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Plasma Membrane ATPase of Red Beet Forms a Phosphorylated Intermediate[1]

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ABSTRACT

When a plasma membrane-enriched fraction isolated from red beet (*Beta vulgaris* L.) was incubated in the presence of 40 micromolar [γ-^{32}P] ATP, 40 micromolar $MgSO_4$ at pH 6.5, a rapidly turning over phosphorylated protein was formed. Phosphorylation of the protein was substrate-specific for ATP, sensitive to diethylstilbestrol and vanadate, but insensitive to azide. When the dephosphorylation reaction was specifically studied, KCl was found to increase the turnover of the phosphorylated protein consistent with its stimulatory effect upon plasma membrane ATPase. The protein-bound phosphate was found to be most stable at a pH between 2 and 3 and under cold temperature, suggesting that the protein phosphate bond was an acyl-phosphate. When the phosphorylated protein was analyzed with lithium dodecyl sulfate gel electrophoresis, a labeled polypeptide with a molecular weight of about 100,000 daltons was observed. Phosphorylation of this polypeptide was rapidly turning over and Mg-dependent. It is concluded that the phosphorylation observed represents a reaction intermediate of the red beet plasma membrane ATPase.

The primary transport event in higher plant cells is the efflux of protons from the cytoplasm to the cell exterior (23, 26), and the induced proton motive force can then serve to drive the transport of other solutes through secondary co- and counter transport mechanisms (13, 23). It is generally believed that a plasma membrane-bound ATPase may provide the enzymic machinery responsible for driving proton extrusion (15 and references therein). Although the plant plasma membrane ATPase has not been fully purified and its transport properties demonstrated in reconstituted membrane systems, much indirect evidence supports its role as a primary energy transducer at the plasma membrane. There is strong evidence that the energy source for transport is ATP (20, 22) and when plasma membrane fractions are isolated from higher plants, a monovalent cation-stimulated ATPase is enriched (15, 16). Orthovanadate, an inhibitor of plasma membrane ATPase (11), has been shown to inhibit proton extrusion and potassium uptake without affecting ATP levels (9) and, recently, evidence for ATP-dependent proton transport has been reported for isolated membrane vesicles, presumably of plasma membrane origin (27). It is presently unclear whether monovalent cations are transported directly by the enzyme (15, 20) in a manner analogous to the animal cell Na^+,K^+-ATPase (8) or whether their transport occurs through secondary carriers in response to the proton motive force (15).

Currently, the known ATP-linked transport mechanisms can be assigned to one of two categories (18 and references therein). The first type of transport enzyme consists of many small subunits, is freely reversible, and does not form a phosphorylated intermediate during the course of ATP hydrolysis. The F_1F_o-transport ATPases of mitochondria, chloroplasts, and bacteria are examples of this first type of transport enzyme (18). The second type of transport enzyme consists of a collection of at most three subunit types (generally one or two) and is specifically phosphorylated when ATP is hydrolyzed. The animal cell Na^+,K^+-ATPase (8), sarcoplasmic reticulum Ca^{2+}-ATPase (10), gastric H^+,K^+-ATPase (25), and the fungal cell plasma membrane H^+-ATPase (12) are examples of this second form of transport enzyme. Recent work by Briskin and Leonard (5, 6) suggests that the plasma membrane ATPase of higher plants may belong to the second class of transport enzyme since the ATPase of a plasma membrane fraction from corn roots was shown to form a phosphorylated intermediate when incubated with [γ-^{32}P]ATP.

In the work presented here, we report that the plasma membrane ATPase from red beet storage tissue forms a phosphorylated intermediate similar to that observed for the corn root enzyme (5, 6). The phosphorylation occurred on a 100,000 D polypeptide, and the stability properties of the protein-phosphate bond suggest that it is an acyl phosphate group.

MATERIALS AND METHODS

Plant Material. Red beet (*Beta vulgaris* L.) storage roots were purchased commercially. The tops of the plants were removed and the storage roots were stored in moist vermiculite at 5°C until use.

Isolation of Plasma Membrane-Enriched Fractions. Plasma membrane fractions were isolated as described by Briskin and Poole (7). Beet roots were rinsed with distilled H_2O and then cut into sections. All subsequent steps were carried out at 2 to 4°C. The storage tissue was homogenized with a Braun juice extractor in a medium containing 250 mM sucrose, 3 mM EDTA, 0.5% (w/v) PVP-40, 70 mM Tris-HCl (pH 8), and 4 mM DTE.[2] One ml of homogenizing media was used per g of tissue. The homogenate was filtered through four layers of cheesecloth and 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 a Beckman Ti70 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 concentration of about 1 mg/ml and treated with 0.25 M KI to remove nonspecific phosphatase. Following incubation for 20 min on ice, the KI-treated membranes were centrifuged at 80,000*g* (32,500 rpm) for 30 min in a Beckman Ti70 rotor. The KI-extracted membrane pellet was suspended in suspension buffer and layered on a 25/30% sucrose discontinuous gradient consisting of 4 ml of 25% (w/w) sucrose layered over 8

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[2] Abbreviations: DTE, dithioerythritol; LDS, lithium dodecyl sulfate; DES, diethylstilbestrol.

ml of 30% (w/w) sucrose. Gradient solutions were buffered with 1 mM Tris-Mes (pH 7.2) and contained 1 mM DTE. The gradient was centrifuged at 115,000*g* (26,000 rpm) for 2 h in a Beckman SW 41Ti rotor. Following centrifugation, membranes enriched in K^+-stimulated ATPase were recovered at the 25/30% sucrose interface and immediately frozen under liquid N_2 for up to 4 d without significant loss in activity.

Phosphorylation. Phosphorylation was performed essentially by the method of Post and Sen (24). The assay was carried out at ice temperature in a 1.0 ml reaction volume containing 40 μM [γ-^{32}P] ATP (45–70 mCi/mmol), 40 μM $MgSO_4$, 30 mM Tris-Mes (pH 6.5; titrated from 0.3 M stocks to desired pH), 2 mg carrier BSA (Sigma, fraction V), and 40 to 70 μg of membrane protein. 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 ice-cold 10% TCA containing 40 mM NaH_2PO_4, 5 mM $Na_4P_2O_7$, and 1 mM ATP (disodium salt). Rapid mixing was provided by a small magnetic stir bar. The quenched reaction mixture was 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 with a chilled glass rod in 0.5 ml of the quenching solution. The suspended pellets were washed by centrifugation at 27,000*g* (15,000 rpm SS-34 rotor) after the addition of 25 ml of quenching solution. The final washed pellets were suspended in 1.0 ml of distilled H_2O and aliquots were transferred to scintillation vials. Radioactivity was determined by liquid scintillation spectroscopy in 5 ml of a dioxane cellosolve scintillation cocktail (5). Any variations on the procedure are indicated in "Results."

When phosphorylated samples were prepared for LDS gel electrophoresis, the final wash step was performed in 8 ml of 30 mN HCl instead of 25 ml of quenching solution.

Assay of Released ^{32}Pi. [^{32}P]Inorganic phosphate released from [γ-^{32}P]ATP was determined under the identical conditions as phosphorylation except that NaH_2PO_4 and $Na_4P_2O_7$ were omitted from the quench solution and the quench volume was reduced to 9 ml. Following centrifugation at 27,000*g* (15,000 rpm, 15 min, Sorvall SS-34 rotor), a 1.0-ml aliquot of the supernatant was removed and mixed with an equal volume of 5% ammonium molybdate in 2.0 N H_2SO_4. The [^{32}P]phosphomolybdate complex was extracted according to Mardh and Zetterquist (19) using isobutanol-benzene (1/1). Three ml of isobutanol-benzene were added and the tubes were vortexed vigorously. After phase separation was completed, a 2-ml aliquot of the organic phase was removed and radioactivity was determined in 10 ml of scintillation cocktail.

LDS-Polyacrylamide Gel Electrophoresis. LDS gel electrophoresis was carried out by the method of Lichtner and Wolf (17) on 5.6% acrylamide slab gels containing 0.2% (w/w) LDS and buffered to pH 2.4 with 50 mM Tris-citrate. At this pH, the Tris base has no buffering capacity but serves instead as an organic counter-ion during electrophoresis. Phosphorylated protein samples were adjusted to 1% (w/w) LDS, 50 mM Tris-citrate (pH 2.4), 2% (v/v) β-mercaptoethanol, 4 M urea, 20% (v/v) glycerol, 10 μg/ml pyronin Y, and incubated at room temperature for 10 min. Ten μl of sample (about 70 μg protein) were applied per slab gel lane, and the slab gels were electrophoresed at 25 mamp per gel for 2.5 h at 2°C. The tank buffer contained 50 mM Tris-citrate (pH 2.4) and 0.2% (w/w) LDS.

Following electrophoresis, the gels were immediately dried on Whatman No. 1 paper and then subjected to autoradiography for 36 h against Kodak XAR-5 X-ray film with Cronex 'Lightning Plus' intensifying screens at −80°C.

For the determination of mol wt, 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 by Briskin and Leonard (5). A linear relationship was found for the standards tested, except at low mol wt (Fig. 1).

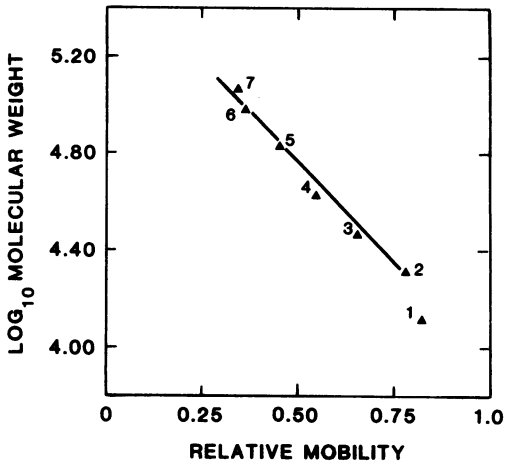

FIG. 1. Mol wt standards for LDS gel electrophoresis. Protein samples were dissolved in electrophoresis buffer containing 4 M urea, and electrophoresis was carried out as described in "Materials and Methods." Relative mobility was determined with pyronin Y as tracking dye. Protein standards were each present as 5 μg/lane: (1), α-lactalbumin (14,000 D); (2), soybean trypsin inhibitor (21,500 D); (3), carbonic anhydrase (31,000 D); (4), ovalbumin (43,000 D); (5), BSA (68,000 D); (6), phosphorylase b (92,500 D); (7), β-galactosidase (116,250 D).

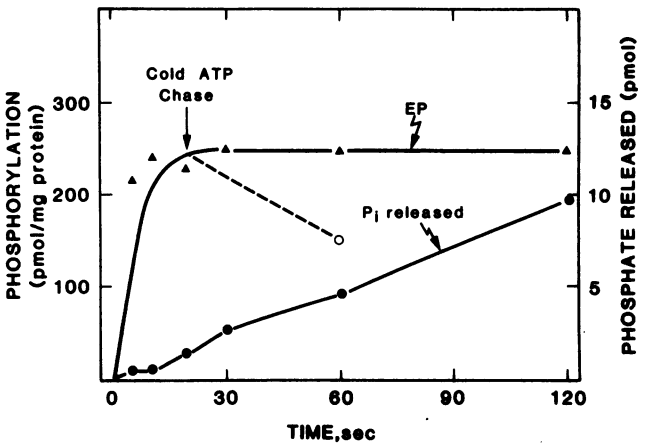

FIG. 2. Time course of [^{32}P]phosphate incorporation and [γ-^{32}P]ATP hydrolysis by a red beet plasma membrane fraction. Determination of ^{32}P phosphorylation and [^{32}P]phosphate release were carried out as described in "Materials and Methods." At the time indicated, 1.5 μmol ATP (Tris-salt, pH 6.5) was added and the reaction was quenched after 40 s to give the data indicated by the dashed lines.

Protein Assay. Protein was determined by the method of Peterson (21) following a TCA precipitation to eliminate interference by DTE.

The data shown are for representative experiments which have been repeated one or more times.

RESULTS

Phosphorylation Time Course. The time course of [^{32}P]phosphate incorporation into TCA-precipitable protein was determined for the plasma membrane-enriched fraction from red beet storage roots (Fig. 2). This fraction contains a monovalent cation-stimulated ATPase activity with properties (7) similar to the ATPase activity that occurs in plasma membrane fractions from corn and oat roots (15, 16). Phosphate incorporation reached a plateau within seconds and this steady-state level was maintained for the duration of the time course. When an excess of unlabeled

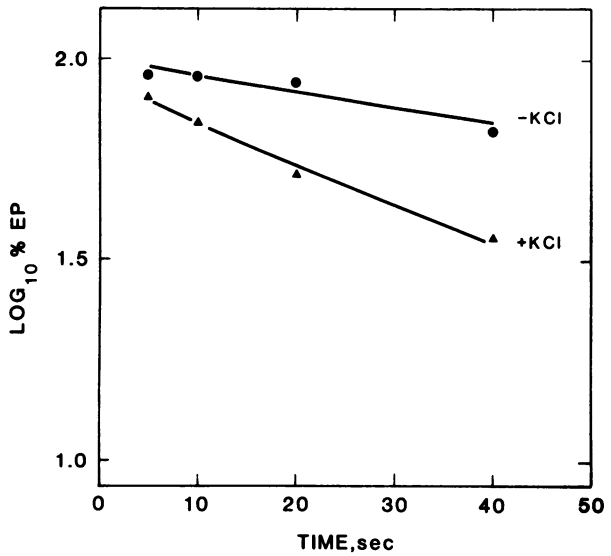

FIG. 3. Time course of the dephosphorylation reaction for a red beet plasma membrane fraction. Phosphorylation was carried out as described in "Materials and Methods." After 20 s, 1.5 μmol ATP (Tris-salt, pH 6.5) and 50 μmol KCl (if indicated) was added. The steady-state level of phosphorylation at 20 s (202.4 pmol/mg protein) was taken as time 0 and the reactions were quenched at the times indicated. 'Best fit' lines were determined by linear regression, and first-order rate constants were calculated as $K_d = -2.303$ (slope). The rate constants for dephosphorylation were 0.0091 and 0.023 s^{-1} in the absence and presence of KCl, respectively.

ATP (Tris-salt, pH 6.5) was added, the steady-state phosphorylation level rapidly declined. Under the conditions where a steady-state level of phosphorylation was maintained, [^{32}P]inorganic phosphate accumulated indicating that net hydrolysis of [γ-^{32}P] ATP was occurring. Both the nature of the time course and the response of the steady-state phosphorylation to an unlabeled ATP chase indicate that the protein-bound phosphate is rapidly turning over and most likely represents the covalent reaction intermediate of an enzyme (5, 14, 28).

The nature of the time course also suggests that, unlike the plasma membrane preparation from corn roots (5), substantial levels of protein kinase activity were not present in the plasma membrane preparation from red beet. A minor component of protein kinase activity could only be detected when the time course was carried out in excess of 6 min (data not shown). This activity did not interfere with analysis of the rapidly turning over protein-bound phosphate as long as the duration of the experiments did not exceed 2 min. It is unclear at the present time whether the low level of protein kinase activity associated with this preparation is an inherent property of red beet plasma membrane or whether this is a result of the KI extraction step (*i.e.* removal of membrane peripheral proteins) performed during the membrane isolation.

Dephosphorylation Reaction. The turnover of the phosphorylated protein was specifically studied by examining the time course of the dephosphorylation reaction (Fig. 3). This was done by adding an unlabeled ATP chase to phosphorylation reactions at steady-state (20 s phosphorylation), and the reactions were quenched at increasing times following the chase. Under these conditions, the phosphorylation reaction becomes unmeasurable and dephosphorylation is observed. The breakdown of the phosphorylated protein was exponential with time indicating a first order kinetic relationship (Fig. 3). This is consistent with the unimolecular breakdown of an enzyme reaction intermediate, and the slope of the plot can be related to the first-order rate constant for this process. When the unlabeled ATP chase was carried out 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 phosphorylated protein. Calculation of the rate constant for dephosphorylation indicated a 2.5-fold increase with the addition of KCl. This effect of KCl on the turnover of the phosphorylated protein is similar to that observed for the phosphorylated intermediate of the corn root plasma membrane ATPase (6) and is consistent with the enzymic properties of the ATPase activity of this preparation. The beet root plasma membrane ATPase has substantial velocity in the presence of Mg alone, and KCl causes a roughly 2-fold increase in this velocity (7).

General Characteristics of the Phosphorylation. The type of pulse-chase technique presented in the previous sections was used to assess the substrate specificity of the phosphorylation reaction (Table I). In this case, various unlabeled nucleoside phosphates were added after phosphorylation reached steady-state (20 s) and the reactions were quenched after an additional 40 s. The most effective nucleoside phosphate for reducing the steady-state phosphorylation level was ATP, indicating that the phosphorylation reaction was relatively substrate-specific for ATP. Substrate specificity for ATP is a characteristic property of the plasma membrane ATPase activity that has been correlated with transport in plants (15, 16) and is also a property of electrogenic ATP-dependent transport observed for sealed membrane vesicles from plant cells (27).

It was observed that steady-state phosphorylation was reduced by ADP to an extent greater than could be accounted for by its hydrolysis as a substrate (7). This may result from an ATP/ADP exchange reaction in which the high-energy phosphorylated intermediate reacts with ADP to resynthesize ATP (8, 10, 12). This reaction has been demonstrated for both the Na^+,K^+-ATPase (8) and sarcoplasmic reticulum Ca^{2+}-ATPase (10) of animal cells.

The effect of various inhibitors on phosphorylation was examined in Table II. Phosphorylation was insensitive to the mitochondrial ATPase inhibitor NaN_3 but was inhibited by sodium vanadate. The most effective inhibitor of phosphorylation was found

Table I. *Effect of Adding Various Unlabeled Nucleoside Phosphates on the Steady-State ^{32}P-Phosphorylation Level for a Red Beet Plasma Membrane Fraction*

Phosphorylation was performed as described in "Materials and Methods." After 20 s, 1.5 μmol of the indicated nucleoside phosphate was added. The reaction was quenched 40 s after the addition.

Addition[a]	^{32}P-Phosphorylation	
	pmol/mg protein	*% control*
Control (no addition)	220.2	100
ATP	125.5	57
ADP	138.7	63
GTP	173.9	79
CTP	200.4	91
UTP	187.2	85
ITP	198.2	90

[a] ATP was added as the Tris salt (pH 6.5) while all other nucleoside phosphates were added as sodium salts.

Table II. *Effect of Various Inhibitors on the Level of Steady-State ^{32}P-Phosphorylation for a Red Beet Plasma Membrane Fraction*

Phosphorylation was carried out as described in "Materials and Methods" for 20 s with the indicated additions.

Addition	^{32}P-Phosphorylation	
	pmol/mg protein	*% control*
Control (no addition)	216.3	100
1 mM sodium azide	209.5	97
100 μM sodium vanadate	159.3	74
500 μM sodium vanadate	107.4	50
100 μM DES	48.6	23

to be DES. Both DES and sodium vanadate have been shown to be inhibitors of the plasma membrane ATPase of higher plants (3, 11). The extent of vanadate inhibition on phosphorylation was less than that found for the phosphorylated intermediate of the corn root plasma membrane ATPase where 60% inhibition was found with 100 μM vanadate (6). In this study, 500 μM vanadate only produced about 50% inhibition of steady-state phosphorylation.

Phosphorylation also appeared to be less sensitive to vanadate than previously reported ATPase activity for this preparation (7). This lack of correspondence between vanadate inhibition of phosphorylation and ATPase which has also been observed for the fungal plasma membrane ATPase (1) may be a reflection of the mode of action of this inhibitor (1, 8). Differences in the assay conditions for phosphorylation (20 s, 40–70 μg protein, ice temperature) and ATPase (30 min, 10–20 μg protein, 38°C) may also contribute to the lack of direct correspondence for this inhibitor.

Stability of the Protein-Bound Phosphate. The stability properties of the acid-denatured phosphoenzyme were examined in order to assess the type of phosphoprotein linkage (Fig. 4). Phosphorylation reactions were quenched at steady-state (20 s), the TCA pellets were suspended in buffers of increasing pH, and the samples were incubated at room temperature for 30 min. Following incubation, the samples were washed by centrifugation and the radioactivity that remained bound to protein was determined. This value was compared to control samples where the radioactivity was determined immediately following phosphorylation. Assuming that the nonenzymic release of protein-bound phosphate will follow a first-order relationship (4), the data are expressed in terms of first-order phosphohydrolysis rate constants (Fig. 4). Maximal stability was found when the denatured phosphoenzyme was incubated at pH 2.4. At a pH either lower or higher than this value, increased hydrolysis of the protein-bound phosphate occurred.

The temperature stability of the denatured phosphoenzyme at pH 2.4 was also examined (Fig. 5). Following phosphorylation, the denatured phosphoprotein was incubated at pH 2.4 with increasing temperature for 30 min and the samples were analyzed in a manner similar to the above. The phosphoprotein demonstrated decreased stability as the temperature of incubation was raised.

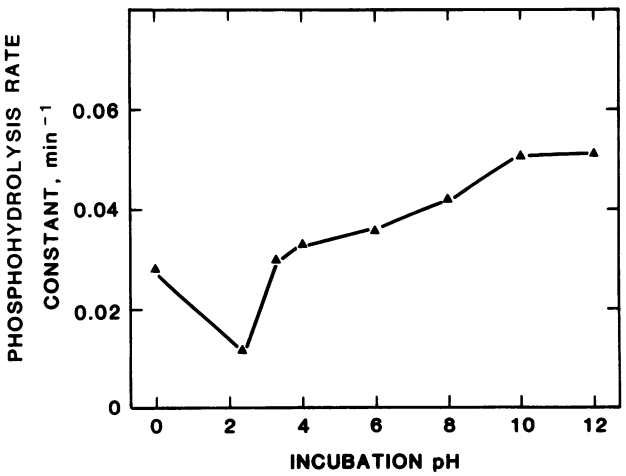

FIG. 4. The pH stability of the phosphorylated protein. Phosphorylation was carried out for 20 s as described in "Materials and Methods," and the initial TCA pellets were suspended in 1.0 ml of buffer at the indicated pH. The samples were incubated for 30 min at room temperature and then 25 ml of ice-cold quench solution was added. The samples were centrifuged at 27,000*g* (15 min), and the radioactivity remaining in the TCA pellet was determined as described in "Materials and Methods." The radioactivity remaining bound to protein was compared to control samples where the radioactivity was determined immediately following phosphorylation. The phosphohydrolysis rate constant was calculated as $K_{phoshydrol} = -(\ln EP_{30}/EP_0)/30$. The treatments used were: pH 0, 2 N HCl; pH 2.4, 3, 4, 6, 100 mM Na-citrate; pH 8, 100 mM Tris-HCl; pH 10, 12, 100 mM Na-borate.

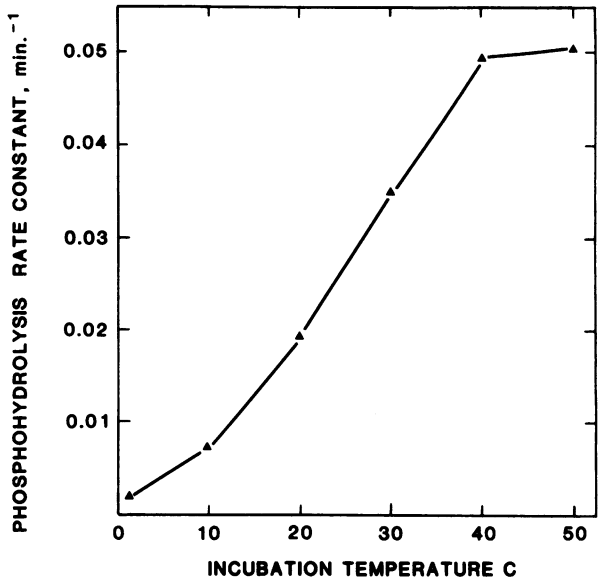

FIG. 5. The effect of temperature on the stability of the phosphorylated protein. Phosphorylation was carried out for 20 s as described in "Materials and Methods" and the initial TCA pellets were suspended in 1.0 ml of 100 mM Na-citrate (pH 2.4). Samples were incubated at the indicated temperature for 30 min, and then 25 ml of ice-cold quench solution was added. Following centrifugation at 27,000*g* (15 min), the radioactivity remaining bound to protein was determined as described in "Materials and Methods." The phosphohydrolysis rate constant was determined as described for Figure 4 by comparison to a control sample where the radioactivity was determined immediately following phosphorylation.

Stability of the denatured phosphoprotein at a pH between 2 and 3 (2, 4, 28) and under low temperature (28) is consistent with the protein-phosphate bond being an acyl phosphate. Further support for the presence of an acyl-phosphate group comes from the observation that a large portion of the protein-bound phosphate can be discharged in the presence of 0.25 M hydroxyl amine at pH 5.2 (data not shown; see 28). The acyl phosphate bond is the characteristic type of protein phosphate group found for those transport ATPases that form phosphorylated intermediates (1, 8, 10, 12, 25).

LDS-Gel Electrophoresis. The phosphorylated protein from red beet plasma membrane was analyzed by polyacrylamide gel electrophoresis in the presence of LDS (Fig. 6). This system, described by Lichtner and Wolf (17), was especially suitable for analyzing the phosphoenzyme since it can be used at both low pH and low temperature, conditions where the phosphorylated protein is most stable.

An autoradiograph of the gel demonstrated that the phosphorylated protein was present as a band with a mol wt of about 100,000 D (Fig. 6) when compared to protein standards (Fig. 1). Although this system allowed clear resolution of the phosphorylated protein, solubilization of membrane protein was difficult as indicated by the amount of radioactivity that remained at the sample well. The identity of the labeled material remaining at the origin of the gel is unclear, although much of this labeling would appear to be nonenzymic in nature since it was present in samples boiled for 5 min prior to phosphorylation. The presence of radioactive label at the origin, however, does raise the possibility that other proteins in addition to the plasma membrane ATPase may be phosphorylated in this preparation. The radioactivity that migrated just behind the tracking dye was also nonenzymic in

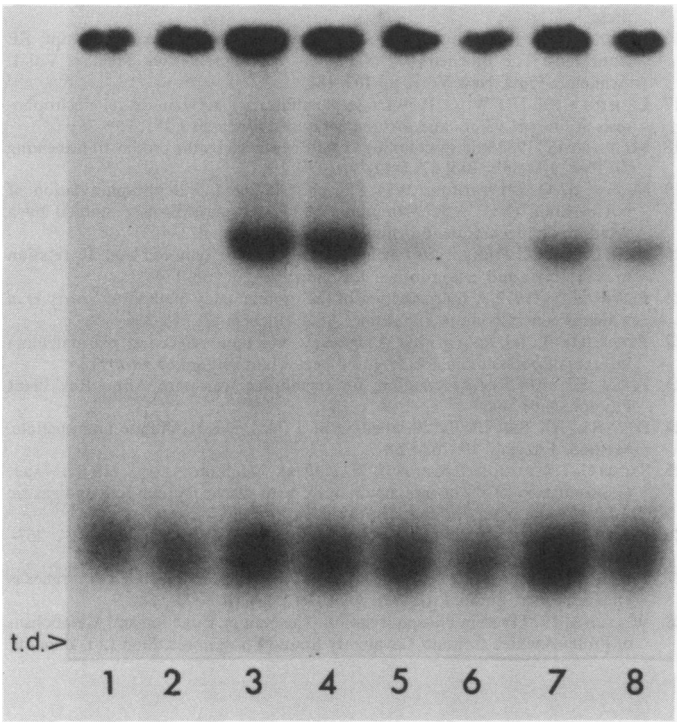

FIG. 6. Gel autoradiograph of the phosphorylated protein for a red beet plasma membrane fraction. Phosphorylation and electrophoresis were carried out as described in "Materials and Methods." The dried gel was placed against X-ray film for 36 h at −80°C with a Cronex 'Lightning Plus' intensifying screen. The position of the pyronin Y tracking dye is indicated by t.d. Approximately 70 μg of membrane protein was applied per slab gel lane. Lanes 1 and 2, membrane sample boiled 5 min prior to phosphorylation. Lanes 3 and 4, 20-s phosphorylation. Lanes 5 and 6, 20-s phosphorylation followed by a 40-s chase with 1.5 μmol of unlabeled ATP (Tris-salt, pH 6.5). Lanes 7 and 8, 20-s phosphorylation followed by a 40-s chase with 10 μmol of EDTA (Tris-salt, pH 6.5).

nature since it occurred when a membrane sample was boiled for 5 min prior to phosphorylation. This most likely represents residual [γ-^{32}P]ATP and [^{32}P]inorganic phosphate not removed by the washing steps during sample preparation.

Phosphorylation of the 100,000 D polypeptide showed rapid turnover since the labeling of this band was decreased by an unlabeled ATP chase (Fig. 6). Phosphorylation was Mg-dependent since the labeling of the polypeptide was decreased by a chase with 10 mM Tris-EDTA (pH 6.5). This also demonstrates that the dephosphorylation reaction does not require Mg since turnover could occur in the presence of an excess of EDTA.

DISCUSSION

The results of this study provide evidence that the plasma membrane ATPase of red beet storage root tissue forms a phosphorylated intermediate during the course of ATP hydrolysis. When a plasma membrane fraction from red beet was incubated in the presence of [γ-^{32}P]ATP, radioactive phosphate was incorporated into TCA-insoluble protein. Both the nature of the time course and the response of the steady-state phosphorylation to an unlabeled ATP chase are consistent with rapid turnover of the protein-bound label (Fig. 2). Phosphorylation, like plasma membrane ATPase (7, 15), was substrate-specific for ATP (Table I) and inhibited by DES and sodium vanadate (Table II). When the dephosphorylation reaction was specifically studied, KCl was found to increase the turnover of the phosphorylated protein (Fig. 3) consistent with its stimulatory effects upon the ATPase activity associated with this preparation (7). The phosphorylated protein was found to be most stable at a pH between 2 and 3 (Fig. 4) and at low temperature (Fig. 5) consistent with the presence of an unstable acyl phosphate bond (2, 4, 28) common to those transport ATPases that form phosphorylated intermediates (1, 8, 10, 12, 24).

From the data presented in this study, it is possible to examine the correspondence between the breakdown of the phosphoenzyme and ATP hydrolytic activity in the absence of KCl. From Figure 2, the slope of a 'best fit' line for the plot of [^{32}P]inorganic phosphate released with time and the protein concentration value for the samples (40 μg/assay) allow an estimate of ATP hydrolysis at 40 μM Mg-ATP and ice temperature. This rate of hydrolysis is 7.5 nmol/mg·h. The rate of hydrolysis based on phosphoenzyme breakdown can be calculated using the equation: $V = K_d \cdot [EP]$ where K_d is the dephosphorylation rate constant, V is the hydrolysis rate, and $[EP]$ is the level of steady-state phosphorylation for the dephosphorylation time course (Fig. 3). With a dephosphorylation rate constant of 0.0091 s^{-1} and a steady-state phosphorylation level of 202.4 pmol/mg protein, the velocity of phosphoenzyme hydrolysis is 6.6 nmol/mg·h. Considering the variability of the data, it is reasonable to conclude that the phosphoenzyme is kinetically competent to account for a major portion of the total ATP hydrolytic activity of this preparation.

When the phosphorylated protein was analysed by LDS gel electrophoresis, a labeled polypeptide with a mol wt of about 100,000 D was observed (Fig. 6). Evidence that this polypeptide represented the phosphorylated intermediate was shown by its phosphorylation being enzymic in nature, rapidly turning over, and Mg-dependent. Since the phosphorylated intermediates of the transport ATPases from animal (8, 10, 25) and fungal (12) cells occur on a polypeptide of about this mol wt, structural similarity between these transport enzymes and the plasma membrane ATPase of higher plants may be implied.

The general properties of the phosphoenzyme for the red beet plasma membrane ATPase are similar to those observed for the corn root plasma membrane ATPase (5, 6). However, the beet root preparation shows about a 4-fold higher steady-state phosphorylation level than the corn root preparation (5). Inasmuch as both preparations have roughly similar ATPase activities at 38°C (and possibly also at 40 μM ATP and ice temperature), a dramatic difference might be expected for the observed dephosphorylation rate constant. This indeed is the case. In the absence of KCl, the dephosphorylation rate constant for the corn root enzyme is 0.028 s^{-1} (6) while this value is only 0.0091 s^{-1} for the red beet enzyme. Therefore, no simple relationship between ATPase activity and phosphorylation level can be assumed when plasma membrane fractions from different plant species are compared.

The high steady-state phosphorylation level and slow turnover make the red beet plasma membrane fraction an excellent system to study ATPase phosphorylation. High phosphorylation levels imply a greater incorporation of radioactive label for a given amount of protein and thus a higher signal to noise ratio. Slow turnover allows the radioactive label to be retained over greater periods of time when the dephosphorylation reaction is studied, giving greater precision in estimating rate constants. Further studies are underway with the red beet system to identify the specific amino acid moiety of the protein phosphorylated and elucidate the reaction mechanism of the enzyme.

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