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Electron Transport through Photosystem I Stimulates Light Activation of Ribulose Bisphosphate Carboxylase/ Oxygenase (Rubisco) by Rubisco Activase

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ABSTRACT

The activation state of ribulose bisphosphate carboxylase/ oxygenase (rubisco) in a lysed chloroplast system is increased by light in the presence of a saturating concentration of ATP and a physiological concentration of CO_2 (10 micromolar). Electron transport inhibitors and artificial electron donors and acceptors were used to determine in which region of the photosynthetic electron transport chain this light-dependent reaction occurred. In the presence of DCMU and methyl viologen, the artificial donors durohydroquinone and 2,6-dichlorophenolindophenol (DCPIP) plus ascorbate both supported light activation of rubisco at saturating ATP concentrations. No light activation occurred when DCPIP was used as an acceptor with water as electron donor in the presence of ATP and dibromothymoquinone, even though photosynthetic electron transport was observed. Nigericin completely inhibited the light-dependent activation of rubisco. Based on these results, we conclude that stimulation of light activation of rubisco by rubisco activase requires electron transport through PSI but not PSII, and that this light requirement is not to supply the ATP needed by the rubisco activase reaction. Furthermore, a pH gradient across the thylakoid membrane appears necessary for maximum light activation of rubisco even when ATP is provided exogenously.

Light activation of rubisco[1] has been demonstrated in leaves (15, 18, 25, 29) and intact chloroplasts (2, 10). The light responses of the activation of rubisco, the enzyme which catalyzes photosynthetic CO_2 assimilation (16), and the rate of CO_2 assimilation have been shown to proceed in tandem (18, 25, 29). Several enzymes involved in C_3 photosynthetic carbon metabolism in addition to rubisco are also light activated, but unlike rubisco these enzymes are activated by the ferredoxin/thioredoxin system (5). Rubisco, *in vivo*, is activated by the rubisco activase system in an ATP-dependent process (19). Before the discovery of rubisco activase (24), the role of light in rubisco activation was proposed to be the establishment of an alkaline pH and an increase in Mg^{2+} concentration within the chloroplast stroma (9, 14). An additional role for light in rubisco activation, ATP synthesis, became evident when the rubisco activase requirement for ATP was demonstrated (26). Rubisco activation state was found to be correlated with ATP concentration in isolated chloroplasts (22) but not in intact leaves (4). Thus, while ATP is required for rubisco activation *in vivo*, it is not the sole regulator of activation state.

Previous studies (23, 27, 30) suggested a role for thylakoid membranes in the activation of rubisco, and early experiments with the rubisco activase system (20, 24) required the presence of thylakoid membranes and light in reconstituted chloroplast assays. However, considering the rubisco activase requirement for ATP, the role of the thylakoid membrane seemed to be defined. Recently we demonstrated a requirement for light and photosynthetic electron transport for full activation of rubisco by rubisco activase in lysed chloroplasts at physiological concentrations of CO_2, even though ATP was supplied exogenously at saturating concentrations (6). Activation occurred in the presence of MV, indicating the ferredoxin/ thioredoxin system was not involved. These results implied a more direct involvement of the thylakoid membrane in the activation of rubisco *in vivo*.

The objectives of the present study were to determine the location of the light-dependent reaction within the thylakoid membrane that leads to increased rubisco activation, and also to determine if a transthylakoid pH gradient was required for this stimulation of activation in the presence of saturating concentrations of exogenously added ATP.

MATERIALS AND METHODS

Rubisco Activation in Lysed Chloroplasts

Intact chloroplasts were prepared from hydroponically grown spinach (*Spinacea oleracea* L. cv American Hybrid 424) plants (6) by the method of Robinson and Portis (22). Chloroplasts were diluted by adding one volume intact chloroplasts to four volumes ice-cold lysis buffer (5 mM Tricine-NaOH [pH 8.0], 20 mM NaCl). Diluted chloroplasts were kept on ice until immediately before each experiment, within 3 h of preparation of the intact chloroplasts.

Rubisco activation reactions were performed at physiological CO_2 concentrations (10 μM) by modification of the method previously described (6), as indicated below. Chloro-

[1] Abbreviations: rubisco, ribulose bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; DBMIB, dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone); DHQ, durohydroquinone; DCPIP, 2,6-dichlorophenolindophenol; MV, methyl viologen.

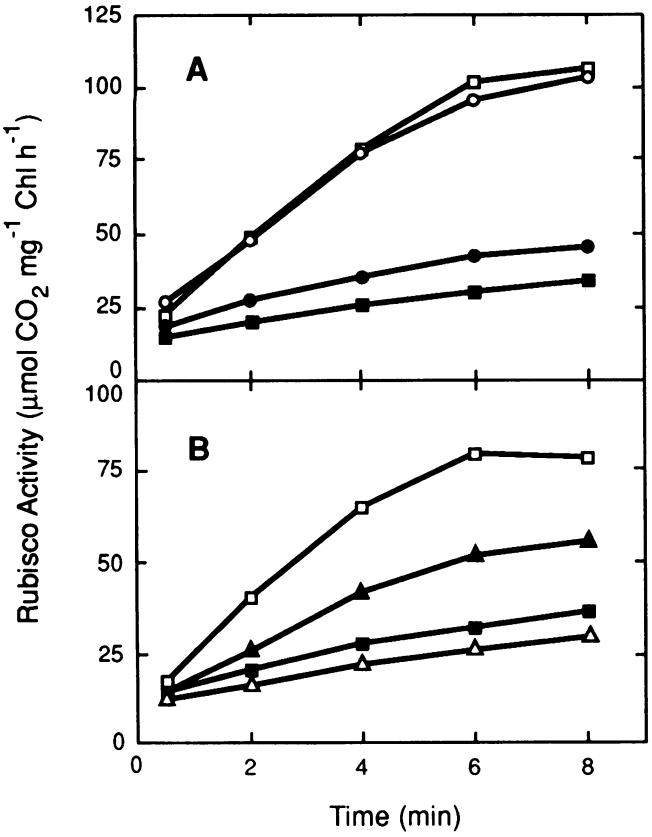

Figure 1. Activation of rubisco in lysed chloroplasts in the presence and absence of DCMU. A), Chloroplasts with no additions in the light (□) and dark (■), and with 20 μM DCMU, 0.1 mM MV, 0.2 mM DCPIP, and 1.5 mM ascorbate in the light (○) and dark (●); B), chloroplasts with no additions in the light (□) and dark (■), and with 20 μM DCMU, 0.1 mM MV, plus (▲) or minus (△) 0.5 mM DHQ in the light.

plasts were further diluted in buffer (125 mM Tricine-NaOH [pH 8.0]) to a concentration of 1 mg Chl mL^{-1}. This step, and the dilution described above, caused lysis of the chloroplasts. After a 5 min dark incubation at 25°C to allow deactivation of endogenous rubisco, RuBP was added to a concentration of 5 mM, followed by a second dark incubation of 1 min. Depending on the experiment, various electron transport inhibitors, donors, acceptors, and ionophores were then added at the indicated concentrations, and the activation reaction was initiated at time zero with the addition of $NaHCO_3$, $MgCl_2$, ATP, and an ATP-regenerating system (phospho*enol*pyruvate and pyruvate kinase). Reactions were conducted in 1.5 mL vials at 25°C. For activation in the light, irradiation (1000 μmol photons m^{-2} s^{-1}) was begun at time zero. Dark activation reactions were conducted under a green safe light (6). Final concentrations in the activation reactions were: 100 mM Tricine-NaOH (pH 8.0), 0.08 mg Chl mL^{-1} (0.025 mg Chl mL^{-1} in Fig. 3), 4 mM RuBP, 0.75 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM ATP, 2 mM phospho*enol*pyruvate, and 20 units mL^{-1} pyruvate kinase. The final dilution of chloroplasts was 1:25 to 1:50, depending on the initial concentration of intact chloroplasts. Chl was extracted in 80% (v/v) acetone and quantified by the method of Arnon (1).

Electron Donors, Electron Acceptors, and Ionophores

The inhibitors DCMU and DBMIB were used to block electron transport between PSII and PSI. Both compounds were dissolved in a 1:1 (v/v) mixture of ethanol and ethylene glycol. The artificial electron donor DHQ was prepared in ethanol-ethylene glycol from duroquinone as described by Izawa and Pan (12). DCPIP was dissolved in water, and when used as an electron donor, ascorbate was included. When used as an acceptor, ascorbate was omitted. MV was dissolved in water and used as an electron acceptor. Nigericin was dissolved in ethanol, and valinomycin in acetone. Final concentrations of these compounds in the activation reactions were: 20 μM DCMU, 5 μM DBMIB, 0.5 mM DHQ, 0.2 mM DCPIP, 1.5 mM ascorbate, 0.1 mM MV, 0.5 μM nigericin, and 1 μM valinomycin. When using ionophores, KCl was present at 30 mM. Ethanol-ethylene glycol was present at 2% in all experiments and acetone was present at 0.25% in the valinomycin experiments. At these concentrations, neither ethanol-ethylene glycol nor acetone had a detectable effect on rubisco activation. The sites of action of the artificial donors and acceptors are indicated below. Artificial acceptors, donors, and ionophores were from Sigma.[2]

Determination of Electron Transport

Oxygen evolution was used as a measure of electron transport in the presence of 5 μM DBMIB. Components of the rubisco activation reaction, as described above, were placed in an oxygen electrode maintained at 25°C. Water served as the electron donor and, when used as an electron acceptor,

[2] Mention of a trademark, propriety product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

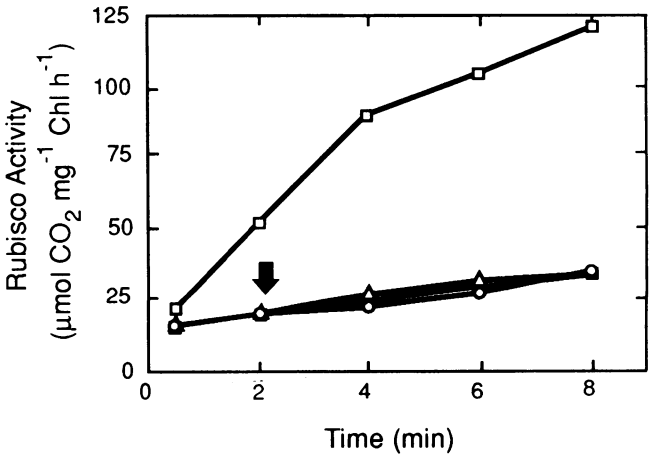

Figure 2. Activation of rubisco in lysed chloroplasts in the presence and absence of DBMIB. Chloroplasts with no additions in the light (□) and dark (■), and with 5 μM DBMIB plus (○) or minus (△) 0.2 mM DCPIP added after 2 min (arrow) in the light. Activation rates in the dark (■) and in the presence of DBMIB, with or without DCPIP, (○, △) were virtually identical; therefore, the curves are coincident and some symbols are obscured.

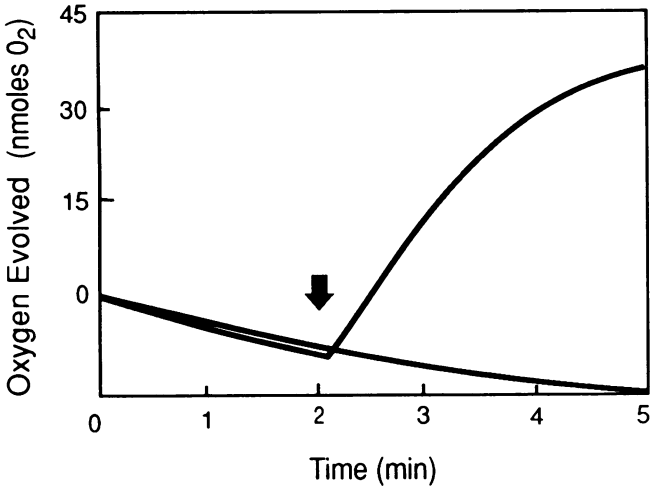

Figure 3. Electron transport, measured as O_2 evolution, in lysed chloroplasts in the light following addition (arrow) of 0.2 mM DCPIP as an electron acceptor. DBMIB was present at 5 μM, and lysed chloroplasts were present at 0.025 mg Chl mL^{-1}. The initial rate of O_2 evolution was 65 μmol O_2 mg^{-1} Chl h^{-1}.

DCPIP was added after 2 min to a concentration of 0.2 mM. Lysed chloroplasts were present at 0.025 mg Chl mL^{-1}.

Assay of Rubisco Activity

Fifty microliter aliquots were removed from the rubisco activation reactions at the times indicated and assayed for rubisco activity. The activity assay reaction contained 110 mM Tricine-NaOH (pH 8.0), 10 mM $NaH^{14}CO_3$ (1 Ci mol^{-1}), 11 mM $MgCl_2$, and 0.5 to 0.9 mM RuBP. Assays, conducted at 25°C, were initiated with the addition of the reaction suspension aliquots and terminated after 0.5 min by addition of 4 N formic acid in 1 N HCl. Samples were dried at 70°C overnight and acid-stable ^{14}C-products were determined by liquid scintillation spectroscopy.

RESULTS

Light stimulated the activation of rubisco in a lysed chloroplast system in the presence of the electron transport inhibitor DCMU, artificial electron donors, and MV as an electron acceptor. When DCPIP and ascorbate were used as an electron donor couple, activation of rubisco in the light was the same as in the control (Fig. 1A). In the dark, activation in the presence of DCPIP and ascorbate was slightly greater than the dark control. In a similar experiment using DHQ as the electron donor, activation in the light was about one-half of the activation in the control (Fig. 1B), and activation in the dark was somewhat less than the dark control (data not shown). In the presence of DCMU and MV but without an added electron donor, light activation did not occur (Fig. 1B).

When the electron transport inhibitor DBMIB was used and water served as the electron donor, light activation of rubisco did not occur regardless of whether or not DCPIP was present as an electron acceptor (Fig. 2). The absence of light activation was not caused by a direct effect of DBMIB or DCPIP on the rubisco-rubisco activase system since 5 μM DBMIB inhibited activation less than 25% in a standard rubisco activase assay with purified rubisco and purified rubisco activase (data not shown). Also, as shown in Figure 1A, rubisco was activated in lysed chloroplasts in the presence of DCPIP. These observations demonstrate that the complete absence of light activation in the presence of DBMIB and DCPIP in Figure 2 was not the result of direct DBMIB or DCPIP inhibition of rubisco or rubisco activase.

To determine if electron transport was occurring between water (as a donor) and DCPIP (as an acceptor) in the presence of DBMIB, conditions which did not support light activation of rubisco (Fig. 2), oxygen evolution was measured. In the absence of an added electron acceptor no oxygen evolution occurred (Fig. 3). However, when DCPIP was added after 2 min in the light, oxygen evolution was observed (Fig. 3). The reactions conducted in an oxygen electrode (Fig. 3) contained all the reagents as the corresponding rubisco activation assays (Fig. 2) including RuBP, ATP, and the ATP-regenerating system. When oxygen evolution was measured in the absence of RuBP, ATP, and the regenerating system (data not shown), the results were identical to those in Figure 3. The data in Figures 2 and 3 demonstrated that electron transport occurred but rubisco was not light activated in lysed chloroplasts in the presence of DBMIB and DCPIP.

The effect of ionophores on light activation of rubisco was investigated in the lysed chloroplast system without added electron donors or acceptors. Nigericin caused complete inhibition of light activation (Fig. 4). Valinomycin (1 μM) did not prevent light activation, but activation was only about 50% of the control (Fig. 4). At 0.5 μM valinomycin (data not shown) inhibition was the same as at 1 μM, indicating that increasing the valinomycin concentration above 1 μM would not have resulted in greater inhibition of activation than shown in Figure 4. Gramicidin (0.5 μM) plus NH_4Cl (1.5 mM) inhibited light activation of rubisco about the same as 0.5 μM nigericin (data not shown).

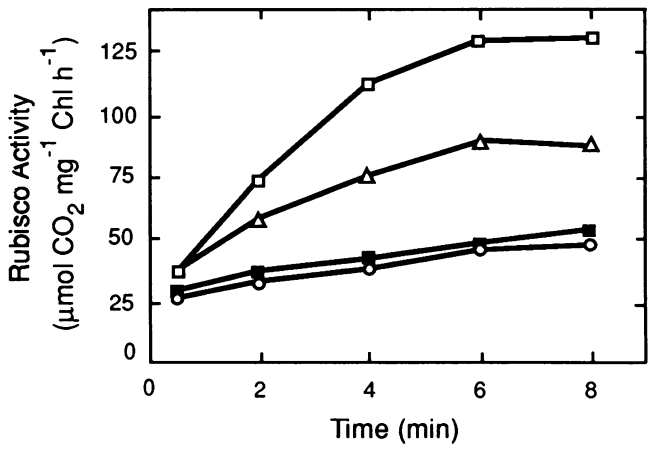

Figure 4. Inhibition of light activation of rubisco in lysed chloroplasts by ionophores. Chloroplasts with no ionophore in the light (□) and dark (■), and with 0.5 μM nigericin (○) or 1.0 μM valinomycin (△) in the light. KCl was added to a concentration of 30 mM in all reactions.

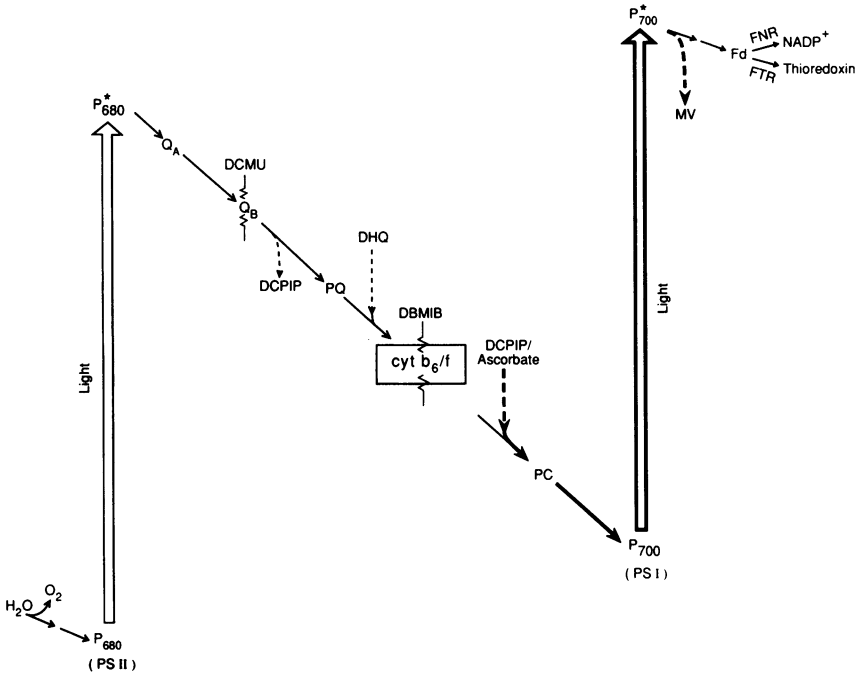

Figure 5. A simple Z scheme representing the path of electrons through PSII and PSI, and the sites of action of the artificial electron donors and acceptors used in the present experiments. Inhibition of electron transport by DCMU and DBMIB is indicated. Artificial donors (DHQ, DCPIP/ascorbate) are indicated by broken lines directed into the scheme, and artificial acceptors (DCPIP, MV) are indicated by broken lines directed away from the scheme. Electron transport through the region indicated by the heavy lines (DCPIP/ascorbate → MV) supports light activation of rubisco by rubisco activase. P_{680}, P_{700}, reaction center Chl of PSII and PSI, respectively; Q_A, Q_B, PSII quinone electron acceptors; PQ, plastoquinone; Cyt b_6/f, Cyt b_6/f complex; PC, plastocyanin; Fd, ferredoxin; FNR, ferredoxin-$NADP^+$ oxidoreductase; FTR, ferredoxin-thioredoxin reductase.

DISCUSSION

Activation of rubisco by rubisco activase in a lysed chloroplast system at physiological concentrations of CO_2 (10 μM) and saturating concentrations of ATP and Mg^{2+} was observed to be stimulated by light (6). Although it was clear that a light-dependent reaction was involved in rubisco activation, the data (6) were from experiments that utilized whole-chain electron transport and therefore the location of the light-dependent reaction within the thylakoid membrane was not identified. In the present experiments, electron transport inhibitors and artificial electron donors and acceptors were used to functionally isolate PSI and PSII and thus localize the site of the light-dependent reaction (Fig. 5).

In the presence of DCMU, PSII-dependent reduction of the secondary quinone acceptor, Q_B, is prevented (17). Thus, the physiological electron donor, water, can no longer serve as the electron donor to PSI and an artificial donor must be used to allow PSI electron transport. Two different PSI electron donors were used with MV as the electron acceptor (11). DHQ substitutes for plastoquinone as the donor to the Cyt b_6/f complex (11, 12), and electron transport from DHQ to MV supported the light activation of rubisco. DCPIP, in the presence of ascorbate as an electron reservoir, donates electrons to the Cyt b_6/f-plastocyanin region (11), and electron transport from DCPIP/ascorbate to MV also supported the light activation of rubisco. Activation was greater with DCPIP/ascorbate as the donor; however, this may have been due to the relative efficiencies of the artificial donors rather than to the different sites to which electrons were donated. In the presence of DCMU and MV but without an added electron donor, there was no light activation of rubisco. These results (Fig. 1) support the conclusion that in the light electron transport through PSI stimulates the activation of rubisco in the presence of saturating concentrations of ATP.

The inhibitor DBMIB is a plastoquinone antagonist and blocks electron transport through the Cyt b_6/f complex (28). Electron transport through PSII will occur in the presence of DBMIB if an artificial acceptor is included. DCPIP in the absence of ascorbate can be used as the PSII electron acceptor (11), intercepting electrons directly from the PSII reaction center. No PSII-dependent light activation of rubisco occurred when water and DCPIP were used as the electron donor/acceptor couple in the presence of DBMIB. The response of rubisco activation was the same under these conditions as both in the dark, and in the light with DBMIB but no added electron acceptor (Fig. 2). Since PSII electron transport was shown to occur from water to DCPIP (Fig. 3), we conclude that electron transport through PSII does not support light activation of rubisco. However, when electron transport through both PSII and PSI was utilized (water as donor and MV as acceptor), light activation of rubisco was observed (6). The region of the electron transport chain that supports light activation of rubisco, and is common to all experiments in which light activation was observed (Fig. 1; ref. 6), is between the site where DCPIP/ascorbate donates electrons and where MV accepts electrons, and is indicated by the heavy lines in Figure 5.

As reported earlier (6) and shown here in Figures 1, 2, and 4, endogenous rubisco in the lysed chloroplast system was activated in the dark if rubisco activase (endogenous), RuBP, CO_2, Mg^{2+}, and ATP were present. However, the rate of dark activation is very slow, and activation in the light was quite clearly more rapid.

The pH gradient established across the thylakoid membrane as a result of photosynthetic electron transport (17) can be dissipated by nigericin, which allows the exchange of H^+ for K^+ (7). The light-stimulated activation of rubisco in lysed chloroplasts was completely inhibited by nigericin, but only

partially inhibited by valinomycin, which renders the membrane permeable to K^+ but not to H^+ (7). Since high rates of electron transport still occur when nigericin is used (8), inhibition of the light activation of rubisco seems to be the result of collapse of the pH gradient. Previous reports (21, 23) suggested involvement of a transthylakoid pH gradient in light activation of rubisco by rubisco activase. Since the thylakoid pH gradient is utilized to synthesize ATP in the chloroplast (13), the requirement for a pH gradient appeared to be synonymous with the requirement for ATP in the activation of rubisco by rubisco activase (26). In the present experiments ATP was supplied exogenously (with a regenerating system) yet a transthylakoid pH gradient was still necessary for light activation.

Chloroplast thylakoid membranes consisted of appressed, or stacked, regions enriched in PSII, and nonappressed regions enriched in PSI (3). The requirement for electron transport through PSI in light activation of rubisco is consistent with the location of the PSI complex within the nonappressed regions of the thylakoid membranes, since these regions are exposed to the stroma whereas the appressed regions are not (17). This arrangement thus permits access of the soluble stromal components to the region of the thylakoid membrane which mediates the stimulation of rubisco activation.

The observations reported here and earlier (6) firmly establish the requirement for light to attain maximum rubisco activation at physiological concentrations of CO_2 by the rubisco activase system, even when saturating ATP is supplied exogenously. Associated with light activation is the apparent requirement for electron transport through the PSI region of the electron transport chain, and the establishment of a transthylakoid pH gradient. The mechanism by which electron transport and the pH gradient are sensed and translated into a higher rubisco activation state remains to be determined.

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