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Regulation of Ribulose-1,5-Bisphosphate Carboxylase Activity in *Alocasia macrorrhiza* in Response to Step Changes in Irradiance[1]

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JEFFREY R. SEEMANN*, MIKO U. F. KIRSCHBAUM, THOMAS D. SHARKEY, AND ROBERT W. PEARCY
Department of Biochemistry, University of Nevada, Reno, Nevada 89557 (J.R.S.), *Department of Botany, University of California, Davis, California 95616* (M.U.F.K., R.W.P.), *and Department of Botany, University of Wisconsin, Madison, Wisconsin 53706* (T.D.S.)

ABSTRACT

The regulation of ribulose-1,5-bisphosphate (RuBP) carboxylase (Rubisco) activity and pool sizes of RuBP and P-glycerate were examined in the tropical understory species *Alocasia macrorrhiza* following step changes in photon flux density (PFD). Previous gas exchange analysis of this species following a step increase in PFD from 10 to 500 micromoles quanta per square meter per second suggested that the increase in photosynthetic rate was limited by the rate of increase of Rubisco activity for the first 5 to 10 minutes. We demonstrate here that the increase in photosynthetic rate was correlated with an increase in both the activation state of Rubisco and the total k_{cat} (fully activated specific activity) of the enzyme. Evidence presented here suggests that a change in the pool size of the naturally occurring tight binding inhibitor of Rubisco activity, 2-carboxyarabinitol 1-phosphate, was responsible for the PFD-dependent change in the total k_{cat} of the enzyme. RuBP pool size transiently increased after the increase in PFD, indicating that photosynthesis was limited by the capacity for carboxylation. After 5 to 10 minutes, RuBP pool size was again similar to the pool size at low PFD, presumably because of the increased activity of Rubisco. Following a step decrease in PFD from 500 to 10 micromoles quanta per square meter per second, Rubisco activity declined but at a much slower rate than it had increased in response to a step increase in PFD. This slower rate of activity decline than increase was apparently due to the slower rate of 2-carboxyarabinitol 1-phosphate synthesis than degradation and, to a lesser degree, to slower deactivation than activation. RuBP pool size initially declined following the decrease in PFD, indicating that RuBP regeneration was limiting photosynthesis. As Rubisco activity decreased, RuBP slowly increased to its original level at high PFD. The slow rate of activity loss by Rubisco in this species suggests a biochemical basis for the increased efficiency for CO_2 assimilation of successive lightfleck use by species such as *A. macrorrhiza*.

Many understory plant species accomplish a considerable portion of their daily carbon gain during and immediately following transitory lightflecks which penetrate the overstory (3, 4, 14). Carbon gain during these lightflecks depends on the length of the lightfleck (6) and the induction state of the leaf (5, 9). Induction can occur during a daily series of lightflecks so that those which occur later are utilized more efficiently than those occurring early (6). The capacity of a leaf to assimilate carbon during lightflecks is in part determined by the rate at which this induction occurs. Further, the rate at which the induction state is lost following the end of the lightfleck will affect the capacity of the plant to respond to any subsequent lightflecks.

At the biochemical level, the capacity of a leaf for carbon acquisition is the result of the activity of the carboxylating enzyme, Rubisco.[2] The *in vivo* activity of this enzyme is a function of the total amount of enzyme, mechanisms which regulate its activity, and the concentration of its substrates, CO_2 and RuBP. Rubisco activity is regulated by light, a process which can involve both the tight binding inhibitor CA1P (1, 8, 22, 25) and the carbamylation (activation) of a specific lysine residue on the enzyme (12). This latter process is apparently regulated by another enzyme, Rubisco activase (17). Measurement of the light-dependent induction of CO_2 assimilation in intact leaves suggests that part of the response may be due to changes in the activity of Rubisco (9, 10, 16, 18).

In this study, we examined the regulation of Rubisco activity in leaves subjected to step changes in PFD and compared the responses of the enzyme to those observed for whole leaf gas exchange that were reported by Kirschbaum and Pearcy (9). We also examined the consequences of this regulation on pool sizes of some metabolites of photosynthesis. We used *Alocasia macrorrhiza*, an Australian tropical understory species that has been extensively studied as an example of a lightfleck-utilizing species (2, 5, 6, 27). We demonstrate the importance of the regulation of Rubisco activity during lightflecks and that changes in both CA1P pool size and activation state may be involved simultaneously in effecting such regulation in *A. macrorrhiza*.

MATERIALS AND METHODS

Plant Material. *Alocasia macrorrhiza* (L.) G. Don was grown from seed collected in a rainforest area near Atherton, Queensland, Australia. The plants were initially grown in a glasshouse in Davis, CA, under shade cloth which gave 20 to 30 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1} during midday. Details of the growth conditions are given in Kirschbaum and Pearcy (9). Either 2 weeks or 2 d prior to the experiment, plants were transferred to Reno, NV, and were maintained in controlled environment facilities at 27/15°C day/night and 20 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1} for a 12 h day. Plants were watered daily with one-half strength Hoagland solution.

Experimental Protocol. For the "low light to high light" experiment, an attached leaf of a plant that had previously been at low PFD (approximately 10 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1}) for at least

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[2] Abbreviations: Rubisco, RuBP carboxylase (EC 4.1.1.39); CA1P, 2-carboxyarabinitol 1-phosphate; PGA, P-glycerate; PFD, photon flux density; RuBP, ribulose 1,5-bisphosphate; k_{cat}, catalytic constant.

several hours was placed in the freeze-clamp gas exchange cuvette, where it received 7 to 10 μmol quanta·m^{-2}·s^{-1}, 180 mbar O_2, and 290 μbar CO_2 at 23 to 24°C. At time zero, the PFD was increased in a single step to 400 to 500 μmol quanta·m^{-2}·s^{-1}. Leaf temperature increased to 26 to 27°C. After a specified length of time, the leaf was freeze-clamped (<0.25 s to 0°C following interruption of the light) (for details see Ref. 26). The resulting two 3 cm^2 punches were immediately stored in liquid nitrogen until analysis.

For the "high light to low light" experiment, plants growing at 20 μmol quanta·m^{-2}·s^{-1} in the growth room were transferred to a shaded greenhouse about 1 h prior to their use. In the greenhouse, the plants received up to (but not exceeding) 500 μmol quanta·m^{-2}·s^{-1}. An attached leaf was then placed in the freeze-clamp cuvette at 400 to 500 μmol quanta·m^{-2}·s^{-1}, 180 mbar O_2, 290 μbar CO_2, and 26 to 27°C. Leaves were kept at a high PFD until a constant rate of CO_2 assimilation was obtained, about 10 to 20 min. The PFD was then reduced in a single step to 7 to 10 μmol quanta·m^{-2}·s^{-1}. Leaf temperature decreased to 23 to 24°C. After a specified length of time, the leaf was freeze-clamped and samples stored as described above. Each time point in Figures 1 through 4 is the mean ± SE of 3 to 4 freeze-clampings. Values for zero time and the longest time points on Figures 3 and 4 were taken from the 20 min and zero time points, respectively, of Figures 1 and 2.

Rubisco and Metabolite Measurements. Measurements of Rubisco activation, Rubisco k_{cat}, and RuBP and PGA pool sizes were made as described by Seemann and Sharkey (24). For Rubisco measurements, a 3 cm^2 portion of leaf frozen in liquid nitrogen was rapidly ground in 3 mL of ice-cold 100 mM Bicine (pH 7.8) prepared CO_2 free, containing 5 mM $MgCl_2$, 5 mM DTT, and 1 mM EDTA. This extract was microfuged for 10 s and immediately assayed for the "initial" (*in vivo*) Rubisco activity. We found that Rubisco activation state in these extracts was particularly sensitive to warming, with very brief periods of time above ice temperature promoting deactivation. A portion of this extract was also brought to 10 mM HCO_3^-, 20 mM $MgCl_2$ (10% dilution) and reassayed after 10 min on ice. This activity is referred to as the "total" activity. Similar total activities resulted if activation was carried out at room temperature. Rubisco assays were conducted for 30 s at 25°C in 100 mM Bicine (pH 8.2), 20 mM $MgCl_2$, 15 mM $NaH^{14}CO_3$ (30 GBq·mol^{-1}), and 1.5 mM RuBP generated from ribose 5-phosphate immediately prior to assay.

Determination of the Rubisco catalytic site concentration in these extracts was made using ^{14}C-carboxyarabinitol 1,5-bisphosphate. Leaves used in these experiments averaged 6.1 μmol Rubisco catalytic sites·m^{-2}. The k_{cat} of Rubisco is expressed in units of mol CO_2 fixed·mol^{-1} Rubisco·s^{-1} and abbreviated s^{-1}. The 'total k_{cat}' was calculated using the total activity of the enzyme and the 'initial k_{cat}' calculated using the initial activity. The total k_{cat} is the fully carbamylated specific activity of the enzyme as influenced by the level of tight binding inhibitors in the leaf, while the initial k_{cat} is the specific activity of the enzyme as affected by the state of activation and/or the level of tight binding inhibitors in the leaf (23). The activation state of Rubisco is [initial k_{cat}/total k_{cat} (×100)] and represents an indirect measure of the apparent carbamylation state of catalytic sites that do not have inhibitor bound to them. However, it is possible that factors other than carbamylation may affect this measure. These three measures of Rubisco performance (initial k_{cat}, total k_{cat}, activation state) allow the *in vivo* activity of the enzyme to be assessed (initial k_{cat}) and the influence of the two regulatory mechanisms to be resolved (total k_{cat} − inhibitors; activation state − putative carbamylation).

RuBP and PGA pool sizes were determined on the matching 3 cm^2 leaf portion following extraction in 3% perchlorate and are expressed on the basis of the Rubisco catalytic site content. The pool size of CA1P in leaves was measured as described by Seemann *et al.* (22).

RESULTS

Low Light to High Light. During photosynthetic induction in *A. macrorrhiza*, the leaf's biochemical capacity for CO_2 assimilation increased for the first 5 to 10 min following a step increase in PFD (9). To investigate whether this increase in photosynthetic capacity was the result of an increase in the activity of Rubisco, leaves were freeze-clamped at a low PFD (7–10 μmol quanta·m^{-2}·s^{-1}) and at different times following an increase in PFD to 500 μmol quanta·m^{-2}·s^{-1} (Fig. 1). The Rubisco activation state in leaves acclimated to the low PFD was 55 ± 6% (Fig. 1A). This result suggests that at low PFD, 55% of the catalytic sites to which any tight binding inhibitor was not bound were activated, since the assay for activation measures only those sites to which a tight binding inhibitor is not bound (23). Following the increase in PFD, activation rose rapidly, with a $t_{1/2}$ of approximately 2 min. Maximum activation (90 ± 3%) was reached after 10 to 20 min.

The depression of the k_{cat} of fully activated Rubisco (total k_{cat}) from its intrinsic maximum is a measure of the extent to which catalytic sites have a tight binding inhibitor bound to them (11, 23). In low PFD acclimated plants, the total k_{cat} was 13.2 ± 0.6 s^{-1} (Fig. 1B), about 65% of the maximum value measured for *A.*

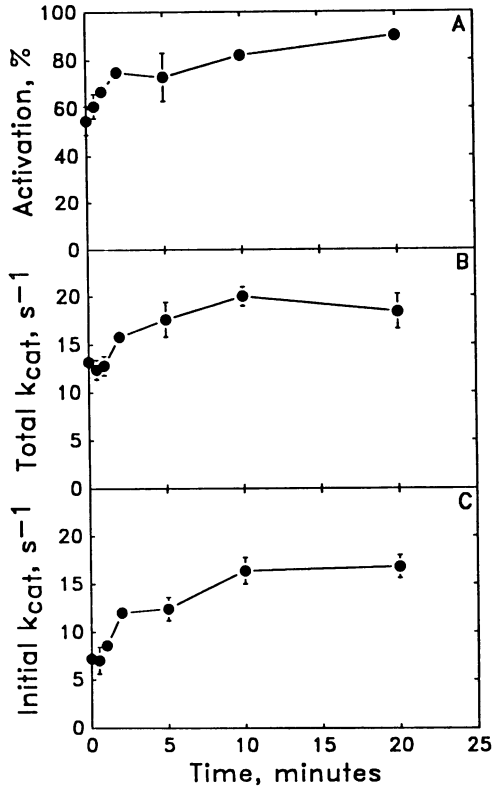

FIG. 1. Time course of the response of Rubisco activity following a step increase in PFD from 7 to 10 up to 500 μmol quanta m^{-2} s^{-1} at zero time. Points are mean ± SE, n = 3 to 4. Error bars smaller than the point size are not shown. A, Response of Rubisco activation state, determined as [initial k_{cat}/total k_{cat}] (×100). B, Response of the total k_{cat} of Rubisco, mol CO_2 fixed·mol^{-1} Rubisco·s^{-1}. This value was determined using the activity of the CO_2-Mg^{2+} activated enzyme, the catalytic site content as determined by ^{14}CABP binding and mol wt = 550,000. C, Response of the initial k_{cat} of Rubisco, in units as in B. This value was determined as in B except that the initial (in vivo) activity of Rubisco was used.

macrorrhiza of 20 s^{-1}, which is similar to that of other species. This result indicates that in low light about 35% of the Rubisco catalytic sites had a tight binding inhibitor bound to them and were not available for catalysis, just as a deactivated site is not. The total k_{cat} of Rubisco remained unchanged during the first 2 min after the increase in PFD and then rose to its maximum value at 10 min with a $t_{1/2}$ of about 2.5 min, suggesting that an inhibitor was released from the enzyme.

In a separate experiment, we assayed for the presence of tight binding inhibitors of Rubisco in freeze-clamped samples from leaves of *A. macrorrhiza* that had been equilibrated at either 10 or 500 μmol quanta·m^{-2}·s^{-1}. Leaves at the high PFD contained little or no CA1P or similar inhibitors of Rubisco activity. Leaves at the low PFD contained substantial quantities of an acid-stable compound with the characteristics of CA1P and capable of inhibiting Rubisco activity (data not shown). CA1P levels were sufficient to account for the observed reduction in the total k_{cat}.

The potential *in vivo* activity of Rubisco is determined by the number of catalytic sites which are activated and do not have a tight binding inhibitor such as CA1P bound to them. The appropriate measure of this activity, which takes into account both activation and tight binding inhibitor (*e.g.* CA1P) effects on Rubisco activity, is the initial k_{cat}; the k_{cat} of the enzyme determined using the initial activity value. The response of the initial k_{cat} to an increase in PFD is shown in Figure 1C and indicates that essentially all of the change in Rubisco activity, as mediated jointly by activation state and CA1P, occurred within 10 min with a $t_{1/2}$ of about 2 to 3 min.

The time courses of RuBP and PGA pool sizes before and after the increase in PFD are shown in Figure 2. RuBP pool size was initially 1.6 ± 0.3 mol·mol^{-1} Rubisco catalytic sites under steady-state low light conditions (Fig. 2A). One minute after switching to high light the RuBP pool was 4.5 ± 1.5 mol·mol^{-1}. RuBP pool size then began to decrease and returned to a constant level within 5 to 10 min. The PGA pool size decreased from 4.2 ± 1.4 to 0.5 ± 0.2 mol·mol^{-1} within the first 30 s following the increase in the PFD and remained low (Fig. 2B).

High Light to Low Light. The response of Rubisco activity in *A. macrorrhiza* to a step decrease in PFD is shown in Figure 3. At time zero, leaves which had acclimated to 500 μmol quanta·m^{-2}·s^{-1} were stepped to 10 μmol quanta·m^{-2}·s^{-1} and then freeze-clamped after different time intervals. Rubisco activation fell from 90 ± 3 to 55 ± 3% following the step decrease in PFD (Fig. 3A). This response began immediately upon lowering the PFD and took from 30 to 45 min for the new steady state activation state to be reached. The total k_{cat} remained unchanged for the first 15 min following the decrease in PFD and then began to decline (Fig. 3B). This decline suggests that CA1P was being synthesized, and we measured a large increase in the pool size of a tight binding inhibitor of Rubisco in low light leaves (see above). After 45 min, the total k_{cat} was 16.0 ± 1.0 s^{-1}, still somewhat above its steady-state value of 13.2 ± 1.0 s^{-1} at low PFD. The initial k_{cat}, representing the combined effects of activation and CA1P, declined over time in a linear fashion (Fig. 3C). This response was not quite complete after 45 min. These results indicate that the activity loss following a decrease in PFD is much slower than the gain in activity in response to an increase in PFD.

RuBP pool size decreased immediately following the step decrease in PFD (Fig. 4A) and then slowly increased toward the low PFD steady state value seen previously (Fig. 2A). This rise in the RuBP pool size was quite slow, being only partially complete after 45 min. PGA pool size increased markedly in the first 30 s at low PFD (0.9 ± 0.2 to 8.5 ± 0.3 mol·mol^{-1}) and remained high for the following several minutes (Fig. 4B). PGA content then declined and after 15 to 20 min at a low PFD was stable at a value similar to that in Figure 2B.

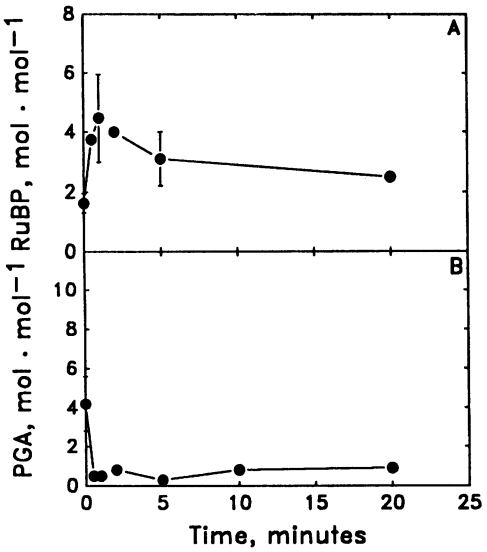

FIG. 2. Time course of metabolite pool sizes (mol metabolite·mol^{-1} Rubisco catalytic sites) following a step increase in PFD from 7 to 10 up to 500 μmol quanta m^{-2} s^{-1} at zero time. Points are mean ± SE, n = 3 or 4. Error bars smaller than the point size are not shown. A, Response of the RuBP pool size; B, response of the PGA pool size.

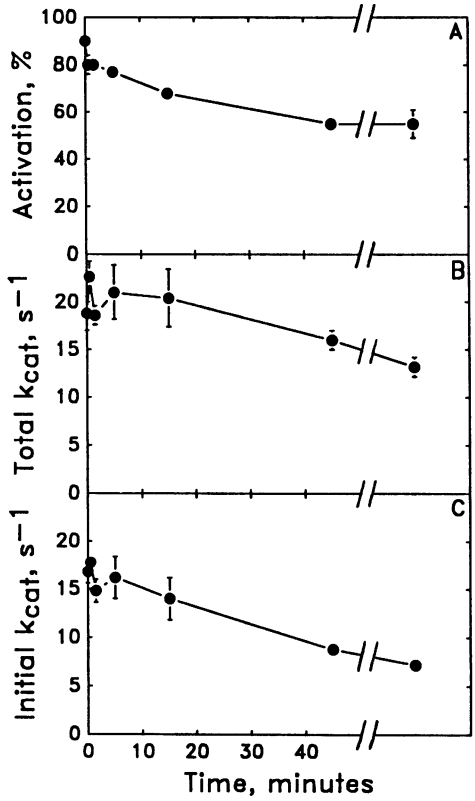

FIG. 3. Time course of the response of Rubisco activity following a step decrease in PFD from 500 μmol quanta m^{-2} s^{-1} to 10 μmol quanta m^{-2} s^{-1} at zero time. See Figure 1 legend for details. Points are mean ± SE, n = 3 or 4. Error bars smaller than the point size are not shown. Values for the zero and longest time points are taken from the 20 min and zero time points, respectively, in Figure 1. A, Response of Rubisco activation state; B, response of the total k_{cat} of Rubisco; C, response of the initial k_{cat} of Rubisco.

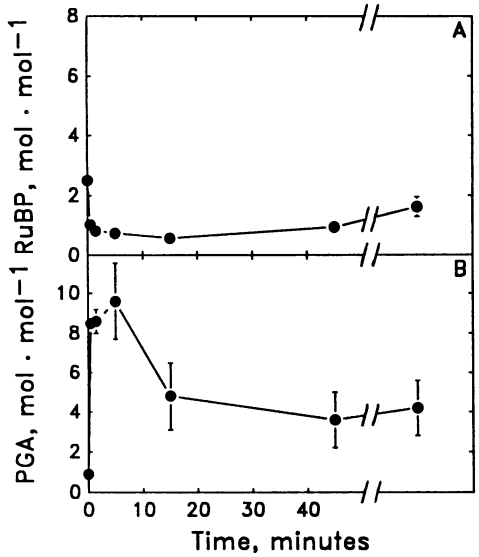

FIG. 4. Time course of metabolite pool sizes following a step decrease in PFD from 500 to 10 μmol quanta m^{-2} s^{-1} at zero time. Points are mean ± SE, n = 3 or 4. Error bars smaller than the point size are not shown. Values for the zero and longest time points are taken from the 20 min and zero time points, respectively, in Figure 2. A, Response of the RuBP pool size; B, response of the PGA pool size.

DISCUSSION

The induction requirement for maximum photosynthetic rate appears to be universal in photosynthetic CO_2 exchange (7, 10, 18, 20). The gas exchange kinetics of photosynthetic induction under fluctuating light conditions have been extensively examined in a number of species native to understory environments, including *A. macrorrhiza* (5, 6, 9, 15). These results indicate that both stomatal and carboxylation limitations are important in determining the rate of induction, but carboxylation limitations probably predominate during the first 5 to 10 min (9) because leaf conductances in the shade understory are usually quite high (2). Available evidence also indicates that the rate at which the induced state is lost plays a critical role in determining the capacity of understory plants to use successive lightflecks (6).

Evidence presented above indicates that light regulation of Rubisco activity occurs by a combination of activation state and CA1P concentration changes in *A. macrorrhiza*, similar to tobacco (21) and sugar beets (11). A step increase in PFD produced a 55 to 65% increase in both Rubisco activation (Fig. 3A) and total k_{cat} (Fig. 3B). The kinetics of these two responses were relatively similar, although there appears to be a short lag in the light-induced increase of the total k_{cat}, possibly due to a lag in CA1P degradation. The initial k_{cat} of Rubisco rose from 7.2 ± 0.4 to 16.4 ± 1.4 s^{-1} in the first 10 min following the increased PFD (Fig. 3C). This means that the capacity of the leaf for CO_2 assimilation increased by 128% during this time period. This change corresponds to a similar increase in photosynthetic capacity observed in intact leaves of *A. macrorrhiza* that had been preconditioned to have open stomates (9). Thus, the rate at which Rubisco is activated and CA1P is metabolized appear to be important factors in determining the rate of induction of photosynthesis in *A. macrorrhiza*.

The pool sizes of RuBP and PGA are a consequence of the Rubisco activity and the PFD-dependent capacity for PGA reduction and RuBP regeneration. A step increase in PFD produced a rapid increase in the RuBP pool size, followed by a gradual return to values similar to those originally present at low PFD (Fig. 2A). This transient increase in RuBP, caused by an increased capacity for RuBP production and a constant capacity for carboxylation, was then apparently damped by an approximately equal increase in the capacity for consumption of RuBP by Rubisco, *i.e.* Rubisco activity increased. This regulation of Rubisco activity, resulting from changes in both activation state and CA1P concentration, appears to regulate the RuBP pool at a relatively constant level in *A. macrorrhiza* in response to varying PFD, consistent with the suggestion for *Xanthium strumarium* by Mott *et al.* (13) (see also Ref. 11). The significance of such regulation may be related to the overall regulation of inorganic phosphate concentration (26). The PGA pool size was not regulated in a fashion similar to RuBP under these conditions (Fig. 2B).

Following a step decrease in PFD, changes in activation state and CA1P concentration again jointly played a role in controlling Rubisco activity. However, the kinetics and relative effects of each of these regulatory mechanisms were different than with an increase in PFD. Activation state fell with a $t_{1/2}$ of approximately 12 min, several times slower than the light-induced increase in activation. An even greater difference in the kinetics of the total k_{cat} decline, representative of the rate of CA1P synthesis, was observed with a reduction as opposed to an increased PFD. A significant reduction in the Rubisco total k_{cat} following a step decrease in PFD did not occur until more than 15 min after the change (Fig. 3B). After 45 min, the total k_{cat} was still 20% higher than its steady-state low light value.

The initial k_{cat} declined in a nearly linear fashion from 16.8 ± 1.2 to 8.8 ± 0.4 s^{-1} 45 min after the decrease in PFD (Fig. 3C). The slow rate of decline of the initial k_{cat} appears to be a consequence of the extremely slow rate of CA1P synthesis in this species. In contrast, the rate of CA1P synthesis and decrease in Rubisco total k_{cat} in *Phaseolus vulgaris* following a reduction in PFD is extremely rapid (23). The slow rate at which Rubisco activity is decreased by CA1P synthesis in *A. macrorrhiza* thus apparently slows the rate of induction loss. These slower rates of deactivation and CA1P synthesis may explain why subsequent lightflecks produce higher rates of photosynthesis than the initial lightfleck following a period of low PFD (15). In forest understories, periods of relatively frequent lightflecks are typically separated by periods with few or no lightflecks (14). The slow rate of CA1P synthesis may act as a means of ensuring carryover of the induced state from one lightfleck to the next.

With a step reduction in PFD, the pool size of RuBP in *A. macrorrhiza* initially declined (Fig. 4A), as has been observed in a number of species (13, 16, 19). There was also a very large and rapid increase in the PGA pool size (0.9 ± 0.2 to 8.5 ± 0.3 mol PGA·mol^{-1} Rubisco catalytic sites) (Fig. 4B), which has also been observed in spinach (19). These rapid changes in RuBP and PGA pool sizes result from a high carboxylation capacity and a low RuBP regeneration capacity. Photosynthesis is thus limited by the capacity to make RuBP following the decrease in PFD. The slow rise in RuBP pool size at low PFD (Fig. 4A) probably results from the slow reduction in Rubisco activity (Fig. 3C) as brought about by deactivation and CA1P synthesis (Fig. 3, A and B). This regulation of Rubisco activity by these two mechanisms in response to a decrease in light once again allows RuBP pool size to be controlled. Prinsley *et al.* (19) have suggested that the high PGA pool size, which occurs under such conditions, may also contribute to reducing Rubisco activity by acting as a competitive inhibitor of carboxylation with respect to RuBP.

CONCLUSIONS

The rate of increase of whole leaf photosynthetic CO_2 assimilation in response to a step increase in PFD is often limited by the rate at which the activity of Rubisco increases. In *A. macrorrhiza*, this rate is determined by the rate at which both Rubisco catalytic sites are activated and the tight-binding Rubisco inhibitor, CA1P, is degraded. Following a step decrease in PFD, the

rate of activity loss by Rubisco as mediated by activation and CA1P is much slower than the rate of decline of photosynthesis. Under these conditions the rate of photosynthesis is limited by the capacity for RuBP regeneration and the change in Rubisco activity is only regulatory and does not limit photosynthesis. These changes in Rubisco activity, as brought about by activation/deactivation and CA1P degradation/synthesis produce a relatively constant RuBP pool size following acclimation to a new PFD. The rate of activity loss by Rubisco is also much slower than the rate at which its activity increases during induction, primarily as a consequence of the slow rate of CA1P synthesis in this species. This slow rate of activity loss by Rubisco may provide the plant with a greater capacity to take advantage of succeeding lightflecks.

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