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Involvement of Stromal ATP in the Light Activation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in Intact Isolated Chloroplasts

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ABSTRACT

Light activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) and stromal ATP content were measured in intact isolated spinach chloroplasts. Treatments which decreased stromal ATP, such as incubation with the ATP analog β,γ-methylene adenosine triphosphate or with the energy transfer inhibitor phloridzin inhibited the light activation of rubisco. In the absence of added inorganic phosphate (Pi), light activation of rubisco was inhibited, coincident with low stromal ATP. Addition of methyl viologen restored both stromal ATP and rubisco activity to levels observed in the presence of Pi. Activation of rubisco was inhibited in the presence of 2 millimolar dihydroxyacetone phosphate or 3-phosphoglycerate and stromal ATP was also decreased under these conditions. Both were partially restored by increasing the Pi concentration. The strong correlation between activation state of rubisco and stromal ATP concentration in intact chloroplasts under a wide variety of experimental conditions indicates that light activation of rubisco is dependent on ATP and proportional to the ATP concentration. These observations can be explained in terms of the rubisco activase protein, which mediates activation of rubisco at physiological concentrations of CO_2 and ribulose-1,5-bisphosphate and is dependent upon ATP.

Five enzymes of the photosynthetic carbon reduction cycle are known to be light activated, *i.e.* the activity which can be extracted from illuminated leaves or chloroplasts is higher than that in the dark, even when both are measured under optimal conditions. For four of these enzymes (glyceraldehyde-3-P dehydrogenase, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, and phosphoribulokinase) light activation involves thioredoxin and thioredoxin reductase and is dependent on reducing equivalents from the chloroplast thylakoid electron transport chain (16). Rubisco[1] is also light activated but the thioredoxin system is not involved in this case. *In vitro*, rubisco can be spontaneously activated by incubation at alkaline pH with CO_2 and Mg^{2+}, resulting in carbamylation of the enzyme (7, 8, 10). In isolated chloroplasts, light activation of rubisco has been shown to depend on CO_2, stromal Mg^{2+} concentration, and the light-dependent alkalization of the chloroplast stroma (1, 3) indicating that light activation of the enzyme *in vivo* may occur by a similar mechanism.

There are, however, several features of the light activation of rubisco *in situ* which cannot be readily understood in terms of the known spontaneous activation of the purified enzyme by CO_2 and Mg^{2+}. The activation state of rubisco increases with increasing light intensity, saturating at similar values to photosynthesis, whereas alkalization of the stroma and light-induced increases in stromal Mg^{2+} are saturated at much lower light intensities (12, 19). The K_{act} (CO_2) for spontaneous activation of purified rubisco is 25 to 30 μM (4, 7), which is three times higher than the CO_2 concentration thought to exist in the chloroplast *in vivo* (13). Activation of purified rubisco is strongly inhibited by physiological concentrations of RuBP (4, 10). In leaves the activation state of rubisco varies with external CO_2 concentration in the dark, but not in the light (19). Finally, light activation of rubisco in isolated chloroplasts only occurs in the presence of Pi in the suspending medium (3, 9) whereas the spontaneous activation of rubisco by CO_2 and Mg^{2+} does not require Pi or other chloroplast metabolites.

Somerville *et al.* (20) isolated the *rca* mutant of *Arabidopsis* in which light activation of rubisco was absent even though the rubisco isolated from this plant could be spontaneously activated by CO_2 and Mg^{2+} in the normal manner. Light activation of fructose-1,6-bisphosphatase, which also depends on light-dependent increases in stromal pH and Mg^{2+} concentration, was unaffected in the *rca* mutant (19). Salvucci *et al.* (18) identified two polypeptides which were absent in the *rca* mutant and demonstrated that a stromal protein, termed rubisco activase, was necessary for light activation of rubisco in a reconstituted chloroplast system. This indicated that light activation of rubisco *in vivo* is mediated by a specific chloroplast protein and is not a spontaneous process. Using partially purified rubisco activase, Portis *et al.* (13) demonstrated activation of rubisco *in vitro* at physiological concentrations of CO_2 and RuBP. Recently, Streusand and Portis (21) found that ATP was required for this process and that ADP inhibited rubisco activation. To investigate whether rubisco activation *in vivo* also requires ATP and whether changes in ATP concentration are reflected by changes in the activation state of rubisco, we have determined light activation of rubisco in intact isolated chloroplasts under various conditions which alter the stromal ATP concentration.

MATERIALS AND METHODS

Plant Material. Spinach (*Spinach oleracea* L. cv American Hybrid 424) seeds were germinated in moist peat then transferred

[1] Abbreviations: Rubisco, ribulose bisphosphate carboxylase/oxygenase; AMP-PCP, β,γ-methylene adenosine triphosphate; DHAP, dihydroxyacetone phosphate; MV, methyl viologen; PGA, 3-phosphoglycerate; RuBP, ribulose-1,5-bisphosphate; Pi, inorganic phosphate.

to hydroponic culture. The plants were grown in controlled environmental chambers under the following conditions: 12-h light period, 20°C, 60 to 70% RH, 12-h dark period, 20°C; light intensity 350 μE m^{-2} s^{-1} (PAR) provided by fluorescent and incandescent lamps.

Chloroplast Isolation. All procedures were carried out at 0°C. Leaves (15–20 g) were ground for 3 s in a Polytron[2] blender with 150 ml of medium A: 330 mM sorbitol, 5 mM $MgCl_2$, 10 mM $Na_4P_2O_7$, 4 mM ascorbate adjusted to pH 6.5 with HCl. The brei was squeezed through two layers of Miracloth containing a layer of cotton wool and the filtrate was centrifuged for 1 min at 1500*g* in a swing-out rotor. The supernatant was discarded and the pellets resuspended in 9 ml of medium B: 330 mM sorbitol, 50 mM Hepes-KOH, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MgCl_2$ (pH 7.6) plus 0.2% BSA and placed into two centrifuge tubes. Using a syringe, each was underlayered with the same medium with 40% (v/v) Percoll, then the tubes were again centrifuged at 1500*g* for 1 min. Broken chloroplasts formed a band at the top of the Percoll layer, whereas intact chloroplasts were pelleted by this procedure. The supernatants were discarded and the pellets resuspended in medium B plus 0.2% BSA. The total isolation procedure took 15 to 20 min. Chloroplasts were greater than 95% intact based on penetration of ferricyanide (6), and exhibited rates of CO_2-dependent O_2 evolution of 200 to 250 μmol mg^{-1} Chl h^{-1}, measured at 25°C with saturating light and 4 mM $NaHCO_3$, 0.4 mM Pi, and 700 U ml^{-1} catalase.

Light Activation of Rubisco. All measurements were made at 25°C. Darkness refers to experiments conducted under green safelights (light intensity less than 0.1 μE m^{-2} s^{-1} (PAR). Unless stated otherwise, chloroplasts equivalent to 40 μg Chl were added to medium B with 0.4 mM Pi, 0.5 mM $NaHCO_3$, and 700 U ml^{-1} catalase in a total volume of 1 ml and kept in the dark for 2 min before turning on the light. At intervals, 50 μl samples were withdrawn and rapidly injected into 450 μl of assay medium: 50 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 0.5 mM RuBP, 0.1% (v/v) Triton-X-100, and 4.6 mM $NaH^{14}CO_3$ (0.5 Ci mol^{-1}). After 0.5 min, the reaction was terminated by adding 100 μl of 4 N formic acid plus 1 N HCl. The samples were dried, redissolved in 0.5 ml of 0.1 N HCl, and mixed with 4.5 ml of scintillation cocktail to measure acid-stable radioactivity. A sample of the assay medium was diluted with 0.5 M Tris base to determine total radioactivity.

In the experiments reported, chloroplasts were incubated with near-physiological CO_2 concentration (0.5 mM $NaHCO_2$, pH 7.6) to minimize any spontaneous activation of rubisco by CO_2. However, qualitatively similar results were obtained when chloroplasts were incubated in saturating CO_2 (4 mM $NaHCO_3$). Maximal rubisco activity, measured by incubating lysed chloroplasts in 10 mM $MgCl_2$ and 10 mM $NaHCO_3$ (pH 8.0) for 10 min prior to assay was 400 to 450 μmol mg^{-1} Chl h^{-1}.

Chloroplast ATP. Chloroplasts were incubated as described for rubisco measurements and 50 μl samples were injected into 50 μl of 2 N $HClO_4$, rapidly mixed, and then stored on ice. The extracts were centrifuged for 1 min at 12,000*g* to remove protein and the supernatants were neutralized to pH 7.5 with K_2CO_3. The precipitate of $KClO_4$ was removed by centrifugation. ATP in the supernatant was determined using the luciferin-luciferase bioluminescence assay (14). Reaction medium was made by dissolving 80 mg of Boehringer CLS reagent in 10 ml of distilled water. The concentration of ATP standards was determined spectrophotometrically at 260 nm before diluting with 5 mM Hepes-KOH (pH 7.0) to give a solution of 0.25 μM ATP. Each determination was made by adding 10 μl of chloroplast extract to 200 μl reaction medium in a 0.4 ml microcentrifuge tube. After measuing light emission in an Aminco photometer, 10 μl of 0.25 μM ATP was added as an internal standard.

Chemicals. RuBP was synthesized from ribose-5-phosphate as described previously (4). Scintillation cocktail was Optifluor from Packard Instrument Company. All other biochemicals were from Sigma.

RESULTS

Inhibition of ATP Synthesis. We sought methods of decreasing stromal ATP without inhibiting the light-induced alkalization of the stroma, which would also inhibit rubisco activation (1, 3). The chloroplast adenine nucleotide transporter is an obligatory counter-exchange type carrier and incubation of chloroplasts with ATP analogs results in their uptake in exchange for the endogenous chloroplast nucleotides (17). Once outside the chloroplast, these endogenous nucleotides are diluted more than 1000-fold in the suspending medium and cannot effectively compete with the analog for re-entry on the transporter. Incubation of spinach chloroplasts in the dark for 5 min at 25°C with the nonmetabolisable ATP analog, AMP-PCP, inhibited CO_2-dependent oxygen evolution by more than 90% (data not shown). This treatment also inhibited light activation of rubisco in the chloroplasts (Fig. 1). In control chloroplasts, rubisco activity increased 2.8-fold in the light, reaching maximal activity 6 to 8 min after turning on the light. In the treated chloroplasts, rubisco activity increased in the first 1 to 2 min, reaching a level 1.5-fold higher than in the dark, but subsequently declined so that activity was below dark levels after 8 min in the light. Stromal ATP levels for the same chloroplasts are also shown in Figure 1. In control chloroplasts there was a rapid increase in ATP following illumination followed by a decline to steady state levels with the onset of CO_2 fixation. Chloroplasts preincubated with AMP-PCP had lower levels of ATP in the dark and there was only a small increase in the light.

Phloridzin is an energy-transfer inhibitor in chloroplasts and inhibits ATP synthesis without decreasing stromal pH (14). Addition of phloridzin inhibited light activation of rubisco in chloroplasts in a concentration-dependent manner (Fig. 2). As with the ATP analog, there was an initial increase in rubisco activity following illumination but it was less than in the control chloroplasts and activity subsequently declined. In the presence of 15 mM phloridzin, rubisco activity was below dark levels after 8 min in the light. The changes in rubisco activity paralleled changes in stromal ATP (Fig. 2). With 5 mM phloridzin the increase in ATP following illumination was slower and the subsequent decline was greater than in the control. At 15 mM phloridzin the light-induced increase in ATP was only half that in the control and by 4 to 6 min stromal ATP was below dark levels.

Phosphate Requirement. In the absence of added phosphate, CO_2-dependent oxygen evolution was not maintained. After 1 to 2 min in the light the rate declined due to consumption of the endogenous chloroplast Pi and after 10 min illumination, rates were less than 10% of control chloroplasts (incubated with 0.4 mM Pi in the suspending medium; data not shown). Light activation of rubisco was also inhibited in the absence of added Pi (Fig. 3) as has been observed previously (3, 9). There was an initial increase in rubisco activity in the first 1 to 2 min of light followed by a continued decline, with activity reaching dark levels after 8 to 10 min illumination in the absence of Pi. Stromal ATP also declined rapidly in chloroplasts without added Pi (Fig. 3). This decline in ATP was prevented if chloroplasts were incubated in the absence of Pi but with the addition of methyl viologen, which inhibits CO_2 fixation and the concomitant consumption of Pi and at the same time provides an electron

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

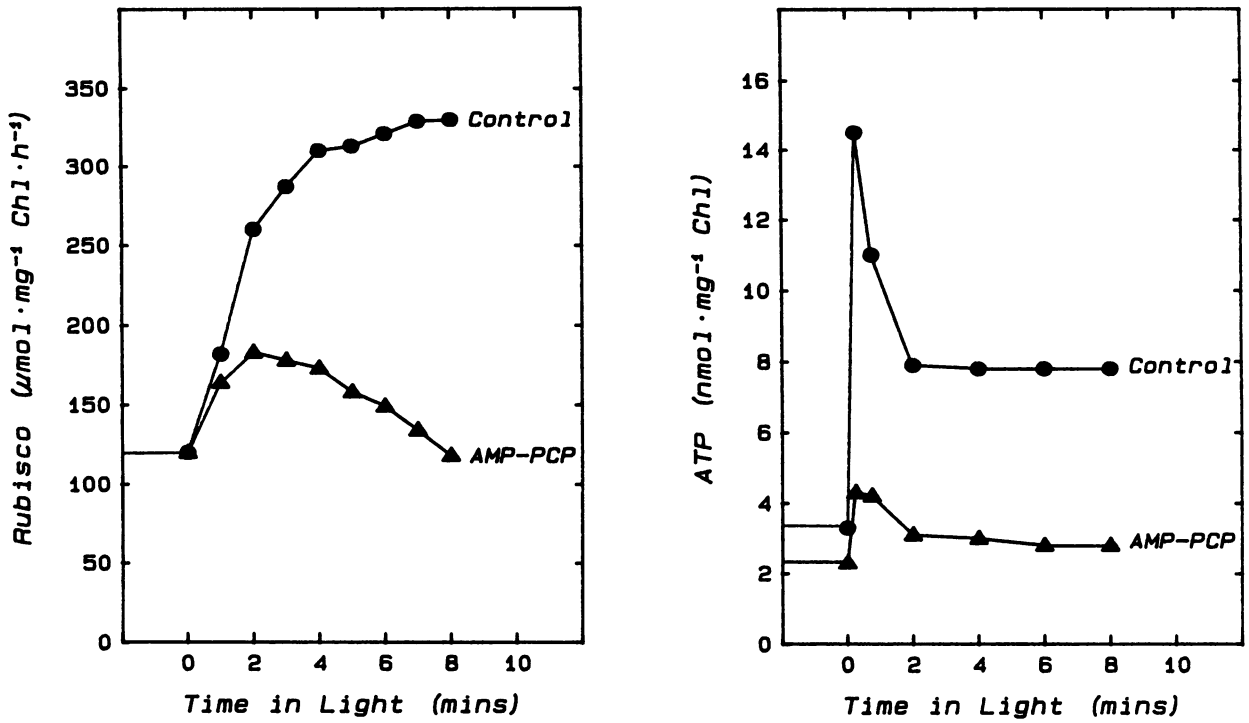

FIG. 1. Light activation of rubisco and stromal ATP in isolated spinach chloroplasts incubated in the presence and absence of 0.5 mM AMP-PCP. The chloroplasts were preincubated for 5 min in the dark before starting illumination. For determination of ATP in the chloroplast stroma, the chloroplasts were separated from the medium by centrifugation through silicone oil in the light (*cf.* Ref. 14).

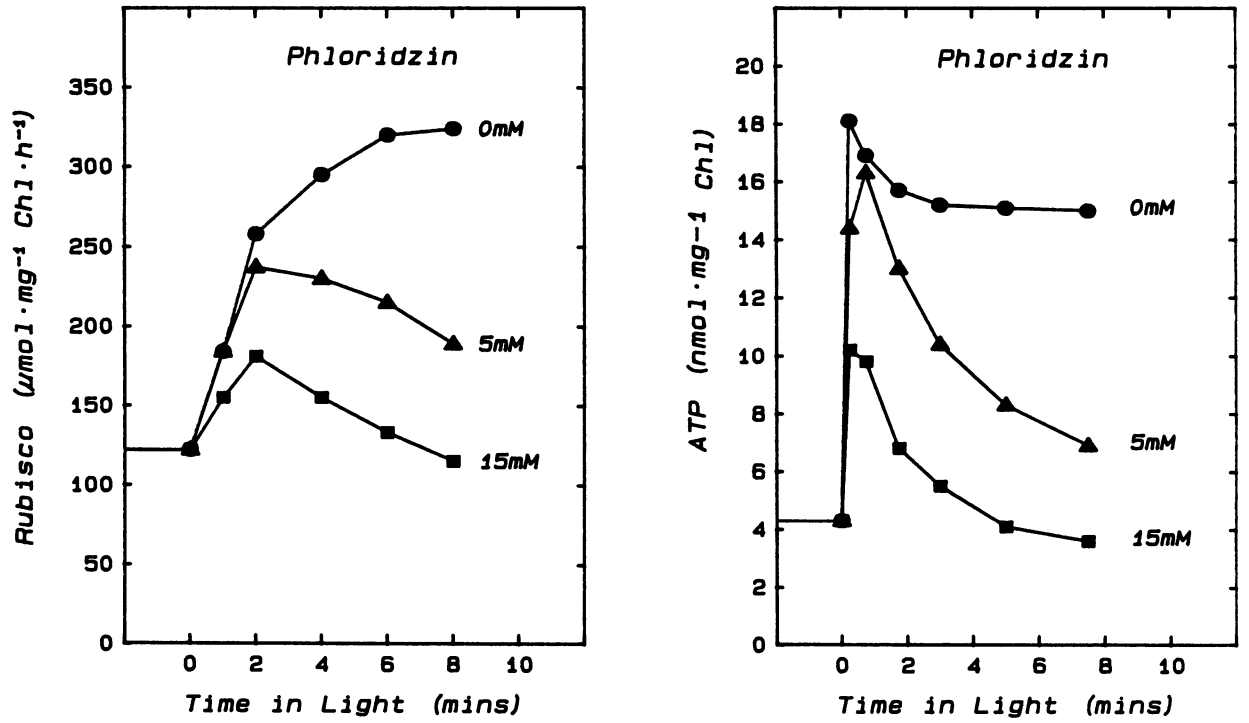

FIG. 2. Light activation of rubisco and stromal ATP in isolated chloroplasts in the presence of varying concentrations of phloridzin. The phloridzin was dissolved in methanol and all samples contained methanol at a final concentration of 2% (v/v).

acceptor to drive photophosphorylation. In the presence of methyl viologen, high ATP levels were maintained in the light even in the absence of added Pi (Fig. 3) and light activation of rubisco was restored and even exceeded that observed in the +Pi control. D,L-Glyceraldehyde, an inhibitor of CO_2 fixation, also allowed maintenance of high ATP levels in the absence of added Pi, but in this case light activation of rubisco was only 67% of that in the +Pi control (data not shown), possibly due to some direct inhibition of rubisco activation by this compound (1). Glycerate should act as a sink for ATP, even in the presence of methyl viologen, since its conversion to 3-phosphoglycerate would consume ATP but not be dependent on NADPH. As shown in Figure 3, addition of glycerate and methyl viologen in the absence of added Pi reduced both stromal ATP and light

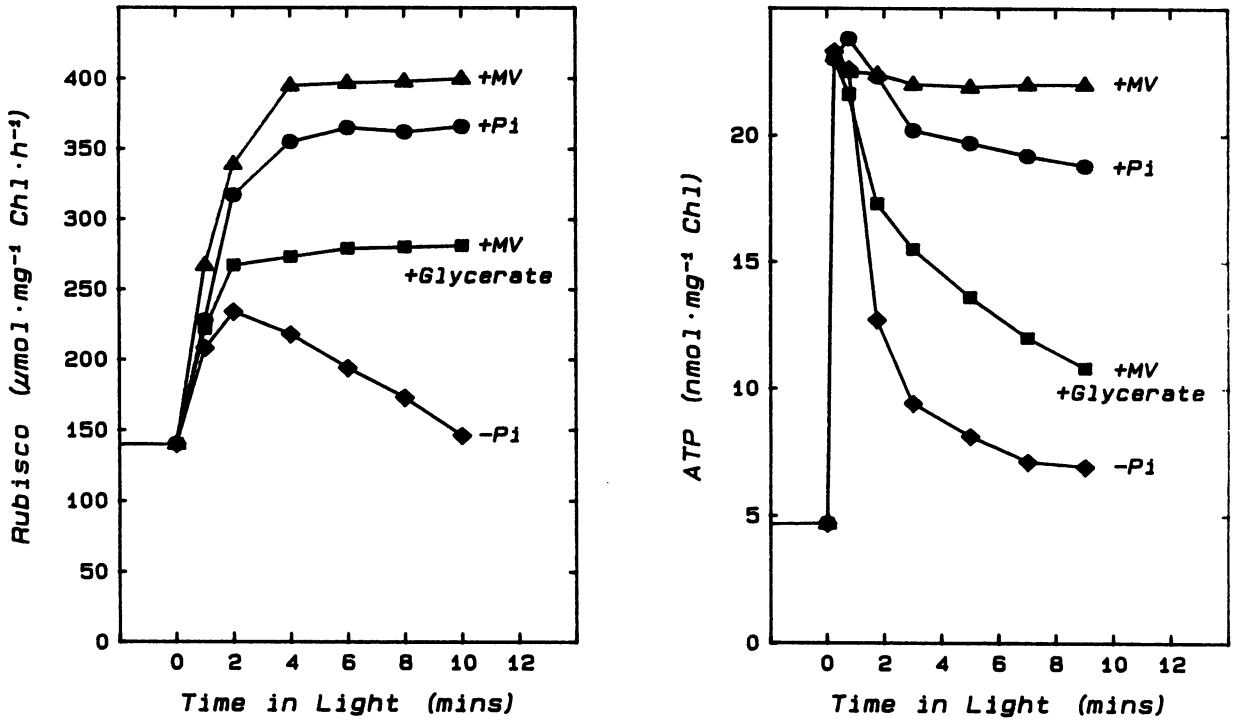

FIG. 3. Light activation of rubisco and stromal ATP in isolated chloroplasts in the presence and absence of 0.4 mM Pi. Other additions (0.1 mM methyl viologen and 2 mM D-glycerate as indicated) were made in the absence of added Pi.

activation of rubisco.

Effect of DHAP and PGA. DHAP and PGA have previously been observed to inhibit light activation of rubisco in isolated chloroplasts (2, 3, 9). In the presence of 0.2 mM Pi, 2 mM DHAP almost completely inhibited light activation of rubisco and also decreased stromal ATP (Fig. 4). This would have resulted, at least in part, from inhibition of Pi uptake via the phosphate transporter. Addition of superoptimal Pi (2 mM) inhibited CO_2 fixation but maintained high levels of stromal ATP and rubisco activity. In the presence of DHAP, addition of 2 mM Pi partially restored light activation of rubisco and stromal ATP levels (Fig. 4). Virtually identical results were obtained with 3-phosphoglycerate (data not shown).

In Figure 5, rubisco activity is plotted against stromal ATP for chloroplasts illuminated for 8 to 10 min under the various conditions shown in Figures 2 to 4. There is a strong correlation between rubisco activity and stromal ATP and the relationship appears to be linear over the range of values measured. In the two experiments with DHAP (Fig. 4) rubisco activity was lower than would have been predicted from the measured ATP level (two lower triangles in Fig. 5). This may reflect an additional effect of DHAP or its metabolites on light activation of rubisco.

DISCUSSION

The experiments reported here show a very strong correlation between light activation of rubisco and stromal ATP levels in isolated chloroplasts (Fig. 5). In a complex system such as the chloroplast, changes in one metabolite invariably result in changes in the levels of other metabolites, and it could be argued that some other factor, also dependent on ATP concentration, determines rubisco activity. For example, in the absence of Pi (Fig. 3) or in the presence of high external concentrations of DHAP or PGA (Fig. 4), stromal Pi would also be low and it may be this which determines rubisco activation. However, this would not be the case with the energy transfer inhibitor, phloridzin (Fig. 2) or the ATP analog, AMP-PCP (Fig. 1). The fact that the correlation of rubisco activation with stromal ATP concentration was observed under such a wide variety of conditions suggests a direct link between the two. We suggest that this link is the rubisco activase protein. It has recently been demonstrated that purified rubisco can be activated *in vitro*, in the presence of physiological levels of CO_2 and RuBP, by partially purified rubisco activase (13). This reconstituted system requires ATP and is inhibited by ADP (21). It is clear from the present results that changes in stromal ATP in isolated chloroplasts are also accompanied by changes in rubisco activation state, providing further evidence for the participation of the rubisco activase protein in the activation process *in vivo*.

The mechanism of rubisco activase is not yet known nor is the function of ATP in this process. Nevertheless, it is clear from the data of Figure 5 that rubisco activation varies continuously with stromal ATP concentration over a physiologically meaningful range. Using a stromal volume of 25 μl mg^{-1} Chl (14) the ATP levels of 4 to 24 nmol mg^{-1} Chl in Figure 5 would correspond to stromal ATP concentrations of 0.16 to 0.96 mM. With partially purified rubisco activase in the presence of an ATP-regenerating system, there was a hyperbolic relationship between ATP concentration and activity which saturated at 0.6 to 0.8 mM ATP (21). However, in the absence of a regenerating system activity was linearly related to ATP concentration up to 1.2 mM ATP, presumably as a result of inhibition by ADP generated in the reaction. The linear relationship between ATP and rubisco activation (Fig. 5) indicates that ADP inhibition of activase also occurs in intact chloroplasts. The ATP concentration within the chloroplast is a result of the balance between its synthesis by photophosphorylation and consumption by the carbon cycle. Since the total adenine nucleotide pool is constant over short time periods, decreased ATP also implies increased ADP, and it is not yet clear whether it is the concentration ATP *per se* or the ATP/ADP ratio which determines rubisco activation state. Changes in rubisco activation were much slower than changes in stromal ATP, and the rapid fluctuations in ATP following the onset of illumination were not directly reflected by changes in rubisco activity. Stromal ATP concentrations were highest 0.25

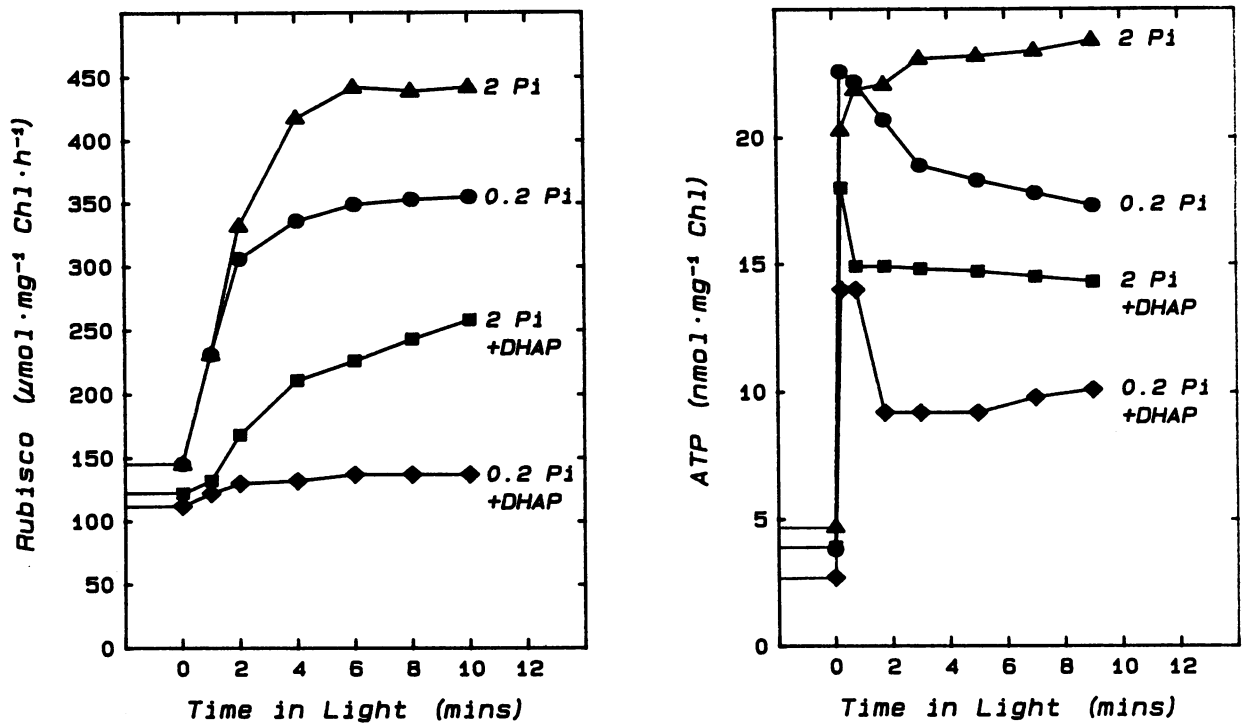

FIG. 4. Light activation of rubisco and stromal ATP in isolated chloroplasts in the presence and absence of 2 mM DHAP with 0.2 mM or 2 mM Pi.

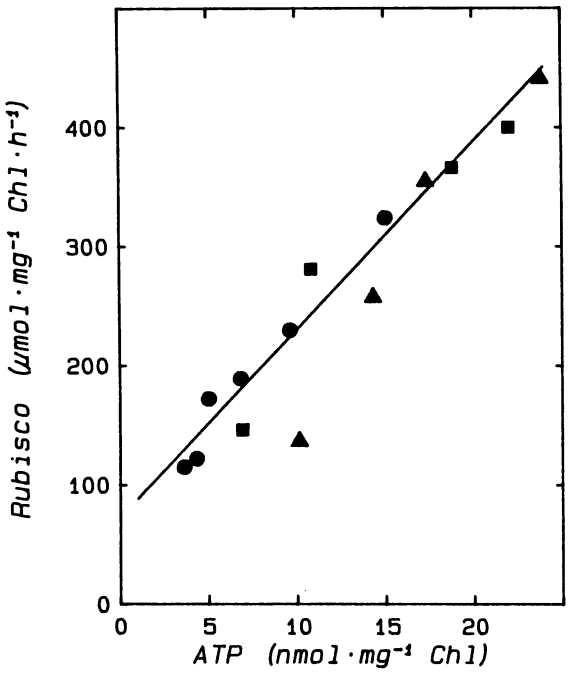

FIG. 5. Relationship between rubisco activity and stromal ATP. The data plotted are from Figure 2 (circles), Figure 3 (squares), and Figure 4 (triangles) after 8 or 10 min illumination. The line was fitted by linear regression and had a correlation coefficient of 0.984.

to 0.75 min after illumination and subsequently declined with the onset of CO_2 fixation, whereas maximum rubisco activity was only observed after 2 to 8 min in the light. This indicates a relatively slow interaction of ATP with rubisco activase which may act to damp out rapid fluctuations in stromal ATP.

This is the first instance of an enzyme of the Calvin Cycle whose activation state appears to be dependent on stromal ATP concentration. Both the 3-phosphoglycerate kinase and phosphoribulokinase reactions are dependent on the concentrations of ATP and ADP, but this is an effect on the rate of catalysis and not an alteration of the activation state of the enzyme (5, 15). Light activation of other Calvin Cycle enzymes is dependent on the ferredoxin-thioredoxin reductase system, modulated by levels of metabolites. The dependence of rubisco activation state *in situ* on ATP may explain changes in rubisco activation with varying light intensity (12, 19) and the lack of response to CO_2 concentration in the light (11, 19). It also provides an explanation for the Pi requirement for light activation in isolated chloroplasts (3, 9) and the inhibition of activation by DHAP, PGA, and glycerate (2, 3, 9). Further experimentation will be needed to establish whether changes in chloroplast ATP levels in leaves also result in changes in the activation state of rubisco.

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