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Light-Dependent Changes in Ribulose Bisphosphate Carboxylase Activase Activity in Leaves[1]

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ABSTRACT

An assay for the activity of ribulose bisphosphate carboxylase (Rubisco) activase in crude leaf extracts was developed. The assay is based on a spectrophotometric assay of Rubisco, and activase activity (in nanomoles activated Rubisco per minute per milligram chlorophyll) was calculated from the rate of increase in Rubisco activity over time. Activase activity measurements were made using samples from spinach (*Spinacia oleracea*) leaves undergoing (a) steady-state photosynthesis at various photon flux density (PFD) values and (b) nonsteady-state photosynthesis following an increase from darkness to a high PFD. Analysis of these samples showed that steady-state Rubisco activase activity was relatively low in darkness, increased with PFD, and saturated below 300 micromoles per square meter per second. Rubisco activity (measured spectrophotometrically) was also found to be low in darkness and to increase with PFD, but it saturated at much higher PFD values (approximately 1000 micromoles per square meter per second) along with the rate of photosynthesis. Following an increase in PFD from darkness to 650 micromoles per square meter per second, activase activity increased more or less linearly over a period of 5 to 6 minutes, after which it was constant. Rubisco activity, however, increased more slowly. The light-dependence of Rubisco activase is consistent with previous gas-exchange data showing two interdependent processes in the activation of Rubisco following an increase in PFD.

Leaves in both natural and agricultural environments are often exposed to large changes in PFD[2] because of mutual shading and the movement of clouds. Many leaves, therefore, receive a large fraction of their total incident PFD as "sunflecks," *i.e.* periods of high PFD surrounded by periods of lower PFD (9). Under many conditions, photosynthesis may respond relatively slowly to a sudden increase in PFD (3, 16), and this can decrease the amount of carbon gained by the leaf under fluctuating light conditions relative to the carbon gain calculated by assuming photosynthesis responds instantaneously to a change in PFD (9). There is now good evidence that a large portion of this slow increase in photosynthesis is caused by the rate at which Rubisco activity increases following an increase in PFD (15, 16). Furthermore, the distribution of sunflecks in both forest and agricultural systems is often such that Rubisco activity is relatively low (10, 11), so the effect of slow Rubisco activation on the total carbon gain during a sunfleck will be large (10).

In many plants, light-dependent increases in Rubisco activity are caused by the addition of activator CO_2 and Mg^{2+} to the enzyme (7). The conversion of the ER to the ECM, termed activation, appears to be largely catalyzed by the enzyme Rubisco activase *in vivo* (13). The possibility exists, therefore, that the catalytic activity of Rubisco activase could have a measurable effect on the rate of Rubisco activation and therefore on the amount of carbon gained by a leaf in a fluctuating-light environment.

This possibility is supported by gas-exchange data showing that light-dependent increases in Rubisco activity are slower following a transition from very low PFD to high PFD than following a transition from moderate PFD to high PFD (4). Woodrow and Mott (17) have used a series of gas-exchange experiments to demonstrate that this phenomenon can be explained by the existence of two sequential, light-dependent steps in the activation of Rubisco. Their data show that the first step saturates at a relatively low PFD (near 135 μmol m^{-2} s^{-1}) and that the second step saturates in parallel with the rate of photosynthesis. They suggested that the first of the two steps in the activation of Rubisco could be a light-dependent increase in the activity of the enzyme Rubisco activase. The second slow step is most likely the activase-catalyzed activation of Rubisco.

To test this hypothesis, we developed an assay for the activity of Rubisco activase in crude leaf extracts. We then used this assay to investigate the effect of PFD on activase activity in leaves under steady-state conditions and in the nonsteady state following an increase from darkness to a high PFD.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea* L.) plants were grown hydroponically in aerated, half-strength Hoagland solution using controlled-environment chambers. PFD was 350 μmol m^{-2} s^{-1} with a 10-h photoperiod, and day and night temperatures were 25 and 20°C, respectively.

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[2] Abbreviations: PFD, photon flux density; ER, Rubisco-RuBP complex; RuBP, ribulose-1,5-bisphosphate; ECM, Rubisco-CO_2-Mg^{2+} complex; PGA, 3-phosphoglyceric acid.

Rubisco and Chemicals

Rubisco was isolated from spinach leaves as described previously (5). The purified enzyme was stored as a precipitate in 50% saturating ammonium sulfate at −80°C. NADH was obtained from Boehringer Mannheim. All other chemicals were obtained from Sigma.

Gas Exchange and Freeze-Clamp

Spinach leaves used for experiments were placed in a clamp-on leaf chamber that had separate circular chambers above and below the leaf. Gas-exchange data were measured with a standard single-pass gas-exchange system that has been described previously (8). When the desired gas-exchange conditions existed in the chamber, the leaf was quick-frozen by firing a stainless steel cutting tube cooled with liquid nitrogen through the chamber. This quick-kill technique has been described elsewhere (2). The frozen leaf discs were stored at −80°C until used.

Preparation of Enzyme Extracts

Frozen leaf discs were ground in a mortar and pestle chilled by liquid nitrogen and then transferred to a glass homogenizer for further grinding. The glass homogenizer contained 1 mL of 100 mM Bicine (pH 8.0), 5 mM $MgCl_2$, 15 mM DTT, 1 mM EDTA, 1 mM benzamidine, 1 mM PMSF (dissolved in isopropyl alcohol), 10 μM leupeptin, and 0.4 mM ATP. An aliquot (200 μL) of the extract solution was added to 800 μL of acetone for spectrophotometric Chl determination. The remaining extract was immediately transferred to a microcentrifuge tube and spun at 13,000*g* for 30 s. The supernatant was assayed to determine the activity of Rubisco and Rubisco activase.

Rubisco and Rubisco Activase Assays

Each spectrophotometric assay of Rubisco and Rubisco activase was carried out in a cuvette containing 0.5 mL 50 mM Bicine (pH 8.0), 15 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM NaCl, 2 mM RuBP, 5 mM DTT, 5 mM ATP, 0.2 mM NADH, 5 mM phosphocreatine, 10 units mL^{-1} creatine phosphokinase, 10 units mL^{-1} glyceraldehyde-3-phosphate dehydrogenase, 10 units mL^{-1} 3-phosphoglycerate kinase, and leaf extract solution containing 1.2 μg Chl. The reaction was started as described by Lan and Mott (5) except that the Rubisco activase was from the leaf extracts instead of purified activase. Following the initiation of the reaction by adding Rubisco-RuBP complex (0.66 μM), absorbance (340 nm) was recorded digitally to a computer file every 2 s for 2 min. These values were converted to NADH concentration (molar) using an extinction coefficient of 6.22 mM^{-1}.

ER was made by adding RuBP to CO_2- and Mg^{+2}-free, inactivated, purified Rubisco (2). The concentration of Rubisco was determined from the activity of the fully activated preparation assuming a turnover number of 3.3 s^{-1}.

Calculation of Rubisco and Activase Activity

Rubisco and Rubisco activase activities were calculated by approximating the NADH *versus* time data with a second-order polynomial. The first derivative of the polynomial approximation, the rate of NADH consumption, was used as a measure of PGA production, and Rubisco activity was calculated assuming two PGA molecules produced per RuBP consumed. The second derivative of the polynomial approximation, the rate of change in NADH consumption, was used as a measure of Rubisco activase activity. A turnover number of 3.3 s^{-1} for Rubisco was used to calculate the moles of the ECM form of Rubisco produced per time from the increase in Rubisco activity per time (see Lan and Mott [5] for a complete discussion).

RESULTS

The spectrophotometric assay described here measures both activase-catalyzed and uncatalyzed Rubisco activation. These two processes can be resolved, however, by adding ER (in the same concentration to be used in the subsequent assay) to the reaction mixture in the absence of the leaf extract. Under these conditions, the rate of NADH oxidation was found to be relatively low and to increase very slowly over time (Fig. 1, a and b, ◇). The uncatalyzed rate of Rubisco activation was calculated from this rate of NADH oxidation, which was determined from a second-order polynomial approximation to the data (Fig. 1, a and b, lines through the diamonds). This statistical fitting of the data was necessary to calculate accurately the rate at which NADH oxidation increased over time.

In calculating activase activity, it was also necessary to account for the rate of ECM formation (both catalyzed and uncatalyzed) that was caused by the ER contained in the activase extract itself. To make this correction, a separate control was performed with only the leaf extract and the coupling enzymes present in the reaction mixture. The resulting rate of NADH oxidation under these conditions was lower than that achieved with exogenous ER and increased slowly with time (Fig. 1, a and b, △). The initial rate of NADH oxidation was used to calculate the activity of Rubisco in the extract, and the rate at which NADH oxidation increased was used to calculate the rate of ECM formation that was due to the ER contained in the leaf extract. As described above, second-order polynomials were used to approximate the data (Fig. 1, a and b, lines through the triangles) and to calculate the first and second derivatives of the data.

To determine the rate at which the exogenously added ER was converted to ECM by the Rubisco activase contained in the leaf extract, ER and the leaf extract were added to the reaction mixture containing the coupling enzymes. The resulting time course for NADH oxidation (Fig. 1, a and b, ○) was approximated with a polynomial (Fig. 1, a and b, line through circles) and the second derivative of this polynomial was used to calculate the total rate at which ER was converted to ECM in the reaction mixture. The rate of uncatalyzed ECM formation and rate of ECM formation due to the ER in the leaf extract (see above) were then subtracted from this total rate to yield the activase activity of the leaf extract. This means of correction is valid as long as the concentration of ER used in the assay is less than the K_m(ER) for Rubisco activase (2.7 μM (5). This condition is adhered to in all of the measurements described in this paper.

The range of activase concentrations over which the assay

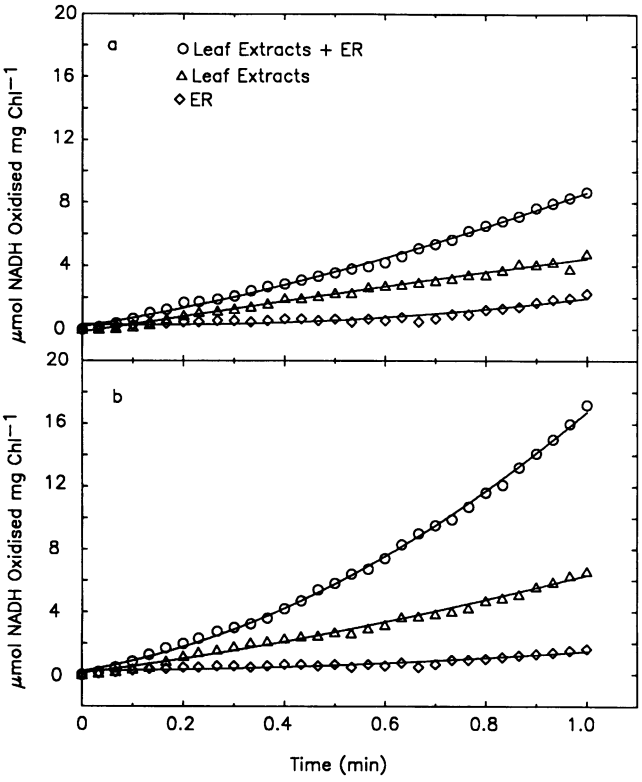

Figure 1. Time courses for NADH oxidation in reaction mixtures containing only the leaf extract (◇), only exogenous ER (△), or both the leaf extract and ER (○). The leaf was quick-frozen in darkness (a) or at 215 μmol m^{-2} s^{-1} and extracted as described in "Materials and Methods." RuBP (2 mM) was added to deactivated, purified Rubisco to form ER. The specific activity of Rubisco was 1.5 μmol RuBP min^{-1} mg^{-1} Rubisco. The reaction mixture contained saturating levels of Mg^{2+}, CO_2, and ATP, and an ATP-regenerating system. The reaction was initiated by adding exogenous ER (0.66 μM in the reaction mixture) in the presence (○) and absence (◇) of the leaf extract or by adding the leaf extract (1.2 μg Chl in the reaction mixture) in the absence of ER (△). The lines through the points show the second-order polynomial approximations of the data from which Rubisco activity and Rubisco activase activity were calculated (see "Materials and Methods").

system accurately represented enzyme activity was investigated by adding different amounts of leaf extract to the assay. Figure 2 shows that activase activity was linearly related to the amount of extract added to the assay up to an activase concentration equivalent to 2 μg Chl. The relationship most probably became nonlinear for larger amounts of extract because the relatively high optical density of the reaction mixtures made it difficult to accurately measure the rate of NADH oxidation.

As a test of the assay for activase activity, we determined the activase activity of leaf extracts taken from illuminated leaves as a function of the concentration of ER added to the reaction mixture (Fig. 3). Because the correction for the ER contained within the leaf extracts was accurate only at ER concentrations below the K_m value, it was not possible to use high concentrations of ER in the crude extract assay. An

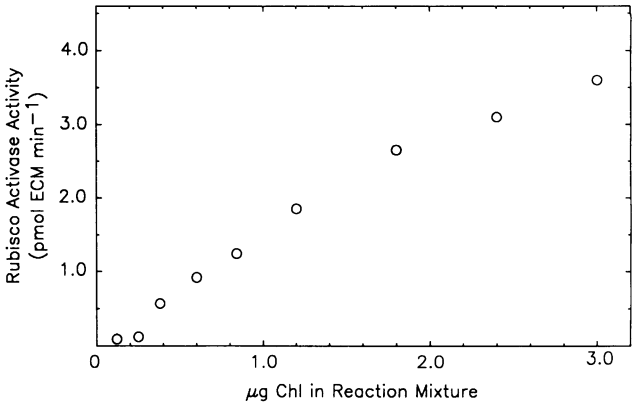

Figure 2. Activase activity in the leaf extracts as a function of the amount of leaf extract. The leaf was quick-frozen directly from an illuminated (approximately 350 μmol m^{-2} s^{-1}) growth chamber.

accurate determination of kinetic parameters, therefore, was not possible, but a least-squares fit of the data to the Michaelis-Menten equation yielded a K_m(ER) of 2.5 μM.

The effect of PFD on photosynthesis rate, Rubisco activity, and Rubisco activase activity in intact leaves was determined by placing leaves in the gas-exchange system and allowing them to reach steady-state at different PFD values. The rate of photosynthesis was recorded for each leaf before it was quick-frozen for analysis with the Rubisco activase assay. Figure 1, a and b show representative data for Rubisco and Rubisco activase assays of extracts from leaves held in darkness and 215 μmol m^{-2} s^{-1}, respectively. The assays show clearly that the activity of Rubisco activase, as determined by the curvature of the line through the circles (after subtracting the controls), was much higher in leaves held for at least 1 h at 215 μmol m^{-2} s^{-1} than in leaves held in darkness. In both cases, the rate of uncatalyzed ECM formation, as determined from the curvature of the line through the diamonds, was low. In darkness (Fig. 1a), the activity of Rubisco, as determined from the slope of the line through the triangles, was very low, indicating a correspondingly low amount of the

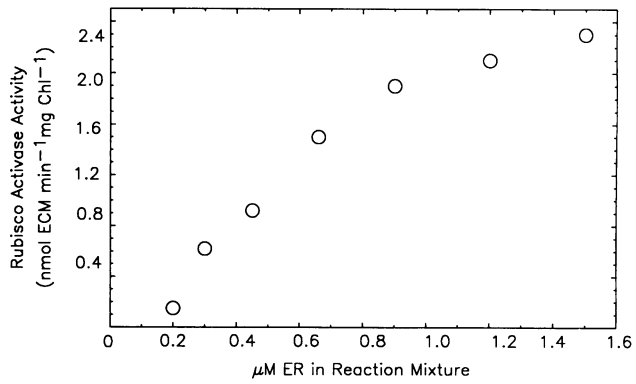

Figure 3. Activase activity in leaf extracts as a function of exogenously added ER concentration. The concentration of the leaf extracts was 1.2 μg Chl in the reaction mixture. The leaf sample was obtained as in Figure 2.

ECM form of Rubisco. At a PFD of 215 μmol m^{-2} s^{-1}, however, the activity of Rubisco (Fig. 1b, slope of the line through the triangles) was somewhat higher, indicating a greater proportion of Rubisco in the ECM form.

A series of experiments similar to those shown in Figure 1, a and b revealed that Rubisco activase activity was low in darkness, increased with PFD, and saturated below a PFD of 300 μmol m^{-2} s^{-1} (Fig. 4c). Rubisco activity was also low in darkness and increased with PFD, but saturated at a PFD of approximately 1000 μmol m^{-2} s^{-1} (Fig. 4b).

Similar experiments were conducted to determine the kinetics for the increases in Rubisco and Rubisco activase activity following a transition from darkness to a PFD of 650 μmol m^{-2} s^{-1} (Fig. 5, a and b). In these experiments, leaves were held in darkness for at least 45 min before the PFD was increased to 650 μmol m^{-2} s^{-1}. The leaf tissue was quick-frozen at different times after the increase in PFD, and extracts of the tissue were used to determine Rubisco and Rubisco activase activities. Rubisco activity was found to increase continuously over 10 min (Fig. 5a), and activase activity was found to increase in a roughly sigmoidal fashion for approximately 6 min, after which activity appeared to level off (Fig. 5b).

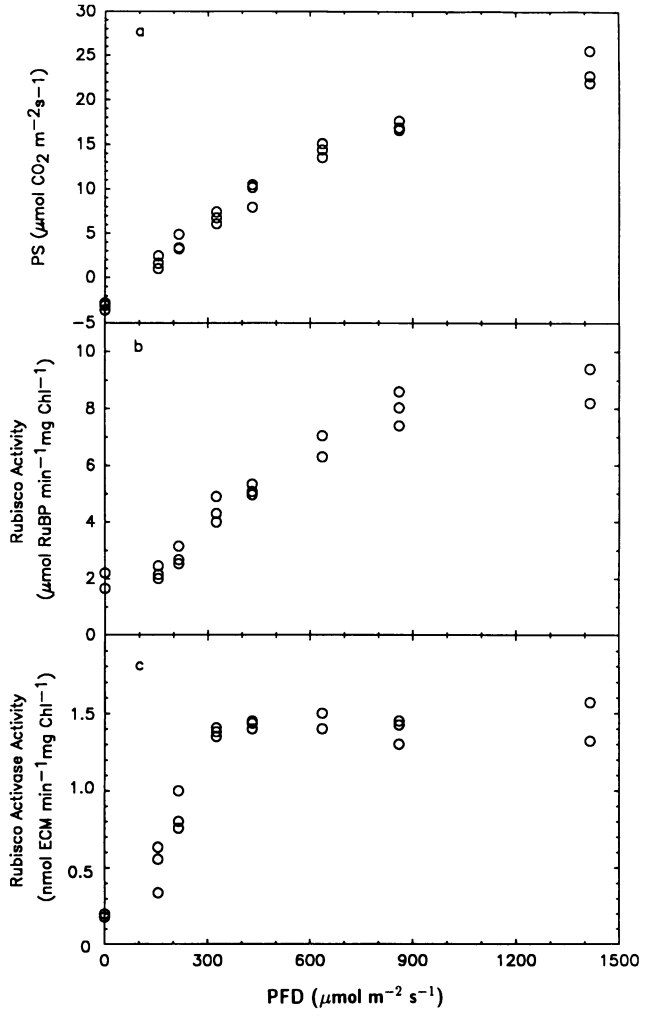

Figure 4. Effect of PFD on (a) photosynthesis rate, (b) Rubisco activity, and (c) Rubisco activase activity. Spinach leaves were exposed to various PFD values for at least 1 h and then quick-frozen. Each point represents data for a separate leaf that was quick-frozen and assayed for Rubisco and Rubisco activase activities.

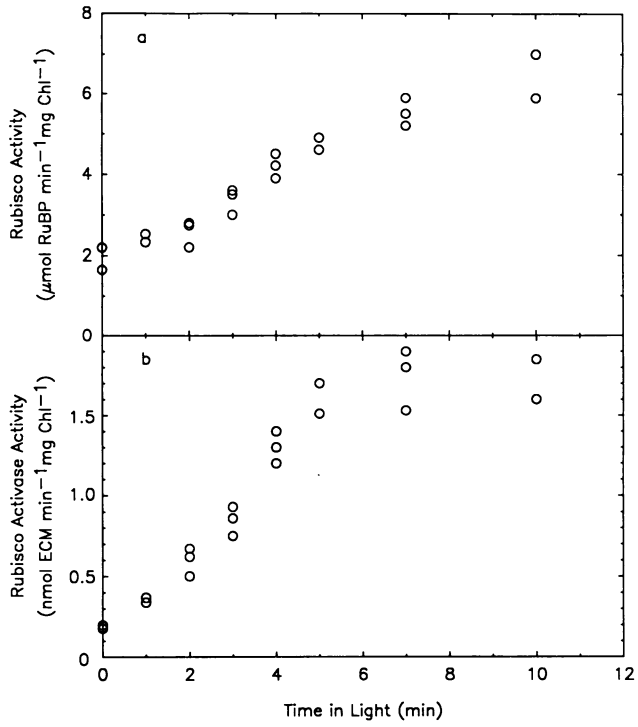

Figure 5. Time courses for the increase in (a) Rubisco activity and (b) activase activity following a transition from darkness to 690 μmol m^{-2} s^{-1}. Spinach leaves were placed in darkness for at least 1 h and then illuminated at 690 μmol m^{-2} s^{-1} for various times before being quick-frozen and assayed for Rubisco activity. Each point represents a separate leaf.

DISCUSSION

The assay of Rubisco activase activity used in this study is based on that described by Lan and Mott (5). It involves determining the rate at which the ER form of Rubisco is converted to the ECM form by measuring the appearance of the ECM form indirectly as the increase in catalytic activity of Rubisco over time. Rubisco activity is determined by coupling the production of PGA to NADH oxidation and determining the concentration of NADH spectrophotometrically as a function of time (see Lilley and Walker [6]). The rate of NADH oxidation per time is then determined from the slope of the curve relating NADH oxidized to time, and this rate is converted to Rubisco activity by assuming two NADH molecules oxidized per RuBP carboxylated. (The assay mixture contains a high concentration of CO_2 and a low concentration of O_2, so the oxygenation reaction will be negligible.)

When this assay is used to determine only Rubisco activity, the slope of the NADH *versus* time curve can usually be assessed graphically or by subtraction. Calculation of Rubisco

activase activity, however, involves determining the rate at which Rubisco activity increases over time; *i.e.* the rate of change in the slope of the NADH *versus* time curve. This parameter is very sensitive to minor fluctuations in the spectrophotometric determinations of NADH *versus* time and is difficult to quantify graphically or by subtraction. To solve this problem, polynomial approximations of the spectrophotometer readings over time were used to "smooth" the data and allow a more rigorous determination of the second derivative of the data. These polynomial approximations to the data had very low residual values (typically less than 0.3%) and are shown in Figure 1 as the lines through each set of points. Visual examination of these graphs confirms that the polynomial approximations accurately represent the raw data.

Assays of Rubisco activase using a crude leaf extract are more complex than those using purified enzymes for at least three reasons. First, the substrate ER is present in the activase extract in varying amounts and contributes an unknown fraction of the total rate of ECM formation. Second, other NADH-consuming reactions present in the extract may contribute to the total rate of NADH oxidation. And third, because the leaf extract is not optically clear and has some absorbance at 340 nm, there is an upper limit to the amount of extract, and therefore activase, that can be added to the reaction mixture without causing unacceptable noise in the absorbance reading.

The first and second problems mentioned above were corrected for by determining the rate of ECM production in the absence of added ER. This was accomplished by assaying the rate of NADH oxidation in the absence of added ER (Fig. 1, a and b, Δ) and determining the second derivative of the curve. This rate of ECM production was typically very low, and it was subtracted from the rate of ECM production in the presence of added ER. This correction is valid because activase activity will be approximately proportional to ER concentration at the low ER concentrations that were present in the assay.

The third problem of signal-to-noise ratio in the spectrophotometer was overcome by assuming that activase activity was constant over the first minute of the assay, and using a second-order polynomial to approximate the data. The assumption of constant activase activity over the first minute is supported by the data of Lan and Mott with the purified enzyme (5) and is quite reasonable considering the low activase concentration in the assay. Moreover, previous assays of Rubisco activase using radiometric techniques (14) have implicitly incorporated this assumption.

Despite these disadvantages, the data presented indicate that the assay provides a reliable estimate of the activase activity contained within a crude leaf extract. Several facts support this conclusion. First, activase activity was linearly dependent on the amount of crude extract that was added to the reaction mixture except for very large amounts of extract. The nonlinearities at high extract concentrations were probably because of interference in the spectrophotometer readings by pigments in the crude extract. Second, the dependence of activase activity on [ER] was consistent with the K_m(ER) value of 2.7 μM reported for the purified enzyme (5).

The dependence of photosynthesis rate and Rubisco activity on PFD found in this study compares well with previously published data (*e.g.* ref. 12). Both parameters were low in darkness, increased with PFD, and saturated at a similar and relatively high PFD value. Rubisco activase activity was also found to be low in darkness and to increase with PFD, but it saturated at much lower PFD values than did photosynthesis and Rubisco activity (Fig. 4). The raw data shown in Figure 1, a and b are particularly instructive: they clearly show a slight increase in Rubisco activity (as calculated from the slope of the line through the triangles) from darkness to 215 μmol m^{-2} s^{-1}, and a large increase in Rubisco activase activity (as calculated from the curvature of the line through the circles) over the same range.

The difference in the light-dependence of Rubisco and Rubisco activase activities is interesting with regard to the regulation of Rubisco activity. Because activase activity appears to saturate at a much lower PFD than Rubisco, the amount of active activase is not the means by which the proportion of active Rubisco is controlled. At higher PFD values, modulation of the level of other substrates, such as ATP, most probably regulate activase activity, and therefore the amount of active Rubisco. If, under most natural conditions, the PFD in full shade is sufficient to maintain activase in a fully active state, then the activation of Rubisco during a sunfleck would proceed relatively rapidly. This may be significant for the total carbon gain of plants growing in environments where the PFD changes frequently.

The mechanism for the light-dependent changes in activase activity found in this study is as yet unknown. Activase activity *in vitro* requires several substrates (13), but the concentrations of these substrates were held approximately constant in the assay mixture. Therefore, the differences in activity must be due to a slow modification of the enzyme in the chloroplast. Our laboratory has recently found that activase undergoes light-dependent protein phosphorylation in the presence of thylakoids, and this process may be linked to the changes in activity found in this study.

The light-dependent changes in Rubisco activase activity reported in this study are consistent with data of several other studies. Campbell and Ogren (1) have reported a light-dependent increase in Rubisco activity in lysed chloroplasts that were supplemented with saturating ATP concentrations. Although there are several plausible explanations for this effect, one possibility is a light-dependent increase in Rubisco activase activity.

Furthermore, Woodrow and Mott (17), using gas-exchange techniques, have shown that the increase in Rubisco activity following an increase in PFD consists of two interdependent steps, and they suggested that the first step could be a light-dependent increase in the activity of Rubisco activase. In that study, both steps in the Rubisco activation process were shown to be light dependent, with the first saturating at a PFD of approximately 135 μmol m^{-2} s^{-1} and occurring over a time frame of 6 to 8 min. Those values are consistent with the light-dependent increase in activase activity observed in this study. The two studies, taken together, strongly suggest that light activation of activase occurs and can have a marked effect on the rate at which photosynthesis responds to an increase in PFD and, therefore, on the total carbon gain under conditions of fluctuating light.

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