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K^+ Stimulation of ATPase Activity Associated with the Chloroplast Inner Envelope[1]

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ABSTRACT

Studies were conducted to characterize ATPase activity associated with purified chloroplast inner envelope preparations from spinach (*Spinacea oleracea* L.) plants. Comparison of free Mg^{2+} and Mg·ATP complex effects on ATPase activity revealed that any Mg^{2+} stimulation of activity was likely a function of the use of the Mg·ATP complex as a substrate by the enzyme; free Mg^{2+} may be inhibitory. In contrast, a marked (one- to twofold) stimulation of ATPase activity was noted in the presence of K^+. This stimulation had a pH optimum of approximately pH 8.0, the same pH optimum found for enzyme activity in the absence of K^+. K^+ stimulation of enzyme activity did not follow simple Michaelis-Menton kinetics. Rather, K^+ effects were consistent with a negative cooperativity-type binding of the cation to the enzyme, with the K_m increasing at increasing substrate. Of the total ATPase activity associated with the chloroplast inner envelope, the K^+-stimulated component was most sensitive to the inhibitors oligomycin and vanadate. It was concluded that K^+ effects on this chloroplast envelope ATPase were similar to this cation's effects on other transport ATPases (such as the plasmalemma H^+-ATPase). Such ATPases are thought to be indirectly involved in active K^+ uptake, which can be facilitated by ATPase-dependent generation of an electrical driving force. Thus, K^+ effects on the chloroplast enzyme *in vitro* were found to be consistent with the hypothesized role of this envelope ATPase in facilitating active cation transport *in vivo*.

The chloroplast inner envelope represents an osmotic barrier and is the limiting membrane restricting ion movement between the cytoplasmic and chloroplast stromal compartments of the cell (10). For more than two decades, it has been known that one intrinsic protein component of the inner envelope is an ATPase (10, 25). Although this enzyme has been the focus of many previous studies, its physiological role *in situ* is still a point of controversy and conjecture.

It has been speculated that this enzyme acts as an active H^+ efflux/K^+ influx transport system (15). In a model developed by Maury *et al.* (15), the *in situ* physiological role attributed to this putative active transport system was the maintenance of a steady-state pH gradient between the cytoplasm and chloroplast stroma in the illuminated leaf. The significance of this hypothesized role of the ATPase is underscored by the work of Heldt and coworkers (29). They demonstrated that optimal activity of photosynthetic carbon reduction cycle enzymes is dependent on light-induced stromal alkalization and the maintenance of a pH gradient (by about 1 unit) between the illuminated stroma and the cytoplasm. However, it has been asserted that the physiological role of the envelope ATPase may be some function other than H^+/K^+ transport. Also, the evidence supporting the role as an H^+/K^+ active transport system is only indirect and somewhat controversial.

Nguyen and Siegenthaler (19, 20) speculated that this envelope ATPase may act as a calmodulin-activated (light-dependent) Ca^{2+} uptake system or an energy-dependent mechanism for uptake into the stroma of cytoplasmically synthesized polypeptides. The evidence supporting the active H^+/K^+ transport model was developed in studies of the intact isolated chloroplast. Oligomycin (which has been shown to inhibit the envelope ATPase and not the chloroplast coupling factor; 15, 19) has been found to inhibit photosynthesis of intact chloroplasts (15). Photosynthetic inhibition caused by envelope ATPase inhibitors has been associated with a decrease in stromal pH (22) and stromal K^+ (15, 30) and is reversed by treatments that increase stromal pH (15, 22, 30).

This model of envelope ATPase involvement in the maintenance of an alkaline stromal pH is not supported by work done by Robinson (24) with the intact isolated chloroplast. In that study, treatments that significantly reduced stromal [ATP] were found to have no effect on stromal pH in the light. However, we have raised the possibility that active K^+ and/or H^+ pumping across the envelope of the isolated chloroplast may not be directly linked to counterflux of the respective other cation under all assay conditions (10, 18).

In light of these aforementioned controversies, the previously developed speculation (4, 10) that the envelope ATPase may act as a transport protein facilitating active H^+ and/or K^+ movement requires further study. Membrane ATPases that are involved (either directly as a K^+ pump or indirectly as an H^+ pump with H^+ movement linked to K^+ flux) in active K^+ transport are characterized by a significant K^+ stimulation of ATP hydrolytic activity (3, 5, 14, 25, 28). However, ATPase activity associated with purified chloroplast inner envelope membrane vesicles has been specifically found to be insensitive to K^+ (10, 11, 16, 19).

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It was the objective of the work reported here to reinvestigate the ATPase activity associated with preparations of purified chloroplast inner envelope vesicles. As part of our overall attempt to characterize the nature of the system of chloroplast envelope proteins that facilitate and regulate K^+ and H^+ transport (30, 31), we present data that are consistent with the model of envelope ATPase involvement in K^+ transport.

MATERIALS AND METHODS

Isolation of Chloroplast Inner Envelope Vesicles

Approximately 5 kg nonsenescing leaves from spinach (*Spinacia oleracea*) was deribbed, washed, and spun dry. Spinach was either obtained at local markets or, alternatively, harvested from field plots at the Rutgers vegetable research farm in New Brunswick, NJ. Spinach harvested from the research farm was from seeds (var Melody) planted in August and harvested in October or planted in April and harvested in June. (It should be noted that all experiments were repeated at least twice with different membrane preparations; no differences other than total membrane protein yield were noted in preparations derived from market *versus* research farm plants.) Batches of approximately 200 g leaves were ground with approximately 600 mL grind medium (0.33 M sorbitol, 50 mM Hepes-NaOH [pH 6.8], 2 mM Na_2EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) twice for 5 s in a Waring blender. The slurry was passed through a metal strainer and two layers of Miracloth before centrifugation in 250-mL bottles at 1500*g* for 10 min using a GSA rotor and a RC5C Sorvall (Wilmington, DE) centrifuge. Pellets were resuspended in approximately 400 mL grind medium and layered (25 mL/tube) onto cushions of 25 mL grind medium made up to 40% (v/v) Percoll and 0.5% (w/v) BSA in 50-mL centrifuge tubes. After centrifugation at 2500*g* for 5 min in an HS4 rotor, the intact chloroplasts in the pellets were resuspended (at 2–3 mg/mL) in 30 to 50 mL hyperosmotic envelope medium (10 mM Tricine-NaOH [pH 7.5], 2 mM Na_2 EDTA, 2 mM DTT, and 0.6 M sucrose).

Inner envelope membranes were purified from the intact chloroplasts using a protocol adapted from Keegstra and Yousif (12). Chloroplasts were ruptured by exposure to two freeze/thaw cycles (freezing at −20°C for a minimum of 1.5 h followed by thawing at room temperature). The envelope medium containing ruptured chloroplasts was then diluted by adding 2 volumes of envelope medium containing no sucrose. After gentle shaking for 5 min at 2°C, the medium was centrifuged in 50-mL tubes at 6000*g* for 30 min (HS4 rotor) to pellet thylakoid membranes. Supernatants were decanted and centrifuged again at 40,000*g* in an SS-34 rotor for 1 h to pellet chloroplast envelope membrane vesicles. The mostly yellow pellets (which occasionally had some thylakoid contamination observed as a slight green coloration) were resuspended in 30 mL envelope medium that had 0.2 M sucrose. The volume of envelope medium used to resuspend the pellet was dependent on loading six 13-mL ultracentrifuge tubes with 5 to 6 mL each. These 13-mL tubes contained a discontinuous sucrose gradient made up with envelope medium throughout, a bottom layer of 2 mL with 1.0 M sucrose, followed by 3 mL with 0.8 M sucrose, and 2 mL with 0.46 M sucrose. After the resuspension of crude envelope vesicles was loaded on top of these gradients, they were centrifuged at 180,000*g* in a SW-40 rotor and Beckman L8–70M ultracentrifuge for 3 h. Chloroplast inner envelope vesicles were recovered as a yellow band at the 1.0 M/0.8 M sucrose interface by careful removal with a Pasteur pipette. The pooled inner envelope membrane fractions were diluted 10-fold with 0.2 M sucrose envelope medium, pelleted by centrifugation for 30 min at 40,000*g* in an SS-34 rotor (RC5C Sorvall centrifuge) resuspended in a small volume of the same medium, and stored at −80°C. Protein concentration was measured by a modified Lowry technique using a Sigma kit; protein concentration was typically maintained at 2 to 3 mg protein/mL in the final resuspension.

Measurement of ATPase Activity

ATPase activity associated with the chloroplast envelope was measured using two different procedures: an NADH-linked spectrophotometric assay involving coupling enzymes and a colorimetric determination of Pi release. The NADH-linked procedure was similar to that recently used by Coves *et al.* (7) in their work with the chloroplast envelope ATPase and is based on a protocol originally reported by Chang and Penefsky (6). Purified envelope membranes were diluted 10-fold with 20 mM Tricine-Tris (pH 8.0) and 0.2 M sucrose, pelleted (30 min at 40,000*g*), and resuspended in the same medium. Assay medium (1 mL; 50 mM Tricine-Tris [pH 8.0], 0.2 M sucrose, 2 mM Tris-ATP, 0.7 mM Na-phospho*enol*pyruvate, 0.2 mM NADH, 30 units/mL pyruvate kinase, and 20 units/mL lactate dehydrogenase with cation additions as noted in figure legends and table headings) was added to a cuvette, with the reaction started by addition of chloroplast envelope membranes equivalent to 50 to 100 μg protein; the final volume was 1.2 mL. ATPase activity was monitored as NADH oxidation at 340 nm in a Shimadzu (Princeton, NJ) UV-160A spectrophotometer.

Alternatively, ATPase activity was estimated by measuring Pi release using a method adapted from LeBel *et al.* (13). Envelope vesicles equivalent to 100 μg protein were added to 1.5-mL microfuge tubes containing 50 mM Tricine-Tris (pH 8.0), 0.2 M sucrose, and 2 mM Tris-ATP (final concentrations) in a total volume of 1.2 mL with additions as noted. Tubes were incubated at 25°C for 15 min before addition of 100 μL 100% TCA. After the tubes were vortexed and centrifuged (10 min at 16,000*g* on an IEC Centra-M microfuge), 1 mL of the supernatant was added to tubes that contained 3 mL of solution A (0.25% $CuSO_4$ and 0.6% NaOAC in 2 N acetic acid), 0.5 mL of solution B (5% [w/v] $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$), and 0.5 mL solution C (2% [w/v] *p*-methylaminophenol sulfate and 5% [w/v] $NaSO_2$). Stocks of solutions A and B are stable for months; solution C was made up weekly and kept in the dark. After the tubes were incubated at room temperature for 15 min, A_{870} was measured. ATPase-dependent Pi release was calculated from a standard curve generated using Pi salts after subtraction of blanks (samples processed without addition of envelope vesicles) in each experiment. All reagents were purchased from Sigma except for Hepes (Re-

search Organics, Cleveland OH) and *p*-methylaminophenol sulfate (Kodak, Rochester, NY).

RESULTS AND DISCUSSION

Cation Effects on Chloroplast Envelope ATPase

The ATPase associated with the chloroplast envelope is typically referred to as an "Mg^{2+}-dependent" enzyme (7, 10, 15). Previous studies (1) have demonstrated that the Mg^{2+} dependency of other transport ATPases is not due to the presence of a catalytic site on the enzyme for Mg^{2+} but, rather, because the Mg·ATP complex, as opposed to free ATP, is the actual substrate for the enzyme. Our initial characterization of the ATPase activity associated with the chloroplast inner envelope, therefore, focused on this Mg^{2+} dependency.

The identification of Mg·ATP, rather than ATP, as the substrate for an ATPase is classically based on the monitoring of ATPase activity in the presence of a high level of ATP and increasing Mg^{2+} (1). An analysis such as this is shown in Figure 1. In the presence of 5 mM ATP, increasing reaction medium Mg^{2+} (up to 5 mM) resulted in a substantial stimulation in the rate of ATP hydrolysis. Optimal enzyme activity occurred at equimolar (5 mM) concentrations of Mg^{2+} and ATP (*i.e.* maximal levels of the Mg·ATP complex). As [Mg^{2+}] is increased past this point (allowing for the presence of free Mg^{2+} in the reaction solution), inhibition of enzyme activity was noted (Fig. 1). Based on results similar to those shown in Figure 1, Balke and Hodges (1) concluded that plasmalemma ATPase activity is actually inhibited by free Mg^{2+}, and any stimulatory effect of Mg^{2+} on enzyme activity is due to the requirement for Mg·ATP as a substrate. The chloroplast envelope ATPase is likely affected by Mg^{2+} in a similar fashion. It should be noted that previous work (16, 19, 20) has shown that both Mn^{2+} and Ca^{2+}, in addition to Mg^{2+}, can stimulate envelope ATPase activity. The stability constants for ATP complexes with all of these divalent cations are high (8); Mn^{2+} and Ca^{2+} also likely affect ATPase activity by forming a metal/nucleotide complex that acts as a substrate.

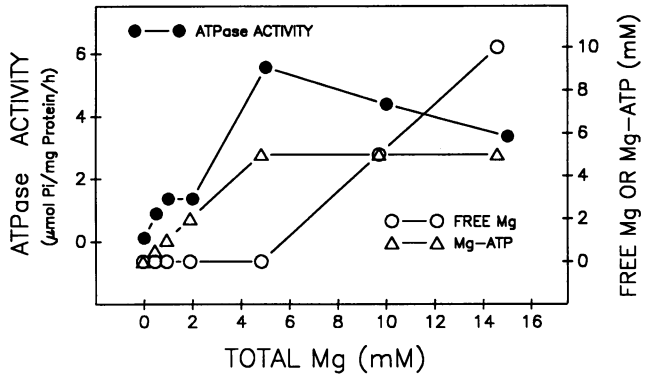

Figure 1. ATPase activity associated with purified preparations of chloroplast inner envelope at varying Mg^{2+} added to the assay medium. All assays included 5 mM Tris-ATP and 50 mM K^+-gluconate. At each Mg^{2+} concentration, relative concentrations of free Mg^{2+} (○) and Mg·ATP complex (△) were calculated according to binding constants cited by Dixon (6). In this experiment, ATPase activity was measured using the direct Pi release assay.

Table I. *K^+ Stimulation of ATPase Activity Associated with Purified Preparations of Chloroplast Inner Envelope*

Mg^{2+} (5 mM) and K^+ (50 mM) were added as the chloride salts. Activity was measured using the NADH-linked assay in all experiments. Because the ATPase activity in the presence of Mg^{2+} and the extent of K^+ stimulation varied considerably from preparation to preparation, results from a large number of replicate experiments are shown in this table. Data are presented as means of three replications ± SE. Whenever determined, ATPase activity in the absence of both Mg^{2+} and K^+ was close to zero. For example, in experiment 1, ATPase activity in the absence of Mg^{2+} and K^+ was 0.25 ± 0.25 μmol Pi/mg protein/h; this level of activity was 7% of the rate in the presence of Mg^{2+}.

Experiment No.	ATPase Activity		Stimulation[a]
	Mg	Mg + K	
	μmol Pi/mg protein/h		%
1	3.38 ± 0.03	4.54 ± 0.17	34
2	1.28 ± 0.13	4.35 ± 0.28	240
3	2.40 ± 0.20	5.33 ± 0.07	122
4	1.71[b]	4.37[b]	156
5	3.03 ± 0.28	10.52 ± 0.36	233
6	5.48 ± 0.24	8.17 ± 0.25	49
7	3.20 ± 0.17	8.27 ± 0.35	158
Average	2.93	6.51	122

[a] % Stimulation = $\frac{Rate_{Mg+K} - Rate_{Mg}}{Rate_{Mg}}$. [b] Only one measurement was made for each treatment in this experiment.

In four separate publications (10, 11, 16, 19) by several different research groups, it has been reported that K^+ has no effect on the envelope ATPase. We found a markedly different K^+ response in our preparations. In a number of experiments (Table I), K^+ was found to stimulate (by one- to twofold) ATPase activity. In light of the previous reports showing no significant K^+ response, we undertook extensive studies to rule out a number of artifacts as responsible for the K^+ stimulation of ATPase activity associated with our inner envelope membrane preparations.

The NADH-linked assay used in the experiments shown in Table I has been presented in review articles (21) as appropriate for measurement of other K^+-stimulated ATPases. However, K^+ has been found to stimulate the activity of pyruvate kinase, one of the linking enzymes in the assay (2). In control experiments (using ADP as a starting substrate), we found our assay to be sensitive to K^+ in the absence of added envelope protein (data not shown). This effect of K^+ on the assay enzymes should not much affect the measure of ATPase activity in the presence of added ATP and envelope protein. The activity of the linking enzymes, in the presence or absence of added K^+, should be far in excess of the rate of ATPase-dependent ADP generation. Supporting this contention, we found a similar extent of K^+ stimulation of ATPase activity despite increasing the concentration of linking enzymes in the assay mixture by fivefold (data not shown).

In continued experiments, we definitively ruled out the linking enzymes as responsible for the K^+ stimulation of ATPase activity in our membrane preparations by directly

monitoring ATP-dependent phosphate release. As shown in Figure 2, a marked stimulation of the ATPase by added K^+ was still noted when activity was monitored using the direct phosphate assay. Another artifact can be ruled out from the results shown in Figure 2. Cations were typically added to our assay mixture as chloride salts. As shown in Figure 2, K^+ added as the gluconate salt stimulated ATPase activity to at least as great an extent as KCl, discounting chloride additions as mediating enhanced ATPase activity.

Previous studies (3) have demonstrated that the plasmalemma ATPase is stimulated by K^+ in a fashion similar to that demonstrated here with our chloroplast inner envelope preparations. This point raises the possibility that K^+ stimulation of activity in our preparations (Table I, Fig. 2) is due to contamination by plasmalemma ATPase. Extensive studies were undertaken to address this possibility.

Keegstra and Yousif (12) suggested that analysis of gels run with inner envelope preparations can allow for an evaluation of their purity (*i.e.* cross-contamination by other membrane fractions). Contamination by plasmalemma ATPase in our envelope preparations was evaluated by analysis of polypeptide patterns on PAGE. Representative peptide patterns are shown in Figure 3. Comparison of these results with previously published (7, 12) peptide patterns of purified chloroplast inner envelope protein run on PAGE indicates a great deal of homology. For example, Coves *et al.* (7) found major bands at 30, 37 (this major band is the envelope ATPase; 28), 54 (standard Rubisco large subunit contamination), and 110 kD as representative of purified inner envelope preparations. These peptides are present as major bands in our preparations. Noticeably absent in our gels is a major band at 100 kD, which, if it were present, would be evidence of contamination by the plasmalemma ATPase (4). It can be concluded from

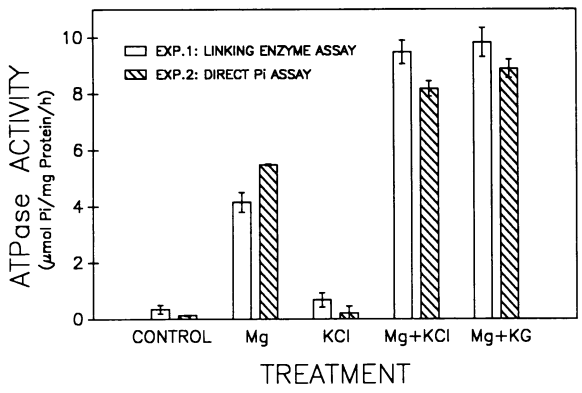

Figure 2. K^+ stimulation of chloroplast envelope ATPase. ATPase activity of envelope preparations was determined using either the NADH-linking enzyme assay or the direct measure of Pi release. Measurements were made in the presence and absence of Mg^{2+} (5 mM) and 50 mM chloride or gluconate (KG) salts of K^+. Each treatment value represents the mean of three replications and is shown ± SE. KCl stimulation of ATPase activity was also found using the direct Pi assay when experiment 2 was repeated a number of times. In these repeat experiments, rates in the presence of 5 mM $MgCl_2$ of 2.30 ± 0.25 and 0.93 ± 0.08 increased to 5.20 ± 0.10 and 2.19 ± 0.24 μmol Pi released/mg protein/h, respectively, when 50 mM KCl was added.

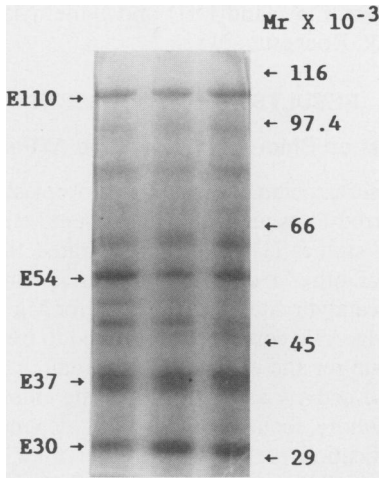

Figure 3. Patterns of Coomassie blue-stained chloroplast envelope proteins separated using SDS-PAGE. Protein extraction, 8% acrylamide gel preparation, and protein-resolving conditions essentially followed the procedures described by Piccioni *et al.* (23). Numbers on the right of the figure refer to mol wt standards in thousands. Numbers on the left preceded by "E" refers to the mol wts of a series of major envelope proteins presented by Coves *et al.* (7) as representative of chloroplast inner envelope protein preparations. The three lanes of a slab gel shown in this figure are from different envelope preparations.

the PAGE analysis shown in Figure 3, therefore, that no contamination of our preparations by the plasmalemma ATPase is evident and that a polypeptide of a molecular mass similar to the envelope ATPase (37 kD) is found in abundance.

The possibility that K^+-dependent ATPase activity in our envelope membrane preparations was due to the presence of plasmalemma ATPase contamination was evaluated in another manner. The plasmalemma ATPase has a relatively low pH optimum of about 6.5 to 7.0 (28), and K^+ enhancement of basal activity (*i.e.* in the presence of Mg^{2+} and ATP) also is optimal at a low pH of about 6.0 to 6.5 (5, 28). ATPase activity associated with the chloroplast inner envelope, in contrast, has a pH optimum close to 8.0 (18). In a number of experiments using two different buffering systems, optimal Mg^{2+}-dependent ATPase activity in our preparations (Fig. 4A) and K^+ stimulation above the level of activity found in the presence of Mg^{2+} alone (Fig. 4, A and B) were found to occur at pH 8.0.

No significant K^+ stimulation was evident at pH values <7.0 (Fig. 4). It can be concluded, then, that the K^+-stimulated ATPase activity in our preparations had a pH optimum that was representative of the chloroplast envelope ATPase. The data in Figure 4 also indicate that plasmalemma ATPase activity was likely not present. Plasmalemma contamination of our inner envelope membrane preparations was also probed by assaying our membrane vesicles for glucan synthase activity. This experiment (data not shown) indicated that there was no detectable activity of this plasmalemma marker enzyme in our preparations.

The data presented in Figures 1 to 4 and Table I offer

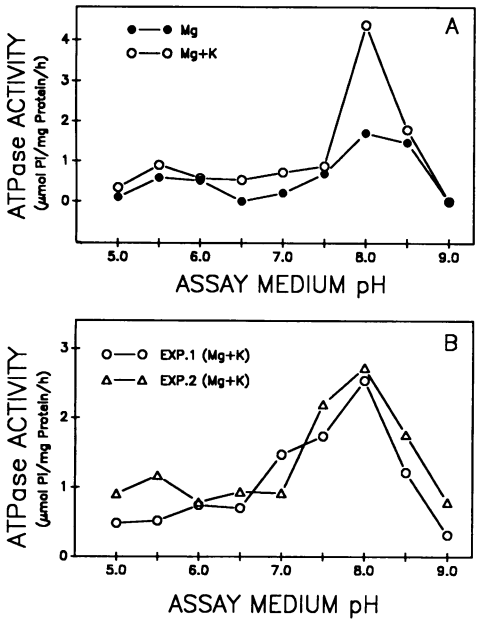

Figure 4. Assay medium pH effects on envelope ATPase activity. Activity was measured using the NADH-linked assay in the presence of 5 mM $MgCl_2$ (●) or 5 mM Mg^{2+} and 50 mM KCl (○, △). Two different buffer systems were used; 40 mM Tris-Mes (A) and 40 mM Tricine-Tris (B). Each data point represents a single measurement in these experiments.

substantial evidence that the characterization prevalent in the literature of the chloroplast envelope ATPase as an Mg^{2+}-stimulated activity insensitive to K^+ may be misleading. This enzyme may more appropriately be classified as a K^+-ATPase that uses divalent cation-chelated ATP as a substrate.

Inhibitor Sensitivity

Although not tested in the presence of K^+, chloroplast envelope ATPase activity has been well characterized with regard to differential response to a range of inhibitors. Typically, the activity has been found to be insensitive to DCCD (10, 18), an inhibitor of the thylakoid-coupling factor. We also found that ATPase activity of our envelope vesicles, both in the presence and absence of K^+, is insensitive to *N,N'*-dicyclohexylcarbodiimide (data not shown). Although McCarty *et al.* (16) reported that ATPase activity associated with pea chloroplast envelopes is insensitive to oligomycin, other researchers (15, 19) noted a 25 to 50% inhibition of activity in the presence of 20 μg/mL oligomycin. Representative of a number of experiments, data shown in Figure 5A show a 20% sensitivity of ATPase activity to oligomycin in the absence of K^+. Vanadate at concentrations approaching 1 mM has been found to completely inhibit ATPase activity in envelope vesicles (18, 19); at lower concentrations, only a slight effect has been noted (15, 16, 18). At 0.1 mM vanadate, we found no significant inhibition of ATPase activity in the absence of K^+ (Fig. 5B). Interestingly, we found much more marked effects of the inhibitors in the presence of K^+. As shown in Figure 5, A and B, oligomycin and vanadate had a greater inhibitory effect on the K^+-stimulated component of the total ATPase activity associated with the chloroplast envelope. These data suggest that, if there was more than one ATPase in the chloroplast envelope, these inhibitors were specifically affecting the K^+-stimulated component of total activity in the envelope membrane preparations. It should be noted that the data shown in Figure 5A indicate that K^+ apparently increased the inhibitory effect of oligomycin. One possible explanation for this effect could be that K^+ increased inhibitor binding to the ATPase. The presence of monovalent cations has been found to increase the binding of inhibitors to other ATPase pumps (26).

In an earlier study (30), we speculated from inhibitor experiments with intact chloroplasts that there may be more

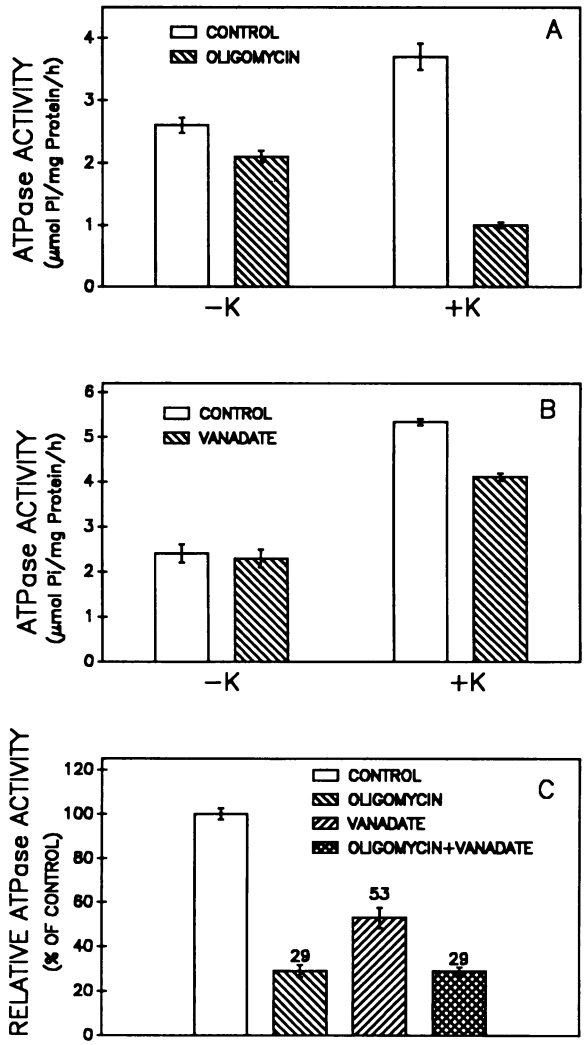

Figure 5. Oligomycin and vanadate effects on envelope ATPase activity in the presence and absence of K^+. The NADH-linked assay was used in all experiments, and in all cases 5 mM $MgCl_2$ was included in the assay medium. Oligomycin, vanadate, and K^+ were at 20 μg/mL, 0.1 mM, and 50 mM, respectively. For C, the numbers above the bars represent the rate of ATPase activity for that treatment as a percentage of the control rate. The control rate for the experiment shown in C was 5.4 μmol Pi/mg protein/h. For all experiments, results are shown as means of three replications ± SE.

than one type of ATPase in the chloroplast envelope. In light of the fact that E_1-E_2-type transport ATPases, such as the plasmalemma H^+ ATPase, are typically sensitive to vanadate and insensitive to oligomycin (4), we hypothesized that there may be different ATPases in our envelope preparation that were sensitive to either vanadate or oligomycin. Data presented in Figure 5C suggest otherwise. In this experiment, oligomycin inhibited K^+-stimulated ATPase activity by 71%. In the same preparation, vanadate inhibited activity by 47%. However, addition of both components together inhibited activity still by only 71%, a level of inhibition similar to that found in the presence of oligomycin alone. The effects of these inhibitors are not additive. The results shown in Figure 5C, then, are consistent with the hypothesis that these inhibitors affected the same, K^+-stimulated ATPase in our chloroplast envelope preparations. These data, however, do not completely rule out the possibility that our chloroplast inner envelope preparations contain several different K^+-stimulated ATPases.

An alternative explanation for the results shown in Figure 5C is that oligomycin somehow prevented vanadate inhibition of a second ATPase. Although we cannot completely rule out this possibility, there are no reports in the literature of this latter effect, and it is hard to imagine how one inhibitor prevented a different compound from acting on a second ATPase. The more plausible explanation seems to be that the inhibitors act on the same, K^+-stimulated ATPase. The possible establishment of this point, vanadate and oligomycin inhibition of the same K^+-stimulated ATPase, is intriguing. Vanadate sensitivity (with no effect by oligomycin; 15) is diagnostic for the E_1-E_2 K^+-stimulated plasmalemma ATPase (4). The sensitivity of K^+-stimulated chloroplast envelope ATPase activity to both inhibitors suggests that, although it may have some functional similarities to E_1-E_2-type ATPase such as the plasmalemma enzyme, there must be some structural distinctions allowing for differential sensitivity to inhibitors.

The data presented in Figure 5 also lend further support to the contention made here that the ATPase activity stimulated by K^+ in our preparations is, in fact, derived from the chloroplast inner envelope. Sensitivity to both vanadate and oligomycin is not common among known plant ATPases, and it is a characteristic of the envelope enzyme (19). In addition to the data in Figure 5A showing sensitivity of K^+-stimulated ATPase activity in our preparations to a low (20 μg/mL) level of oligomycin, other experiments indicated that higher oligomycin concentrations completely inhibited the rate (data not shown).

In previous work (30), we noted that the Na^+/K^+ pump inhibitor digitoxin inhibited photosynthesis, reduced stromal K^+, and increased stromal H^+ in the intact chloroplast. These effects were attributed to an inhibition of a chloroplast envelope ATPase that was thought to be involved (either directly or indirectly) in H^+ efflux/K^+ influx. It was of interest, therefore, to evaluate the effects of digitoxin on the K^+-stimulated ATPase activity in our envelope preparations. However, we noted no significant effect of this inhibitor on ATPase activity in either the presence or absence of K^+ or with or without treatment of the membrane vesicles with detergent to solubilize the ATPase (data not shown). Na^+/K^+ pump activity in membrane preparations is typically evidenced by a demonstration of digitoxin sensitivity and also by a stimulation of ATPase activity by addition of Na^+ and K^+ together (21). In addition to finding no digitoxin sensitivity, we found that ATPase activity in the presence of Na^+ and K^+ was never greater, and usually much lower, than the rate in the presence of K^+ alone (data not shown). Thus, in our work with purified inner envelope membranes reported here, we found no evidence to support our earlier hypothesis that one component of envelope ATPase activity may be a protein acting similarly to the Na^+/K^+ pump ATPase of animal cell membranes.

Kinetics of K^+ Stimulation

Figure 6A shows the K^+ stimulation of ATPase activity in envelope membrane preparations at a range of K^+ concentrations. These results indicate that the stimulation in activity is substantial at low concentrations. However, detailed analysis of the K^+ response is not possible; the K^+ activation of the enzyme does not obey simple Michaelis-Menton kinetics. This can be ascertained from the Lineweaver-Burk plot shown in Figure 6B. In such a plot, a concave downward curve (with increasing [substrate]) is representative of enzymes that exhibit negative cooperativity kinetics for ligand (in this case K^+) binding. Intriguingly, similar kinetics for K^+-stimulation of activity were demonstrated for the plasmalemma ATPase (14). In their thorough and insightful analysis, Leonard and Hodges (14) pointed out that such kinetics occur when the binding of the ligand to the first subunit of a multisubunit enzyme causes other subunits to have lower affinity for the ligand. Both the plasmalemma K^+-stimulated ATPase (4) and the chloroplast envelope ATPase (17) are thought to exist as oligomeric (or at least dimeric in the case of the plasmalemma ATPase) enzymes in their native membranes. The analysis shown in Figure 6, then, suggests that K^+ interaction with the envelope ATPase may be similar to that previously characterized for the plasmalemma ATPase. Both enzymes display

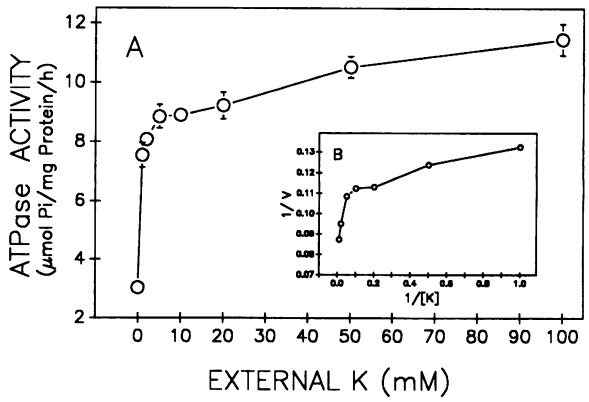

Figure 6. Kinetics of K^+ stimulation of envelope ATPase activity. Enzyme activity was determined using the NADH-linked assay in this experiment, and all assays included 5 mM $MgCl_2$. A, Data are shown as means of three replications ± SE. The same data are shown in B as a Lineweaver-Burk plot. This experiment was repeated a second time; the same downward curving line in the Lineweaver-Burk plot was evident (data not shown).

high affinity (*i.e.* a K_m well below 1 mM) for the cation at low $[K^+]$ with increasing K_m as $[K^+]$ increases.

The kinetics of K^+ stimulation of the plasmalemma ATPase have been correlated with the kinetics of K^+ uptake across the plasmalemma in a number of studies (*cf.* discussion in refs. 3, 4, 14, and 27). This association has been cited as evidence that the plasmalemma ATPase is involved (indirectly or directly) in energy-dependent K^+ uptake into the cytoplasm of plant cells. It is intriguing to note that a similar relationship exists between K^+ stimulation of envelope ATPase activity and light-dependent K^+ uptake into the stroma of the chloroplast. A Lineweaver-Burk plot of K^+ ($^{86}Rb^+$) uptake into chloroplasts generated from the data of Demmig and Gimmler (9) has a concave downward curve displaying kinetics similar to those shown in Figure 6B.

Conclusion

Studies included in this report focused on characterizing ATPase activity associated with purified chloroplast inner envelope preparations. This enzyme has been heretofore referred to as a Mg^{2+}-dependent ATPase insensitive to monovalent cations. Based on the results reported here, we conclude that this enzyme likely is not stimulated by free Mg^{2+} (and, in fact, may be inhibited). Rather, the enzyme appears to be stimulated by millimolar levels of K^+. The identification of this effect has important implications with regard to the physiological role of this enzyme. One- or twofold K^+ stimulation of activity has been noted for the plasmalemma H^+-ATPase. It has been theorized (3, 4, 27) that this K^+ stimulation is related to the physiological role of the H^+-ATPase in indirectly facilitating K^+ import into plant cells. K^+ stimulation of chloroplast envelope ATPase activity of this magnitude certainly does not prove that it acts as a K^+ pump *in situ*. However, this finding does suggest that the enzyme may act indirectly to facilitate energy-dependent K^+ accumulation in the stroma.

The envelope ATPase may act *in situ* as an H^+ efflux pump with linkage to K^+ import occurring via H^+ efflux-induced changes in membrane potential across the envelope. Alternatively, it could act to directly pump K^+ into the chloroplast against an apparent concentration gradient *in situ*. In either case, the activity of this enzyme could be an important mechanism allowing for the maintenance of an alkaline stroma. We (31) and others (9, 15) have theorized that the accumulation of high stromal K^+ reduces the driving force for H^+ import into the chloroplast.

Thus, new evidence has been presented in this report that is consistent with the hypothesized relationship between the activity of this enzyme *in situ* and physiological regulation of monovalent cation fluxes across the chloroplast envelope. However, the definitive identification of this enzyme as a primary K^+ (or H^+) pump awaits further study. Future work should focus on reconstitution of the purified enzyme into liposomes capable of maintaining measurable ion gradients. One last point germane to this analysis is the novel possibility raised by Serrano (27) in a recent review. In reviewing evidence linking the plasmalemma H^+-ATPase to K^+ transport, Serrano (27) raised the possibility that the ATPase molecule might be "tightly associated with a K^+ channel and may actually contain it." We recently demonstrated that the chloroplast inner envelope likely contains a well-regulated (*i.e.* gated) K^+ channel. Perhaps this close association also is present at the organelle membrane.

LITERATURE CITED

1. **Balke NE, Hodges TK** (1975) Plasma membrane adenosine triphosphatase of oat roots. Activation and inhibition by Mg^{2+} and ATP. Plant Physiol **55:** 83–86
2. **Baysdorfer C, Bassham JA** (1984) Spinach pyruvate kinase isoforms. Partial purification and regulatory properties. Plant Physiol **74:** 374–379
3. **Briskin DP** (1986) Plasma membrane H^+-transporting ATPase. Role in potassium ion transport? Physiol Plant **68:** 159–163
4. **Briskin DP** (1990) The plasma membrane H^+-ATPase of higher plant cells: biochemistry and transport function. Biochim Biophys Acta **1019:** 95–109
5. **Briskin DP, Poole RJ** (1983) Characterization of a K^+-stimulated adenosine triphosphatase associated with the plasma membrane of red beet. Plant Physiol **71:** 350–355
6. **Chang TM, Penefsky HS** (1973) Aurovertin, a fluorescent probe of conformational change in beef heart mitochondrial adenosine triphosphatase. J Biol Chem **248:** 2746–2754
7. **Coves J, Pineau B, Joyard J, Douce R** (1988) Solubilization of chloroplast envelope membranes by detergents and its effects on activity of envelope marker enzymes. Plant Physiol Biochem **26:** 151–163
8. **Dawson RMC, Elliott DC, Elliott WH, Jones KM** (1986) Data for Biochemical Research, Ed 3. Oxford University Press, New York, pp 413–414
9. **Demmig B, Gimmler H** (1983) Properties of isolated intact chloroplasts at cytoplasmic K^+ concentrations. I. Light-induced cation uptake into intact chloroplasts is driven by an electrical potential difference. Plant Physiol **73:** 169–174
10. **Douce R, Holtz RB, Benson AA** (1973) Isolation and properties of the envelope of spinach chloroplasts. J Biol Chem **248:** 7215–7222
11. **Joyard J, Douce R** (1976) Preparation et activities enzymatiques de l'enveloppe des chloroplastes d'epinard. Physiol Veg **14:** 31–48
12. **Keegstra K, Yousif AE** (1986) Isolation and characterization of chloroplast envelope membranes. Methods Enzymol **118:** 316–325
13. **LeBel D, Poirier GG, Beaudoin AR** (1978) A convenient method for the ATPase assay. Anal Biochem **85:** 86–89
14. **Leonard RT, Hodges TK** (1973) Characterization of plasma membrane-associated adenosine triphosphatase activity of oat roots. Plant Physiol **52:** 6–12
15. **Maury WJ, Huber SC, Moreland DE** (1981) Effects of magnesium on intact chloroplasts. II. Cation specificity and involvement of the envelope ATPase in (sodium) potassium/proton exchange across the envelope. Plant Physiol **68:** 1257–1263
16. **McCarty DR, Keegstra K, Selman BR** (1984) Characterization and localization of the ATPase associated with pea chloroplast envelope membranes. Plant Physiol **76:** 584–588
17. **McCarty DR, Selman BR** (1986) Partial purification of a nucleoside triphosphatase from the inner membrane of the chloroplast envelope of pea. Arch Biochem Biophys **248:** 523–531
18. **McCarty DR, Selman BR** (1986) Properties of a partially purified nucleoside triphosphatase (NTPase) from the chloroplast envelope of pea. Plant Physiol **80:** 908–912
19. **Nguyen TD, Siegenthaler PA** (1983) Proteins and polypeptides of envelope membranes from spinach chloroplasts. FEBS Lett **164:** 67–70
20. **Nguyen TD, Siegenthaler PA** (1985) Purification and some properties of an Mg^{2+}-, Ca^{2+}- and calmodulin-stimulated ATPase from spinach chloroplast envelope membranes. Biochim Biophys Acta **840:** 99–106
21. **Norby JG** (1988) Coupled assay of Na^+, K^+-ATPase activity. Methods Enzymol **156:** 116–119
22. **Peters J, Berkowitz GA** (1991) Studies on the system regulating

proton movement across the chloroplast envelope. Plant Physiol **95:** 1229–1236

23. **Piccioni R, Bellemare G, Chua N-H** (1982) Methods of polyacrylamide gel electrophoresis in the analysis and preparation of plant polypeptides. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam, The Netherlands, pp 985–1014
24. **Robinson SP** (1985) The involvement of stromal ATP in maintaining the pH gradient across the chloroplast envelope in the light. Biochim Biophys Acta **806:** 187–194
25. **Sabinis DD, Gordon M, Galston AW** (1970) Localization of adenosine triphosphatase activity on the chloroplast envelope in tendrils of *Pisum sativum*. Plant Physiol **45:** 25–32
26. **Schwartz A, Whitmer K, Grupp G, Grupp I, Adams RJ, Lee S-W** (1982) Mechanism of action of digitalis: is the Na,K-ATPase the pharmacological receptor? Ann NY Acad Sci **402:** 253–271
27. **Serrano R** (1989) Structure and function of plasma membrane ATPase. Annu Rev Plant Physiol Plant Mol Biol **40:** 61–94
28. **Sommarin M, Lundborg T, Kylin A** (1985) Comparison of K,Mg-ATPases in purified plasmalemma from wheat and oat. Substrate specificities and effects of pH, temperature and inhibitors. Physiol Plant **65:** 27–32
29. **Werdan K, Heldt HW, Milovancev M** (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. Biochim Biophys Acta **396:** 276–292
30. **Wu WH, Berkowitz GA** (1991) Lidocaine and ATPase inhibitor interaction with the chloroplast envelope. Plant Physiol **97:** 1551–1557
31. **Wu WH, Berkowitz GA** (1992) Stromal pH and photosynthesis are affected by electroneutral K^+ and H^+ exchange through chloroplast envelope ion channels. Plant Physiol **98:** 666–672