Plant Physiol. (1988) 88, 84-91
0032-0889/88/88/0084/08/$01.00/0

Phosphorylation and Dephosphorylation Reactions of the Red Beet Plasma Membrane ATPase Studied in the Transient State[1]

Received for publication December 11, 1987 and in revised form April 14, 1988

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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-^{32}P]ATP and [5',8-^{3}H]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 timecourse 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 Mg^{2+}-dependent and preincubation of the enzyme with free ATP followed by the addition of Mg^{2+} increased the rate of phosphoenzyme formation 2.3-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*,[2] 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 kD 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 Ca^{2+}-ATPase (15, 29), which belong to the E_1E_2 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-^{32}P]-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 ^{32}Pi. 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

[1] Supported by U.S. Department of Agriculture Competitive Grant 86-CRCR-1-1977.

[2] Abbreviations: EP*, ADP-discharged phosphoenzyme intermediate; EP, ADP-insensitive phosphoenzyme intermediate; Pi, inorganic phosphate.

mg/mL, frozen under liquid nitrogen, and then stored at −80°C until use.

Detergent treatment of the plasma membrane fraction for both the extraction of extraneous protein and subsequent solubilization of the plasma membrane ATPase was carried out as described by Briskin and Poole (11). The supernatant containing the plasma membrane ATPase was decanted and the 200,000*g* pellet was discarded. The supernatant fraction was used immediately in kinetic assays.

Measurement of Phosphorylation and Dephosphorylation Reactions. Phosphorylation and dephosphorylation reactions were carried out using a rapid mixing apparatus modified from that described by Kanazawa *et al.* (17). The syringe push blocks were driven by AC push-type solenoids capable of developing 70 oz 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% tolerance 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 Gutfreund (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 H_2O were added to each quenched reaction sample. The absorbance of the 2,4-dinitrophenol 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 over this time range (2, 14, 17).

In the study of ATPase reactions, equal volumes (150 μl) of enzyme 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 NaH_2PO_4, 5 mM $Na_4P_2O_7$, and 1 mM Na_2ATP. Phosphorylation and dephosphorylation reactions were carried out using 70 to 100 μg of solubilized ATPase preparation, 100 μM [γ-^{32}P]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 microfuge. This washing procedure was repeated four times, and the radioactivity associated with the final pellet was determined by liquid scintillation spectroscopy.

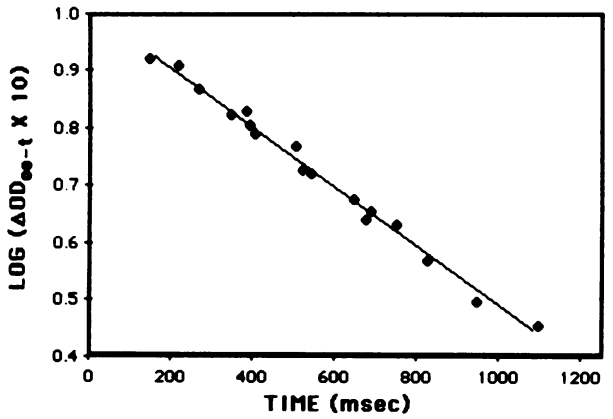

FIG. 1. Alkaline hydrolysis of 2,4-dinitrophenyl acetate at 10°C measured with the rapid quenching apparatus. The reaction was initiated by the rapid mixing of 0.6 N NaOH with 1.25 mM 2,4-dinitrophenyl acetate in 3.8 mM HCl. The reactions were quenched over the indicated time range (90–1200 ms) by rapid mixing with 0.5 N HCl. Following the addition of 0.5 mL of 2 M K-acetate, pH 4.5, and 3 mL H_2O, the absorbance was determined at 360 nm. The data are plotted in a semilogarithmic manner.

Measurement of ADP and Pi Produced during ATP Hydrolysis. 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 supernatant, 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 H_2O (4:2:3:4, v/v) and the location of nucleotide spots was detected under UV light. In this system, ^{32}Pi remains close to the origin while ATP and ADP move substantially with the solvent resulting in a migration of 0.42 and 0.58 (R_F 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-^{32}P]ATP and [5′,8-^{3}H] ATP were used as substrates, and the released [^{3}H]ADP and ^{32}Pi 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^{32}P; 5′,8-^{3}H) in 30 mM Tris-Mes pH 6.5 at 10°C, phosphoenzyme was rapidly formed and reached a steady-state level by 600 ms (Fig. 2). Over this time range, Pi production showed an initial slow phase, which changed into a more rapid, linear rate of production as the phosphoenzyme level reached steady state. In contrast to Pi production, the time course of ADP production showed an initial rapid phase followed by a linear rate. As expected, the rates of Pi and ADP liberation during the steady-state linear phase were equal. The differences in the kinetic

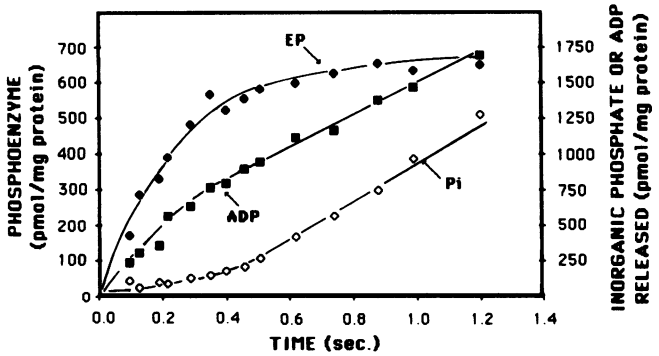

FIG. 2. Phosphoenzyme formation, ADP liberation, and phosphate liberation during the initial pre-steady-state phase of the reaction. The solubilized red beet ATPase (78 μg protein in 150 μL) was rapidly mixed with 150 μL of 200 μM ATP [γ-^{32}P, 5′,8-^{3}H; both at 0.5 Ci/mmol], 200 μM $MgSO_4$, and 60 mM Tris-Mes, pH 6.5, and allowed to react for the indicated period of time at 10°C. The reactions were quenched by rapid mixing with 800 μL of 15% TCA containing 40 mM NaH_2PO_4, 5 mM $Na_4P_2O_7$, and 1 mM Na_2ATP. The quenched reactions were centrifuged in a microfuge for 15 min, and 10 μL aliquots of the supernatant were used for the determination of released ADP and phosphate as described in "Materials and Methods." The TCA pellet was washed an additional four times with 1.0 ml of the quench solution, by repeated centrifugation and suspension, before radioactivity was determined by liquid scintillation spectroscopy.

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:

$$E + ATP \rightarrow E\text{-}P + ADP$$

$$E\text{-}P + H_2O \rightarrow E + P_i$$

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:AT^{32}P) 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 μM [γ-^{32}P]ATP alone, phosphoenzyme formation did not occur. However, when 100 μM $MgSO_4$ 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

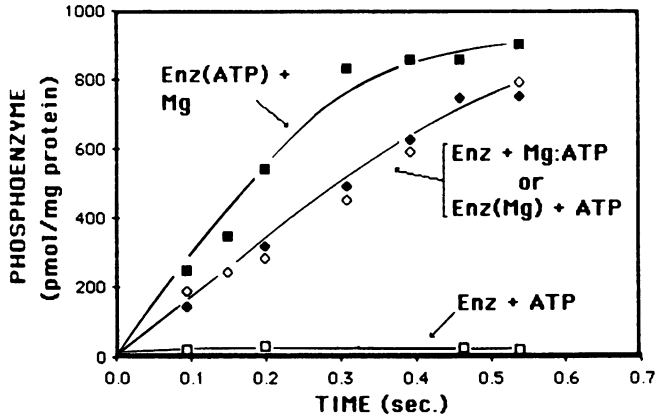

FIG. 3. Role of magnesium in the formation of the phosphorylated intermediate of the red beet plasma membrane ATPase. Phosphorylation reactions were carried out at 10°C with [γ-^{32}P]ATP by the method outlined in the legend for Figure 2. The reactions were initiated by either: (a) mixing 150 μL of the enzyme (84 μg protein) preincubated with [γ-^{32}P]ATP (200 μM) with 150 μL of 60 mM Tris-Mes (pH 6.5) and 200 μM $MgSO_4$ [Enz(ATP) + Mg]; (b) mixing 150 μL of enzyme with 150 μL of 200 μM [γ-^{32}P]ATP, 200 μM $MgSO_4$, and 60 mM Tris-Mes (pH 6.5) [Enz + Mg(ATP)]; (c) mixing 150 μL of enzyme preincubated with 200 μM $MgSO_4$ with 150 μL of 200 μM [γ-^{32}P]ATP and 60 mM Tris-Mes, pH 6.5 [Enz(Mg) + ATP]; or (d) mixing 150 μL of enzyme with 150 μL of 200 μM [γ-^{32}P]-ATP and 60 mM Tris-Mes, pH 6.5 [Enz + ATP]. The reactions were quenched at the indicated time by mixing with 800 μL of 15% TCA, 40 mM NaH_2PO_4, 5 mM $Na_4P_2O_7$, and 1 mM Na_2ATP. The amount of phosphoenzyme intermediate was determined as described in "Materials and Methods."

with 100 μM $MgSO_4$ for 20 s and then initiating the reaction by rapid mixing with 100 μM [γ-^{32}P]ATP did not affect the rate of phosphoenzyme formation. However, if the enzyme was preincubated with [γ-^{32}P]ATP for 20 s and the reaction initiated by rapid mixing with 100 μM $MgSO_4$, 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:

$$E + ATP \underset{\text{Slow}}{\longrightarrow} E(ATP) \overset{Mg^{2+}}{\underset{\text{Fast}}{\longrightarrow}} E\text{-}P + ADP$$

where E(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(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(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 [γ-^{32}P]ATP and excess unlabeled ATP containing $MgSO_4$ was added, a transient 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 μM Mg:ATP at 0°C (5). The assay pH also affects the level of steady-state phosphoenzyme and its rate of turnover. Steady-state phos-

phoenzyme 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 pK_a between 6 and 7. Since this is in the range of the pK_a 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).

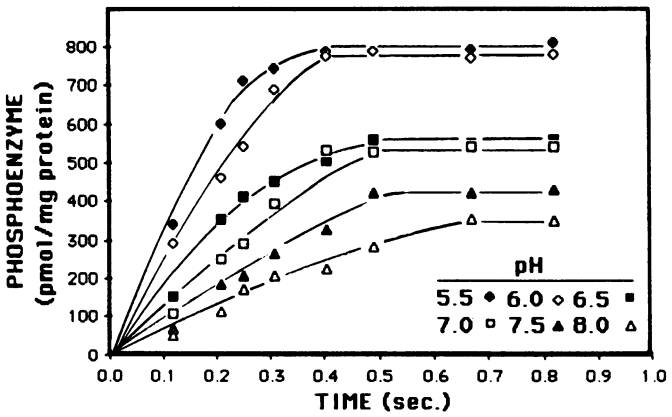

FIG. 4. Effect of pH on the formation of the phosphorylated intermediate of the red beet plasma membrane ATPase. Phosphorylation reactions were carried out at 10°C by mixing 150 μL of 200 μM [γ-^{32}P] ATP, solubilized red beet ATPase (78 μg protein) with 150 μL of 200 μM $MgSO_4$, and 60 mM Tris-Mes at the indicated pH. The reactions were quenched, and the amount of phosphoenzyme intermediate was determined as described in "Materials and Methods."

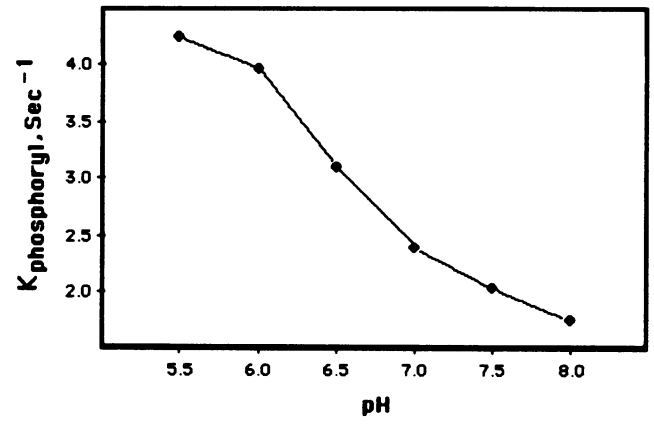

FIG. 5. Plot of the apparent first-order rate constants for phosphoenzyme formation as a function of pH. The apparent first-order rate constants were calculated from the initial portions of the time courses on Figure 4 and were plotted as a function of pH.

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-^{32}P] 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 E_1E_2 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,

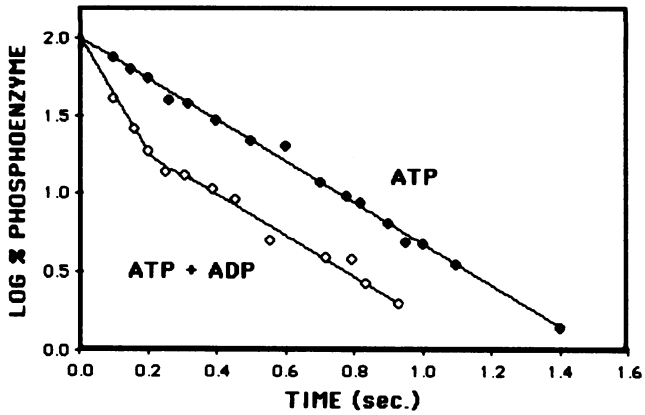

FIG. 6. Dephosphorylation reactions of the red beet plasma membrane ATPase measured in the absence of KCl. The phosphoenzyme was formed at 10°C by mixing 150 μL of the enzyme (98 μg protein) with 150 μL of 200 μM [γ-^{32}P]ATP, 200 μM $MgSO_4$, 60 mM Tris-Mes, pH 6.5, and allowed to reach steady-state levels (3 s). Dephosphorylation was initiated by rapid mixing with 100 μL of 40 mM ATP (Tris salt, pH 6.5) with 60 mM ADP (Tris salt, pH 6.5, when present). This time for the second addition is taken as zero time. At increasing times following the initiation of dephosphorylation, the reaction was stopped by the addition of 700 μL of 17% TCA, 40 mM NaH_2PO_4, 5 mM $Na_4P_2O_7$, 1 mM Na_2ATP, and the amount of phosphorylated protein was determined as described in "Materials and Methods." The rate constants for the decline of phosphorylated protein were determined as −2.303 (slope). For the rapid phase of phosphoenzyme decay with the addition of ATP and ADP, this calculation involved a replot of the difference between data points for this phase and an extrapolation of the slower phase.

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 intermediate(s) 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-^{32}P] 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.

Synthesis of ATP during Discharge of the ADP-Sensitive 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-^{14}C]ADP. The reactions were quenched at increasing times after the excess ATP/[^{14}C]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[^{14}C]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 uncertainities associated with rehydrolysis and recovery, the relative amounts of ^{14}C-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:

$$\xleftarrow{k_{-1}} EP^* \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EP \xrightarrow{k_3}$$

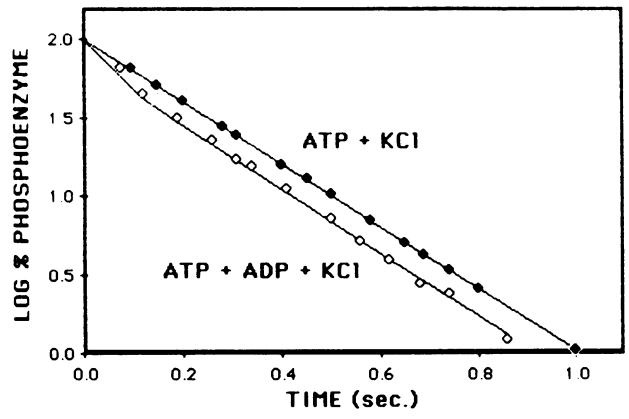

FIG. 7. Dephosphorylation reactions of the red beet plasma membrane ATPase measured in the presence of 50 mM KCl. The phosphoenzyme was formed at 10°C by mixing 150 μL of the enzyme (98 μg protein) with 150 μL of 200 μM [γ-^{32}P]ATP, 200 μM $MgSO_4$, 60 mM Tris-Mes (pH 6.5), and 100 mM KCl and was allowed 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).

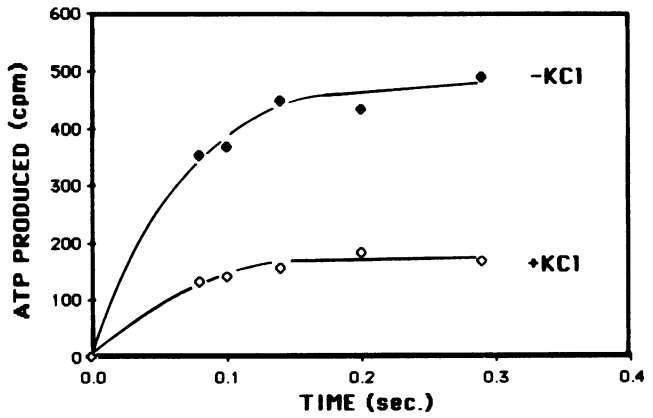

FIG. 8. Synthesis of ATP during the ADP-dependent discharge of the phosphorylated intermediate. A steady-state level of unlabeled phosphorylated intermediate (3 s) was formed for the ATPase (106 μg protein) in the absence or presence of 50 mM KCl as described for Figures 6 and 7. Dephosphorylation of the enzyme was initiated by rapid mixing with 100 μL of 40 mM ATP (Tris salt, pH 6.5) containing 60 mM [8-^{14}C]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 NaH_2PO_4, $Na_4P_2O_7$, and 1 mM Na_2ATP. 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.

For this kinetic relationship the relative amounts of the two phosphoenzyme forms can be described by the equations:

$$d(EP^*)/dt = k_{-2}[EP] - (k_{-1} + k_2)[EP^*] \quad (1)$$

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

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

$$[EP]_{total,\ t} = [EP^*] + [EP] = Ae^{-\alpha t} + Be^{-\beta t} \quad (3)$$

where the slow phase of phosphoenzyme decline is defined by A and α and the fast phase of phosphoenzyme decline is defined by B and β. These four parameters (A, B, α, β) 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 [$d(EP)/dt = 0$] can be applied to Equation 2 so that:

$$[EP^*]/[EP] = (k_{-2} + k_3)/k_2 \quad (4)$$

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} 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:

$$[EP]_{total,t} = Ae^{-\alpha t} \quad (5)$$

where A would represent the total phosphoenzyme present at zero time and α 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_{-2}, and k_3 so that under these conditions, $\alpha = k_3 + k_2$ (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 I). 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_2, this would imply that during steady-state turnover in the absence of KCl phosphoenzyme formation from ATP is substantial compared to k_2 and k_{-1}. 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_2. The presence of 50 mM KCl caused an 11-fold increase in k_2 and changed the ratio of k_2/k_{-2} from 33.4 to 80.4. Thus, this effect of KCl on the displacement 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 I. *Relative Levels of Phosphoenzyme Intermediate Forms and Rate Constants Determined from an Analysis of Dephosphorylation Kinetics*

Condition	Phosphoenzyme Intermediates[a]		Kinetic Rate Constants[a]		
	EP*	EP	k_2	k_{-2}	k_3
	% *total phosphoenzyme*		s^{-1}		
−KCl	65.0	35.0	1.67	0.05	3.06
+KCl	20.9	79.1	18.5	0.23	4.58

[a] 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 Ca^{2+}-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 Mg:ATP) 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 I). Within this scheme, it should be noted that the pathway from the free enzyme (E) to EP* via E(ATP) most likely occurs only in these types of *in vitro* kinetic studies because the true physiological substrate for the enzyme is the Mg:ATP complex (1, 19).

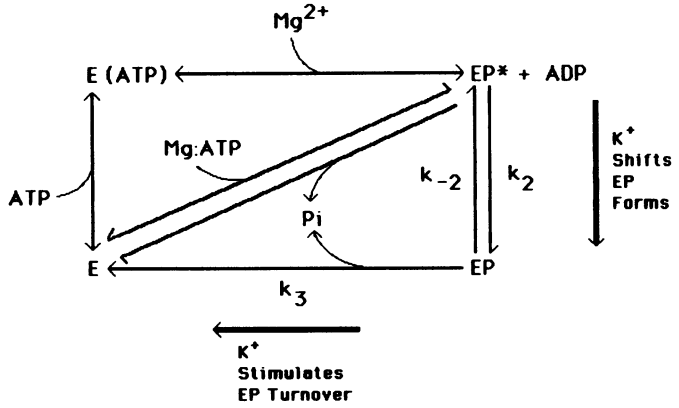

FIG. 9. Proposed reaction mechanism for the red beet plasma membrane ATPase based upon the observations in this study. The rate constants determined in this study (see Table I) and the effects that K^+ exerts on the reaction steps and intermediates are indicated on the figure. See text for discussion.

The proposal that the EP* phosphoenzyme form represents the phosphorylated intermediate that is formed first from the reaction of the enzyme with Mg:ATP 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 Mg:ATP. The degree to which this first EP* phosphorylated 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.* $k_{-1} = k_3$). 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:

$$\overset{k_{-1}}{\leftarrow} \text{EP*} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{EP} \overset{k_3}{\rightarrow}$$

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 $AT^{32}P$ 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:

$$d(\text{EP*})/dt = k_{-2}[\text{EP}] - (k_{-1} + k_2)[\text{EP*}] \quad \text{(A1)}$$

$$d(\text{EP})/dt = k_2[\text{EP*}] - (k_{-2} + k_3)[\text{EP}]. \quad \text{(A2)}$$

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:

$$[\text{EP*}] = C_1 e^{-\alpha t} + C_2 e^{-\beta t} \quad \text{(A3)}$$

$$[\text{EP}] = C_3 e^{-\alpha t} + C_4 e^{-\beta t}. \quad \text{(A4)}$$

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

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

where $A = C_1 + C_3$, $B = C_2 + C_4$ and $A + B = [\text{EP}]_{\text{total}}$ at $t = 0$. The components of these equations are defined as:

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

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

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

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

$$C_3 = C_1 (h + u)/2k_{-2} \quad \text{(A10)}$$

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

where:

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

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

and $[\text{EP*}]_o$ and $[\text{EP}]_o$ 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 defined from Equations A6 to A13 as:

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

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

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 ∞. 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.

Acknowledgment—The author acknowledges the expert guidance given by Paul Krabbe of the electrical engineering shop, Department of Physics, University of Illinois, during the design of the circuitry used in the rapid quench apparatus.

LITERATURE CITED

1. BALKE NE, TK HODGES 1975 Plasma membrane adenosine triphosphatase of oat roots. Activation and inhibition by Mg2+ and ATP. Plant Physiol 55: 83–86
2. BARMAN TE, H GUTFREUND 1964 A comparison of the resolution of chemical and optical sampling *In* B Chance, R Eisenhardt, Q Gibson, K Lonberg-Holm, eds, Rapid Mixing and Sampling Methods in Biochemistry. Academic Press, New York, pp 339–344
3. BRADFORD MM 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 243–254
4. BRISKIN DP 1986 Plasma membrane H^+-ATPase: role in potassium ion transport? Plant Physiol 68: 159–163
5. BRISKIN DP 1986 Intermediate reaction states of the red beet plasma membrane ATPase. Arch Biochem Biophys 248: 106–115
6. BRISKIN DP 1988 Chemical equivalence of phosphoenzyme reaction states in the catalytic mechanism of the red beet (*Beta vulgaris* L.) plasma membrane ATPase. Plant Physiol 88: 78–84
7. BRISKIN DP, RT LEONARD 1982 Partial characterization of a phosphorylated intermediate associated with the plasma membrane ATPase of corn roots. Proc Natl Acad Sci USA 79: 6922–6926
8. BRISKIN DP, RJ POOLE 1983 Characterization of a K^+-stimulated adenosine triphosphatase associated with the plasma membrane of red beet. Plant Physiol 71: 350–355
9. BRISKIN DP, RJ POOLE 1983 Plasma membrane ATPase of red beet forms a phosphorylated intermediate. Plant Physiol 71: 507–512
10. BRISKIN DP, RJ POOLE 1983 Role of magnesium in the plasma membrane ATPase of red beet. Plant Physiol 71: 969–971
11. BRISKIN DP, RJ POOLE 1984 Characterization of the solubilized plasma membrane ATPase of red beet. Plant Physiol 76: 26–30
12. BRONNIKOV GE, SD ZAKHAROV 1983 Microquantitative determination of Pi-ATP and ADP-ATP exchange kinetics using thin layer chromatography on silica gel. Anal Biochem 131: 69–74
13. HELMICH-DE JONG ML, SE VAN EMST-DE VRIES, JJHHM DE PONT, FMAH SCHUURMANS STEKHOVEN, SL BONTING 1985 Direct evidence for an ADP-sensitive phosphointermediate of (K^+ + H^+)-ATPase. Biochim Biophys Acta 821: 377–383
14. HIROMI K 1979 Kinetics of Fast Enzyme Reactions: Theory and Practice. Halsted Press, Tokyo, pp 187–286
15. INESI G, T WATANABE, C COAN, A MURPHY 1982 The mechanism of sarcoplasmic reticulum Ca^{2+}-ATPase. Ann NY Acad Sci 402: 515–534
16. JØRGENSEN PL 1982 Mechanism of the Na+,K+-pump: protein structure and conformations of the pure (Na+,K+)-ATPases. Biochim Biophys Acta 694: 27–68
17. KANAZAWA T, M SAITO, Y TONOMURA 1970 Formation and decomposition of a phosphorylated intermediate in the reaction of Na^+-K^+ dependent ATPase. J Biochem 67: 693–711
18. KLODOS I, JG NØRBY, IW PLESNER 1981 The steady-state kinetic mechanism of ATP hydrolysis catalyzed by membrane-bound (Na+ + K+)-ATPase from ox brain. II. Kinetic characterization of phosphointermediates. Biochim Biophys Acta 643: 463–482
19. LEONARD RT 1984 Membrane associated ATPase and nutrient absorption by roots. *In* PB Tinker, A Lauchli, eds, Advances in Plant Nutrition, Vol I. Praeger Scientific, New York, pp 209–240
20. O'NEILL SD, RM SPANSWICK 1984 Solubilization and reconstitution of a vanadate-sensitive H^+-ATPase from the plasma membrane of *Beta vulgaris*. J Membr Biol 79: 231–243
21. PEDERSEN PL, E CARAFOLI 1987 Ion motive ATPases. I. Ubiquity, properties and significance to cell function. Trends Biochem Sci 12: 146–150
22. POOLE RJ, DP BRISKIN, Z KRATKY, RM JOHNSTONE 1984 Density gradient localization of plasma membrane and tonoplast from storage tissue of growing and dormant red beet. Characterization of proton transport and ATPase activity in tonoplast vesicles. Plant Physiol 74: 549–556
23. SACHS G, T BERLEIDH, E RABON, B WALLMARK, ML BARCELLONA, HB STEWART, G SACCOMANI 1980 The interaction of K+ with gastric parietal cells and gastric ATPase. Ann NY Acad Sci 358: 118–137
24. SCALLA R, A AMORY, J RIGAUD, A GOFFEAU 1983 Phosphorylated intermediate of a transport ATPase and activity of protein kinase in membranes from corn roots. Eur J Biochem 132: 525–530
25. SINGH SP, BVS KESAV, DP BRISKIN 1987 Reconstitution and rapid partial purification of the red beet plasma membrane ATPase. Plant Physiol 69: 617–626
26. STEWART B, B WALLMARK, G SACHS 1981 The interactions of H^+ and K^+ with the partial reactions of gastric (H^+ + K^+)-ATPase. J Biol Chem 256: 2682–2690
27. SZE H 1985 H^+-translocating ATPases: advances using membrane vesicles. Annu Rev Plant Physiol 36: 175–208
28. TIPTON KF, HBF DIXON 1983 Effects of pH on Enzymes. *In* DL Purich, ed, Contemporary Enzyme Kinetics and Mechanism. Academic Press, New York, pp 97–148
29. TONOMURA Y 1986 Energy-Transducing ATPase—Structure and Kinetics. Cambridge University Press, Cambridge
30. VARA F, R SERRANO 1982 Partial purification and properties of the proton translocating ATPase of plant plasma membranes. J Biol Chem 257: 12826–12830
31. VARA F, R SERRANO 1983 Phosphorylated intermediate of the ATPase of plant plasma membranes. J Biol Chem 258: 5334–5336
32. WALLMARK B, S MARDH 1979 Phosphorylation and dephosphorylation kinetics of potassium stimulated ATP phosphohydrolase from hog gastric mucosa. J Biol Chem 254: 11899–11902