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Rubisco Activase Activity in Spinach Leaf Extracts

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Rubisco activase is a chloroplast stromal protein that catalyzes the activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) in vivo. Activation must occur before rubisco can catalyze the photosynthetic assimilation of CO_2. In leaves, photosynthesis and rubisco activation increase with increasing light intensity. Techniques are described that allow the activity of rubisco activase to be measured in extracts of spinach (*Spinacea oleracea* L.) leaf tissue. In this context, rubisco activase activity is defined as the ability to promote activation of the inactive ribulose-1,5-bisphosphate-bound rubisco in an ATP-dependent reaction. Determination of rubisco activase activity in extracts of dark and light treated leaf tissue revealed that the activation state of rubisco activase was independent of light intensity.

Key words: Light-activation — Photosynthesis — Ribulose bisphosphate carboxylase — Rubisco activase — Rubisco activation — *Spinacea oleracea.*

The assimilation of CO_2 during photosynthesis is catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco). Rubisco activity may be affected by several regulatory factors (Portis 1992), but in order to catalyze CO_2 assimilation, rubisco must be in an activated state. Light plays a primary role in the activation of rubisco in the plant, and is therefore also of major importance in regulating the rate of photosynthesis (Ogren 1991). Light-activation of rubisco in the plant is mediated by the protein rubisco activase (Portis 1990), but several details of the light-activation process are not yet understood (Campbell and Ogren 1990a).

The nature of the coupling of rubisco activity to light intensity and rubisco activase activity remains obscure, although the interaction of rubisco activase with thylakoid membranes has been shown to be a necessary component of the photosynthetic light activation system (Campbell and Ogren 1992b). The experiments described here were conducted to assay the activity of rubisco activase in spinach leaves as an alternative to measuring the activity of the purified protein. Also, assay of rubisco activase in extracts of leaf tissue, rapidly collected and frozen in liquid N_2, permits the investigation of the effect of the light intensity at the time of leaf collection on rubisco activase. The sugar phosphate 2-carboxyarabinitol 1-phosphate (CA1P) has been shown to be a confounding factor in the light regulation of rubisco in several species, but not in spinach (Seemann et al. 1990, Servaites et al. 1986), and so was considered to be not important here. Regulatory factors within the chloroplast known to be influenced by light, specifically pH and the concentrations of Mg^{2+} and ATP, were held at constant levels in these experiments so that direct effects of light on rubisco activase activity could be examined.

Results from similar experiments were reported recently by Lan et al. (1992), in which they concluded rubisco activase was light activated. Our data, reported here, do not support their conclusions. A preliminary report of these data were published previously as an abstract (Campbell and Ogren 1992a).

Materials and Methods

Plant growth and leaf tissue sampling—Spinach (*Spinacea oleracea* L. cv American Hybrid 424) was grown hydroponically in growth chambers maintained at 20°C, under mixed incandescent and fluorescent lamps. Light intensity was approximately 250 μmol photons m^{-2} s^{-1} (400–700 nm), except for plants used in photosynthetic electron

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; ER, rubisco-RuBP complex; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate.

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transport measurements (see below), and the photoperiod was 12 h. For most experiments, leaf tissue was removed from plants in the light and rapidly placed into liquid N_2. The tissue was ground to a powder with a pre-cooled mortar and pestle and stored in liquid N_2 until assayed. Leaf extracts were prepared by homogenizing the frozen leaf powder (0.4–0.8 g) in a Ten Broeck tissue grinder with 1 ml grinding buffer (50 mM Tricine-NaOH, pH 8.0, 5 mM dithiothreitol, and 2 mM EDTA). The homogenized tissue was then centrifuged at $2,000 \times g$ at 4°C for 0.5 min. The pellets were discarded and supernatants (subsequently referred to as extracts) assayed as described below. For experiments in which different light intensity treatments were used (Figs. 1 and 4), spinach was removed from the growth chamber, brought to the laboratory, and placed in darkness for 15 h. The light intensity was increased in the order 25, 200, and 1,000 μmol photons m^{-2} s^{-1}, and each light intensity was maintained for 30 min. After treatment, leaf tissue was collected and stored as described above. In these experiments, light was provided by a single 150-watt cool beam floodlight. A water tray with an infrared filter was placed between the light and the leaves to reduce the heat incident to leaves. Light intensity was changed by varying the distance between the light source and the leaves or by inserting layers of cheesecloth between the light and plant.

Assay of rubisco activity—The in vivo rubisco activity (often referred to as initial activity) of leaves exposed to varying light intensities was estimated by assay of leaf extracts. Aliquots equivalent to approximately 0.1 mg soluble leaf protein were added to assay vials containing 100 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ ($3.7\ 10^{10}$ Bq mol^{-1}), and 0.5 mM RuBP. Assays were conducted at 25°C, and were terminated after 0.5 min by addition of 4 M formic acid in 1 M HCl. Samples were dried at 70°C, and acid-stable ^{14}C-products were determined by liquid scintillation spectroscopy. Leaf protein concentration was determined by a dye-binding method (Bradford 1976).

Assay of rubisco activase activity—Rubisco activase activity was assayed as the ability to activate the inactive rubisco-RuBP complex in an ATP-dependent reaction. Activation reactions were carried out as follows. Aliquots of leaf extracts equivalent to approximately 0.03 mg Chl were diluted into 40 mM Tricine-NaOH, pH 8.0, to deactivate the endogenous rubisco. After incubation for 5 min in darkness at 25°C, RuBP was added to a concentration of approximately 8 mM, and a second incubation of 3 min in darkness was used to allow formation of the inactive rubisco-RuBP complex. In past experiments with lysed and reconstituted chloroplasts (Campbell and Ogren 1990a) a 10 min preincubation was used to convert almost all the rubisco to ER. However, we found that the ATP response of rubisco activase activity in leaf extracts was reduced by preincubation for longer than 3 min and so the shorter time was used. In some experiments purified spinach rubisco bound with RuBP was included in the reaction assay buffer following the second incubation. The activation reactions were initiated at time zero min with the addition of a pre-mixed aliquot comprised of Tricine-NaOH, $MgCl_2$, $NaHCO_3$, and with or without ATP and a phosphocreatine-creatine phosphokinase ATP regenerating system (Campbell and Ogren 1992b). The final concentration of the activation reaction mixture was 100 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 10 mM or 0.75 mM $NaHCO_3$, 4 mM RuBP, and, if present, 1 mM ATP, 3 mM phosphocreatine, and 20 units ml^{-1} creatine phosphokinase. Leaf tissue extracts were used at final concentrations of 0.062 to 0.200 mg Chl ml^{-1}, as indicated in the figure legends. The final concentration of exogenous ER, when used, was 0.50 or 0.75 mg rubisco ml^{-1}. Since specificity in the interaction of rubisco with rubisco activase has been demonstrated for several species (Wang et al. 1992), leaf extracts and purified rubisco were both prepared from spinach leaves. When activation reactions were in the light (1,000 μmol photons m^{-2} s^{-1}), illumination was begun at time zero. At the indicated times, aliquots were removed from the activation reaction vials and assayed for rubisco activity as described above. Chlorophyll was estimated by the method of Arnon (1949).

To compare rubisco activase activity of leaves from dark and high light (1,000 μmol photons m^{-2} s^{-1}) treated spinach (Fig. 4), leaf extracts from each treatment were assayed with and without exogenously added ER (0.50 mg rubisco ml^{-1}), in the presence of ATP and the ATP-regenerating system. In these experiments the deactivation step was omitted, and there were no incubation periods prior to initiation of the activation reaction. Leaf extracts were used at a final concentration of 0.062 mg Chl ml^{-1}. With these exceptions, activation assays were as described above.

Purified rubisco, where used, was prepared from spinach leaves as described by Salvucci et al. (1986b), and deactivated by gel filtration through Sephadex G-25 equilibrated with 50 mM Tricine-NaOH, pH 8.0. RuBP, synthesized as described by Jordan and Ogren (1981), was added to a concentration of 0.2 mM to form ER. Purified rubisco concentration was estimated by $A_{280} \times 0.61 =$ mg rubisco ml^{-1} (Wishnick and Lane 1971).

Assay of photosynthetic electron transport—For measurements of photosynthetic electron transport, leaf tissue was collected from spinach grown at approximately 450 μmol photons m^{-2} s^{-1}. Washed thylakoid membranes were prepared from intact chloroplasts as previously described (Campbell and Ogren 1992b). Leaf extracts were prepared from both fresh leaf tissue and liquid N_2-frozen leaf tissue as described above. Photosynthetic electron transport was determined in an O_2 electrode, at 25°C and 1,000 μmol photons m^{-2} s^{-1}. Whole-chain electron transport ($H_2O \rightarrow$ methyl viologen) was assayed in 40 mM

HEPES-NaOH, pH 7.5, 100 mM sorbitol, 30 mM KCl, 1% ethanol, 0.1 mM methyl viologen, and 0.5 μM nigericin. Photosystem I electron transport (ascorbate/DCPIP → methyl viologen) was assayed as above but also included 20 μM DCMU, 0.2 mM DCPIP, and 1.5 mM ascorbate. Washed thylakoid membranes or leaf extracts were added to a concentration of 0.02 mg Chl ml^{-1}. Electron transport rates were measured between 0.25 and 1.75 min.

Results

Exposure to increasing irradiance, from zero up to 1,000 μmol photons m^{-2} s^{-1}, increased rubisco activity in spinach leaves (Fig. 1). This commonly observed response is the light-dependent increased activation state of rubisco. The role of rubisco activase in the activation of rubisco was studied in the experiments presented here using the endogenous enzymes within leaf extracts, rather than purified enzymes or isolated chloroplasts as done previously.

Figure 2 demonstrates the activation of inactive rubisco in leaf extracts by the rubisco activase system. Activation was ATP-dependent, consistent with the requirement of ATP for activity with purified rubisco activase (Streusand and Portis 1987). Endogenous rubisco activase increased the activation of endogenous rubisco (Fig. 2A) and purified rubisco added to leaf extracts as ER (Fig. 2B). The greater enzyme activity seen in Figure 2B resulted from the addition of the purified rubisco. Reactions in Figure 2 were conducted at high bicarbonate concentration which allowed some spontaneous activation of rubisco to occur even in the absence of ATP. Reactions corresponding to those presented in Figure 2 were also conducted at a low bicarbonate concentration of 0.75 mM (data not shown).

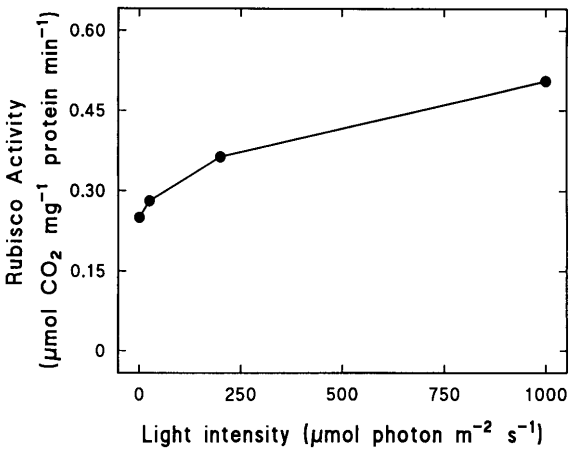

Fig. 1 Rubisco activity in spinach leaves sampled at different light intensities. Chlorophyll concentration in the assays was 0.1–0.2 mg ml^{-1}. The increase in rubisco activity with increasing light intensity is the result of the light-activation of rubisco in intact leaves.

Purified rubisco was activated by endogenous rubisco activase in leaf extracts, but at 0.75 mM bicarbonate, activation of both endogenous and added rubisco was strictly ATP-dependent.

In contrast to past observations with chloroplast systems, exposure of leaf extracts to light during the activation reactions failed to stimulate the rate or degree of rubisco activation (Fig. 3). Measurements of photosynthetic electron transport suggested damage to thylakoid membranes in both fresh and liquid N_2-frozen whole-leaf extracts, compared to thylakoid membranes isolated from intact chloroplasts (Table 1). Whole-chain electron transport was reduced 44% in fresh leaf extracts and 81% in extracts from liquid-N_2 frozen leaves. Loss of PSI activity was only observed following freezing in liquid N_2. Thus while the assay system described here can be used to assay rubisco activase activity in crude leaf extracts, it can not be used to demonstrate the light-dependent stimulation of rubisco observed in intact, lysed, and reconstituted chloro-

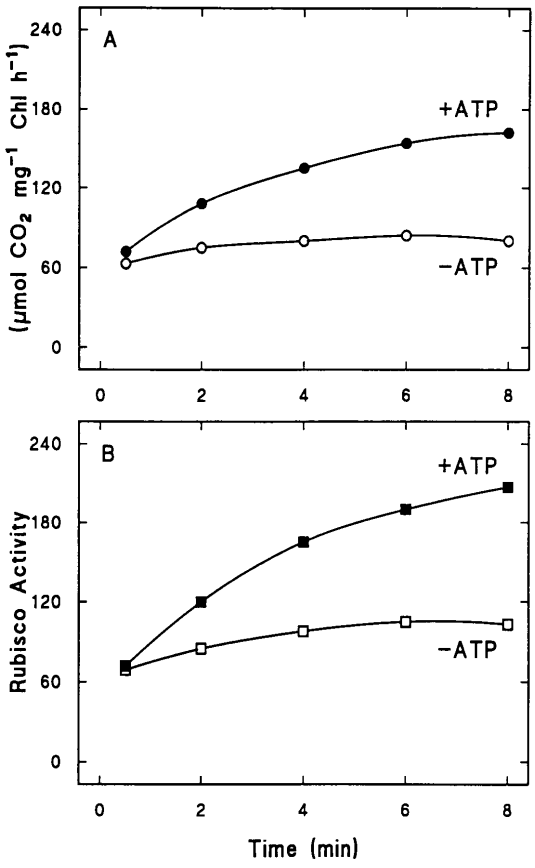

Fig. 2 Activation of rubisco by rubisco activase in leaf extracts at high bicarbonate concentration (10 mM). Reactions were conducted ± ATP as indicated. The source of rubisco was endogenous (A), or endogenous plus exogenous added at 0.5 mg ER ml^{-1} (B). The concentration of chlorophyll in the reactions was 0.1 mg ml^{-1}. Leaf extracts were preincubated with RuBP for 3 min prior to beginning the reaction.

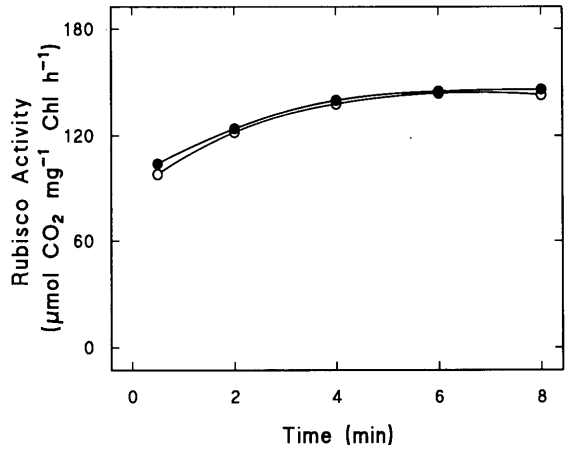

Fig. 3 Activation of rubisco by rubisco activase in leaf extracts in reactions conducted in light (○) or dark (●). Light intensity was 1,000 μmol photons m^{-2} s^{-1}, bicarbonate concentration was 0.75 mM, and the source of rubisco was endogenous plus exogenous added at 0.5 mg ER ml^{-1}. The concentration of chlorophyll in the reactions was 0.1 mg ml^{-1}. All rubisco was inactivated by addition of RuBP prior to beginning the reaction.

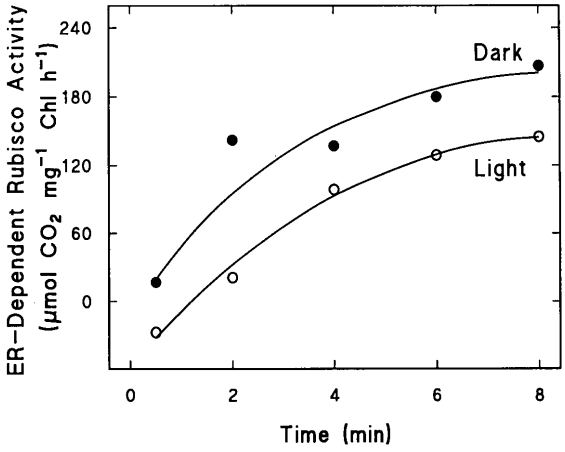

Fig. 4 The time course of the increase in ER-dependent rubisco activity, mediated by rubisco activase, in extracts from dark (●) and light (○) treated leaves. Exogenous rubisco was added to extracts at 0.5 mg ER ml^{-1}, chlorophyll concentration in the reactions was 0.062 mg ml^{-1}, bicarbonate concentration was 10 mM, and light intensity was 1,000 μmol photons m^{-2} s^{-1}. Leaf extracts were not preincubated with RuBP prior to beginning the reactions.

plasts (Campbell and Ogren 1992b).

Extracts prepared from dark treated leaves (15 h in complete darkness), and from leaves subjected to high light intensity (30 min at 25 μmol photons m^{-2} s^{-1}, followed by 30 min at 200 μmol photons m^{-2} s^{-1} and then 30 min at 1,000 μmol photons m^{-2} s^{-1}) were assayed for the ability to activate endogenous rubisco, and also endogenous plus exogenously added purified rubisco (added as inactive ER). The differences between activity in the assay with endogenous rubisco plus exogenous ER, and activity in the assay with endogenous rubisco only, termed ER-dependent rubisco activity, are presented in Figure 4 for both dark and light-treated leaves. The presence of ER-dependent activity indicates that the rubisco activase in leaf extracts activated exogenous inactive ER, and demonstrates the activity of rubisco activase in leaf extracts. Low endogenous rubisco activity in extracts of dark-treated leaves results in greater ER-dependent rubisco activity, compared to extracts from light-treated leaves (Fig. 4). These data are consistent with lower rubisco activity in leaves maintained in darkness and higher rubisco activity in leaves maintained in the light, due to the rubisco activase-mediated light activation of rubisco (Fig. 1, Salvucci et al. 1986a).

Table 1 Rates of photosynthetic electron transport in chloroplast and leaf systems

	Maximal rates of photosynthetic electron transport (μmol O_2 evolved (mg Chl)$^{-1}$ h^{-1})	
	Whole-chain ($H_2O \rightarrow MV$)	PSI (Ascorbate/ DCPIP $\rightarrow$ MV)
Washed thylakoids	432	590
Fresh leaf extract	239	595
Frozen leaf extract	79	282

Rates were measured in washed thylakoid membranes prepared from intact chloroplasts, and in fresh and liquid N_2-frozen leaf extracts. Data were collected between 0.25 and 1.75 min of illumination.

The time course of the increase in ER-dependent rubisco activity (Fig. 4) was the same for dark and light-treated leaves. The rate of activation of ER by rubisco activase in the leaf extracts, indicated by the shape of the activity curves in Figure 4, was the same in both the dark and light extracts. The absence of any difference in the rate of activation of added ER by endogenous rubisco activase from extracts of dark or high light-treated leaves indicates that the activation state of rubisco activase did not change with exposure of leaves to light.

Discussion

The light-dependent activation of rubisco, previously reported by others (Mächler and Nösberger 1980, Perchorowicz et al. 1981, Salvucci et al. 1986a, von Cammerer and Edmonson 1986), is demonstrated in the present experiments using spinach leaves exposed to different light intensities. Rubisco activity increased as light intensity was increased (Fig. 1) in the rubisco activase-mediated process.

Light establishes H^+ and Mg^{2+} concentrations favorable for activation of rubisco within the chloroplast stroma (Heldt 1979), and generates ATP which is required for the activation of rubisco by rubisco activase (Streusand and Portis 1987). There is at least one additional light-requiring reaction involved in regulation of rubisco activation state (Campbell and Ogren 1990a), but details of that mechanism have not been elucidated.

Rubisco activase present in leaf extracts increased the activation state of endogenous rubisco, which had been deactivated by addition of RuBP (Fig. 2). These activation reactions were conducted in the presence of ATP and an ATP-regenerating system, maintaining a constant ATP concentration. When purified rubisco was added to the leaf extract reaction, rubisco activase was able to activate the exogenously added rubisco (added as the rubisco-RuBP form), as well as the endogenous rubisco in the leaf extract, which was deactivated by RuBP (Fig. 2B). This result allows the activity of rubisco activase in leaf extracts to be assayed by following the activation of inactive purified rubisco added to the reaction.

Illumination of leaf extracts during the rubisco activation reactions had no effect on the rate or degree of activation compared to reactions conducted in darkness (Fig. 3). Previous work with lysed chloroplasts (Campbell and Ogren 1990a), and reconstituted systems containing thylakoid membranes from lysed chloroplasts (Campbell and Ogren 1992b), demonstrated an increase in rubisco activity when activation reactions were conducted in light. In the experiments reported here, illumination of the rubisco activation reaction did not stimulate rubisco activity. However, damage to the thylakoid membranes during preparation of whole-leaf extracts was indicated by measurement of photosynthetic electron transport rates (Table 1). Direct freezing of leaf tissue in liquid N_2 without cryoprotectant caused damage to PSI. Previously, electron transport through PSI was shown to be required for the light-stimulation of rubisco activation (Campbell and Ogren 1992b). Thus the inability to observe light-stimulation of the rubisco activation reaction in the present experiments appears to be partly or wholly the result of damage to thylakoid membranes during sample preparation.

Details are lacking regarding certain aspects of the in vivo light activation system used to activate rubisco in plants. Experiments described here were conducted to address the question of whether light can affect the activation state of rubisco activase in leaves. Extracts were prepared from leaves kept in darkness overnight, and from leaves exposed to high light intensity. Activation of the endogenous rubisco in the extracts was measured, and the assays were then repeated with the addition of purified rubisco (added as ER). Since rubisco is light-activated, its activity is lower in leaf tissue sampled in darkness compared to tissue sampled in the light (Fig. 1). Exogenously added ER can be activated by endogenous rubisco activase in extracts prepared from both dark and light treated leaves. The difference in the absolute activities between assays with light and dark leaf extracts are a result of the differences of the endogenous rubisco activity in the dark and light leaf tissue extracts and the method of calculating the ER-dependent rubisco activity. These observations show that rubisco activase from leaves exposed to darkness and to high light were equally effective in increasing the activation state of inactivated rubisco. Based on these results, we conclude that the activation state of rubisco activase does not change with light intensity.

Recently, Lan et al. (1992) reported experimental results which they interpreted as supporting the hypothesis (Woodrow and Mott 1992) that activation of rubisco activase is light dependent. Our results do not support that hypothesis. Lan et al. employed different experimental techniques than were used in experiments presented here, and also used a much lower concentration of leaf extract in their activation reactions. On a chlorophyll basis, the leaf tissue extract concentration in experiments reported here was approximately 25-fold greater than the concentrations used by Lan et al. (1992). Salvucci (1992) found that rubisco activase activity was affected by the concentration of inert polymers that apparently influenced the molecular mass of rubisco activase by promoting its self-association in the presence of the polymers, such as might occur in the high protein concentration environment of the chloroplast stroma. Wang et al. (1993), also reported that the degree of aggregation of rubisco activase affected its activity. Although at the present time it is not obvious why our data and those of Lan et al. (1992) are contradictory, extensive dilution of rubisco activase in their experiments may have affected activity by reduced association of the rubisco activase protein.

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