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Stimulation of thylakoid energization and ribulose-bisphosphate carboxylase/oxygenase activation in *Arabidopsis* leaves by methyl viologen

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The light responses of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) activation and light scattering, a measure of the transthylakoid ΔpH, were found to be similar in *Arabidopsis* leaves. Methyl viologen lowered the light requirements for both activation and scattering, indicating that rubisco activation is related to thylakoid energization status. Nigericin inhibited rubisco activation in spinach protoplasts, providing further evidence for an association between rubisco activation and thylakoid energization. Light scattering and fluorescence quenching signals indicated an overenergization of the thylakoids in an *Arabidopsis* mutant defective in light activation of rubisco.

Ribulose-bisphosphate carboxylase; Thylakoid; Light activation; ΔpH; *(Arabidopsis)*

1. INTRODUCTION

The activation state of rubisco in leaves, determined by rapid extraction, closely correlates with the light response of photosynthesis [1-3]. Although the energetics of the activation process have not been examined in detail, considerable evidence has been presented for the involvement of photosynthetic electron-transport activity in rubisco activation. Early studies with isolated chloroplasts showed that rubisco activation could be inhibited by the electron-transport inhibitor DCMU [4,5] and the uncoupler CCCP [4]. More recently, Weiss [6] suggested that rubisco activation might involve an association between rubisco and specific components in the thylakoid membrane after observing that rubisco activation was the primary site affected by reversible heat inactivation of photosynthesis. Similar conclusions were reached by Taylor and Terry [7] from their studies of the response of photosynthesis to Fe deficiency.

We have recently shown that an association between thylakoids and rubisco activation can be reconstituted in vitro by the addition of rubisco activase, a soluble chloroplast protein [8], to purified rubisco and washed thylakoids in the presence of RuBP, ATP and light [8,9]. Pyocyanine and MV, PS I electron-transport acceptors, were required for activation in the reconstituted system [8,9] and the latter stimulated rubisco light activation in vivo [3]. Rubisco activation in the well-buffered reconstituted assay (unpublished) and in vivo [4,10] is uncoupler-sensitive, suggestive of a requirement for a pH gradient across the thylakoid membrane.

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Abbreviations: rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; DCMU, dichlorophenyldimethylurea; CCCP, carbonyl cyanide dichlorophenylhydrazone; RuBP, ribulose 1,5-bisphosphate; PS, photosystem; MV, methyl viologen

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Although there are no direct methods for measuring ΔpH in leaves, light scattering and pulse modulation fluorometry have been used as non-intrusive techniques for qualitatively determining the energization status of the thylakoids in situ [11–14]. Estimates of ΔpH using these methods correspond to those based on 9-aminoacridine fluorescence measurements of intact chloroplasts [12]. Here, the energetics of rubisco light activation were examined in situ by measuring the responses of light scattering and rubisco activation to irradiance and MV. The results demonstrate a close correlation between thylakoid energization and rubisco activation level.

2. MATERIALS AND METHODS

Arabidopsis thaliana (L.) Columbia wild-type and the mutant *rca* strain (CS 207-1) were grown at 1% CO_2 [15] using a 14 h light/10 h dark photoperiod. Protoplasts were isolated from hydroponically grown spinach [16]. Enzyme activation was measured by rapid extraction and assay [3]. Intact plants or spinach protoplasts were flushed at 25°C with a humidified gas stream consisting of 350 μl CO_2/l and 2% O_2, balance N_2. Protoplasts were lysed by injection into Triton X-100-containing assays. RuBP carboxylase activity was measured as in [3]. Fructose-1,6-bisphosphate phosphatase (FBPase) [17] and ribulose-5-phosphate (Ru5P) kinase activity [18] were measured after a 10 s centrifugation. Results presented are the means for three separate determinations. Three plants were homogenized for each determination. MV was incorporated into leaves by immersing plants in 0.2 mM MV containing 0.05% Triton X-100 [3] or by spraying with 1.0 mM MV in 0.05% Triton X-100.

Light scattering was measured as described by Dietz et al. [13]. *E* quenching, the energy-dependent component of fluorescence quenching, was calculated from fluorescence signals measured with a pulse amplitude modulation fluorometer [13,14].

3. RESULTS

The activation state of rubisco increased when darkened *Arabidopsis* plants were illuminated at near-saturating irradiance (fig.1). Activation was

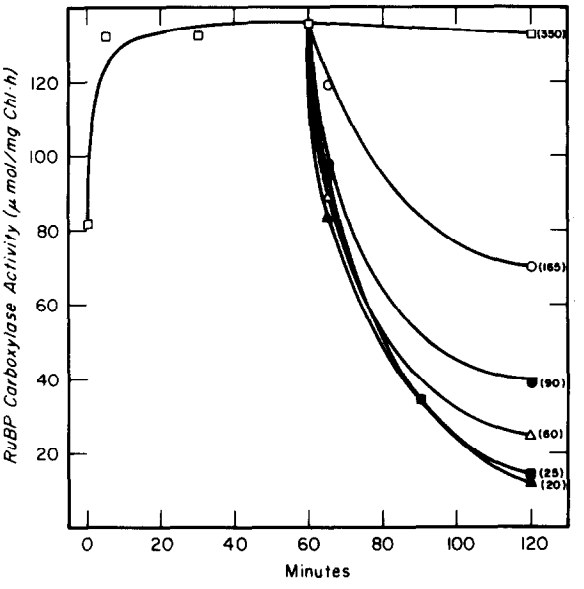

Fig.1. Time course of rubisco activation and deactivation in response to changes in irradiance. *Arabidopsis* plants were incubated in the dark for 60 min (zero time) and then illuminated at 350 μmol photons/m^2 per s. After 60 min, the irradiance was lowered to the levels indicated in parentheses.

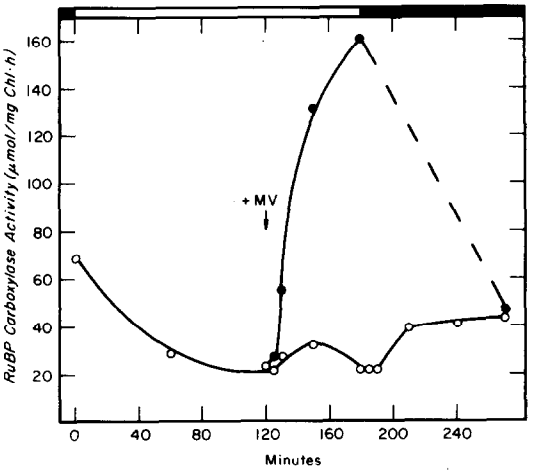

Fig.2. Time course of rubisco deactivation at low irradiance and methyl viologen (MV)-promoted activation. *Arabidopsis* plants were incubated in the dark for 60 min (zero time), illuminated at low irradiance (30 μmol photons/m^2 per s) for 180 min and then darkened for 90 min (○). After 120 min in the light (arrow), half of the plants were sprayed with 0.05% Triton X-100 (○) and the other half sprayed with the Triton X-100 solution containing 1.0 mM MV (●).

rapid, attaining a new steady-state level in less than 5 min. Deactivation of rubisco occurred almost immediately upon decreasing the light intensity, but 60 min were required to reach a steady-state level. At the lowest irradiance, 20 μmol photons/m^2 per s, the activation level was less than 10% of the level at high irradiance and considerably lower than that of dark activation. We have previously proposed an explanation for deactivation of rubisco based on interactions between RuBP and inactive enzyme at low irradiance [3].

Deactivation kinetics similar to those measured for the high-to-low irradiance transition were observed when darkened plants were illuminated at low irradiance (fig.2). Rubisco activation was stimulated at low irradiance when plants were sprayed with MV (fig.2). MV treatment reversed the deactivation process almost immediately and promoted activation to levels approaching those attained at saturating irradiance. Upon darkening, the activation level of rubisco decreased in MV-treated plants to that of control plants.

The effects of low irradiance and MV on activation level were also examined for FBPase and Ru5P kinase, two ferredoxin-thioredoxin activated enzymes [19]. The activation levels of rubisco, FBPase and Ru5P kinase increased 2.2-, 23.4- and 2.3-fold, respectively, when darkened plants were illuminated at saturating irradiance (table 1). Marked deactivation of all three enzymes occurred at low irradiance. At low irradiance, MV caused FBPase and Ru5P kinase to deactivate to an even lower level, as predicted from the mode of action of this acceptor. In contrast, rubisco activation was stimulated by MV.

Table 1

Effect of irradiance and MV on the activation levels of rubisco, FBPase and Ru5P kinase in *Arabidopsis* leaves

Irradiance level	Activation level (μmol/mg Chl per h)		
	Rubisco	FBPase	Ru5P kinase
Dark	42	4	287
350 μmol/m^2 per s	95	103	675
30 μmol/m^2 per s	18	23	417
30 μmol/m^2 per s + MV	63	10	375

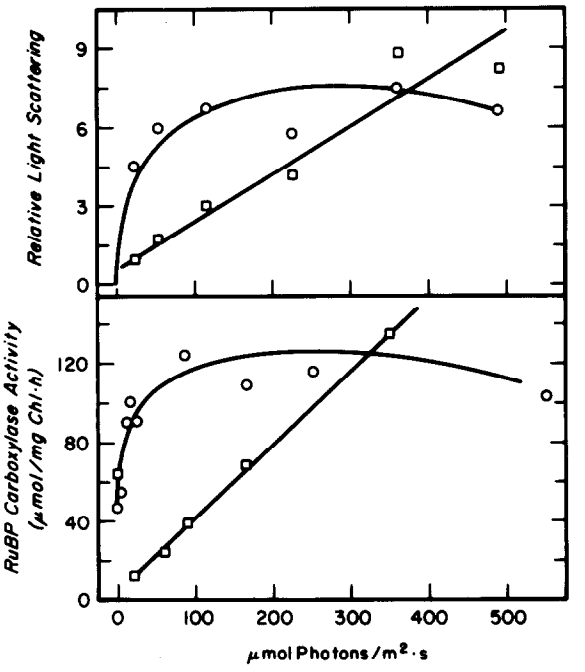

Fig.3. Effect of MV on the response of light scattering (upper) and rubisco activation (lower) to irradiance in *Arabidopsis* leaves. Control plants (□) and plants pretreated with MV (○) were flushed with 2% O_2 containing either 350 (activation) or 580 μl CO_2/l (light scattering).

The light response of rubisco activation in *Arabidopsis* closely matched the response of light scattering (fig.3). In an earlier study [3], the light response of rubisco activation in *Arabidopsis* was also shown to be closely correlated with the light response of photosynthesis. MV treatment lowered the light requirement for both rubisco activation and light scattering (fig.3). For MV-treated plants, 100 μmol photons/m^2 per s saturated rubisco activation and light scattering, whereas light levels greater than 400 μmol photons/m^2 per s were required to saturate untreated plants.

Nigericin, a K^+ ionophore, de-energizes the thylakoid membrane by collapsing the pH gradient. When added to spinach protoplasts at low concentrations, nigericin decreased the activation level of rubisco (fig.4). The concentration dependence of this decrease was similar to that observed for inhibition of photosynthesis and rubisco activation in tobacco protoplasts [10].

The *rca* mutant of *Arabidopsis* is incapable of

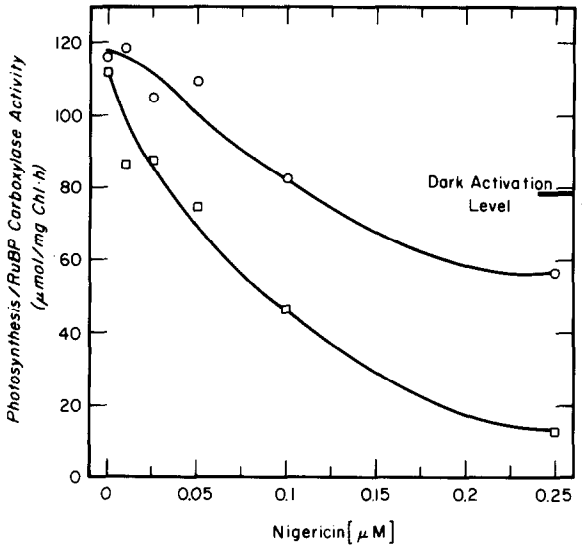

Fig.4. Effect of nigericin concentration on photosynthesis ($^{14}CO_2$ incorporation) (□) and rubisco activation (○) in spinach protoplasts.

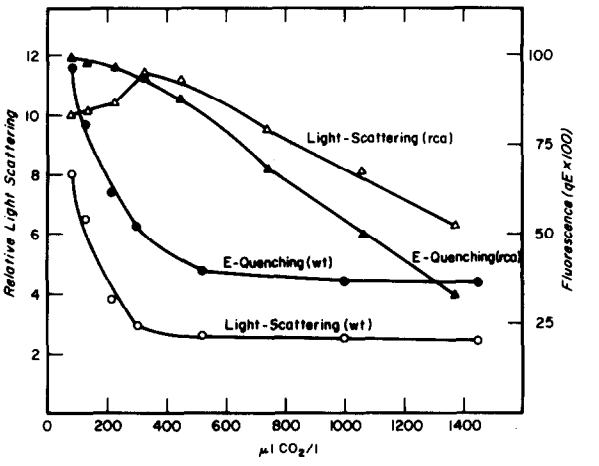

Fig.5. Effect of CO_2 concentration on light-scattering (△,○) and energy quenching fluorescence (▲,●) in wild-type *Arabidopsis* (○,●) and the *rca* mutant (△,▲) at 2% O_2.

activating rubisco in vivo [15] because it lacks rubisco activase [8]. At ambient CO_2, light scattering and *E* quenching in the mutant were considerably greater than in the wild type (fig.5). In response to external CO_2 concentration, light scattering and *E* quenching in the mutant increased linearly as the CO_2 concentration was decreased from 1450 to 350 μl CO_2/l. Maximum values for light scattering and *E* quenching in the mutant were attained at 350 CO_2 μl/l and were relatively unaffected by further decreases in the external CO_2 concentration. Light scattering and *E* quenching in wild-type *Arabidopsis* also increased as the external CO_2 was decreased. However, in contrast to the mutant, light scattering and *E* quenching were very responsive to changes in CO_2 concentration within the range 50–500 μl/l, but showed little change above 500 μl/l (fig.5). Results similar to those observed for wild-type *Arabidopsis* have been reported for spinach [13].

4. DISCUSSION

When added to leaves at limiting irradiance, MV causes inhibition of CO_2 fixation. The consequent inhibition of ATP consumption promotes thylakoid energization. The similarity in the light response curves of rubisco activation and light scattering suggests that MV stimulated rubisco activation at low irradiance either by increased thylakoid energization or increased ATP/ADP ratios or both. Nigericin, an uncoupler of photophosphorylation which dissipates the transthylakoid proton gradient, decreased rubisco activation (fig.4). Since the latter is also uncoupler-sensitive in the reconstituted system, the inhibitory effect of nigericin on rubisco activation must be due either to a collapse of the pH gradient or to inhibition of ATP synthesis or both. It cannot be due to a decrease in stromal alkalization or Mg^{2+} concentration [3].

Recently, it was found that rubisco was activated in a reconstituted system even in the absence of thylakoids and light, if ATP was enzymatically regenerated in order to maintain very high ATP/ADP ratios [20]. In vivo, ATP/ADP ratios rarely exceed values of 3 or 4 even when light intensities are high [21]. At these ATP/ADP ratios, light and thylakoids are required for rubisco activation in the reconstituted system. There are several possibilities to explain the discrepancy between activation in the presence of light and thylakoids and in their absence, but with high ATP/ADP ratios present.

(i) The properties of rubisco activase are modified in the light by energized thylakoids so that activation occurs in the presence of low and physiologically relevant ATP/ADP ratios [20].

(ii) When a considerable part of stromal P_i is converted into phosphate esters including RuBP in vivo, phosphorylation potentials [ATP]/[ADP] [P_i] may be high at low P_i even when ATP/ADP ratios are low. Under these conditions, the equilibrium of reactions of the type

$$ATP + R \rightleftarrows ADP + RP$$

is shifted to the left, but reactions of the type

$$ATP + R' \rightleftarrows ADP + P_i + R''$$

can still be driven to the right. Thus, high phosphorylation potentials, but not necessarily high ATP/ADP ratios, may be required for rubisco activation.

(iii) Ratios of ATP/ADP measured after acidic extraction of adenylates from chloroplasts or leaves may not reflect thermodynamically active ratios, which may be obscured by binding of adenylates to chloroplast constituents.

At the present state of the work, it is unlikely that explanation (iii) can resolve the discrepancy in observations with and without thylakoids. It has been shown that acid extraction of adenylates from chloroplast yields, at very different ATP/ADP ratios, values of the mass action ratio [ATP]-[AMP]/[ADP]^2 which are close to those expected from adenylate kinase equilibrium [22]. This observation argues strongly against the binding hypothesis.

Increasing the O_2 concentration from 2 to 21% at low irradiance increases rubisco activation, but reduces light scattering [23]. This may argue against explanation (ii). Increased O_2 levels provide an alternative electron acceptor and increase photorespiratory RuBP consumption. Photorespiration is an energy sink and decreases thylakoid energization. Decreased thylakoid energization corresponds to a decreased phosphorylation potential, but not necessarily to a decreased ATP/ADP ratio, as P_i should be expected to increase when RuBP decreases. These interactions are further complicated by binding of RuBP to inactive enzyme [24]. Previously we suggested that rubisco activation is determined primarily by the rate at which activase converts the inactive enzyme-RuBP complex to active enzyme [3]. One possible explanation for the observed increase in rubisco activation is that the decrease in the level of RuBP, in response to an increased O_2 level, offsets the lower transthylakoid proton gradient or the lower phosphorylation potential to cause a higher level of enzyme activation.

The *rca* mutant of *Arabidopsis* provided a unique opportunity to evaluate thylakoid energetics in the absence of activase-promoted rubisco activation. In the mutant, the balance between photon flux rate through the electron-transport chain and the rate of CO_2 fixation is disrupted causing overenergization of the thylakoids at ambient CO_2. By increasing the rate of CO_2 fixation [15], higher concentations of CO_2 dissipate the excess energy thus compensating for the limitation imposed by the inability of the system to activate rubisco. These findings suggest a central role for rubisco activase, not only in activating rubisco at physiological substrate levels [9], but also in coordinating the rates of CO_2 fixation via changes in the activation state of rubisco with the rate of electron-transport activity.

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