Plant Physiology, April 1999, Vol. 119, pp. 1379–1385, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Identification of a Ca^{2+}/H^{+} Antiport in the Plant Chloroplast Thylakoid Membrane[1]

William F. Ettinger*, Anne M. Clear, Katheryn J. Fanning, and Mary Lou Peck

Department of Biology, Gonzaga University, E. 502 Boone Avenue, Spokane, Washington 99258

To assess the availability of Ca^{2+} in the lumen of the thylakoid membrane that is required to support the assembly of the oxygen-evolving complex of photosystem II, we have investigated the mechanism of $^{45}Ca^{2+}$ transport into the lumen of pea (*Pisum sativum*) thylakoid membranes using silicone-oil centrifugation. Transthylakoid Ca^{2+} transport is dependent on light or, in the dark, on exogenously added ATP. Both light and ATP hydrolysis are coupled to Ca^{2+} transport through the formation of a transthylakoid pH gradient. The H^{+}-transporting ionophores nigericin/K^{+} and carbonyl cyanide 3-chlorophenylhydrazone inhibit the transport of Ca^{2+}. Thylakoid membranes are capable of accumulating up to 30 nmol Ca^{2+} mg^{-1} chlorophyll from external concentrations of 15 μM over the course of a 15-min reaction. These results are consistent with the presence of an active Ca^{2+}/H^{+} antiport in the thylakoid membrane. Ca^{2+} transport across the thylakoid membrane has significant implications for chloroplast and plant Ca^{2+} homeostasis. We propose a model of chloroplast Ca^{2+} regulation whereby the activity of the Ca^{2+}/H^{+} antiporter facilitates the light-dependent uptake of Ca^{2+} by chloroplasts and reduces stromal Ca^{2+} levels.

The physiology of Ca^{2+} in the plant cell and its distribution among various organelles, gradients, and transient fluxes continues to be the subject of research and the focus of several recent reviews (Evans et al., 1991; Bush, 1993, 1995; Gilroy et al., 1993). However, little attention has been given to the problem of Ca^{2+} transport across the thylakoid membrane. In fact, Ca^{2+} is required for several essential processes inside the chloroplast thylakoid lumen. In particular, Ca^{2+} ions are essential for the function of the OEC, a multimeric complex in the thylakoid lumen responsible for light-dependent oxygen evolution in plants. Functional assembly of PSII and the OEC requires that all essential polypeptides and cofactors are present in the stroma, thylakoid membrane, or thylakoid lumen.

The assembly of the OEC inside the thylakoid lumen requires the assembly of the OE33, OE23, and OE17 polypeptides and the essential inorganic ions Mn^{2+}, Ca^{2+}, and Cl^{-} to the OEC in a light-dependent process (Ghanotakis et al., 1984; Becker et al., 1985; Miller and Brudvig, 1989). Additionally, in saturating light the reaction center D1 protein of PSII rapidly becomes damaged in a process known as photoinhibition (Mattoo et al., 1989). Damage resulting from photoinhibition is repaired by the proteolytic degradation of the D1 protein, followed by the disassembly of the remaining PSII proteins and OEC polypeptides, the resynthesis of D1, and the reassembly of a new PSII core and OEC from existing polypeptides and ions (Broussac et al., 1990; Hundal et al., 1990a, 1990b; Virgin et al., 1990). Therefore, both the initial assembly of PSII and its subsequent reassembly after photoinhibition require Ca^{2+} availability in the chloroplast thylakoid lumen. Furthermore, Ca^{2+} in the thylakoid lumen has been implicated in the formation and maintenance of localized proton domains (Dilley and Chiang, 1989) and the stabilization of the high redox potential form of Cyt b_{559} (McNamera and Gounaris, 1995). All of these processes require the availability of Ca^{2+} in the thylakoid lumen.

Ca^{2+} transported to the thylakoid lumen must originate from the cytosol and be transported through the chloroplast envelope membranes, the stroma, and the thylakoid membrane. It is well documented that Ca^{2+} is actively transported across the chloroplast envelope membranes in the light (Nobel and Packer, 1965; Nobel, 1967, 1969; Muto et al., 1982; Kreimer et al., 1985a, 1985b, 1988). Transenvelope Ca^{2+} movement from the cytosol to the stroma is believed to be energetically coupled to a significant membrane potential across the inner envelope membrane through an electrogenic Ca^{2+} pump (Kreimer et al., 1985b).

Although it is conceivable that Ca^{2+} transport across the thylakoid membrane may be dependent on simple diffusion, the diffusion of a such a large divalent ion across the lipid bilayer of the thylakoid membrane could be a rate-limiting process of OEC biogenesis. Moreover, measurements of transthylakoid membrane potential in the light indicate that there is a 15- to 30-mV positive membrane potential across the thylakoid membrane (Bulychev et al., 1972; Hangarter and Good, 1982). According to the Nernst equation, neither simple nor facilitated diffusion of Ca^{2+} across the thylakoid membrane would be thermodynamically spontaneous unless the ratio of free Ca^{2+} were greater than about 3:1 (stromal:luminal). However, the low-affinity Ca^{2+}-binding site for the photoactivation process of the OEC is reported to have a K_d of 0.3 mM (Miller and Brudvig, 1989; see also Debus, 1992). This suggests that significantly higher Ca^{2+} concentrations are required in the thylakoid lumen for PSII assembly than the 2 to 6 μM free Ca^{2+} in the stroma (Kreimer et al., 1988; Johnson et al., 1995).

[1] This research was funded by a grant to W.F.E. from the M.J. Murdock College Science Research Program.

* Corresponding author; e-mail ettinger@gonzaga.edu; fax 1–509–323–5718.

*Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DCCD, *N,N′*-dicyclohexylcarbodiimide; DCPIP, 2,6-dichlorophenolindophenol; FBPase, Fru-1,6-bisphosphatase; OEC, oxygen-evolving complex of PSII.

In this study we investigated how the essential inorganic ion Ca^{2+} was translocated across the thylakoid membrane to the thylakoid lumen. Using isolated, intact thylakoid membranes we have demonstrated that a significant amount of $^{45}Ca^{2+}$ is translocated across the thylakoid membrane in an energy- and time-dependent process. Light (or ATP in the dark) is necessary to support $^{45}Ca^{2+}$ transport. Furthermore, the activity of the transport process was shown to be sensitive to proton-translocating uncouplers such as CCCP and nigericin/K^+. On the basis of our results we propose that a Ca^{2+}/H^+ antiport actively moves Ca^{2+} from the stroma into the thylakoid lumen in the light. The antiporter provides the concentrations of luminal Ca^{2+} required for the assembly of the OEC and supports other Ca^{2+}-requiring reactions in the thylakoid lumen.

MATERIALS AND METHODS

Materials

We purchased $^{45}Ca^{2+}$ from New England Nuclear. Sigma provided ATP, Percoll, nigericin, CCCP, DCPIP, DCCD, AMP-PNP, tentoxin, and A23187. All other chemicals and reagents were of the highest quality commercially available.

Chloroplast and Thylakoid Isolation

We soaked pea (*Pisum sativum* L. cv Laxton's Progress) seeds in deionized water and planted them 1-cm deep in potting soil. The plants grew in the laboratory under fluorescent lights (18-h days and 6-h nights) and watered as needed with deionized water. Intact chloroplasts and thylakoids were isolated from 12- to 16-d-old pea seedlings essentially as described previously (Cline et al., 1985; Theg et al., 1986). Seedlings were homogenized in a grinding buffer containing 0.05 M potassium-Hepes (pH 7.3), 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM Na_2EDTA, and 0.1% BSA. Intact chloroplasts were isolated by density-gradient centrifugation of the homogenate through a linear Percoll gradient. We isolated the thylakoid membranes from the chloroplasts osmotically. First, they were lysed in 10 mM potassium-Hepes (pH 6.5), and 5 mM $MgCl_2$ on ice for 5 min. The thylakoids were then separated from the stroma by centrifugation and resuspended in an import buffer containing 0.05 M potassium-Tricine (pH 8.0), 0.33 M sorbitol, and 5 mM $MgCl_2$. Chlorophyll concentration was determined by the method of Arnon (1949).

Measurement of ^{45}Ca Transport

We completed the standard Ca^{2+} import reactions in 0.05 M potassium-Tricine (pH 8.0), 0.33 M sorbitol, and 5 mM $MgCl_2$. Thylakoid membranes were added to a final concentration of 0.33 mg chlorophyll mL^{-1}. Other additions to the reactions (ATP, nigericin, and ATPase inhibitors) are indicated in the figure legends. We initiated the reactions by adding 1.5 or 15 μM $^{45}Ca^{2+}$ (12.6 Ci g^{-1}) to the mixture containing import buffer and thylakoids. Reactions run in the light were placed 20 cm from a 60-W incandescent light filtered through 10 cm of a 5% $CuSO_4$ solution at 25°C; dark reactions were placed inside a drawer at 25°C. Reactions were terminated by centrifugation of 60-μL aliquots (20 μg of chlorophyll) through 100 μL of silicone oil, 65% AR-200, and 35% AR-20 (Wacker Silicones, Adrian, MI) layered over 100 μL of 1.5 M perchloric acid in a 0.4-mL microfuge tube. Thylakoid membranes sedimented through the silicone oil layer into the perchloric acid, while the reaction buffer and unincorporated $^{45}Ca^{2+}$ remained in the aqueous layer above the silicone oil (Heldt, 1980). The tubes were then frozen in liquid nitrogen. The frozen tubes were cut through the middle silicone oil layer, and the thylakoid membranes in the lower portion were suspended in a scintillation cocktail (Ecolume, Beckman) before counting. The results were converted from counts per minute to micromoles of Ca^{2+} per milligram of chlorophyll using an average 86% ^{45}Ca-counting efficiency.

RESULTS

Energy-Dependent Transport of Ca^{2+} across the Thylakoid Membrane

We sought to determine the mechanism of $^{45}Ca^{2+}$ transport across the thylakoid membrane by using intact, isolated thylakoids and silicone oil centrifugation. We determined that Ca^{2+} transport proceeds at a significant rate when thylakoids are exposed to light but that the rate is very low in the dark (Fig. 1). The transport reaction reached a plateau after approximately 15 min. Analysis of the rate of Ca^{2+} transport under these conditions indicated that thylakoids are capable of accumulating approximately 2 nmol Ca^{2+} min^{-1} mg^{-1} chlorophyll from external concentrations of 15 μM. There was also a low level of $^{45}Ca^{2+}$ uptake by the membranes that was independent of time or

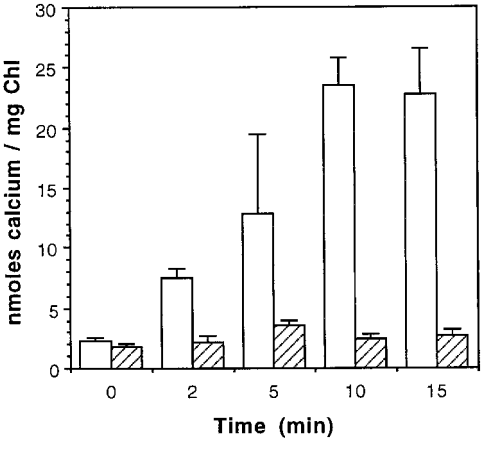

Figure 1. Light-dependent accumulation of $^{45}Ca^{2+}$ across intact, isolated thylakoid membranes. Duplicate transport reactions were initiated by adding $^{45}Ca^{2+}$ (15 μM final concentration) to thylakoid membranes maintained in the light (open bars) or dark (hatched bars). After mixing and at the intervals indicated, 60-μL aliquots were removed from the reactions and the transport reactions were stopped by centrifugation of the membranes through silicone oil. Chl, Chlorophyll.

the state-of-energization of the membranes. This is consistent with Ca^{2+} binding to, or simply diffusing across, the membranes through non-energy-dependent processes.

Light or ATP Hydrolysis Can Facilitate Ca^{2+} Transport

Predominant models of energy-dependent Ca^{2+} transport across membranes rely on either ATP (Ca^{2+}-ATPase) or a ΔpH (Ca^{2+}/H^+ antiport) to support Ca^{2+} transport against its electrical or chemical potentials. The action of light on isolated thylakoid membranes can generate both a ΔpH and ATP through the combined actions of the photosystems and ATP-synthase. Therefore, we tried to determine the means by which light stimulated the uptake of $^{45}Ca^{2+}$ by assessing the ability of ATP to drive the transport reaction in the dark. As shown in Figure 2, 3 mM ATP supported approximately 60% of the import amount in the dark as was achieved in the light in the absence of exogenously added ATP. In the light, added ATP had no significant effect on Ca^{2+} import.

ΔpH Is Necessary for Ca^{2+} Transport

Exogenously added ATP could drive Ca^{2+} transport directly, through a Ca^{2+}-ATPase, or indirectly, through a Ca^{2+}/H^+ antiport coupled to a transmembrane ΔpH generated by ATP hydrolysis by the thylakoid ATP-synthase. A Ca^{2+}/H^+-antiport mechanism would require that ATP hydrolysis be energetically coupled to the Ca^{2+}/H^+ antiport through the formation of a ΔpH. A Ca^{2+}-ATPase would have no such dependence on a ΔpH. Inhibition of the ATP-dependent transport reaction by H^+-translocating uncouplers suggest that Ca^{2+} transport is mediated by a Ca^{2+}/H^+ antiport. Nigericin catalyzes the electroneutral exchange of H^+ and K^+ across membranes and dissipates the thylakoid proton gradient as it transports K^+ into the thylakoid lumen (Shavit et al., 1968). As demonstrated in Figure 3, nigericin/K^+ inhibited the light-dependent import of $^{45}Ca^{2+}$ at concentrations greater than approximately 50 nM. Therefore, the dependence of the reaction on ΔpH strongly suggests that ΔpH was the primary energy source driving Ca^{2+} import through a Ca^{2+}/H^+ antiporter. These results also suggest that the hydrolysis of ATP by the thylakoid ATP-synthase provided the ΔpH to support Ca^{2+} import in the dark.

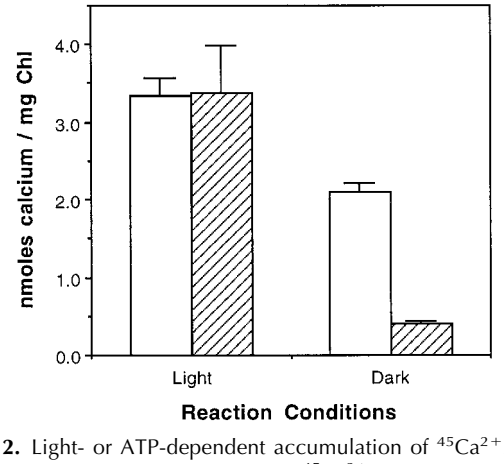

Figure 2. Light- or ATP-dependent accumulation of $^{45}Ca^{2+}$. Transport reactions were initiated by adding $^{45}Ca^{2+}$ (1.5 μM final concentration) to thylakoid membranes suspended in import buffer containing 5 mM $MgCl_2$ and 3 mM ATP. Duplicate reactions were maintained in the dark or light. Reactions were terminated after 15 min. White bars, +ATP; hatched bars, −ATP. Chl, Chlorophyll.

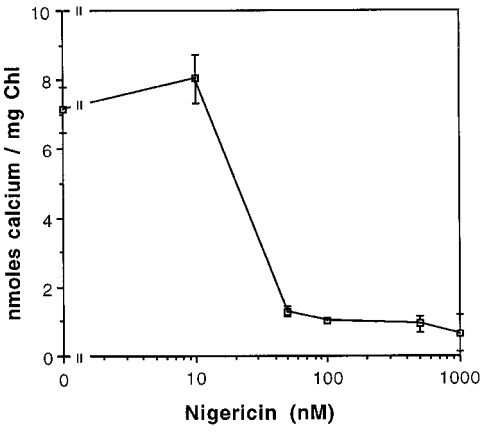

Figure 3. The light-dependent $^{45}Ca^{2+}$ transport reaction is sensitive to nigericin. Reactions were initiated by adding $^{45}Ca^{2+}$ (1.5 μM final concentration) to thylakoid membranes suspended in import buffer in the light. Nigericin was added from an ethanolic stock to the concentration indicated. Potassium, approximately 25 mM, was present in the reaction as the counterion to the Tricine buffer. An equal volume of ethanol (1 μL per 60-μL reaction) was added to all reactions. Reactions were terminated after 15 min. Chl, Chlorophyll.

Light Energy Provides the ΔpH Required for Ca^{2+} Import

If low levels of residual ADP and Pi were trapped in the thylakoid membranes during isolation, and if ADP was subsequently phosphorylated by ATP-synthase in the light, then it is possible that small amounts of ATP could be formed in the light in our standard reactions. To rule out the direct involvement of ATP in the light-dependent transport reaction, we performed the reaction in the presence of various inhibitors of the thylakoid ATP-synthase. Inhibition of either the CF_1 or CF_o portion of ATP-synthase should prevent the formation of ATP in the light. If light-driven Ca^{2+} transport was not altered by the addition of ATPase inhibitors, then these results would confirm that the sole energy source for Ca^{2+} import is ΔpH and not ATP hydrolysis. DCCD blocked the Cf_o channel of the thylakoid H^+-ATPase (Nelson et al., 1977). Tentoxin stopped ATP hydrolysis or synthesis by H^+-ATPase (Steele et al., 1976). AMP-PNP is an ATP analog that inhibited ATP binding to ATPases (Robinson and Wiskich, 1977). We used these inhibitors of ATP-synthase in the light to demonstrate that neither ATP synthesis nor hydrolysis was required for light-dependent $^{45}Ca^{2+}$ import (Fig. 4). These inhibitors stopped ATP-dependent import in the dark, which was expected since they blocked ATP-synthase-dependent ΔpH formation. The slight decrease in the activity of the Ca^{2+}/H^+ antiporter in the presence of DCCD may have been due to the reported sensitivity of the tonoplast

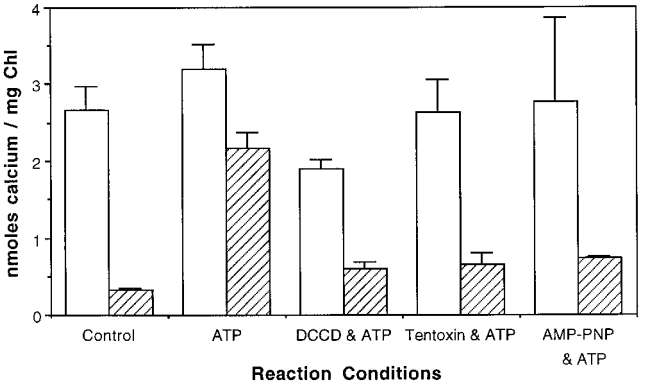

Figure 4. ATP-dependent $^{45}Ca^{2+}$ transport is sensitive to inhibitors of the H^+-ATP synthase. Thylakoid membranes were preincubated with DCCD (100 μM) for 10 min and the membranes were repurified by centrifugation before use. Tentoxin (4 μM) was added to intact chloroplasts, which were then incubated on ice for 1 h before thylakoid membranes were isolated. AMP-PNP was added to a final concentration of 3 mM from an aqueous stock. Transport reactions were initiated by adding $^{45}Ca^{2+}$ (1.5 μM final concentration) to thylakoid membranes suspended in import buffer containing 5 mM $MgCl_2$ and 3 mM ATP in the light (white bars) or in the dark (hatched bars). Reactions were terminated after 15 min. Chl, Chlorophyll.

Ca^{2+}/H^+ antiporter to DCCD (Schumaker and Sze, 1986). Furthermore, these results suggest that a Ca^{2+}-ATPase is not responsible for import, as it likely would have been inhibited by AMP-PNP. These results are consistent with a thylakoid-localized Ca^{2+}/H^+ antiporter.

Characterization of the Ca^{2+}/H^+ Antiporter

If the activity of the Ca^{2+}/H^+ antiporter is dependent on light-stimulated proton pumping, then the reaction rate may be enhanced by the addition of artificial electron acceptors to the isolated thylakoid membranes used in our reactions. However, as shown in Figure 5, attempts to optimize the activity of the Ca^{2+}/H^+ antiporter in intact isolated membranes by the addition of the electron acceptors DCPIP and K_3FeCN_6 proved unsuccessful. We also investigated the effect of several other ions that could be inhibitory. La^{3+} is a well-characterized inhibitor of Ca^{2+} channels and might also interact with this Ca^{2+} antiporter. Likewise, Mn^{2+} that is translocated across the thylakoid membrane by unknown mechanisms may utilize the Ca^{2+}/H^+ antiporter as a transport mechanism. Furthermore, the addition of either $LaCl_3$ or $MnCl_2$ at a concentration of 100 μM had no inhibitory effect on the Ca^{2+}/H^+ antiporter (Fig. 5). Incidentally, the presence or absence of 5 mM $MgCl_2$ had no pronounced effect on the activity of the Ca^{2+}/H^+ antiporter in the light, but $MgCl_2$ was required for optimal activity in the ATP-dependent reaction in the dark (data not shown). Finally, the Ca^{2+} ionophore A23187 and the uncoupler CCCP were shown to inhibit the transport reaction. To ensure that the transport of Ca^{2+} across the thylakoid membrane was applicable to other plant species, we performed sample reactions using thylakoid membranes from chloroplasts isolated from spinach.

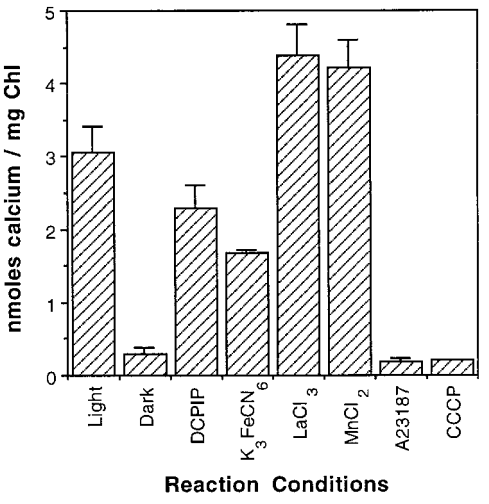

Figure 5. Characterization of $^{45}Ca^{2+}$ transport across the thylakoid membrane. Light-dependent $^{45}Ca^{2+}$ transport was assessed in the presence of DCPIP (10 μM), K_3FeCN_6 (1 mM), Ca^{2+} ionophore A23187 (5 μM), CCCP (5 μM), $LaCl_3$ (100 μM), and $MnCl_2$ (100 μM). One microliter of a concentrated stock of each effector was added to the thylakoid membranes before the reaction was initiated. Transport reactions were initiated by adding $^{45}Ca^{2+}$ (1.5 μM final concentration) to thylakoid membranes suspended in import buffer at 25°C in the light or dark. Reactions were terminated after 15 min. Chl, Chlorophyll.

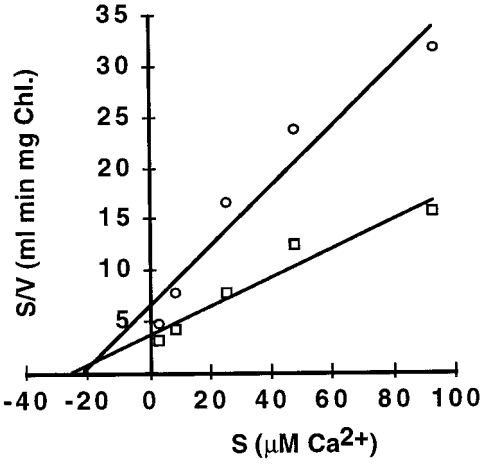

Figure 6. Kinetic analysis of Ca^{2+} uptake by isolated thylakoids(□) and intact chloroplasts (○). A series of reaction solutions were prepared by combining $CaCl_2$ and 3 μM of $^{45}Ca^{2+}$ to a final Ca^{2+} concentration of 3.0, 8.6, 25.5, 48.0, or 93.0 μM. Light-dependent reactions were run in triplicate and initiated by the addition of thylakoid membranes. Reactions were terminated after 1.0, 2.5, and 4.0 min. Reaction rates for each Ca^{2+} concentration were determined by linear-regression analysis of the average Ca^{2+} uptake at the three different time points. Reactions with intact chloroplasts were run in an identical manner, except the reactions were terminated by centrifugation of the membranes through a layer of 100% AR-200 silicone oil. Results shown are the averages of two independent experiments. Chl, Chlorophyll.

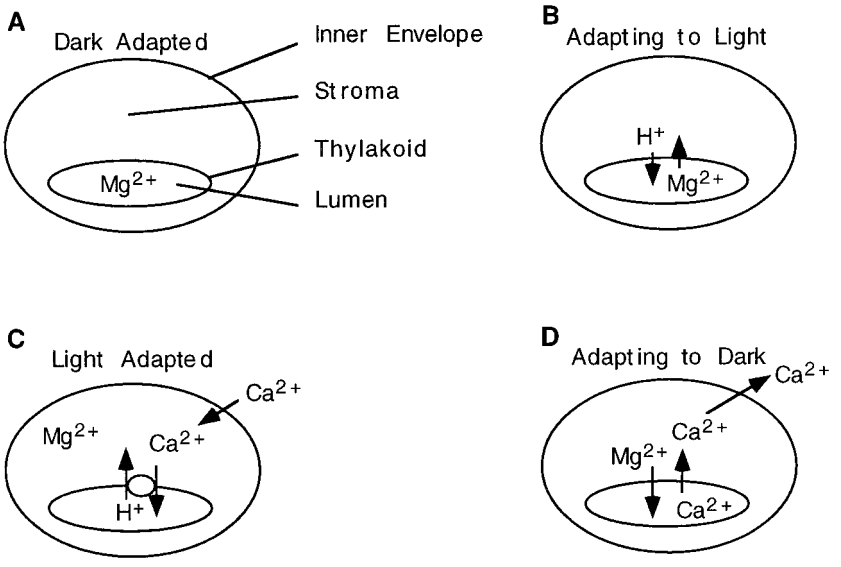

Figure 7. A model of Ca^{2+} flux in the intact chloroplast. Dark-adapted chloroplasts contain micromolar levels of free Ca^{2+} in the stroma, and Mg^{2+} ions are sequestered in the thylakoid lumen (A). The light-stimulated release of Mg^{2+} from the thylakoid lumen reduces the transthylakoid membrane potential (B). The Ca^{2+}/H^+ antiporter pumps Ca^{2+} into the thylakoid lumen and prevents Ca^{2+}-mediated inhibition of CO_2 fixation (C). Adaptation to the dark prompts the electroneutral exchange of Ca^{2+} and Mg^{2+} across the thylakoid membrane and results in Calvin-Benson-cycle inhibition by Ca^{2+} in the dark (D).

Spinach chloroplasts yielded results nearly identical to those of pea (data not shown).

We investigated the kinetics of Ca^{2+} uptake by chloroplast thylakoid membranes and intact chloroplasts by measuring the initial rate of Ca^{2+} uptake as a function of increasing Ca^{2+} concentration (Fig. 6). Analysis of these data demonstrated that the thylakoid transport reaction has a K_m for Ca^{2+} of 24 μM and a V_{max} of 7 nmol min^{-1} mg^{-1} chlorophyll. The K_m for Ca^{2+} of the thylakoid membrane Ca^{2+}/H^+ antiporter is comparable to reported K_m values (10–70 μM) for the Ca^{2+}/H^+ antiporters isolated from higher-plant tonoplast membranes (Schumaker and Sze, 1986; Blackford et al., 1990). The transport of Ca^{2+} across thylakoid membranes and intact chloroplasts showed similar kinetics. Intact chloroplasts have a K_m for Ca^{2+} of 22 μM and a V_{max} of 3 nmol min^{-1} mg^{-1} chlorophyll (Fig. 7). However, the value we obtained for the K_m for Ca^{2+} of the chloroplast Ca^{2+}-transport reaction was significantly lower than the 180 and 188 μM values obtained by Muto et al. (1982) and Kreimer et al. (1985b), respectively. The difference in relative Ca^{2+} K_m values between our data and previously reported values may reflect our differing methods but may also indicate that, like mitochondria, chloroplasts have multiple Ca^{2+}-transport mechanisms (Gunter et al., 1994).

DISCUSSION

We initiated this study to elucidate the mechanism of Ca^{2+} transport across the thylakoid membrane and to assess its availability for assembly into the OEC. We utilized thylakoid membrane fractions prepared by osmotic lysis from freshly isolated Percoll-gradient-purified chloroplasts to ensure membrane purity and stability. Thylakoid membranes prepared the same way have been shown to be competent in the ΔpH- or ATP-dependent transport of the precursor forms of plastocyanin, OE33, OE23, and OE17 and in the integration of the precursor form of the light-harvesting chlorophyll *a/b* protein into the thylakoid membrane (Cline et al., 1992). Uptake of $^{45}Ca^{2+}$ by the thylakoid membranes was monitored by silicone oil centrifugation and liquid-scintillation counting of the recovered membranes (Heldt, 1980). This method has enabled us to demonstrate that the import of a significant amount Ca^{2+} into the thylakoids is time and energy dependent. Our results demonstrate that the transthylakoid ΔpH is necessary and sufficient for Ca^{2+} import across the thylakoid membrane, and our results also strongly suggest that the mechanism for the import of Ca^{2+} is a Ca^{2+}/H^+ antiporter powered by a light- or ATP-induced proton gradient.

Early studies of Ca^{2+} transport across the membranes of isolated chloroplasts were initiated by Nobel and coworkers (Nobel and Packer, 1965; Nobel, 1967, 1969), who demonstrated that Ca^{2+} transport into chloroplast membranes is a light-dependent process that requires ATP and Mg^{2+} and is facilitated by the addition of thiol reagents and PMS. However, these studies did not suggest a mechanism of Ca^{2+} transport across the chloroplast membrane, nor did they separate out the role of the envelope or thylakoid membranes, light, ΔpH, or ATP in the transport process. Studies with intact chloroplasts have been carried out by Muto et al. (1982) and Kreimer et al. (1985a, 1985b, 1988). These studies demonstrated that Ca^{2+} transport from the cytosol into the chloroplast is a light-dependent process. According to their model, the total Ca^{2+} concentration in the chloroplast is 4 to 23 mM. However, much of the stromal Ca^{2+} is bound or sequestered, and the free Ca^{2+} concentration in the stroma is only 2 to 6 μM. Therefore, stroma-free Ca^{2+} is about 10-fold more concentrated than in the cytosol (100–300 nM), and transport must be energetically facilitated by an electrogenic Ca^{2+} pump in the inner envelope membrane (Kreimer et al., 1985a, 1985b, 1988). Other information about transthylakoid ion transport comes from more recent patch-clamp studies demonstrating the existence of thylakoid membrane cation channels that have conductance for K^+, Mg^{2+}, and Ca^{2+} (in order of their decreasing conductance) (Enz et al., 1993; Pottosin and Schönknecht, 1996). However, patch-clamp

studies do not address the direction, energetics, or mechanism of Ca^{2+} transport across the thylakoid membrane.

A H^+/Ca^{2+} antiporter in the thylakoid membrane would certainly supply the Ca^{2+} required for the assembly and maintenance of PSII and other thylakoid functions noted above. Additionally, the transport of Ca^{2+} across the thylakoid membrane may be associated with several important processes in chloroplasts. The transition from dark to light is a key regulatory period for stromal enzymes involved in carbon reduction. However, the role of Ca^{2+} in the regulation of Calvin-Benson-cycle enzymes is more complex. It appears that low levels of Ca^{2+} in the stroma are necessary for the activation of FBPase in the presence of reduced thioredoxin. Ca^{2+} apparently inhibits the catalytic activity of the enzyme (Charles and Halliwell, 1980; Hertig and Wolosiuk, 1980). Thus, there is an apparent discrepancy between the light-dependent transport of Ca^{2+} from the cytosol to the stroma and the activation of Calvin-Benson-cycle enzymes, because Ca^{2+} is known to inhibit the activity of FBPase, and elevated Ca^{2+} levels have a pronounced inhibitory effect on CO_2 fixation. Ca^{2+} accumulated by chloroplasts in the light must be tightly bound in the stroma or sequestered in the thylakoid to prevent the inhibition of CO_2 fixation by Ca^{2+} (Wolosiuk et al., 1993). The thylakoid membrane Ca^{2+}/H^+ antiporter would provide the means to sequester chloroplast Ca^{2+} in the thylakoid lumen in the light and thus prevent the Ca^{2+}-mediated inhibition of Calvin-Benson-cycle enzymes.

Stores of Ca^{2+} in the thylakoid lumen may play an important role in the regulation of Calvin-Benson-cycle enzymes. Recently, Johnson et al. (1995) noted a sharp rise in stromal Ca^{2+} concentrations after the transition of plants from light to dark. This rise in stromal Ca^{2+} peaked 20 to 25 min after the transition from light to dark and may play a role in signaling the light-to-dark transition through the inhibition of Calvin-Benson-cycle enzymes. The peak is believed to represent a change in stromal free Ca^{2+} levels from a basal level of 150 nM to approximately 5 to 10 μM. Such a pronounced increase is unlikely to originate from the cytosol without the expenditure of a great deal of cellular energy in the dark. However, the rapid release of Ca^{2+} from luminal stores could result in dramatic changes in stromal Ca^{2+} concentrations, even if the Ca^{2+} was free to move from the stroma to the cytosol. We propose that the dramatic rise in stromal Ca^{2+} after the light-to-dark transition that was observed by Johnson et al. (1995) is coupled to the release of stores of Ca^{2+} from the thylakoid lumen.

On the basis of our results and the observations by Johnson et al. (1995), we propose that there are dramatic fluxes of Ca^{2+} across the thylakoid membrane that are induced by the transitions from dark to light and light to dark (Fig. 7). When dark-adapted chloroplasts are transferred to the light, there is an initial increase in trans-thylakoid membrane potential to about 60 to 80 mV (lumen positive), which subsequently decays within a few seconds to a steady state of 15 to 30 mV (Bulychev et al., 1972; Hangarter and Good, 1982; Remis et al., 1986). The collapse of the transient membrane potential is caused by the efflux of Mg^{2+} from the thylakoid lumen to the stroma (Fig. 7B). The magnitude of light-induced Mg^{2+} efflux is approximately 26 to 120 nmol Mg^{2+} mg^{-1} chlorophyll (Barber et al., 1974; Hind et al., 1974; Portis and Heldt, 1976; Krause, 1977; Enz et al., 1993).

Several Calvin-Benson-cycle enzymes, including Rubisco, FBPase, and sedoheptulose-1,7-bisphosphatase, are activated by Mg^{2+} ions (Portis and Heldt, 1976), which is in apparent agreement with the light-induced efflux of Mg^{2+} ions from the thylakoid lumen. However, the mechanism by which Mg^{2+} is returned to the thylakoid lumen in the dark has not yet been elucidated. We propose that such a mechanism may be the coupling of the exchange of luminal Ca^{2+} accumulated in the light (Fig. 7C) for stromal Mg^{2+} (Fig. 7D). The counterexchange of these ions would be electrically neutral. Any mechanism for exchanging Ca^{2+} and Mg^{2+} across the thylakoid membrane in the dark would need to be tightly regulated to prevent a futile cycle from forming. This exchange may be regulated by the state of oxidation or by the reduction of thioredoxin, as are several enzymes of the Calvin-Benson cycle (for review, see Wolosiuk, 1993). Cycling of Ca^{2+} and Mg^{2+} across the thylakoid membrane is correlated with the activation of Calvin-Benson-cycle enzymes by Mg^{2+} in the light and their inhibition by Ca^{2+} in the dark.

In conclusion, we have demonstrated the existence of a Ca^{2+}/H^+ antiporter in the thylakoid membrane. This antiporter provides the mechanism and energetics necessary to transport Ca^{2+} into the thylakoid lumen in the light and thereby supply Ca^{2+} for Ca^{2+}-dependent processes in the thylakoid lumen, such as the assembly of the OEC. Additionally, our model of chloroplast Ca^{2+} homeostasis proposes that the uptake of cytosolic Ca^{2+} by chloroplasts in the light does not lead to a pronounced increase in stromal Ca^{2+} but, rather, results in an increase of Ca^{2+} stores in the thylakoid lumen. The Ca^{2+}/H^+ antiporter is critical for transporting stromal Ca^{2+} into the thylakoid lumen in the light, where it would have no inhibitory effect on Calvin-Benson-cycle enzymes. We propose that pools of Ca^{2+} sequestered in the lumen during a period of light are released from the lumen after a period of dark adaptation and complement the activity of thioredoxin in the inhibition of Calvin-Benson-cycle enzymes in the dark.

ACKNOWLEDGMENTS

We would like to thank Dr. Steven Theg for his valuable suggestions and Amanda B. Lehman and Hilary I. Van Hole for their excellent technical assistance. Silicone oils AR-200 and AR-20 were generously donated by Wacker Silicones.

Received August 21, 1998; accepted January 7, 1999.

LITERATURE CITED

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Plant Physiol **24:** 1–15

Barber J, Mills JB, Nicolson J (1974) Studies with cation specific ionophores show that within the intact chloroplast Mg^{2+} acts as the main exchange cation for H^+ pumping. FEBS Lett **49:** 106–110

Becker DW, Callahan FE, Cheniae GM (1985) Photoactivation of NH_2OH-treated leaves: reassembly of released extrinsic PSII polypeptides and relegation of Mn into the polynuclear Mn catalyst of water oxidation. FEBS Lett **192:** 209–214

Blackford S, Rea PA, Sanders D (1990) Voltage sensitivity of H^+/Ca^{2+} antiport in higher plant tonoplast suggests a role in vacuolar calcium accumulation. J Biol Chem **265:** 9617–9620

Broussac A, Zimmermann J-L, Rutherford AW, Lavergne J (1990) Histidine oxidation in the oxygen-evolving photosystem II enzyme. Nature **347:** 303–306

Bulychev AA, Andrianov VK, Kurella GA, Litvin FF (1972) Microelectrode measurements of the transmembrane potential of chloroplasts and its photoinduced changes. Nature **236:** 175–177

Bush DS (1993) Regulation of cytosolic calcium in plants. Plant Physiol **103:** 7–13

Bush DS (1995) Calcium regulation in plant cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol **46:** 95–122

Charles SA, Halliwell B (1980) Action of calcium ions on spinach (*Spinacia oleracea*) chloroplast fructose bisphosphatase and other enzymes of the Calvin cycle. Biochem J **188:** 775–779

Cline K, Ettinger WF, Theg SM (1992) Protein-specific energy requirements for protein transport across or into thylakoid membranes: two lumenal proteins are translocated in the absence of ATP. J Biol Chem **267:** 2688–2696

Cline K, Werner-Washburn M, Lubben TH, Keegstra K (1985) Precursors to two nuclear encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. J Biol Chem **260:** 3691–3696

Debus RJ (1992) The manganese and calcium ions of photosynthetic oxygen evolution. Biochim Biophys Acta **1102:** 269–352

Dilley RA, Chiang GG (1989) Chloroplast thylakoid membrane-bound Ca^{2+} acts in a gating mechanism to regulate energy-coupled proton fluxes. Ann NY Acad Sci **574:** 246–267

Enz C, Steinkanp T, Wagner R (1993) Ion channels in the thylakoid membrane (a patch-clamp study). Biochim Biophys Acta **1143:** 67–76

Evans DE, Briars S-A, Williams LE (1991) Active calcium transport by plant cell membranes. J Exp Biol **42:** 285–303

Ghanotakis DF, Babcock GT, Yocum CF (1984) Calcium reconstitutes high levels of oxygen evolution in polypeptide-depleted photosystem II preparations. FEBS Lett **167:** 127–130

Gilroy S, Bethke PC, Jones RL (1993) Calcium homeostasis in plants. J Cell Sci **106:** 453–462

Gunter TE, Gunter KK, Sheu S-S, Gavin CE (1994) Mitochondrial calcium transport: physiological and pathological relevance. Am J Physiol **267:** 313–339

Hangarter RP, Good NE (1982) Energy thresholds for ATP synthesis in chloroplasts. Biochim Biophys Acta **681:** 397–404

Heldt HW (1980) Measurement of metabolite movement across the envelope and of the pH in the stroma and the thylakoid space in intact chloroplasts. Methods Enzymol **69:** 604–613

Hertig C, Wolosiuk RA (1980) A dual effect of Ca^{2+} on chloroplast fructose-1,6-bisphosphatase. Biochem Biophys Res Commun **97:** 325–333

Hind G, Nakatani HY, Izawa S (1974) Light-dependent redistribution of ions in suspensions of chloroplast thylakoid membranes. Proc Natl Acad Sci USA **71:** 1484–1488

Hundal T, Aro EM, Carlberg I, Andersson B (1990a) Restoration of light induced photosystem II inhibition without de novo protein synthesis. FEBS Lett **267:** 203–206

Hundal T, Virgin I, Styring S, Andersson B (1990b) Changes in the organization of photosystem II following light-induced D1 protein degradation. Biochim Biophys Acta **1017:** 235–241

Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, Haley A, Trewavas A (1995) Circadian oscillations of cytosolic and chloroplastic free calcium in plants. Science **269:** 1863–1865

Krause GH (1977) Light-induced movement of magnesium ions in intact chloroplasts: spectroscopic studies with eriochrome blue SE. Biochim Biophys Acta **460:** 500–510

Kreimer G, Melkonian M, Holtum JAM, Latzko E (1985a) Characterization of calcium fluxes across the envelope of intact spinach chloroplasts. Planta **166:** 515–523

Kreimer G, Melkonian M, Holtum JAM, Latzko E (1988) Stromal free calcium concentration and light-mediated activation of chloroplast fructose-1,6-bisphosphatase. Plant Physiol **86:** 423–428

Kreimer G, Melkonian M, Latzko E (1985b) An electrogenic uniport mediates light-dependent Ca^{2+}-influx into intact spinach chloroplasts. FEBS Lett **180:** 253–258

Mattoo AK, Marder JB, Edelman M (1989) Dynamics of the photosystem II reaction center. Cell **56:** 241–246

McNamera VP, Gounaris K (1995) Granal photosystem II complexes contain only the high redox potential form of cytochrome *b-559* which is stabilized by the ligation of calcium. Biochim Biophys Acta **1231:** 289–296

Miller A-F, Brudvig GW (1989) Manganese and calcium requirements for reconstitution of oxygen-evolution in manganese-depleted photosystem II membranes. Biochemistry **28:** 8181–8190

Muto S, Izawa S, Miyachi S (1982) Light-induced Ca^{2+} uptake by intact chloroplasts. FEBS Lett **139:** 250–254

Nelson N, Eytan E, Notsani B-E, Sigrist H, Sigrist-Nelson K, Gitler C (1977) Isolation of a chloroplast *N,N′*-dicyclohexylcarbodiimide-binding protein proteolipid, active in proton translocation. Proc Natl Acad Sci USA **74:** 2375–2378

Nobel PS (1967) Calcium uptake, ATPase and photophosphorylation by chloroplasts *in vitro*. Nature **214:** 875–877

Nobel P (1969) Light-induced changes in the ionic composition of chloroplasts in *Pisum sativum*. Biochim Biophys Acta **172:** 134–143

Nobel PS, Packer L (1965) Light-dependent ion translocation in spinach chloroplasts. Plant Physiol 633–640

Portis AR, Heldt HW (1976) Light dependent changes of the Mg^{2+} concentration in the stroma in relation to the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts. Biochim Biophys Acta **449:** 434–446

Pottosin II, Schönknecht G (1996) Ion channel permeable for divalent and monovalent cations in native spinach thylakoid membranes. J Membr Biol **152:** 223–233

Remis D, Bulychev AA, Kurella GA (1986) The electrical and chemical components of the proton motive force in chloroplasts as measured with capillary and pH-sensitive electrodes. Biochim Biophys Acta **852:** 68–73

Robinson SP, Wiskich JT (1977) Uptake of ATP analogs by isolated pea chloroplasts and their effect on CO_2 fixation and electron transport. Biochim Biophys Acta **461:** 131–140

Schumaker KS, Sze H (1986) Calcium transport into the vacuole of oat roots: characterization of H^+/Ca^{2+} exchange activity. J Biol Chem **261:** 12172–12187

Shavit N, Dilley RA, San Peitro A (1968) Ion translocation in isolated chloroplasts uncoupling of photophosphorylation and translocation of K^+ and H^+ by nigericin. Biochemistry **7:** 2356–2363

Steele JA, Uchytil TF, Durbin RD, Bhatnagar P, Rich DH (1976) Chloroplast coupling factor I: a species-specific receptor for tentoxin. Proc Natl Acad Sci USA **73:** 2245–2248

Theg SM, Filar LJ, Dilley RA (1986) Photoinactivation of chloroplasts already inhibited on the oxidizing side of photosystem II. Biochim Biophys Acta **849:** 104–111

Virgin I, Ghanotakis DF, Andersson B (1990) Light induced D1-protein degradation in isolated photosystem II core complexes. FEBS Lett **269:** 45–48

Wolosiuk RA, Ballicora MA, Hagelin K (1993) The reductive pentose phosphate cycle for photosynthetic CO_2 assimilation: enzyme modulation. FASEB J **7:** 622–737