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Factors Affecting the Activation State and the Level of Total Activity of Ribulose Bisphosphate Carboxylase in Tobacco Protoplasts[1]

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ABSTRACT

The relationship between the activation state and the level of total activatable activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) was examined in tobacco protoplasts. When darkened protoplasts were illuminated, both activation and total activity increased, but at different rates; the $t_{1/2}$ were 2.3 and 6.7 minutes, respectively. The light response of rubisco activation state and total activity, measured after 15 minutes of illumination, were similar but their responses to light transitions and photosynthetic inhibitors were different. When irradiance was reduced from saturating to subsaturating, deactivation of rubisco in protoplasts was immediate, whereas there was little change in total activity during the first 20 minutes following the transition. The light-induced increases in activation state and total activity were inhibited by nigericin, but activation was more sensitive exhibiting a response similar to that of photosynthesis. Treatment of tobacco protoplasts and leaves with methyl viologen at limiting irradiance increased rubisco activation, but inhibited the light-induced increase in total activity. These results indicate that light activation of rubisco is mechanistically distinct from the light-dependent changes in total activity in tobacco, a species containing carboxyarabinitol 1-phosphate, an endogenous inhibitor of total rubisco activity.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco[2]) (EC 4.1.1.39) catalyzes the initial reactions of the photosynthetic and photorespiratory pathways. As an irreversible reaction at a branch point for two biochemical pathways it is a likely site for regulation (10). *In vitro*, activation of the enzyme by carbamate formation is required for catalytic competence (6, 10) and is regarded as the principle mechanism regulating enzyme activity. Estimates of the activation state of rubisco (the proportion of enzyme in the active form) have been made following rapid extraction and assay of chloroplasts, protoplasts, and leaves (1, 4, 7, 12, 15, 21, 22). This activity, which is often referred to as the '*in vivo*' or 'initial activity,' increases upon illumination, and correlates quantitatively with the light response of photosynthesis (15). Thus, control of rubisco activity *in vivo* involves coordinate regulation of the activation state of the enzyme with the rate of electron transport activity (14, 15).

Light activation of rubisco generally has been attributed to an increased proportion of carbamylated enzyme in the light. However, certain features of rubisco activation *in vivo* are inconsistent with the spontaneous activation mechanism described for the isolated enzyme (for discussion, see Refs. 10, 15, and 27). The identification of rubisco activase, a soluble protein required for the activation of rubisco (14), provided an explanation for the discrepancies observed between the activation requirements of the isolated enzyme and its activity *in vivo*. Reconstituted assays using partially purified activase demonstrated that rubisco activase promoted light-dependent activation of rubisco at physiological substrate levels (13, 14). The activase protein, which appears to be ubiquitous in higher plants (16), is not expressed in a rubisco light-activation mutant of *Arabidopsis* (14–16). Results from comparative studies with the mutant and wild type (15, 22) are consistent with the proposal that rubisco activase is a principle component in the regulation of the *activation state* of rubisco *in vivo* (13–16).

In addition to modulation of the activation state of the enzyme, a second level of regulatory control on rubisco has been found in some but not all plant species (9, 15, 17–19, 25, 26). The mechanism involves modulation of the catalytic activity of the enzyme by a substrate analog, CA 1-P (3). This compound inhibits catalysis noncompetitively by binding tightly to the catalytic site of activated rubisco (3, 17, 18). CA 1-P was initially detected by comparing 'total' activities of rubisco, measured after incubation with high levels of CO_2 and Mg^{2+}, in extracts from illuminated and darkened leaves (9, 25). From these indirect measurements of potential activity and more direct analysis (17, 18), it appears that the amount of this inhibitor bound to the enzyme is high in the dark and at low irradiance and below detectable levels at high irradiance. Thus, changes in the concentration of CA 1-P, like changes in activation state, afford a level of light-dependent control on rubisco.

The presence of two mechanisms for regulating rubisco raises the question of their relative importance in controlling photosynthesis. In species exhibiting light/dark changes in total rubisco activity, illumination of darkened leaves caused increases in both activation state and total activity, which occurred simultaneously and with similar kinetics (17, 19, 25). Upon darkening, however, the activation state decreased more rapidly than total activity (19). In the present study we have used protoplasts isolated from tobacco, a species which exhibits inhibition by CA 1-P (18, 26), to examine activation and total activity in response to light levels and light/dark transitions and to determine the relative effects of photosynthetic inhibitors on these activities. Previous studies

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[2] Abbrevations: rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; CA 1-P, carboxyarabinitol 1-phosphate; RuBP, ribulose-1,5-bisphosphate; MV, methyl viologen.

with *Phaseolus* have shown that there is a direct relationship between total activity and the inhibitor/catalytic site ratio (17) indicating that total activity can be used as a reliable indicator of the amount of CA 1-P bound to the enzyme. The use of protoplasts for measurements of rubisco activation obviates problems associated with leaf extraction since assays can be performed immediately upon lysis of the protoplast (21, 22). The data from the present study demonstrate that light activation of rubisco is mechanistically distinct from changes in total activity.

MATERIALS AND METHODS

Plant Material. Tobacco plants, *Nicotiana rustica* var pulmila, were grown in vermiculite in cylinders suspended in half-strength Hoagland solution. Plants were grown at 300 μmol photons/m^2·s, 60% relative humidity with a 14 h, 26°C/10 h, 22°C light/dark regime.

Protoplast Isolation. Protoplasts were isolated from 4 to 5 week old plants by a modification of the method of Somerville *et al.* (23). Plants were transferred at the end of the dark period to low light (15 μmol photons/m^2·s) and washed with distilled and deionized water. Following removal of the lower epidermis, leaf segments were incubated at 23°C for 1 h on a solution of 0.5 M sorbitol, 1.0 mM $CaCl_2$, 0.2 mM EDTA, and 10 mM 2-(*N*-morpholino)ethane sulfonic acid (Mes)-NaOH (pH 5.5) containing 0.1% w/v BSA, 1.3% w/v cellulysin, 1.3% w/v macerase (CalBiochem).[3] Enzymes were desalted prior to use. Protoplasts were collected by centrifugation, and purified by flotation through 29% v/v Percoll in 0.5 M sorbitol, 1.0 mM $CaCl_2$, 0.1% w/v BSA, and 20 mM Mes-NaOH (pH 6.0), overlayed with the buffer solution. Protoplasts were removed from the interface, resuspended in 0.5 M sorbitol, 1.0 mM $CaCl_2$, and 10 mM Mes-NaOH (pH 6.0), collected by centrifugation and stored in the dark on ice.

Measurement of Photosynthesis and Rubisco Activity in Tobacco Protoplasts. Photosynthetic O_2 evolution was measured at 25°C in a Hansatech O_2 electrode (Decagon Instruments, Pullman, WA). For photosynthesis measurements, protoplasts were suspended at 20 or 50 μg Chl/ml in 0.45 M sorbitol, 1.0 mM $CaCl_2$, 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES)-KOH (pH 7.6), and 1.0 mM $NaHCO_3$ containing 250 units of carbonic anhydrase. For rubisco assays, protoplasts were suspended at 50 μg Chl/ml in the above solution containing 0.19 mM $NaHCO_3$ and were flushed continuously at 200 ml/min with a humidified gas stream containing 290 μl CO_2/L, 21% O_2, balance N_2. One hundred μl aliquots were rapidly removed at the designated time and lysed into detergent-containing assays at 25°C for the measurement of rubisco activity. Activation state, the 'initial' activity of RuBP carboxylase, was determined in a 30 s assay initiated by the addition of the protoplasts to 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (1 μCi/μmol), 0.1% v/v Triton X-100, 0.1% w/v casein, 2 mM DTT, 0.4 mM RuBP, and 100 mM *N*-(tris-[hydroxymethyl]methyl) glycine (Tricine)-NaOH (pH 8.0), in a total volume of 0.5 ml. Total activity was measured by incubating the lysed protoplasts in the above assay mixture minus RuBP to fully activate rubisco; after 5 min, 30 s assays were initiated with RuBP. All assays were terminated by the addition of 100 μl of 4 N HCOOH in 1 N HCl, taken to dryness *in vacuo* and ^{14}C-dpm were determined by liquid scintillation spectrometry.

Measurement of Rubisco Activity in Tobacco Leaves. Leaf discs were sampled from intact plants following treatment under the conditions described in the text and rapidly frozen in liquid N_2. Three discs from three separate plants were used for each determination. Discs were rapidly homogenized at 4°C in a Ten Broeck glass homogenizer containing 10 mM $MgCl_2$, 0.5% w/v ascorbate, 0.5% BSA, 10 mM DTT, and 100 mM Tricine (pH 8.0). One hundred μl aliquots were removed for the determination of activation and total activity as described above. For experiments involving MV, entire leaves were treated with 0.2 mM MV in 0.05% Triton X-100 as described previously (15). The results reported are the mean of two separate determinations.

RESULTS

The activation state and the level of total activatable rubisco activity increased 4- and 2-fold, respectively, during induction of tobacco protoplasts at saturating irradiance (Fig. 1). The increase in activation occurred immediately upon illumination and approached saturation after 6 min ($t_{1/2}$, ~2.3 min). A 1 to 2 min lag was evident in the response of total rubisco activity to irradiance and the increase in activity required approximately 15 min to reach saturation ($t_{1/2}$ ~6.7 min).

Upon darkening, there was an immediate decrease in the activation state of rubisco (Fig. 1). Deactivation of rubisco was slower than activation ($t_{1/2}$, 4 min) and 20 min were required for the enzyme to deactivate to the dark level (Fig. 1). A slight decrease in total rubisco activity occurred upon darkening but the rate was considerably slower than that of deactivation. After 30 min, the level of total activatable rubisco activity was still 60% of that at high light.

Measurements of total activity required a 5 min incubation period prior to assay. Since we assume that changes in total activity reflect changes in the amount of CA 1-P bound to rubisco (17), it was important to conduct mixing experiments to test if metabolism of the inhibitor can occur during the incubation period. When darkened protoplasts (*i.e.* containing CA 1-P) and illuminated protoplasts were added simultaneously in a 1:1 ratio to the assay, the total activity measured in the mixed assay was the arithmetic mean of the activities measured separately (data not shown). These results indicated that, once the protoplasts were lysed, further metabolism of the inhibitor did not occur during the incubation period, even though the enzymes involved in degradation were present in the extract and presumably activated by the exposure to light.

In Figure 2, the time courses of deactivation and reactivation of rubisco and the accompanying changes in total activity level were examined following transitions in light intensity. Changes in activation state and total activity, similar to those measured

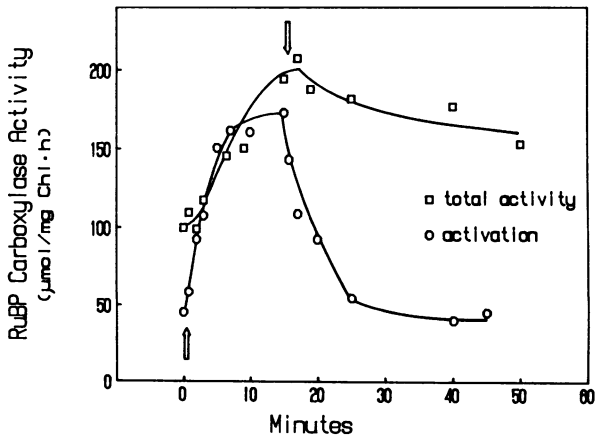

FIG. 1. Time course of rubisco activation (○) and total activity (□) in tobacco protoplasts. Protoplasts were incubated in the dark for 2 min prior to the onset of illumination with 1000 μmol photons/m^2·s (zero time ↑) and, after 15 min, were darkened (↓).

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or 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.

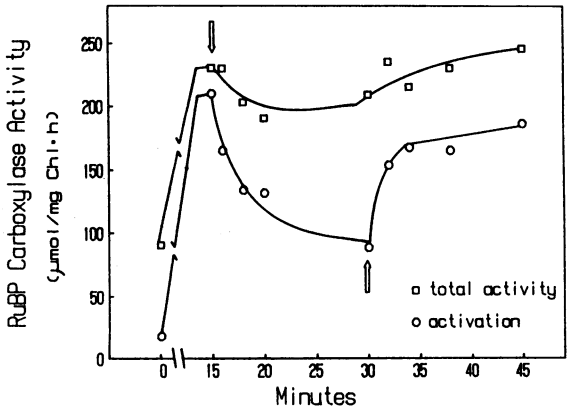

FIG. 2. Time course of rubisco activation (○) and total activity (□) in tobacco protoplasts in response to light transitions. Protoplasts were incubated in the dark for 2 min prior to the onset of illumination with 1000 μmol photons/m^2·s (zero time) and, after 15 min of irradiation, the light intensity was decreased to 30 μmol photons/m^2·s (↓). After 15 min at subsaturating irradiance, the intensity was increased to 1000 μmol photons/m^2·s (↑).

upon darkening, were observed following a decrease from saturating to subsaturating irradiance. When the light intensity was increased after 15 min at low irradiance, the activation state increased 2-fold. Total rubisco activity also increased when the irradiance was changed from subsaturating to saturating, but only slightly, matching the slight drop in total activity which occurred during the subsaturating light period. It also was observed that 4-fold changes in the rates of photosynthetic O_2 evolution occurred during these transitions. Photosynthesis required less than 2 min to reach a new steady state rate following a change in the light intensity.

The light responses of rubisco activation, total activity, and photosynthesis were similar when measured in protoplasts that were isolated from darkened tobacco leaves (Fig. 3A). Light saturation of all three responses occurred at about 300 μmol photons/m^2·s, the light level used for growth. When the light response of activation and total activity were measured in protoplasts that were preilluminated for 15 min at saturating irradiance prior to exposure to the various light levels, differences between the two responses were apparent (Fig. 3B). For preilluminated protoplasts, rubisco activation decreased upon transition from saturating to subsaturating irradiance, the extent dependent on the light intensity (data not presented). As a result, the light response of the rubisco activation state following 15 min deactivation was similar to that measured upon illumination of darkened protoplasts. In contrast, total rubisco activity remained at a constant high level under changing light intensities when protoplasts were preilluminated at saturating irradiance prior to reducing the light intensity.

Nigericin, a K^+/H^+ exchanger, uncouples photophosphorylation from electron transport by collapsing the pH gradient. When added to isolated protoplasts at 0.05 to 0.5 μM, nigericin caused inhibition of photosynthetic O_2 evolution (Fig. 4). This range of concentrations was used to examine the effects of nigericin on rubisco activation state and total activity. In order to examine the effect of nigericin on activation independent of changing total activities, protoplasts were preilluminated at saturating irradiance for 30 min, darkened, and then illuminated. This treatment relieves dark inhibition of total rubisco activity (Fig. 1).

At high nigericin concentrations, both activation and total rubisco activity were inhibited markedly (Fig. 4). At lower concentrations, activation was inhibited to a much greater degree than total activity: the K_i was 0.20 μM for activation and 0.35 μM

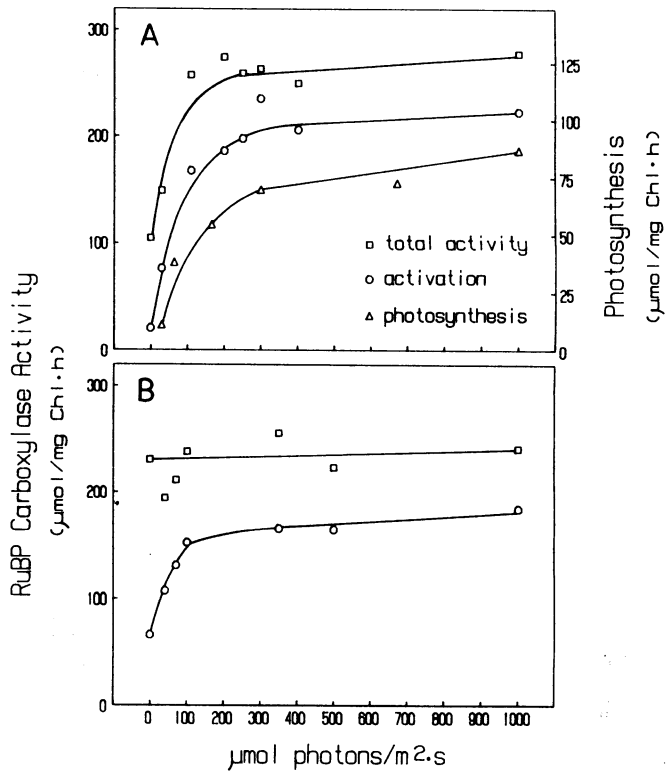

FIG. 3. Light response of rubisco activation (○), total activity (□), and photosynthesis (△) in tobacco protoplasts starting from darkness (A) or after preillumination (B). A, Protoplasts were incubated in the dark for 2 min prior to the onset of illumination at the indicated irradiances. Rubisco activation state and total activity were determined after 15 min of illumination. B, Protoplasts were preilluminated at 1000 μmol photons/m^2·s and after 15 min, the irradiance was changed to the indicated levels. Rubisco activation state and total activity were determined 15 min after changing the light intensity.

for total activity. Low concentrations of nigericin were sufficient to substantially inhibit both photosynthesis and rubisco activation and their responses to nigericin concentration were nearly identical. In contrast, low concentrations of nigericin had only a slight inhibitory effect on total activity.

At a subsaturating irradiance, methyl viologen, a photosystem I electron acceptor, inhibited both activation and total rubisco activity in tobacco protoplasts isolated from darkened leaves (Fig. 5A). When protoplasts were preilluminated for 15 min at saturating irradiance, darkened for 30 min, and then treated with methyl viologen and illuminated at a low irradiance, a slight stimulation of the rubisco activation state occurred (Fig. 5B). Total activity, however, decreased independent of methyl viologen from the high level induced during the preillumination period.

As reported by others (11), protoplasts were rather impermeable to methyl viologen and relatively high concentrations (≥10 mM) were required to obtain inhibition of photosynthesis. Previous work with *Arabidopsis* indicated that concentrations as low as 0.2 mM are effective with leaves if added with 0.05% Triton X-100 (15). For this reason, the methyl viologen experiments described above were repeated with intact leaves using a lower concentration of methyl viologen (Table I). For leaves darkened for 14 h, the rubisco activation state measured at low irradiance was increased slightly by methyl viologen while the level of total activity was decreased. At saturating irradiance, both activities were lower in leaves treated with methyl viologen. In leaves that were preilluminated at saturating irradiance prior to treatment with methyl viologen (to relieve inhibition of total

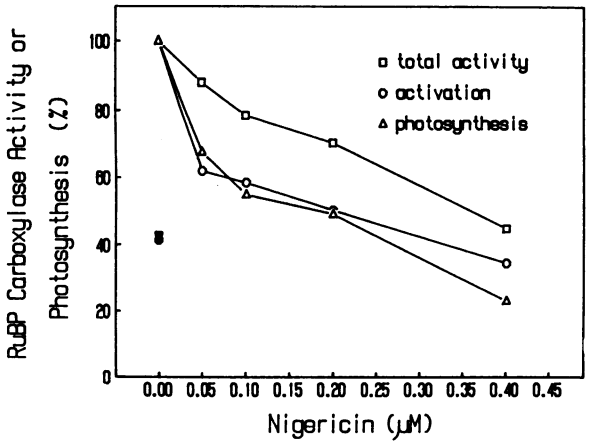

FIG. 4. Response of rubisco activation (○), total activity (□), and photosynthesis (△) to nigericin concentration in tobacco protoplasts. Rubisco activation state was measured in protoplasts that were preilluminated for 15 min at saturating irradiance (1000 μmol photons/m^2·s), darkened for 30 min, and then illuminated for 15 min at 1000 μmol photons/m^2·s. Total activity was measured after 15 min in protoplasts that were incubated in the dark for 2 min prior to the onset of illumination at 1000 μmol photons/m^2·s. Nigericin, at the indicated concentrations, was added just prior to illumination. Control rates for activation, total activity, and photosynthesis were 172, 184, and 67 μmol/mg Chl·h, respectively. Dark values for activation (●) and total activity (■) are indicated.

activity), there was a marked stimulation of the activation state measured at low irradiance. In contrast, total rubisco activity in these leaves was not affected by methyl viologen.

DISCUSSION

Several lines of evidence from this and previous studies indicate that different mechanisms regulate activation state and total activity. First, the activation state of rubisco exhibits light modulation in species which do not contain CA 1-P (17) or show changes in the level of total activatable rubisco activity (15). Second, the kinetics of activation and especially deactivation differed from the light-induced changes in total rubisco activity (19; Fig. 1). Third, activation state and total activity differed in their sensitivity to the uncoupler, nigericin, and in their response to MV. Thus, regulation of total activity via the concentration of CA 1-P appears to be distinct from the regulatory mechanism controlling the activation state.

Since activation state and total activity increase upon illumination, both have been implicated in the regulation of photosynthesis (12, 15, 20). Several previous studies have shown that the activation state is closely correlated with photosynthesis (7, 12, 15), even in plants which exhibit CA 1-P inhibition of rubisco (19). This relationship between activation state and photosynthesis continues through the lifecycle of the plant (8) and is evident in the parallel response of photosynthesis and activation to low O_2 (20), Fe-deficiency (24), and high temperature (28). For transitions from high to low irradiance, our results with tobacco protoplasts indicate that changes in the activation state of the enzyme adjust rubisco activity to the incident light intensity independent of changes in total activity (Fig. 3). Carboxyarabinitol 1-phosphate appears to be metabolized rapidly in the light, but synthesized only slowly in the dark or at low light (19). Therefore, once the chloroplast has been illuminated at high irradiance, no further control is exerted on rubisco by CA 1-P and enzyme activity is modulated almost entirely by changes in activation state (Fig. 3). Control of total activity returns only after exposure to prolonged darkness or low light (19), after deactivation has substantially reduced the activation state to a low level.

While there appears to be little effect of total activity on the rubisco activation state once plants have been exposed to saturating light, a relationship between inhibition of total activity and activation state was demonstrated for protoplasts isolated from plants that were darkened for several hours. When these protoplasts were treated with methyl viologen, no increase in the activation state occurred at limiting irradiance. However, when inhibition of total activity was relieved by exposure to high light, treatment with methyl viologen caused the activation state in tobacco protoplasts and leaves to increase. A similar effect of this compound on rubisco activation has been reported previously for *Arabidopsis*, a noninhibitor plant (15). Thus, under some conditions, total activity may limit the level of activation in plants which contain CA 1-P.

Questions remain concerning the physiological significance of rubisco regulation by CA 1-P. Seemann *et al.* (17) and Gutteridge *et al.* (3) have suggested that the binding of CA 1-P to rubisco may function as a mechanism to maintain the enzyme in the activated state in the dark, keeping the enzyme primed for activity in the light. However, this proposal is not supported by the data in Figure 2 which show that inhibition of total activity has no effect on the rate of rubisco activation. Furthermore, in order to catalyze CO_2 fixation at rates adequate for photosynthesis, rubisco must remain in a highly activated state at air-levels of CO_2 and millimolar concentrations of RuBP, conditions which cause deactivation of the isolated enzyme (2, 5). A regulatory mechanism based on stabilization of activated rubisco by CA 1-P is unable to account for activation under steady state conditions in the light, since the concentration of this inhibitor,

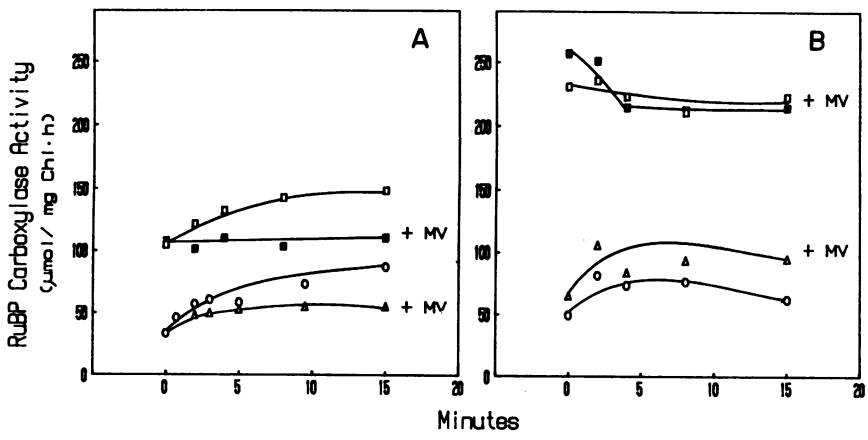

FIG. 5. Time course of rubisco activation (△, ○) and total activity (■, □) at subsaturating irradiance in response to 10 mM methyl viologen in tobacco protoplasts starting from darkness (A) or after preillumination at saturating irradiance (B). A, Protoplasts were incubated in the dark for 2 min prior to the onset of illumination at 30 μmol photons/m^2·s. B, Protoplasts were preilluminated for 15 min at saturating irradiance (100 μmol photons/m^2·s, darkened for 15 min, and illuminated with 30 μmol photons/m^2·s. MV was added just prior to illumination at subsaturating irradiance (△, ■).

Table I. *Effect of 0.2 mM MV on Activation State and Total Activity of Rubisco Extracted from Tobacco Leaves*

Plants were either maintained in the dark for 14 h (no preactivation) or were taken from darkness and illuminated at 1000 μmol photons/m^2·s for 30 min and then darkened for 30 min (preactivation) prior to illumination at low (30 μmol photons/m^2·s) or high (1000 μmol photons/m^2·s) irradiance.

Treatment Condition	Activation		Total Activity	
	Control	MV	Control	MV
	μmol/mg Chl·h			
No preactivation				
Dark	151		168	
30 min low irradiance	184	192	208	149
10 min high irradiance	693	489	543	499
Preactivation				
30 min dark	497		644	
30 min low irradiance	359	552	459	414

which is required in amounts stoichiometric with the active site concentration, is negligible under these conditions (17, 18). If CA 1-P were present in the light in sufficient concentration, the activated enzyme would be rendered catalytically inactive by this inhibitor.

Regulation of the activation state of rubisco has been ascribed to the activity of a specific chloroplast protein, rubisco activase (13–15). This protein appears to be ubiquitous among higher plants (16), indicating that it is probably a central regulator of rubisco activation. An *Arabidopsis* mutant, which lacks rubisco activase activity (14, 16), requires elevated CO_2 levels and cannot be grown in air (22). Based on previous observations with the mutant, we have suggested that the physiological significance of activase is 3-fold. First, by lowering the K_{act} (CO_2), rubisco activase promotes a high level of rubisco activation in the light at air-levels of CO_2 (13–15). Second, rubisco activase appears to prevent RuBP deactivation and facilitates activation of inactive rubisco in the presence of physiological levels of RuBP (13, 14). Third, rubisco activase is dependent on thylakoid reactions for full activity and thus provides a mechanism to explain the coordinate regulation of rubisco activation with photosynthetic electron transport activity (13–15, 24, 28).

To date, the mechanistic aspects of CA 1-P synthesis and degradation have not yet been elucidated. The experiments with nigericin and MV conducted here and elsewhere (15) suggest that there exists a much closer association between rubisco activation (via activase) and the thylakoid-bound reactions involved in photosynthetic electron transport than exists between these reactions and total activity (CA 1-P degradation). The differing sensitivities of activation state and total activity to nigericin indicate that CA 1-P degradation probably is not mediated by rubisco activase, otherwise identical responses to this uncoupler would be expected. Similarly, the effects of MV on activation state and total activity were opposite, since this compound caused inhibition of total activity but stimulated rubisco activation. The inhibitory effect of MV on total activity was similar to the effect of this compound on Fd-thioredoxin activated enzymes (11) and may indicate a similar mechanism for light-activation of CA 1-P degradation.

From the data presented here, we conclude that modulation of the activation state of rubisco in tobacco, a species which synthesizes CA 1-P, is mechanistically distinct from changes in total activity. The available evidence indicates that light dependent changes in the activation state both in CA 1-P-containing and in noninhibitor species is mediated by rubisco activase (16) and that this regulatory protein can fully account for activation of rubisco at physiological conditions (13–15). Our measurements of activation state reflect the amount of rubisco that is activated and catalytically competent. In plant species that synthesize CA 1-P, a large percentage of the activated enzyme can be rendered catalytically incompetent if bound by inhibitor (3, 17, 18). Changes in these various forms of rubisco may affect the levels of RuBP and other chloroplast phosphoesters that bind to the enzyme and could act as a mechanism to maintain homeostasis in the face of slowly changing environmental conditions.

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