Phosphorylation and Dephosphorylation Reactions of the Red Beet Plasma Membrane ATPase Studied in the Transient State

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ABSTRACT

The reaction mechanism of the solubilized red beet (Beta vulgaris L.) plasma membrane ATPase was studied with a rapid quenching apparatus. Using a dual-labeled substrate ([γ-32P]ATP and [5',8-3H]ATP), the pre-steady-state time course of phosphoenzyme formation, phosphate liberation and ADP liberation was examined. The time course for both phosphoenzyme formation and ADP liberation showed a rapid, initial rise while the time course for phosphate liberation showed an initial lag. This indicated that ADP was released with formation of the phosphoenzyme while phosphate was released with phosphoenzyme breakdown. Phosphoenzyme formation was Mg²⁺-dependent and preincubation of the enzyme with free ATP followed by the addition of Mg²⁺ increased the rate of phosphoenzyme formation 10-fold. This implied that phosphoenzyme formation could result from a slow reaction of ATP binding followed by a more rapid reaction of phosphate group transfer. Phosphoenzyme formation was accelerated as the pH was decreased, and the relationship between pH and the apparent first-order rate constants for phosphoenzyme formation suggested the role of a histidyl residue in this process. Transient kinetics of phosphoenzyme breakdown confirmed the presence of two phosphoenzyme forms, and the discharge of the ADP-sensitive form by ADP correlated with ATP synthesis. Potassium chloride increased the rate of phosphoenzyme turnover and shifted the steady-state distribution of phosphoenzyme forms. From these results, a minimal catalytic mechanism is proposed for the red beet plasma membrane ATPase, and rate constants for several reaction steps are estimated.

The plasma membrane ATPase has the capability for coupling ATP hydrolysis to proton translocation (20, 25, 30). In vivo, it is proposed that this enzyme provides the driving force for solute transport at the plasma membrane, in the form of an inwardly directed proton electrochemical gradient (4, 19, 27). Studies on the mechanism of this important transport enzyme have shown that the overall reaction of ATP hydrolysis proceeds with the formation of a covalent phosphorylated intermediate (6, 9, 24, 31). From studies on the plasma membrane ATPase from red beet (Beta vulgaris L.) storage tissue, it was shown that this intermediate was composed of at least two phosphoenzyme forms which differ in their sensitivity to exogenous ADP or KCl (5). One phosphoenzyme form, designated EP*, was rapidly discharged by the addition of exogenous ADP while the other phosphoenzyme form, designated EP, was unaffected by ADP. 

While the addition of 50 mM KCl enhanced the rate of EP breakdown, the effect of KCl on EP* was not investigated. Potassium has been shown to stimulate both ATP hydrolysis (see Leonard [19] and references therein) and phosphoenzyme turnover (5, 7, 9, 24) in plant plasma membrane ATPase preparations. The occurrence of these phosphoenzyme forms and their association with a 100 kDa catalytic subunit (7, 9, 24, 31) is similar to what has been observed for other ATPases such as the animal cell Na,K-ATPase (16, 29) and sarcoplasmic reticulum Ca²⁺-ATPase (15, 29), which belong to the E₁E₂ class of transport enzyme (see Pedersen and Carafoli [21] and Tonomura [29] and references therein).

In our previous studies on the mechanism of the red beet plasma membrane ATPase, the manner in which phosphorylation reactions were carried out seriously limited the kinetic information which we could obtain (see Briskin and Poole [9] for details). The reactions were performed manually at ice temperature, over a time range extending from 3 to 60 s. Under these conditions, the ATP hydrolytic reaction was slowed sufficiently that steady-state phosphoenzyme levels and dephosphorylation kinetics could be examined using [γ-32P]ATP (9-11). However, it was difficult to carry out a large number of determinations and treatments because of limitations associated with the methods used in the processing of samples prior to the determination of protein-associated radioactivity and released ⁳²P. Furthermore, it was not possible to carry out a detailed examination of phosphoenzyme formation in the pre-steady state because, even at ice temperature, this reaction was too rapid to be accurately observed by this approach (9).

In this communication, a fast reaction technique (rapid quenching) was used to study the catalytic mechanism of the red beet plasma membrane ATPase. This approach allowed a characterization of partial reactions for both phosphoenzyme formation and breakdown in the catalytic cycle of the enzyme. From the results of this study, a reaction scheme is proposed for the enzyme, and rate constants are estimated for several of the proposed reaction steps.

MATERIALS AND METHODS

Preparation of the Solubilized Red Beet Plasma Membrane ATPase. Red beet (Beta vulgaris L., cv Detroit Dark Red) storage roots were purchased commercially. The tops of the plants were removed and the storage tissue was placed in moist vermiculite and maintained at 2 to 4°C until use. All storage tissue used was stored at least 10 d to ensure uniformity in membrane isolation (22). Plasma membrane enriched fractions were isolated according to Briskin and Poole (8, 11) with the minor modifications described in the previous paper (6). The membranes banding at the 28%/35% (w/w) sucrose gradient interface were recovered using a Pasteur pipet, diluted to 70 mL with suspension buffer, and then centrifuged at 80,000 g for 30 min. The final membrane pellet was suspended to a protein concentration of about 8 to 10
absorbance
addition
range

enzyme and substrate solutions were mixed and allowed to react of production was found. Thus, the instrument can be used in the

as shown in Figure 1, when the reaction was carried out with

by mixing initiating the reaction (90-1200 ms), the reaction was quenched

by mixing push-type solenoids capable of developing 70 oz of

of push at 110 v A.C. Timing was controlled by a solid state
timing circuit containing N556 TTL dual timing chips connected

in the monostable mode. All circuit components were 1% toler-
ance grade, and the output from the timing circuit was monitored

using a Tektronix storage oscilloscope. The coupling between

the timing circuit and the solenoid syringe drivers was mediated

through the use of a solid-state optical relay system containing

light emitting diodes. This optical coupling ensured complete

isolation of logic circuitry from the line current-driven, fluid-

handling devices. Temperature control was maintained by a

water-jacket and pumped circulator. Complete details on the
design and construction of this apparatus are available upon
request.

in order to test the apparatus, the alkaline hydrolysis reaction
of 2,4-dinitrophenyl acetate was measured at 10°C as described
by Barman and Guthreind (2). The reaction was started by the

rapid mixing of 150 µL of 0.6 N NaOH with 150 µL of 1.25 mM
2,4-dinitrophenyl acetate in 3.8 mM HCl. At various times after

initiating the reaction (90-1200 ms), the reaction was quenched

by mixing with 800 µL of 0.5 N HCl. Subsequently, 0.5 mL of 2
M K-acetate, pH 4.5, and 3 mL of H2O were added to each

quenched reaction sample. The absorbance of the 2,4-dinitro-
phenol released by alkaline hydrolysis was measured at 360 nm.

As shown in Figure 1, when the reaction was carried out with

this apparatus, a first-order kinetic profile for 2,4-dinitrophenol
production was found. Thus, the instrument can be used in the

study of ATPase reactions, equal volumes (150 µl) of

phosphoenzyme

enzymes and substrate solutions were mixed and allowed to react

for 90 to 1200 ms at 10°C, and the reaction was then stopped by

rapid mixing with 800 µL of a quench reagent containing ice
cold 15% TCA, 40 mM NaH2PO4, 5 mM Na2HPO4, and 1 mM
Na2ATP. Phosphorylation and dephosphorylation reactions were

were carried out using 70 to 100 µg of solubilized ATPase preparation,

100 µM [γ-32P]ATP (0.5 Ci/mmol), 30 mM Tris-Mes (assay pH),

and other reagents as indicated in the figure legends for each

individual experiment. Dephosphorylation was initiated by the

rapid mixing of a 100-fold excess of unlabeled ATP (Tris salt,

assay pH) containing additional reagents as indicated. The

quenched reaction solution was expelled into a chilled test tube

and then transferred to a chilled 1.5 mL microfuge tube. Following

centrifugation in a microfuge (fixed angle rotor) at full speed

for 15 min (2-4°C), the supernatant was decanted and, in some

experiments, used for the determination of released ADP or

inorganic phosphate. The pellet was initially suspended in 100
µL of the quenching solution, an additional 900 µL of quenching
solution was added, and the tubes were centrifuged in the micro-
fuge. This washing procedure was repeated four times, and the

radioactivity associated with the final pellet was determined by

liquid scintillation spectroscopy.

Measurement of ADP and Pi Produced during ATP Hydroly-
sis. The quantitation of radioactive ADP and Pi produced during

phosphorylation reactions was carried out by TLC as described
by Bronnikov and Zakharov (12). Ten µL aliquots of the super-

natant, produced by centrifugation of the quenched reaction

samples, were spotted onto silica gel thin layer plates containing

UV indicator. Chromatography was carried out in a solvent

consisting of dioxane, isopropanol, 25% ammonium hydroxide,

and H2O (4:2:3:4, v/v) and the location of nucleotide spots was
detected under UV light. In this system, 32Pi remains close to the

origin while ATP and ADP move substantially with the solvent

resulting in a migration of 0.42 and 0.58 (Rv values), respectively.
The identity of the spots was verified by comigration with
authentic standards. The radioactive spots were cut from the
plate and counted by liquid scintillation spectroscopy in 5 mL
of Aquasol (New England Nuclear).

Protein Assay. Protein was determined by the method of
Bradford (3) using BSA as a standard.

RESULTS AND DISCUSSION

Time Course for Phosphoenzyme Formation, ADP Liberation
and Pi Liberation During the Pre-Steady-State Phase of
Reaction. The time course for phosphoenzyme formation, ADP
liberation, and Pi liberation by the red beet plasma membrane
ATPase was examined by carrying out reactions over a time
interval of 95 to 1200 ms (Fig. 2). The solubilized ATPase was
used in kinetic studies because it had a greater activity and higher
phosphorylation level than the native enzyme used in previous
work (11). In addition, the use of an enzyme preparation not
associated with membranes ensured that uniform access to ligand
binding sites would occur. In order to simultaneously monitor
inorganic phosphate and ADP liberation during the initial phase
of the phosphorylation reaction, both [γ-32P]ATP and [5',8-3H]
ATP were used as substrates, and the released [3H]ADP and 32Pi
were quantified following separation by TLC.

When the ATP hydrolytic reaction was initiated by mixing
the enzyme preparation (78 µg protein) with 100 µM Mg:ATP ([γ-32P];
[5',8-3H]) ATP were used as substrates, and the released [3H]ADP and 32Pi
were quantified following separation by TLC.

Fig. 1. Alkaline hydrolysis of 2,4-dinitrophenyl acetate at 10°C meas-
ured with the rapid quenching apparatus. The reaction was initiated by the
rapid mixing of 0.6 N NaOH with 1.25 mM 2,4-dinitrophenyl acetate
in 3.8 mM HCl. The reactions were quenched over the indicated time
range (90-1200 ms) by rapid mixing with 0.5 N HCl. Following the
addition of 0.5 mL of 2 M K-acetate, pH 4.5, and 3 mL H2O, the
absorbance was determined at 360 nm. The data are plotted in a
semilogarithmic manner.
profiles for ADP and Pi production during the pre-steady-state phase of the reaction indicate the order in which the products are released from the enzyme (see Hiromi [14] and references therein). The lag in the inorganic phosphate production indicates that it was being produced as a result of phosphoenzyme breakdown while the burst in ADP production indicates that this product was being produced with the formation of the phosphoenzyme intermediate. The profiles for ADP and inorganic phosphate production would suggest that ATP hydrolysis occurs by the following minimal reaction path:

\[ E + ATP \rightarrow E-P + ADP \]
\[ E-P + H_2O \rightarrow E + P_i \]

where E and E-P represent ATPase and phosphoenzyme, respectively. In previous studies on the mechanism of the red beet plasma membrane ATPase (5, 9), it was implied that inorganic phosphate was produced as a result of phosphoenzyme breakdown because of the close correspondence between the measured rate of Pi production and the rate predicted by multiplying the level of steady state phosphoenzyme by its rate of turnover ("kinetic competence"). However, in the previous work (5), it was not possible to determine the point in the reaction where ADP was being released.

Role of Magnesium in the Formation of Phosphoenzyme. In previous studies (10), it was observed that an addition of excess EDTA to steady-state phosphoenzyme of the red beet ATPase (formed with Mg:ATP) caused dephosphorylation similar to that observed by adding excess unlabeled ATP. From this observation, it was proposed that Mg\(^{2+}\) was required for the formation of phosphoenzyme but not for its subsequent turnover. Using the rapid quench apparatus, the role of Mg\(^{2+}\) in phosphoenzyme formation was directly examined for the solubilized red beet ATPase (Fig. 3). When the enzyme was mixed with 100 \(\mu\)M [\(^{32}\)P]ATP alone, phosphoenzyme formation did not occur. However, when 100 \(\mu\)M MgSO\(_4\) was included with the radioactive substrate and mixed with the enzyme, the phosphoenzyme formed rapidly (\(k = 3.24\) s\(^{-1}\)), consistent with a requirement for Mg\(^{2+}\) in the phosphorylation reaction. Preincubating the enzyme with 100 \(\mu\)M MgSO\(_4\) for 20 s and then initiating the reaction by rapid mixing with 100 \(\mu\)M [\(^{32}\)P]ATP did not affect the rate of phosphoenzyme formation. However, if the enzyme was preincubated with [\(^{32}\)P]ATP for 20 s and the reaction initiated by rapid mixing with 100 \(\mu\)M MgSO\(_4\), the rate of phosphoenzyme formation (\(k = 7.46\) s\(^{-1}\)) was increased about 2.3-fold. This result can be explained if the overall phosphorylation reaction is considered in terms of two consecutive processes:

\[ E + ATP \rightarrow E(\text{ATP}) \]
\[ E(\text{ATP}) \rightarrow E(\text{ATP}) + Mg^{2+} \]
\[ \text{E-P + ADP} \]

where E(\text{ATP}) represents an enzyme-substrate complex involving ATP bound to the ATPase. In this scheme, the ATPase can bind free ATP to form the complex E(\text{ATP}); however, the presence of Mg\(^{2+}\) is required for the transfer of the terminal phosphate group of ATP to form the phosphoenzyme. Compared to the Mg\(^{2+}\)-dependent phosphoryl transfer reaction, the binding of ATP to the enzyme would be a relatively slow process. Therefore, if the enzyme was pretreated with free ATP to form E(\text{ATP}) and the reaction initiated with Mg\(^{2+}\), a more rapid initial rate of phosphoenzyme formation would occur than when the slow binding of ATP limits the initial phase of the reaction. The ability of the red beet ATPase to bind free ATP and carry out enzyme phosphorylation upon the addition of Mg\(^{2+}\) was suggested by earlier studies of the native enzyme in plasma membrane fractions (5, 10). When the enzyme was pretreated with free [\(^{32}\)P]ATP and excess unlabeled ATP containing MgSO\(_4\), an additional burst of phosphorylation was observed.

Effect of pH on the Formation of Phosphoenzyme. The pH optimum for red beet plasma membrane ATPase activity has been shown to be about 7.0 when assayed in the absence of 50 mM KCl with either 3 mM Mg:ATP at 38°C (8, 11) or 40 \(\mu\)M Mg:ATP at 5°C (5). The assay pH also affects the level of steady-state phosphoenzyme and its rate of turnover. Steady-state phos-
phosphoenzyme was maximal at pH 6.0 while the rate of phosphoenzyme turnover was increased as the pH was increased from pH 5.5 to 8.5 (5). Using transient state kinetic methods, the effect of pH on the formation of phosphoenzyme in the solubilized ATPase was examined (Fig. 4). As the pH was increased from 5.5 to 8.0, both the initial rate of phosphoenzyme formation and the steady-state phosphoenzyme level were decreased. Since the level of phosphoenzyme present during the steady-state turnover of the intermediate reflects the balance of phosphoenzyme formation and breakdown, the reduction of phosphoenzyme level with increasing pH could reflect the combined effects of an increasing rate of phosphoenzyme turnover (5) and a decreasing rate of phosphoenzyme formation. If the initial portion of the curves for phosphoenzyme formation are assumed to follow first order kinetics, then apparent first-order rate constants can be calculated for the rate of phosphoenzyme formation at each pH value. When these rate constants were plotted as a function of pH (Fig. 5), a sigmoid curve was produced that resembled the titration curve for a single ionizable group with a pKₐ between 6 and 7. Since this is in the range of the pKₐ of the imidazole side chain of histidine, these data could imply that such a group may have a role in controlling the rate of phosphoenzyme formation (see Tipton and Dixon [28] and references therein).

**Fig. 4.** Effect of pH on the formation of the phosphorylated intermediate of the red beet plasma membrane ATPase. Phosphorylation reactions were carried out at 10°C by mixing 150 μL of 200 mM [γ-32P]ATP, solubilized red beet ATPase (78 μg protein) with 150 μL of 200 mM MgSO₄, and 60 mM Tris-Mes at the indicated pH. The reactions were quenched, and the amount of phosphoenzyme intermediate was determined as described in "Materials and Methods."

**Fig. 5.** Plot of the apparent first-order rate constants for phosphoenzyme formation as a function of pH. The apparent first-order rate constants were calculated from the initial portions of the time courses on Figure 4 and were plotted as a function of pH.

**Dephosphorylation of the Phosphorylated Intermediate.** In order to observe the dephosphorylation reactions of the red beet plasma membrane ATPase, the enzyme was phosphorylated to a steady-state level (3 s) in the presence of 100 μM Mg[γ-32P]ATP at pH 6.5, and then a 100-fold excess of unlabeled ATP (with or without additional reagents) was rapidly mixed with the enzyme. Following addition of unlabeled ATP, the reactions were stopped at various times by the rapid addition of the TCA quenching reagent. This experimental approach allowed an examination of the effects of different reaction ligands on the dephosphorylation steps as well as an analysis of the interconversion of phosphoenzyme forms.

As shown in Figure 6, when this type of “pulse/chase” experiment was carried out in the absence of KCl, a single-exponential decline (k = 3.06 s⁻¹) of the phosphorylated intermediate was observed. When 15 mM unlabeled ADP was included with the unlabeled ATP chase, the time course for dephosphorylation became biphasic, containing an initial rapid phase (k = 9.19 s⁻¹) followed by a slower phase (k = 3.11 s⁻¹), which occurred at a rate similar to the dephosphorylation observed in the absence of ADP. Based upon previous studies on the mechanism of the red beet plasma membrane ATPase (5), it was suggested that the phosphorylated intermediate was actually present in two forms which could be distinguished by differences in sensitivity to the addition of exogenous ADP. One form (designated EP⁺) was discharged by the addition of ADP while the other form (designated EP⁻) was insensitive to ADP. The presence of these two phosphoryl enzyme forms in the catalytic cycle is a common feature of those ATPases which belong to the E₁,E₂ class of enzyme (see Pedersen and Carafoli [21] and Tonomura [29] and references therein). Therefore, this rapid phase of phosphoenzyme decline that was produced by including ADP with the unlabeled ATP chase most likely reflects a rapid, selective discharge of EP+. The extent of this discharge was dependent upon the concentration of ADP present in the unlabeled ATP chase.
and no further increases were observed when the ADP concentration was 15 mM or greater (data not shown). Therefore, the data presented in Figure 6 represent a profile for the maximal discharge of EP*. According to the mathematical analysis described below, extrapolation of the slow linear phase of phosphoenzyme breakdown to zero time (under conditions where EP* is fully discharged) should give an estimate of the percentage of total EP. Under the conditions of this assay (100 μM Mg:ATP, pH 6.5, 10°C), the total phosphorylated intermediate is composed of about 65% EP* and about 35% EP.

A characteristic property of the plasma membrane ATPase from red beet (8) and other plant species (see Leonard [19] and references therein) is the stimulation of activity by K+. Initial studies on the role of K* in the mechanism of the plant plasma membrane ATPase have suggested that K+ stimulation of ATPase activity most likely occurs through an increase in the rate of phosphoenzyme turnover (7, 9, 24). However, these studies only examined the effects of K* on the phosphoenzyme intermediates formed in the absence of K+, by carrying out a pulse-chase type experiment and including KCl in the unlabeled ATP chase. In order to examine the overall effects of K* on phosphoenzyme turnover and the relative levels of the two phosphoenzyme forms, a pulse-chase experiment was performed where the red beet ATPase was initially phosphorylated to a steady-state level in the presence of 100 μM Mg:ATP[γ-32P] and 50 mM KCl at pH 6.5. When dephosphorylation was examined by the addition of excess unlabeled ATP (with 50 mM KCl), the phosphorylated intermediate showed a single-exponential decline (k = 4.58 s⁻¹) which is about 1.5-fold faster than dephosphorylation in the absence of KCl (Fig. 7). This would be consistent with previous observations that K+ appears to increase the rate of phosphoenzyme turnover. While the inclusion of 15 mM ADP with the unlabeled ATP chase caused the dephosphorylation time course to assume a biphasic profile, the maximum amount of EP* was substantially reduced. Extrapolation of the slower phase of phosphoenzyme decline (k = 4.81 s⁻¹) to zero time would suggest that EP* and EP represent about 21% and 79% of the total phosphorylated intermediate. Therefore, in addition to increasing the rate of phosphoenzyme turnover, it would appear that KCl causes a redistribution of the relative amount of each phosphoenzyme form in the catalytic cycle of the enzyme.

Phosphoenzyme Form. While the above experiments and a previous study (5) indicated that the addition of ADP caused the rapid discharge of a portion of the total phosphoenzyme, the nature of this effect was uncertain. The addition of ADP could accelerate a direct breakdown of EP* to release inorganic phosphate or be involved in ATP resynthesis by accepting enzyme-bound phosphate. In order to test this latter possibility, experiments were carried out as above except that the enzyme was phosphorylated to steady-state with unlabeled Mg:ATP, and the unlabeled ATP chase contained [γ-32P]ADP. The reactions were quenched at increasing times after the excess ATP/[32P]ADP addition and following centrifugation, aliquots of the supernatant were subjected to thin layer chromatography to allow separation of ADP and ATP. The spots that comigrated with authentic ATP were cut from the TLC plate and counted by liquid scintillation spectroscopy.

For phosphorylation reactions carried out either in the absence or presence of 50 mM KCl, it was possible to detect rapid ATP production following the ATP[32P]ADP addition (Fig. 8). For both reactions, maximal ATP production occurred within about 150 ms. While these results can only be considered as representing a qualitative estimate of ATP resynthesis, due to uncertainties associated with rehydrolysis and recovery, the relative amounts of [32P]-label recovered as ATP qualitatively correlate with the relative levels of EP* present under the conditions of each reaction. This result would suggest that the discharge of EP* involves ATP resynthesis by the transfer of enzyme-bound phosphate to the added exogenous ADP.

Mathematical Analysis for Dephosphorylation Kinetics and the Estimation of Rate Constants. For the analysis of the dephosphorylation reactions and the estimation of kinetic rate constants, the mathematical approach described by Klodos et al. (18) and Helmich-de Jong et al. (13) was utilized. In this scheme, a minimal model that describes the above observations would be:

\[
\frac{k_1}{k_2} \text{EP*} \xrightarrow{k_2} \text{EP} \xrightarrow{k_3} \text{KCl}
\]

![Fig. 7. Dephosphorylation reactions of the red beet plasma membrane ATPase measured in the presence of 50 mM KCl. The phosphoenzyme was formed at 10°C by mixing 150 μL of the enzyme (98 μg protein) with 150 μL of 200 μM [γ-32P]ATP, 200 μM MgSO4, 80 mM Tris-Mes (pH 6.5), and 100 mM KCl and was allowed to reach steady-state levels (3 s). Dephosphorylation of the ATPase was examined in the absence and presence of exogenous ADP as described for Figure 6. The rate constants for the decline of phosphorylated protein were determined as -2.303 (slope).](image-url)

![Fig. 8. Synthesis of ATP during the ADP-dependent discharge of the phosphorylated intermediate. A steady-state level of unlabeled phosphorylated intermediate (3 s) was formed for the ATPase (106 μg protein) in the absence or presence of 50 mM KCl as described for Figures 6 and 7. Dephosphorylation of the enzyme was initiated by rapid mixing with 100 μL of 40 mM ATP (Tris salt, pH 6.5) containing 60 mM [γ-32P]ADP (50 mCi/mmol), and the reaction was quenched at the indicated time by the addition of 700 μL of 17% TCA containing 40 mM NaH2PO4, Na2HPO4, and 1 mM Na2ATP. Following centrifugation in a microfuge, 10 μL aliquots of the supernatant were subjected to TLC as described in “Materials and Methods” to determine the amount of radioactive label recovered as ATP.](image-url)
For this kinetic relationship the relative amounts of the two phosphoenzyme forms can be described by the equations:

\[
d(EP^*)/dt = k_2[EP] - (k_1 + k_3)[EP^*] \tag{1}
\]

\[
d[EP]/dt = k_2[EP^*] - (k_2 + k_3)[EP]. \tag{2}
\]

If this relationship is considered as a two-compartment model without input to EP* (i.e. the 32P-labeled ATP substrate is effectively diluted out by the unlabeled ATP chase), it can be shown that the biphase decline of phosphoenzyme that occurs during dephosphorylation in the presence of excess ATP and ADP can be described as the sum of two exponentials:

\[
[EP]_{\text{total}} = [EP^*] + [EP] = Ae^{-\alpha t} + Be^{-\beta t} \tag{3}
\]

where the slow phase of phosphoenzyme decline is defined by \( A \) and \( \alpha \) and the fast phase of phosphoenzyme decline is defined by \( B \) and \( \beta \). These four parameters (\( A, B, \alpha, \beta \)) can be defined in terms of the kinetic rate constants and relative concentrations of the two phosphoenzyme forms (see "Appendix"). Since these two phases of phosphoenzyme decline occur with sufficient difference in rate to allow a graphic separation (\( \alpha < \beta \)), \( A \) can be determined by extrapolation of the slow phase of phosphoenzyme decline to zero time. With the data for dephosphorylation being plotted in terms of the log % EP remaining versus time (Figs. 6 and 7), this value for \( A \) gives an estimate of the percentage of the total phosphoenzyme present as EP under these conditions (see "Appendix"). From this value, the percentage of phosphorylated enzyme present as EP* can also be calculated (i.e. \( 100 - \% \) EP). It should also be noted that since EP represents the only phosphoenzyme form that has both "input" and "output" in this model the steady-state approximation \([dEP/dt = 0]\) can be applied to Equation 2 so that:

\[
[EP^*]/[EP] = (k_2 + k_3)/k_2 \tag{4}
\]

When dephosphorylation is carried out with excess unlabeled ATP in the absence of ADP, a single-exponential decline of the phosphorylated intermediate is observed. While this would initially appear to be inconsistent with this type of model, these results could occur if, under these conditions, \( k_1 = k_3 \) (18) or \( k_1 = k_2 \) is so small that turnover is primarily determined by \( k_3 \). In either case, the decline of phosphoenzyme could then be described in terms of a single compartment model:

\[
[EP]_{\text{total}} = Ae^{-\alpha t} \tag{5}
\]

where \( A \) would represent the total phosphoenzyme present at zero time and \( \alpha \) would equal \( k_3 \). However, when dephosphorylation is carried out in the presence of excess ADP so that EP* is fully discharged, \( k_1 \) is maximal and greater than \( k_2, k_3 \), and \( k_3 \) so that under these conditions, \( \alpha = k_3 + k_5 \) (see Refs. 13 and 18 for detailed discussion).

Using the above methods of analysis, rate constants and phosphoenzyme levels were estimated from the kinetics of dephosphorylation carried out in the absence or presence of 50 mM KCl (Table 1). Due to uncertainties about the magnitude of direct EP* breakdown to release inorganic phosphate, no attempt was made to determine \( k_1 \). In the absence of KCl, about 65% of the total phosphoenzyme intermediate is present as the EP* form. Since \( k_2 \) is small relative to \( k_3 \), this would imply that during steady-state turnover in the absence of KCl phosphoenzyme formation from ATP is substantial compared to \( k_3 \) and \( k_5 \). The inclusion of 50 mM KCl not only increased the rate of phosphoenzyme breakdown (\( k_3 \)), as previously described (7, 9), but also caused a displacement in the relative amounts of the two phosphoenzyme forms. In the presence of 50 mM KCl, most of the phosphorylated intermediate was now present as the EP form, accounting for about 79% of the total. This shift in the relative amounts of the two phosphoenzyme forms appears to be related to a K+-dependent change in \( k_3 \). The presence of 50 mM KCl caused an 11-fold increase in \( k_3 \) and changed the ratio of \( k_2/k_3 \) from 33.4 to 80.4. Thus, this effect of KCl on the steady-state phosphorylation of the phosphoenzyme forms appears to be a major aspect of the interaction of this cation with the enzyme when compared to the 1.5-fold increase in the rate of phosphoenzyme turnover.

### Table 1. Relative Levels of Phosphoenzyme Intermediate Forms and Rate Constants Determined from an Analysis of Dephosphorylation Kinetics

<table>
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<th>Condition</th>
<th>Phosphoenzyme Intermediates*</th>
<th>Kinetic Rate Constants*</th>
</tr>
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<tr>
<td>EP*</td>
<td>EP</td>
<td></td>
</tr>
<tr>
<td>% total phosphoenzyme</td>
<td>100%</td>
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</tr>
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<td>-KCl</td>
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*See text and "Appendix" for a description of the mathematical methods used in the calculation of these parameters from plots of dephosphorylation kinetics (Figs. 6 and 7).

### GENERAL DISCUSSION

A characteristic feature of the plasma membrane ATPase of higher plant cells is the formation of a phosphoenzyme intermediate during the course of ATP hydrolysis (4, 5, 7, 9, 24, 31). In this communication, a transient state kinetic study of the formation (phosphorylation) and breakdown (dephosphorylation) of the phosphoenzyme was carried out for the red beet plasma membrane ATPase using a rapid quenching apparatus. This kinetic technique has proven useful in the previous characterization of the reaction mechanism of animal cell Na, K-ATPase (see Kanazawa et al. [17] and Tonomura [29] and references therein), the sarcoplasmic reticulum Ca2+-ATPase (see Tonomura [29] and references therein), and the gastric mucosal H,K-ATPase (26, 32). The results of this study confirmed several aspects of the mechanism of the ATPase (presence of phosphoenzyme intermediate, binding of free ATP, presence of EP* and EP phosphoenzyme forms) suggested from previous studies on the enzyme using different experimental approaches (5, 9, 10). The enhancement of phosphoenzyme formation at lower pH values together with the previous observation that phosphoenzyme breakdown is accelerated at high pH values (5) suggests that the pH optimum of 7.0 observed for ATP hydrolysis in the absence of KCl represents a compromise between pH effects on these two processes. Enhancement of phosphoenzyme formation at low pH could also imply that a protonation event may determine this process. When the rate constant for phosphoenzyme formation was plotted as a function of pH, a profile was produced suggesting the role of a histidyl moiety in this process. This amino acid could either have some role in ATP (or MgATP) binding to the enzyme or be involved in protonation reactions relevant to ATPase-mediated proton translocation across the membrane.

The data obtained from this study allow a further definition of the mechanism of the red beet plasma membrane ATPase at the level of partial reactions which constitute the overall process of ATP hydrolysis. A minimal catalytic cycle for the plasma membrane ATPase incorporating these partial reactions is presented in Figure 9. Kinetic rate constants have been calculated for several of the steps in this proposed kinetic mechanism (Table 1). Within this scheme, it should be noted that the pathway from the free enzyme (E) to EP* via E(APT) most likely occurs only in these types of in vitro kinetic studies because the true physiological substrate for the enzyme is the MgATP complex (1, 19).
The proposal that the EP* phosphoenzyme form represents the phosphorylated intermediate that is formed first from the reaction of the enzyme with MgATP is based upon the observed ADP sensitivity of this intermediate form (5, 21, 29). That the sensitivity of this intermediate to ADP is related to ATP synthesis argues for the reversal of the reaction where this phosphoenzyme is formed from the free enzyme and MgATP. The degree to which this first EP* phosphorphanyme form breaks down directly to release phosphate and regenerate the free enzyme is uncertain in this study. However, one explanation for the observed single-exponential decay of phosphoenzyme during a pulse-chase experiment with excess unlabeled ATP would be to have an equivalent rate of breakdown of both the EP* and EP phosphoenzyme forms (i.e. $k_1 = k_5$). The degree to which the direct breakdown of EP* contributes to phosphate production in both the absence and presence of KCl will be the focus of future kinetic studies.

While our previous studies had indicated that K* stimulation of ATPase activity occurred through an increase in the rate of phosphoenzyme turnover (5, 9, 10), this effect appears to be relatively minor when compared to the effect of this cation on the displacement of the two phosphoenzyme forms. These multiple effects of K* within the mechanism of the ATPase may have some bearing on the complex nature of the kinetic profile observed for K*-stimulation of ATP hydrolysis (see Leonard [19] and references therein). Based upon the preliminary observation that K* stimulated the rate of phosphoenzyme turnover for the plant ATPases and that this effect was similar to that observed for other ATPases (i.e. Na,K-ATPase; gastric H,K-ATPase) that transport this cation directly (23, 29), it was suggested that the plant ATPase might mediate direct K* transport (see Briskin [4] and references therein). Clearly, this proposal needs to be reevaluated in terms of the current kinetic observations on the mechanism of the enzyme. Due to the importance of K* in the physiology of plant cells (see Leonard [19] and references therein), there is currently a great need to determine whether the plasma membrane ATPase has the capacity to directly transport K*.

**APPENDIX**

When the enzyme is phosphorylated to steady-state levels and then the radiolabeled ATP is diluted out, the reaction scheme:

$$k_1 \quad \text{EP*} \quad k_2 \quad k_3 \quad \text{EP} \quad k_2$$

outlines the possible transitions for the EP* and EP phosphoenzyme reaction states assuming that the production of labeled EP* is prevented (100-fold dilution of the ATP3P label) and minimal phosphorylation of the enzyme occurs from inorganic phosphate (DP Briskin, unpublished results). The kinetic descriptions of the possible transitions for EP* and EP are:

$$d[EP^*/dt] = k_2[EP] - (k_1 + k_2)[EP^*] \quad (A1)$$

$$d[EP]/dt = k_5[EP^*] - (k_2 + k_3)[EP]. \quad (A2)$$

As pointed out by Klodos et al. (18), this can be described according to a two-compartment model with no input, so that the solutions to these equations are:

$$[EP^*] = C_1e^{-\alpha t} + C_2e^{-\beta t} \quad (A3)$$

$$[EP] = C_3e^{-\alpha t} + C_4e^{-\beta t} \quad (A4)$$

In terms of the total phosphorylated intermediate, which is the component measured during transient kinetic experiments:

$$[EP]_{\text{total}} = [EP^*] + [EP] = Ae^{-\alpha t} + Be^{-\beta t} \quad (A5)$$

where $A = C_1 + C_3$, $B = C_2 + C_4$ and $A + B = [EP]_{\text{total}}$ at $t = 0$.

The components of these equations are defined as:

$$\alpha = 1/2(k_{-1} + k_2 + k_{-2} + k_3 - h) \quad (A6)$$

$$\beta = 1/2(k_{-1} + k_2 + k_{-2} + k_3 + h) \quad (A7)$$

$$C_1 = \frac{2k_2[EP]_o + (h - u)[EP^*]_o}{2h} \quad (A8)$$

$$C_2 = [EP]_o - C_1 \quad (A9)$$

$$C_3 = C_1 (h + u)/2k_2 \quad (A10)$$

$$C_4 = [EP]_o - C_3 \quad (A11)$$

where:

$$h = (u^2 + 4k_2k_{-3})^{1/2} \quad (A12)$$

$$u = (k_{-1} + k_2) - (k_{-2} + k_3) \quad (A13)$$

and [EP*]o and [EP]o refer to the amounts of each phosphoenzyme form present at steady-state before the unlabeled ATP chase. The components $A$ and $B$ in Equation A5 can be defined from Equations A6 to A13 as:

$$A = \frac{(h - u + 2k_2)[EP^*]_o + (h + u + 2k_{-3})[EP]_o}{2h} \quad (A14)$$

$$B = \frac{(h + u - 2k_2)[EP^*]_o + (h - u - 2k_{-3})[EP]_o}{2h} \quad (A15)$$

Under the conditions of the unlabeled ATP + ADP chase, no further decrease in EP* occurred when the ADP concentration exceeded 15 mm, and the slow phase of phosphoenzyme breakdown was only slightly larger than that observed in the absence of ADP. According to Klodos et al. (18) and Helmich-de Jong et al. (13), an analysis of the expression for $A$ (Equation A14) shows that as $k_{-1}$ increases, $A$ decreases, and that $dA/dk_{-1}$ approaches 0 as $k_{-1}$ approaches $\infty$. Thus, the decrease in $A$ with increasing $k_{-1}$ (i.e. increasing exogenous ADP) will reach a plateau, and, at this level, extrapolation of the slow phase of phosphoenzyme decline to zero time will yield a value which approximates the relative level of the EP phosphoenzyme form.

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LITERATURE CITED


