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Direct Measurement of ATP-Dependent Proton Concentration Changes and Characterization of a K^+-Stimulated ATPase in Pea Chloroplast Inner Envelope Vesicles[1]

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Inner envelope membrane vesicles prepared from pea (*Pisum sativum* L. var Laxton's Progress No. 9) chloroplasts have K^+-stimulated ATPase activity with a pH optimum of 8.4. ATP addition to inner envelope vesicles loaded with pyranine caused a decrease in pyranine fluorescence that was consistent with internal acidification. The transmembrane pH change induced by the addition of 5 mM ATP was about 0.4 unit. Measurement of phosphate released by ATP hydrolysis paralleled the pH change, indicating that intravesicular acidification was linked to ATPase activity. Vanadate, molybdate, *N*-ethylmaleimide, and dithiothreitol inhibited ATP-dependent vesicle acidification completely, whereas ATPase activity was only partially inhibited. These data indicate that pea chloroplast inner envelope vesicles contain a proton translocating ATPase and that the pyranine-loading method can be utilized to study directly ATP-dependent H^+ transport across these membranes.

The inner envelope of the chloroplast was shown by cytochemical staining techniques to have Mg^{2+}-dependent ATPase activity (Sabnis et al., 1970). Divalent cations are required for ATPase activity with Mg^{2+} and Mn^{2+} serving equally well (McCarty et al., 1984; Nguyen et al., 1987). These researchers also found that ATPase activity was inhibited by vanadate, molybdate, and *N*-ethylmaleimide. Inhibition by vanadate, a transition state analog of phosphate, is a characteristic of all P-type ATPases, which form a covalent phosphorylated intermediate (Pederson and Carafoli, 1987). However, unlike P-type ATPases, the enzyme of the chloroplast inner envelope was not inhibited by *N,N'*dicyclohexylcarbodiimide or ouabain (McCarty et al., 1984; Nguyen et al., 1987).

The activity of this ATPase was first proposed to be linked to H^+ fluxes across the chloroplast envelope by Maury et al. (1981), who noted that Mg^{2+} addition to intact illuminated chloroplasts caused acidification that was reversed by K^+ addition. H^+ fluxes across the envelope were proposed to be regulated by two mechanisms: (a) an active, oligomycin-sensitive H^+ efflux and (b) a reversible Mg^{2+}-dependent, oligomycin-insensitive H^+/K^+ exchange. Studies using isolated chloroplast inner envelopes seem to agree with the possibility that H^+ fluxes across the envelope are regulated by two separate processes. Early studies using isolated chloroplast envelopes indicated that ATPase activity was insensitive to oligomycin (McCarty et al., 1984) and was not stimulated by K^+ (Douce et al., 1973; Nguyen et al., 1987). A recent study indicates that there is a K^+-stimulated ATPase in the chloroplast inner envelope and that this activity is inhibited by oligomycin (Wu and Berkowitz, 1992).

The current evidence for an active H^+ efflux out of the chloroplast envelope is based on indirect observations. The reversal of Mg^{2+}-induced acidification of the stroma of actively photosynthesizing chloroplasts was measured by the equilibration of radioactive bicarbonate ions across the envelope (Huber and Maury, 1980; Maury et al., 1981). In chloroplast inner envelope vesicles methylamine uptake was used to demonstrate that the vesicles accumulated protons in the presence of ATP (Berkowitz and Peters, 1993). Measurement of ATPase activity by either a coupled NADH-linked spectrophotometric assay or the colorimetric determination of phosphate released by ATP hydrolysis have not been definitively linked to H^+ movement across the inner envelope (Wu and Berkowitz, 1992). In all of these assays there is a general inability to measure directly and to quantify the pH changes occurring because of H^+ efflux as the result of ATPase activity.

Use of the membrane-impermeant fluorescent probe pyranine has several advantages when measuring pH changes within inner envelope vesicles. Pyranine entrapped within the lumen of unilamellar phospholipid vesicles is a reliable indicator of the internal aqueous H^+ concentration (Biegel and Gould, 1981). Pyranine loaded into chloroplast inner envelope vesicles was used to measure proton-linked activities of glycolate/H^+ co-transport (Howitz and McCarty, 1988). Subsequent work linked the change in pyranine fluorescence to the change in pH associated with glycolate or D-glycerate proton/substrate symport activity (Young and McCarty, 1993). Pyranine is very sensitive to small changes in pH, and these changes can be quantified, allowing for the

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Abbreviations: DPX, *p*-xylene bispyridinium dibromide; F_{is}, fluorescence of pyranine at a pH-insensitive excitation wavelength; F_s, fluorescence of pyranine at a pH-sensitive excitation wavelength; pyranine, 8-hydroxypyrene-1,3,6-trisulfonate.

direct measurement of vesicle acidification (Renganathan et al., 1993; Young and McCarty, 1993). In addition, pyranine fluorescence can be quenched by DPX, which can be used in vesicle preparations to eliminate contributions from external pyranine (Smolarsky et al., 1977).

Entrapped pyranine was utilized to measure ATP-dependent acidification of inner envelope membrane vesicles and to characterize an apparent pea inner envelope H^+-ATPase system.

MATERIALS AND METHODS

Materials

Pyranine and DPX were purchased from Molecular Probes (Eugene, OR). All other reagents were of the highest grades commercially available.

Plant Material

Pea (*Pisum sativum* L. cv Laxton's Progress No. 9) plants were grown from seeds for 16 to 18 d in vermiculite in a controlled environment growth cabinet set for 16-h day (20°C)/8-h night (20°C) periods.

Chloroplast Isolation

Chloroplasts were isolated essentially as described by Joy and Mills (1987). After centrifugation through Percoll, the chloroplasts were resuspended in 0.6 M Suc, 10 mM K-Hepes (pH 8.0), 2 mM EDTA, and 2 mM DTT and centrifuged for 7 min at 1560*g*. The pellet was resuspended in the same buffer, and the chloroplasts were stored at −70°C until they were used for envelope preparation.

Preparation of Envelope Vesicles

Inner envelope vesicles were prepared according to the method of Keegstra and Yousif (1986). Frozen, intact chloroplasts, equivalent to between 80 and 120 mg of Chl, were thawed at room temperature, refrozen at −20°C, and thawed again. Chloroplast rupture was facilitated by gentle homogenization in a glass-Teflon homogenizer. The homogenate was centrifuged at 3150*g* for 15 min. The supernatants were collected and centrifuged at 27,000*g* for 90 min. Pellets were resuspended in 0.2 M Suc, placed on top of a Suc step gradient, and centrifuged at 105,000*g* for 18 h. Inner envelope vesicles were recovered from the 1.10 to 1.13 g/mL interface. All of the above operations were performed at 4°C.

Pyranine Loading of Inner Envelope Vesicles

Inner envelope membrane suspensions were loaded with pyranine essentially as described by Young and McCarty (1993), with the exception that the resuspension buffer used contained 1.0 mM K-Hepes (pH 8.0), 5 mM $MgCl_2$, and 50 mM KCl.

Fluorescence Measurements

Inner envelope vesicles (1 mL, 50–300 μg of protein) were diluted into 3 mL of elution buffer containing 100 mM Suc, 10 mM Na-Hepes (pH 8.0), 5 mM $MgCl_2$, and 50 mM KCl. Stock solutions of ATP and inhibitors were prepared in the same buffer and titrated to pH 8.0. Vesicle suspensions were placed in a 1-cm cell in an Olis-modified SLM-SPF-500 spectrofluorometer. Pyranine fluorescence emission was monitored at 512 nm with excitation at either 466 nm (F_s) or 413 nm (F_{is}) or by an excitation scan from 350 to 500 nm. All slits were set at 10 nm with a cutoff filter (LP47; Oriel Co., Stamford, CT) placed in the emission window.

Assays

Protein was determined using the modified TCA-Lowry procedure of Bensadoun and Weinstein (1976). Phosphate was determined by the colorimetric procedure (Taussky and Schorr, 1953). ADP and ATP levels were determined by HPLC analysis (Moal et al., 1989). Western immunoblot analysis was performed according to standard procedures (Villalba et al., 1992). Antiserum against plasmalemma H^+-ATPase was a gift from Dr. R. Serrano (Universidad Politécnica, Valencia, Spain).

RESULTS AND DISCUSSION

ATPase Activity in Pea Inner Envelopes

Isolated pea inner envelope membranes catalyze Mg^{2+}-stimulated ATPase activity at rates similar to those published previously (Table I; McCarty et al., 1984; Nguyen et al., 1987). Although the researchers reported that K^+ had no effect on ATPase activity in their preparations, we found significant stimulation by K^+ (Table I). The rate of K^+-stimulated ATPase activity was similar to values reported for plasmalemma ATPase (Bennett et al., 1984; Briskin and Poole, 1983). In our pea preparations the rate was approximately 5 to 10 times higher than rates reported for chloroplast inner envelopes prepared from spinach (Wu and Berkowitz, 1992).

K^+-stimulated ATPase activity associated with the plasmalemma membrane operates optimally at a pH of 6.5 (Briskin and Poole, 1983). The pH optimum of K^+-stimulated ATPase activity in pea inner envelope preparations was

Table I. *K^+ stimulation of ATPase activity in pea chloroplast inner envelope membranes*

The reaction mixture (2 mL) contained 10 mM Na-Hepes (pH 8.0), 5 mM ATP, 100 mM Suc, 150 to 300 μg of inner envelope membrane protein, and potassium and magnesium salts at the indicated concentrations. The reaction was run for 15 min at 25°C and stopped with 0.5 mL of 0.5 N TCA. Pi was assayed as described in "Materials and Methods." Values represent means of four experiments (±SE).

Additions	Phosphate Formation	Percentage of Control
	μmol mg protein^{-1} h^{-1}	
None	0.9 ± 0.2	2
+ $MgCl_2$ (5 mM)	6.0 ± 2.1	14
+ KCl (50 mM)	29.5 ± 3.3	71
+ $MgCl_2$ and KCl (control)	41.5 ± 3.5	100

approximately 8.4 (Fig. 1), indicating that activity at the assay pH (8.0) was not due to contaminating fragments from the plasmalemma. In addition, western immunoblot analysis using antisera against plasmalemma H^+-ATPase did not react with pea chloroplast inner envelope ATPase (data not shown). The alkaline pH optimum, measured here as ADP formation from ATP hydrolysis, is in agreement with a previous report in which Pi release from ATP hydrolysis in spinach preparations was assayed (Wu and Berkowitz, 1992).

The chloroplast inner envelope ATPase is reported to act as an nucleotide triphosphatase having high affinity for other nucleotide triphosphates (McCarty et al., 1984). When ADP alone was added to our preparations of inner envelope vesicles, the rate of ADP hydrolysis was less than 30% of the rate of ATP hydrolysis. In a separate experiment in which ATP was added as the hydrolyzable substrate, subsequent hydrolysis of ADP to AMP was less than 10% of ADP production (Fig. 1). These results indicate that during ATP hydrolysis ADP was poorly utilized as a substrate for ATPase activity.

Vesicle Acidification and Calibration

When small aliquots of HCl are added to pyranine-loaded pea inner envelope vesicles, the excitation spectrum changes (Fig. 2A). With excitation at 413 nm pyranine fluorescence is completely insensitive to the change in proton concentration within the vesicles. This isosbestic point is taken as the wavelength for F_{is}. The fluorescence change due to acidification was greatest at an excitation wavelength of 466 nm, and this wavelength was used as F_s. These F_s and F_{is} excitation wavelengths correspond closely to those previously reported (Young and McCarty, 1993). A plot of F_s/F_{is} versus pH,

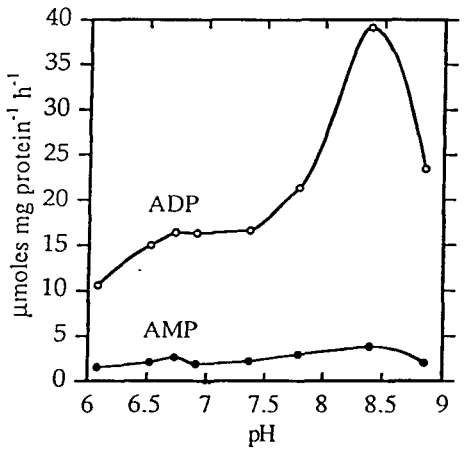

Figure 1. pH activity curve for ATPase in pea inner envelopes. The reaction mixture contained 10 mM Bis-Tris (pH 6–7.5) and 10 mM K-Hepes (pH 7.75–9.00), 5 mM ATP, 5 mM $MgCl_2$, 50 mM KCl, 100 mM Suc, and 150 to 300 μg of inner envelope membrane protein. The reaction was run for 15 min at 25°C; then a 100-μL aliquot was taken and placed into 150 μL of methanol. The resulting precipitate was spun down, and the supernatant was collected and taken to dryness in a vacuum concentrator. The residue was resuspended in 100 μL of deionized water and used for HPLC analysis with a C_{18} column (Moal et al., 1989).

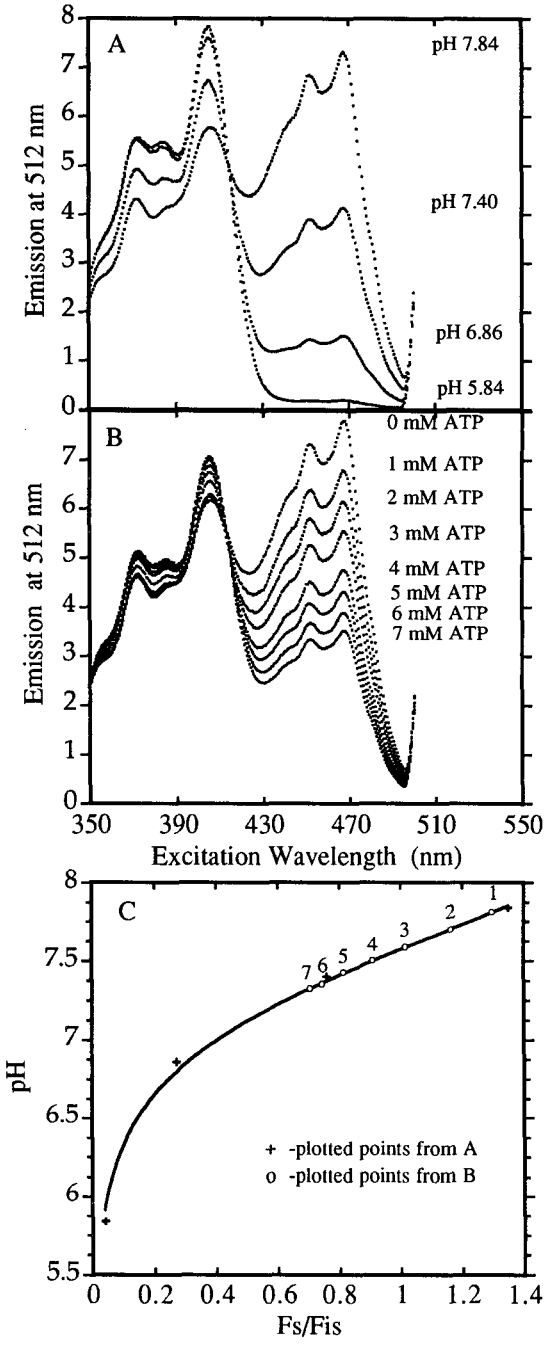

Figure 2. pH calibration curve for pyranine-loaded pea inner envelope vesicles. Vesicles (140 μg protein/mL) loaded with 5 mM pyranine, 1 mM K-Hepes (pH 8.0), 5 mM $MgCl_2$, and 50 mM KCl were assayed at 25°C in a buffer containing 10 mM K-Hepes (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 5 mM DPX, and 100 mM Suc. The excitation spectra were recorded between 350 and 500 nm with the emission monitored at 512 nm in an Olis-SLM (SPF-500) spectrofluorometer. pH was measured directly in the cuvette after addition of 2-μL aliquots of 0.1 N HCl (A). F_s (466 nm) and F_{is} (413 nm) data from the emission spectra were utilized in Equation 1 to produce the pH calibration curve (C). The effect of added ATP on the excitation spectra of pyranine was measured in B. From the pH calibration curve the pH of the vesicle interior could be determined for each addition of ATP (C).

determined by direct measurement, produced the curve in Figure 1C. The curve fits the equation

$$pH = pK_a + \log\left\{\frac{(F_s/F_{is})/(F_s/F_{is})_{max}}{1 - [(F_s/F_{is})/(F_s/F_{is})_{max}]}\right\} \quad (1)$$

with a correlation of 0.997. From the plot of this curve the pK_a was determined to be 7.65 and $(F_s/F_{is})_{max}$ was 2.19. $(F_s/F_{is})_{max}$ represents the value at which essentially all of the pyranine is in its deprotonated form (Young and McCarty, 1993). The determination of pK_a and $(F_s/F_{is})_{max}$ is variable among envelope preparations, requiring that a calibration curve be determined for each preparation. This may be due to variations in the amount of protein used, since pyranine will bind to proteins. However, the relationship between pyranine protonation and pH is approximately linear between pH 7.0 and 8.0 (Fig. 2C).

Additions of ATP also caused a decrease in fluorescence at F_s 466 (Fig. 2B), indicating that protonation of internal pyranine occurred. Internal acidification in pea inner membrane vesicles could be a direct result of ATP transport across the inner envelope (Robinson and Wiskich, 1977). Either by proton symport or hydroxyl antiport, the interior of the vesicles could become more acidic. Chloroplasts from mature spinach leaves transport adenylates at very low rates (Heldt, 1969); yet, significant ATP-induced internal acidification was detected in spinach envelope vesicles (data not shown). Alternatively, protons may be translocated directly via an H^+-ATPase across the inner envelope membrane. The inner envelope membrane vesicles are predominantly in the inside-out orientation (Cline et al., 1985). For acidification to occur within the vesicles by ATPase activity suggests that the ATPase active site must be predominantly on the outside of the vesicles. In intact chloroplasts this would be equivalent to an ATPase located on the inside (stromal) of the inner envelope that translocates protons into the cytosol, as suggested by Maury et al. (1981).

The changes in pyranine fluorescence caused by ATP addition are similar to those caused by HCl, indicating that the decrease in fluorescence was not due to direct ATP quenching of pyranine fluorescence. In solution, addition of millimolar concentrations of ATP were found to have no effect on pyranine fluorescence. ATP did not cause shifts in the F_s and F_{is} excitation wavelengths. Thus, the HCl curve may be used as a calibration curve for pyranine protonation resulting from ATPase activity. At concentrations greater than 5 mM ATP the pH changes became progressively smaller as the nonlinear response of pyranine fluorescence was reached (Fig. 2C). At 5 mM ATP the calculated change in pH was approximately 0.4 unit.

Kinetics of ATP-Dependent Vesicle Acidification

ATP-dependent vesicle acidification was rapid, coming to a new equilibrium in 30 s or less. The newly established fluorescence level was steady for at least several minutes (Fig. 3). The small fluorescence decrease noted when buffer alone was added can be accounted for by dilution. The ATP-dependent pH changes due to internal vesicle acidification did not reach saturation over the range of ATP additions (Fig. 4). Gramicidin, a protonophore, reversed the intravesicular acidification caused by ATP addition (Fig. 5). This reversal of the pH change indicates that acidification of the vesicle interior results from H^+ production and transport into the vesicles during ATP hydrolysis.

The ATPase activity of the inner envelope was suggested to be linked to H^+ movement across the membrane (Maury et al., 1981; Berkowitz and Peters, 1993). Phosphate release from ATP has been used as a measure of ATPase activity (Briskin and Poole, 1983; Bennett et al., 1984; McCarty et al., 1984; Nguyen et al., 1987; Wu and Berkowitz, 1992). Both ATPase activity and intravesicular acidification exhibited very similar dependencies on ATP concentration (Fig. 4).

Effect of Inhibitors

ATPase activity in pea inner envelopes, measured as phosphate release from ATP, was previously shown to be inhibited by vanadate, molybdate, and *N*-ethylmaleimide (McCarty et al., 1984; Nguyen et al., 1987). Similar results were found in our preparations for K^+-stimulated ATPase activity (Table II) with approximately 50% inhibition at 1 mM inhibitor concentration.

Vanadate inhibited phosphate release from ATP by approximately 40% (Table II), but it completely inhibited proton translocation (Fig. 5). Chloroplast inner envelopes may contain more than one type of ATPase, including a proton-translocating ATPase. It has also been proposed that ATPase

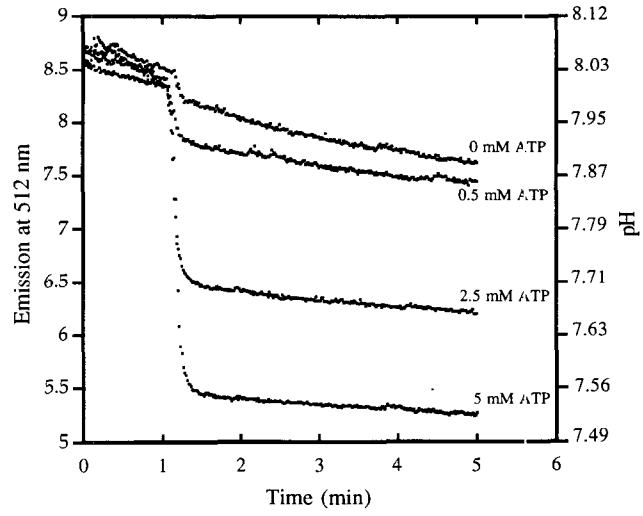

Figure 3. Kinetics of ATP-dependent vesicle acidification. Pea chloroplast inner envelope vesicles were loaded with pyranine as described in "Materials and Methods." Vesicles (1 mL) containing 140 μg of protein were incubated at 25°C in 2 mL of buffer containing 10 mM K-Hepes (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 5 mM DPX, and 100 mM Suc. Pyranine fluorescence was measured at an emission wavelength of 512 nm with excitation at 466 nm (F_s). pH values were calculated from the standard curve using Equation 1. ATP at the indicated concentrations was added after 1 min. After 5 min the assay was stopped and 1.0 mL of 0.5 N TCA was added to the cuvette. Pi was subsequently measured by a colorimetric procedure (Taussky and Schorr, 1953).

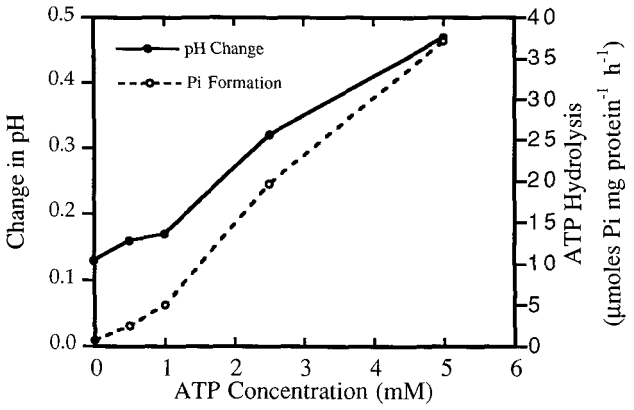

Figure 4. Relationship between ATP-dependent pH changes and ATP-dependent phosphate formation in pyranine-loaded pea inner envelope vesicles. Pea vesicles were assayed as described in Figure 3. Pi was measured as described in "Materials and Methods."

activity may be linked to potassium transport (Maury et al., 1981; Berkowitz and Peters, 1993). This or other ATPases could represent the vanadate-insensitive activity. Vanadate sensitivity indicates that the proton-linked ATPase activity measured in pea inner envelopes is not of tonoplast or mitochondrial origin (Bennett et al., 1984; Sze, 1985; Pederson and Carafoli, 1987). In addition, no proton translocation was seen in the presence of pyrophosphate, an indication of the lack of contamination by tonoplast vesicles (Rea and Sanders, 1987).

Molybdate is a poor inhibitor of K^+-stimulated ATPase activity of the plasmalemma (Briskin and Poole, 1983; Bennett et al., 1984; Sommarin et al., 1985). Nguyen et al. (1987) showed that phosphate release from ATP in inner envelope preparations was partially sensitive to molybdate. In our preparations molybdate inhibited phosphate formation from ATP approximately 50% (Table II) but inhibited proton translocation completely (data not shown).

The K^+-stimulated activity of plasmalemma ATPase has been shown to be sensitive to sulfhydryl-modifying reagents

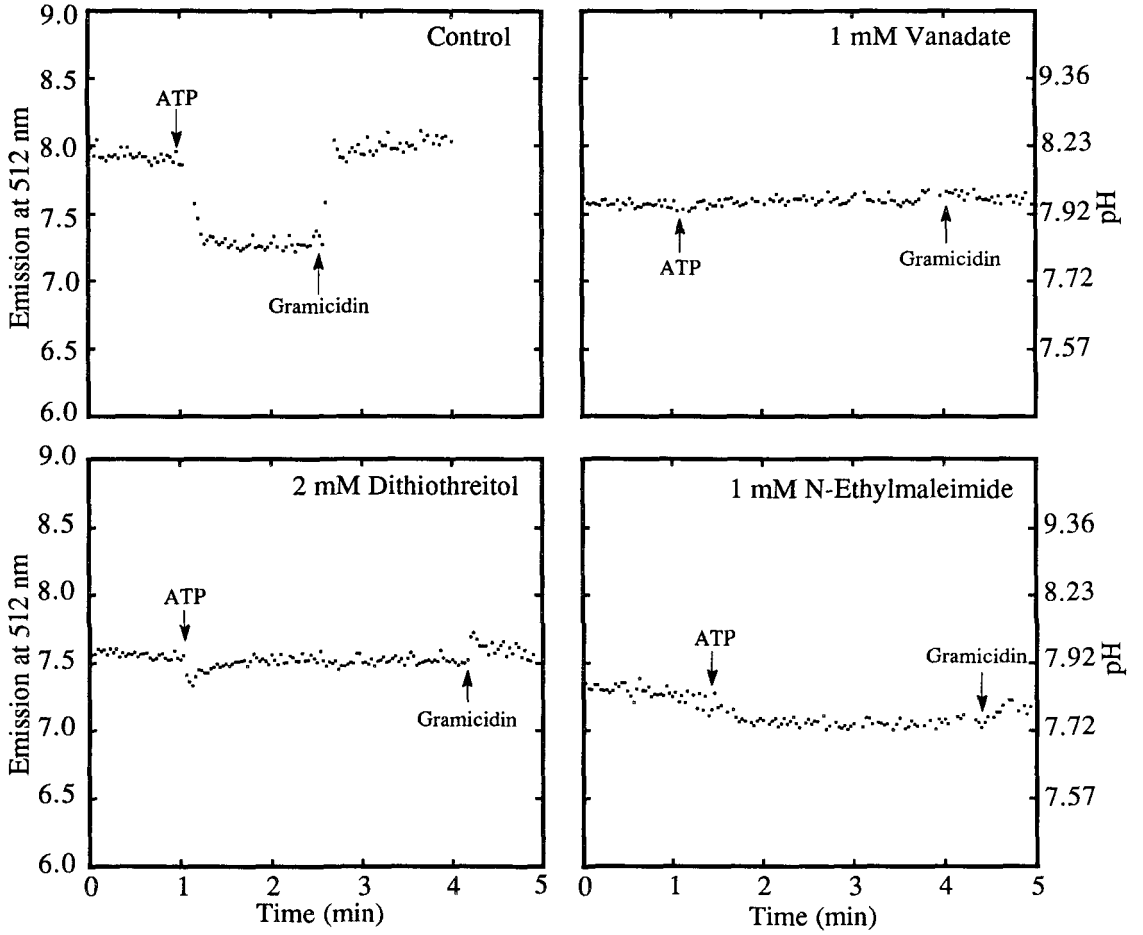

Figure 5. Effect of inhibitors on ATP-dependent vesicle acidification. Pea chloroplast inner envelope vesicles were loaded with pyranine as described in "Materials and Methods." Vesicles (1 mL) containing 50 μg of protein were incubated at 25°C in 2 mL of buffer containing 10 mM K-Hepes (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 5 mM DPX, and 100 mM Suc. Vesicles were preincubated with inhibitors at the given concentrations. ATP (5 mM) and gramicidin (5 μM) were added at the indicated times. pH values were calculated from a standard curve using Equation 1.

Table II. *Effect of inhibitors on the ATPase activity of pea chloroplast inner envelope membranes*

The reaction mixture (2 mL) contained 10 mM K-Hepes (pH 8.0), 5 mM ATP, 5 mM $MgCl_2$, 50 mM KCl, 100 mM Suc, 150 to 300 μg of inner envelope membrane protein, and inhibitors at the indicated concentrations. The reaction was run for 15 min at 25°C and stopped with 0.5 mL of 0.5 N TCA. Pi was assayed as described in "Materials and Methods." Values represent means of four experiments (±SE).

Inhibitor	Phosphate Formation	Inhibition
	μmol mg protein^{-1} h^{-1}	%
Control	38 ± 2	0
+ Vanadate (1 mM)	23 ± 5	40
+ Molybdate (1 mM)	20 ± 5	47
+ DTT (2 mM)	18 ± 5	53
+ *N*-Ethylmaleimide (1 mM)	15 ± 6	60

(Sommarin et al., 1985). *N*-Ethylmaleimide inhibits ATPase activity in chloroplast inner envelopes (Sabnis et al., 1970; Nguyen et al., 1987). This sulfhydryl-alkylating reagent inhibited ATPase activity in our inner envelope preparations by 60% (Table II) and strongly inhibited proton translocation (Fig. 5).

Remarkably, DTT at just 2 mM inhibited both ATPase activity (Table I) and ATP-dependent proton translocation (Fig. 5) in inner envelope vesicles. The failure of others to detect K^+-stimulated ATPase activity in chloroplast inner envelopes (McCarty et al., 1984; Nguyen et al., 1987) could be traced to the extraordinary sensitivity of this activity to DTT, a common additive to buffer and assay media. Since plasmalemma and tonoplast H^+-ATPases are not inhibited by low concentrations of DTT, it is unlikely that the ATPase and ATP-linked acidification are the result of contamination from these membranes. The apparent sensitivity of the inner envelope ATPase to sulfhydryl modifiers and to sulfhydryl reduction in particular might indicate a means by which the chloroplast can regulate the activity of this enzyme (i.e. through a possible Fd/thioredoxin reduction system). In mammalian systems an H^+-K^+-ATPase has been shown to be sensitive to reduction by 2-mercaptoethanol and DTT but only at high concentrations of these reducing agents (Chow et al., 1992).

CONCLUSION

The physiological role of the chloroplast envelope ATPase is not yet clear. This ATPase could be involved in direct H^+ translocation out of the chloroplast as part of a means of maintaining a stromal/cytosolic change in pH (Maury et al., 1981; Berkowitz and Peters, 1993). The measurement of ATP-dependent H^+ translocation using pyranine-loaded inner envelope vesicles tends to support this suggestion. The chloroplast envelope ATPase could participate either directly or indirectly in H^+/K^+ exchange across the membrane (Huber and Maury, 1980; Maury et al., 1981; Wu and Berkowitz, 1992; Berkowitz and Peters, 1993). Potassium stimulates the ATPase activity of pea inner envelope preparations, but it remains to be determined whether it is due to an activation of the enzyme or due to H^+/K^+ exchange across the membrane. Previous methods measuring proton transport across the chloroplast inner envelope membrane have been qualitative (Berkowitz and Peters, 1993). The pyranine-loading method indicates that proton translocation across the inner envelope can be directly measured and quantitated. The involvement of the chloroplast inner envelope ATPase in cation transport across the membrane could be investigated similarly by loading specific ion fluorescent probes into vesicles.

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