (74.1)	43.3 (91.4)
ΔKNO_3	4.1 (10.3)	4.7 (16.9)	0 (0)	0.7 (1.5)
ΔNaN_3	4.9 (12.3)	2.7 (9.7)	0.2 (0.6)	0 (0)
ΔNa_2MoO_4	22.9 (57.7)	14.9 (53.6)	5.2 (15.9)	3.2 (6.8)
Protein (mg/fraction)	354.0	36.3	29.5	12.8

Control ATPase assay contained 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl, 30 mM Tris–Mes pH 7.0 and 20 to 40 μg protein. Inhibitor-sensitive components of ATPase activity represent the difference between the control activity and the activity in the presence of 50 μM Na_3VO_4 (ΔNa_3VO_4) or 50 mM KNO_3 (ΔKNO_3) or 10 mM NaN_3 (ΔNaN_3) or 500 μM Na_2MoO_4 (ΔNa_2MoO_4). Values in parentheses represent the percent of the control activity.

cannot be accounted for by the presence of non-specific phosphatase activity. Both nitrate sensitivity, a putative marker for tonoplast ATPase [17] and azide sensitivity, a marker for mitochondrial F_1-ATPase [16 and refs therein] were also decreased to barely measurable levels with each step in the isolation procedure (Table 1).

Taken together, these results demonstrate that the majority of the ATP hydrolytic activity associated with the final gradient purified membrane fraction is inhibited by vanadate yet insensitive to molybdate, azide, and nitrate. This suggests the enrichment of plasma membrane ATPase. In contrast the homogenate and microsomal fractions, presumably similar to the fractions used in previous studies, contained ATP hydrolytic activity that was sensitive to both vanadate and molybdate. This would indicate that these fractions contain substantial levels of non-specific phosphatase activity [16]. As in studies with red beet, membrane associated non-specific phosphatase activity could be reduced by extraction with KI prior to sucrose gradient centrifugation [15].

Table 2. Substrate specificity for the sugarbeet ATPase

Substrate	ATPase activity (μmol Pi/hr·mg)	%
ATP	35.8	100.0
ADP	4.9	13.7
AMP	1.4	3.9
CTP	4.1	11.5
ITP	6.8	18.9
GTP	5.7	15.9
UDP	8.5	23.7
IDP	7.2	20.7
p-Nitrophenyl-P	3.9	10.9

ATPase activity was assayed in the presence of 3 mM substrate, 3 mM $MgSO_4$, 50 mM KCl, and 30 mM Tris–Mes pH 7.

General characteristics of the sugarbeet ATPase

When the phosphohydrolase activity associated with gradient purified membranes was examined with various phosphate compounds, ATP was found to be the preferred substrate for hydrolysis (Table 2). The best alternative substrates were UDP and IDP, which may reflect some contamination of golgi membrane nucleoside diphosphatase activity [18]. Substrate specificity for ATP is a characteristic property of the plasma membrane ATPase of higher plant cells [1–3, 15, 17].

The pH optimum for ATP hydrolysis by the sugarbeet ATPase was 7.0 and the presence of 50 mM KCl did not greatly affect the pH optimum or the level of the activity (Fig. 1A). Only a slight stimulation of the activity by KCl was observed below pH 6.5. This clearly differs from what has generally been observed for the plant plasma membrane ATPase where 50 mM KCl causes a shift in the pH optimum of ATPase from 7.0 to 6.5 and a substantial stimulation of the activity [1, 3]. The reason for this difference is unclear and very interesting since the plasma membrane ATPase isolated from red beet, another variety of the same species, did show the expected response to K^+ [15]. The lack of significant K^+-stimulation in this enzyme preparation is also important in terms of the proposed role that this enzyme might have in ion transport. By analogy to transport enzymes, such as the animal cell Na, K-ATPase and gastric H, K-ATPase, it has been suggested that K^+-stimulation could reflect direct K^+-transport by the plant ATPase (see refs [1] and [3] for further discussion). Therefore, the lack of a response to K^+ may question this proposal. In a recent report by Yoshida *et al.* [19], where the plasma membrane ATPase from orchard grass was studied, very little response to K^+ was observed. Therefore the pH optimum and response to K^+ may vary among species (or even within species), and this may become more apparent as more plant species are investigated.

The activity optimum at pH 7.0 does, however, represent the peak of ATPase activity which is sensitive to Na_3VO_4 (Fig. 1B). A minor peak of molybdate sensitive phosphohydrolase activity occurred at pH 5.5 which may

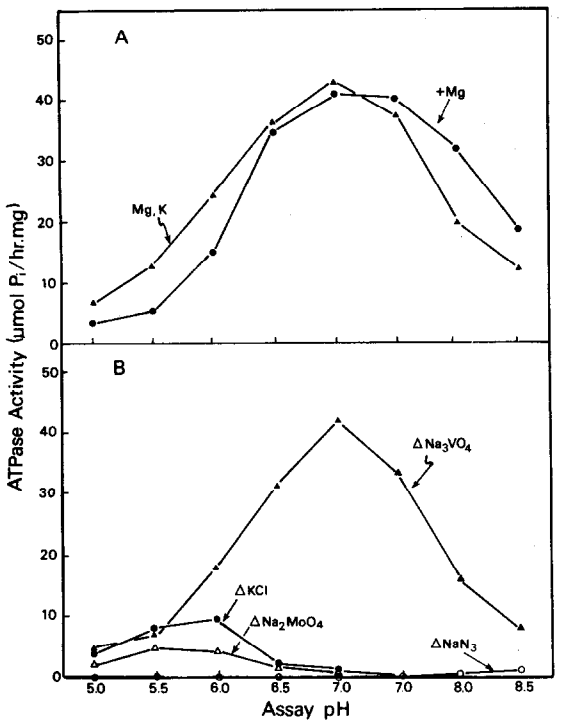

Fig. 1. Effect of assay pH on the ATPase activity associated with sugarbeet membranes (A). The standard assay contained 3 mM ATP, 3 mM $MgSO_4$, 30 mM Tris–Mes pH 7, 20 to 40 μg membrane protein and 50 mM KCl (when present). Inhibitor sensitive components of ATPase activity (B) represent the difference between control activity (containing 50 mM KCl) and the activity in the presence of 50 μM Na_3VO_4 (ΔNa_3VO_4) or 500 μM Na_2MoO_4 (ΔNa_2MoO_4) or 1 mM NaN_3 (ΔNaN_3). ΔKCl represents K^+-stimulated ATPase.

Table 3. Effect of divalent cations on sugarbeet ATPase

Treatment	ATPase activity (μmol/hr·mg)	%
No divalent cation	4.6	9.2
$MgSO_4$	50.2	100.0
$MnSO_4$	21.9	43.6
$CaSO_4$	3.8	7.6
$CuSO_4$	7.1	14.1
$FeSO_4$	7.9	15.7
$ZnSO_4$	2.6	5.2
$MgSO_4$ + 1.5 mM $CaSO_4$	10.5	20.9

ATPase was assayed in the presence of 3 mM ATP (Tris salt pH 7), 3 mM divalent cation (when added), 50 mM KCl and 30 mM Tris–Mes pH 7.2.

Table 4. Effects of phosphohydrolase inhibitors on the sugarbeet ATPase

Treatment	ATPase activity	%
Control	46.6	100.0
5 μg/ml Oligomycin	43.7	93.8
10 μM Ouabain	44.8	96.1
10 μM DCCD	20.6	44.2
100 μM DES	13.1	28.1

The standard ATPase assay was carried out in the presence of the indicated concentration of inhibitor. Both the control ATPase and inhibitor treatment contained 0.95% ethanol.

represent residual phosphatase activity not removed by KI extraction. Only a minor component of azide sensitive ATPase was found at pH 8.5.

The ATPase activity associated with sugarbeet membranes required the presence of a divalent cation for activity and the highest level of activity was observed with Mg^{2+} (Table 3). Other divalent cations, such as Mn^{2+}, Cu^{2+} or Fe^{2+}, could only partially substitute for Mg^{2+}. Maximal enhancement of activity occurred when Mg^{2+} and ATP were present in the 1:1 concentration ratio. When the enzyme was assayed in the presence of Mg^{2+} and K^+, Ca^{2+} acted as an inhibitor (Table 3). These results are similar to what has been observed for the plasma membrane ATPase isolated from other plant species [1–3, 15] and are consistent with the Mg:ATP complex being the true substrate for the enzyme [2, 3, 20]. Calcium ion acts as an inhibitor by interfering with the formation of the Mg:ATP complex [1, 3]. When the activity was measured at various Mg:ATP concentrations, Michaelis–Menten kinetics were observed and a K_m of 0.75 mM for Mg:ATP was found (data not shown).

The effects of phosphohydrolase inhibitors, in addition to those shown in Table 1, were examined for the sugarbeet ATPase (Table 4). The activity was insensitive to oligomycin, another inhibitor of mitochondrial ATPase [13] and also insensitive to ouabain, a specific inhibitor of the animal cell Na^+K^+-ATPase [13]. The activity, however, was sensitive to *N,N'*-dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol (DES). These compounds have been shown to be potent inhibitors of the plasma membrane ATPase in plant cells [1–3, 21].

When the effect of various combinations of Na^+ and K^+ on the sugarbeet ATPase were examined, the synergistic effects reported in previous investigations on sugarbeet could not be reproduced. At three different pH values (pH 6, 7 and 8), no peaks of synergism were found and only slight stimulatory effects of those ions were observed at pH 6.0. Thus the sugarbeet ATPase did not respond to combinations of Na^+ and K^+ as would be expected for an enzyme similar to the animal cell Na^+K^+-ATPase [13].

Phosphorylation

The formation of a rapidly turning over phosphoenzyme during the course of ATP hydrolysis has been shown to be characteristic of the plant plasma membrane ATPase [22–25] and many other transport ATPases present in eucaryotic cells [13 and refs therein]. When the time course of phosphorylation was examined for the sugarbeet membranes, the incorporation of labeled phosphate from (γ-^{32}P) ATP into trichloroacetic acid precipitable protein showed a steady increase with time and

did not reach a steady-state plateau within the duration of the experiment (Fig. 2A). This type of time course is consistent with the view that protein kinase activity is associated with the membrane fraction [26–29]. In contrast, the expected phosphorylation time course for a rapidly turning over phosphoenzyme would rapidly reach a steady-state plateau [23, 27–29]. In corn roots, plasma membrane associated protein kinase activity could be removed by treatment with 0.1% (w/v) deoxycholate so that phosphorylation characteristic of the ATPase phosphoenzyme could be observed [22, 27]. When the membranes from sugarbeet were treated in an identical manner, protein kinase activity could be reduced but not completely eliminated (Fig. 2B). Despite the presence of residual protein kinase activity, the level of phosphorylation at 20 sec could be rapidly reduced by an addition of excess unlabeled ATP (tris salt, pH 7). This indicates that when phosphorylation is measured over a short time interval ($t < 20$ sec), much of the radioactive label is rapidly turning over and most likely represents the covalent intermediate of an enzyme [22, 27].

While deoxycholate extraction reduced the protein kinase activity associated with the sugarbeet membrane preparations, the properties of the ATPase activity were unaffected by the detergent treatment. The ATPase activity associated with deoxycholate treated membranes was Mg^{2+}-dependent, largely unaffected by K^+, substrate specific for ATP and not synergistically stimulated by combinations of Na^+ and K^+ (data not shown). The activity also had the same inhibitor sensitivity as the activity present in the untreated membranes (data not shown). The ability of deoxycholate to affect protein kinase activity without affecting ATPase activity in sugarbeet is similar to what was observed for corn roots [22, 27].

Short-term phosphorylation (10 sec) was then used to characterize the properties of the rapidly turning over portion of the phosphorylation. The phosphorylation was magnesium dependent (Table 6), insensitive to azide and oligomycin but was inhibited by vanadate and DES (Table 5). The presence of Na^+, K^+ or both ions simultaneously in the reaction solution had little effect upon the short-term phosphorylation level (Table 6). This would be expected for an ATPase which is not affected by these cations. In contrast, the Na^+K^+-ATPase of animal cells which demonstrates Na^+-K^+ synergism shows Na^+-dependent phosphoenzyme formation and K^+-stimulated phosphoenzyme breakdown [13 and refs therein].

When the sugarbeet membranes, phosphorylated over a slightly longer time interval, were analysed by lithium dodecyl sulfate (LDS) polyacrylamide gel electrophoresis, two major radioactive bands were observed at M_r 105 000 and 29 000. Phosphorylation of these polypeptides was enzymatic in nature since the radioactive label was absent when the membranes were heat denatured prior to phosphorylation. Phosphorylation of the M_r 105 000 polypeptide demonstrated rapid turnover since the labeling of this band was decreased with an unlabeled ATP chase. The labeling of the M_r 29 000 band, however, remained after treatment with unlabeled ATP. These results suggest that M_r 105 000 band represents an enzyme reaction intermediate while the M_r 29 000 band most

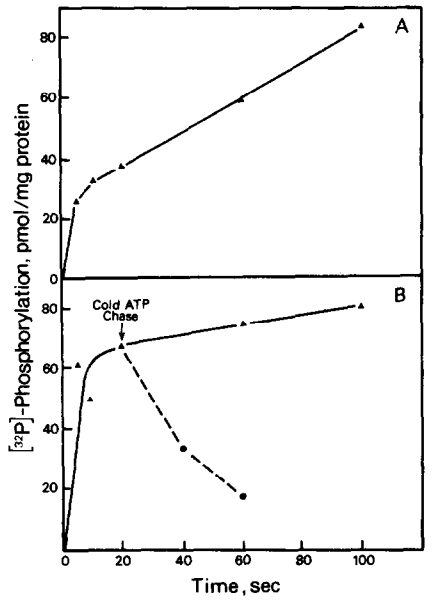

Fig. 2. Time course of radioactive phosphate incorporation for a plasma membrane (A) and 0.1% deoxycholate extracted plasma membrane (B) fraction from sugarbeets. The cold ATP chase was added as 0.1 ml of 150 mM unlabeled ATP (Tris salt pH 7.0).

Table 5. Effect of inhibitors on the short term phosphorylation of a 0.1% DOC extracted membrane fraction from sugarbeet

Treatment	Phosphorylation (pmol/mg)	%
Control	55.5	100.0
200 μM Na_3VO_4	29.4	54.9
1 mM DES	26.8	50.1
1 mM NaN_3	56.6	107.6
10 μg/ml Oligomycin	51.4	96.1

Phosphorylation was carried out for 10 sec in the presence of the indicated concentration of inhibitors. The assays with DES and oligomycin contained 0.95% ethanol. This ethanol concentration did not affect the control phosphorylation level.

Table 6. Effect of $MgSO_4$, NaCl and KCl on the short term phosphorylation of a sugarbeet plasma membrane fraction

Treatment	Phosphorylation (pmol/mg)	%
No ions	5.7	11.8
1.5 mM $MgSO_4$	48.0	100.0
+50 mM NaCl	45.9	95.6
+50 mM KCl	47.3	98.5
+25 mM NaCl, 25 mM KCl	43.8	91.3

Phosphorylation was carried out for 10 sec with the indicated combinations of ions.

likely represents a stable (non-turning over) protein kinase product.

Since the properties of the short-term phosphorylation correspond to the properties of the ATPase, the M_r 105 000 phosphorylated polypeptide probably represents the catalytic subunit of the sugarbeet ATPase [22–25]. Based upon steady-state kinetic studies, Lindberg [10] proposed a reaction scheme for the sugarbeet plasma membrane ATPase in which inorganic phosphate is released before ADP, Na^+ and K^+ are transported, and a phosphorylated intermediate does not occur. Clearly, the results presented in this study are in conflict with this proposed mechanism.

Conclusions

The results of this study are in strong disagreement with several earlier reports [7–12] regarding the properties of the plasma membrane ATPase of sugarbeet. No evidence for a synergistic Na^+, K^+-ATPase was found in plasma membrane enriched fractions where the presence of other phosphohydrolases was quantitated using phosphohydrolase inhibitors. With the exception of the pH optimum and response to monovalent cations, the sugarbeet ATPase demonstrated several properties similar to the plasma membrane ATPase from other species [1, 3] including the formation of a M_r 105 000 phosphorylated intermediate [22–25]. This may suggest that the ability of sugarbeet to tolerate higher levels of Na^+ may not be related to any modification of the plasma membrane ATPase. Research is in progress to develop a sealed vesicle system with this preparation so that the function that this enzyme has in transport can be resolved.

EXPERIMENTAL

Plant material. Sugarbeets (*Beta vulgaris* L., var GWD-2) were grown in field plots adjacent to Utah State University in Logan, Utah. The sugarbeets were planted in early June 1983 and harvested in late October. Following harvest, the tops of the plants were removed, and the storage roots were maintained at 7° until used.

Isolation of plasma membrane enriched fractions. Plasma membrane enriched fractions were isolated as described in ref. [15] with minor modifications. The beet roots were rinsed with H_2O and then cut into sections. All subsequent steps were carried out at 2–7°. The storage tissue was homogenized with an Oster vegetable juice extractor in a medium containing 250 mM sucrose, 3 mM EDTA, 0.5% polyvinyl pyrolidone (PVP-40), 15 mM β-mercaptoethanol, 70 mM Tris–HCl pH 8 and 4 mM dithioerythritol (DTE). Using 1 ml of homogenizing media/g tissue, the homogenate was filtered through four layers of cheesecloth and then centrifuged at 13 000 *g* (10 500 rpm) for 15 min in a Sorvall SS-34 rotor. The pellets were discarded and the supernatant was centrifuged at 80 000 *g* (32 500 rpm) for 30 min in an IEC A-269 rotor to obtain a microsomal pellet. The microsomal pellet was suspended in 250 mM sucrose, 1 mM Tris–Mes pH 7.2, 1 mM DTE (suspension buffer) to a protein concn of *ca* 1 mg/ml and then treated with 0.25 M KI to reduce non-specific phosphatase activity [15]. Following incubation for 20 min at 4°, the KI-treated membranes were centrifuged at 80 000 *g* (32 500 rpm) for 30 min in an IEC A-269 rotor. The KI-extracted membrane pellet was suspended in 0.5 ml of suspension buffer and then layered on a 30/38% sucrose discontinuous gradient consisting of 4 ml of 30% sucrose layered over 8 ml of 38% sucrose (both gradient solns buffered with 1 mM Tris–MES pH 7.2 and containing 1 mM DTE). The gradient was centrifuged at 120 000 *g* (26 000 rpm) for 2 hr in an IEC SB-283 rotor. Following centrifugation, the membranes present at the 30/38% gradient interface were recovered with a Pasteur pipette. The membranes were frozen under liquid N_2 and stored for up to 2 months without significant loss of ATPase activity.

Treatment with deoxycholate. The plasma membrane fraction was treated with 0.1% deoxycholate to reduce protein kinase activity essentially as described in ref. [27]. The membrane fraction was adjusted to a protein concn of *ca* 2–3 mg/ml with suspension buffer. 1 vol of 0.2% deoxycholate (Na salt), 4 mM Na_2 EDTA, 10 mM Na_2ATP, 200 mM KCl, 50 mM Tris–HCl pH 7.5 and 1 mM DTE was added dropwise, on ice, with constant stirring. The detergent-treated membranes were incubated on ice for 20 min and centrifuged at 100 000 *g* (42 000 rpm) for 1 hr in an IEC A237 rotor. The supernatant was decanted, and the deoxycholate treated membranes were suspended in suspension buffer.

Enzyme assay. Phosphohydrolase activity was assayed as described in ref. [15]. ATP ('vanadate free') was purchased from Sigma as the Na^+ salt and converted to the tris salt by cation exchange on Dowex 50 resin. All other substrates were used as Na^+ salts. Phosphate hydrolysing activity was measured in a 1 ml reaction vol. with 3 mM substrate, 3 mM $MgSO_4$ and 30 mM Tris–Mes pH 7. When KCl was present, the final concn was 50 mM. Any variations are indicated in the Results section. The reaction was carried out for 15–20 min at 38°, and the released Pi was determined by the method of ref. [30].

Phosphorylation was performed by the method of ref. [31]. The standard assay was carried out at ice temp. in a 1 ml reaction vol. in the presence of 1.5 mM (γ-^{32}P)-ATP (generally 45–75 mCi/mmol), 1.5 mM $MgSO_4$, 30 mM Tris–Mes pH 7, 2 mg carrier BSA (Sigma fraction V) and 70–100 μg of membrane protein. Any variations are indicated in the Results. The reaction was started by the addition of (γ-^{32}P)-ATP and quenched at the appropriate time by the addition of 25 ml of 10% TCA, 70 mM NaH_2PO_4, 5 mM $Na_4P_2PO_7$ and 1 mM Na_2 ATP. Rapid mixing was provided by a small magnetic stir bar. The quenched samples were centrifuged at 27 000 *g* (15 000 rpm) for 15 min in a Sorvall SS-34 rotor. The supernatant was removed and the pellets were suspended in 0.5 ml of the quenching soln. The suspended pellets were washed by centrifugation at 27 000 *g* after the addition of 25 ml of quenching soln. The final pellets were suspended in 1 ml H_2O and transferred to scintillation vials. Radioactivity was determined by liquid scintillation spectroscopy in 5 ml of liquid scintillation cocktail (Aquasol-2, New England Nuclear). When the phosphorylated samples were prepared for LDS gel electrophoresis, the final wash step was performed in 8 ml of 30 mM HCl instead of 25 ml of quench soln.

LDS-PAGE. Lithium dodecylsulphate (LDS) polyacrylamide gel electrophoresis was carried out by the method of ref. [32] on 5.6% acrylamide slab gels containing 0.2% (w/w) LDS and buffered to pH 2.4 with 50 mM Tris–citrate. Phosphorylated samples were adjusted to 1% LDS, 50 mM Tris–citrate (pH 2.4). 2% β-mercaptoethanol, 4 M urea, 20% glycerol, 10 g/ml pyronin Y (tracking dye) and incubated at room temp. for 10 min. Then 20 μl of sample (*ca* 40 μg protein) were applied per gel lane, and the slab gels were electrophoresed at 20 mA/slab for 5 hr at 4°. The tank buffer contained 50 mM Tris–citrate pH 2.4 and 0.2% LDS.

Following electrophoresis, the radioactive gels were dried immediately on blotter paper and then subjected to autoradiography for 36 hr against Kodak XAR-5 X-ray film with cronex 'Lightning Plus' intensifying screens at −80°. The relative distribution of radioactivity on the gel autoradiograph was

determined by scanning the X-ray film with a Helena Laboratories Auto-gel Scanner. For the determination of M_r, the gel system was calibrated using protein standards solubilized and electrophoresed under the identical conditions as the phosphorylated samples. The standard gel was fixed, stained with Coomassie blue and destained as described in ref. [27].

Protein assay. Protein was determined by the method of ref. [33] following a TCA precipitation to remove interference by DTE.

REFERENCES

1. Leonard, R. T. (1983) in *Metals and Micronutrients, Uptake and Utilization by Plants* (Robb, D. A. and Pierpoint, W. S., eds) p. 71. Academic Press, London.
2. Leonard, R. T. and Hodges, T. K. (1973) *Plant Physiol.* **52**, 6.
3. Leonard, R. T. (1985) in *Advances in Plant Nutrition* (Tinker, P. B. and Lauchli, A., eds) Praeger Scientific, New York (in press).
4. Poole, R. J. (1982) in *Membranes and Transport* (Martinosi, A. N., ed.) Vol. 2, p. 651. Plenum Press, New York.
5. Kylin, A. and Gee, R. (1970) *Plant Physiol.* **45**, 169.
6. Vakhmistrov, D. B., Tikhaya, N. I. and Mishustina, N. E. (1982) *Physiol. Plant.* **55**, 155.
7. Lindberg, S. (1982) *Physiol. Plant.* **54**, 455.
8. Lindberg, S., Hansson, G. and Kylin, A. (1974) *Physiol. Plant.* **32**, 103.
9. Lindberg, S. (1976) *Physiol. Plant.* **36**, 139.
10. Lindberg, S. (1980) *Physiol. Plant.* **48**, 65.
11. Wingstrand, G. and Lindberg, S. (1982) *Physiol. Plant.* **56**, 333.
12. Lindberg, S. and Bjorklund, G. (1977) *Physiol. Plant.* **40**, 275.
13. Hobbs, A. S. and Albers, R. W. (1980) *Annu. Rev. Biophys. Bioeng.* **9**, 259.
14. Kylin, A. and Quatrano, R. S. (1975) in *Ecological Studies. Analysis and Synthesis* (Poljakoff-Mayber, A. and Gale, J., eds) Vol. 15, p. 145. Springer, Berlin.
15. Briskin, D. P. and Poole, R. J. (1983) *Plant Physiol.* **71**, 350.
16. Galleghar, S. R. and Leonard, R. T. (1982) *Plant Physiol.* **70**, 1335.
17. O'Neill, S. D., Bennett, A. B. and Spanswick, R. M. (1983) *Plant Physiol.* **72**, 837.
18. Nagahasi, G. and Kane, A. P. (1982) *Protoplasma* **112**, 167.
19. Yoshida, S., Matsuo, U., Teruo, N., Akira, S. and Gusta, L. (1983) *Plant Physiol.* **72**, 105.
20. Balke, N. E. and Hodges, T. K. (1975) *Plant Physiol.* **55**, 83.
21. Balke, N. E. and Hodges, T. K. (1979) *Plant Physiol.* **63**, 48.
22. Briskin, D. P. and Leonard, R. T. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6922.
23. Briskin, D. P. and Poole, R. J. (1983) *Plant Physiol.* **71**, 507.
24. Vera, F. and Serrano, R. (1983) *J. Biol. Chem.* **258**, 5334.
25. Scalla, R., Amory, A., Riguard, J. and Goffeau, A. (1983) *Eur. J. Biochem.* **132**, 525.
26. Lin, P. C. and Key, J. L. (1980) *Plant Physiol.* **66**, 360.
27. Briskin, D. P. and Leonard, R. T. (1982) *Plant Physiol.* **70**, 1459.
28. Weller, M. (1977) *J. Theor. Biol.* **64**, 391.
29. Weller, M. (1979) *Protein Phosphorylation. The Nature, Function, and Metabolism of Proteins Which Contain Covalently Bound Phosphorus.* Pion, London.
30. Ames, B. N. (1966) *Methods Enzymol.* **8**, 115.
31. Post, R. L. and Sen, A. K. (1967) *Methods Enzymol.* **10**, 762.
32. Lichtner, R. S. and Wolf, H. U. (1979) *Biochem. J.* **181**, 759.
33. Peterson, G. L. (1977) *Analyt. Biochem.* **83**, 346.