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Mechanisms for light-dependent regulation of ribulose-1,5-bisphosphate carboxylase activity and photosynthesis in intact leaves

(CO_2 fixation/enzyme inhibitor/enzyme regulation/light)

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ABSTRACT **The mechanisms involved in the *in vivo* light-dependent regulation of ribulose-1,5-bisphosphate (RbuP_2) carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] activity in intact leaves were studied. In the three species examined, *Phaseolus vulgaris*, *Beta vulgaris*, and *Spinacea oleracea*, the regulated level of RbuP_2 carboxylase activity (assayed *in vitro* with saturating substrate) was highly correlated ($r = 0.96$) with the rate of net CO_2 uptake of the corresponding leaves measured over a wide range of photosynthetic photon flux density (PPFD). However, the mechanisms by which the enzyme was regulated differed between these species. In *Phaseolus*, the inhibitor 2-carboxyarabinitol 1-phosphate (CA*P*) accounted for all of the PPFD-dependent regulation of RbuP_2 carboxylase activity. A similar compound was detected in *Beta*, and changes in its concentration accounted for about half of the PPFD-dependent regulation of enzyme activity in this species. No CA*P* was detected in *Spinacea*, but evidence we obtained suggests that a different inhibitor (possibly RbuP_2) accounts for a significant portion of the PPFD-dependent regulation of enzyme activity in this species. Changes in the activation state of the enzyme were observed with *Beta* and *Spinacea*, while in *Phaseolus* the enzyme was apparently fully activated at all PPFD levels. These results indicate that plant species may differ markedly in the mechanisms they use to regulate RbuP_2 carboxylase activity as PPFD changes. The results also suggest that tight binding inhibitors are a more widespread mechanism for regulation of this enzyme than previously thought. Furthermore, the results establish the importance of such inhibitors in regulating both the activity of RbuP_2 carboxylase and whole leaf photosynthesis over a range of PPFD.**

The rate of photosynthetic CO_2 assimilation by an intact leaf is a direct result of the activity of ribulose-1,5-bisphosphate (RbuP_2) carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing); EC 4.1.1.39]. The activity of this enzyme at any concentration of CO_2 and O_2 may be determined *in vivo* by the concentration of its other substrate, RbuP_2, by the enzyme catalytic site concentration, or by mechanisms that alter the apparent specific activity (k_{cat}) of the enzyme present in the leaf. At least two different mechanisms are known that regulate the apparent k_{cat} of RbuP_2 carboxylase *in vivo*. The first mechanism involves a reversible reaction of CO_2 and Mg^{2+} with a lysine residue to form a carbamate, which is essential for catalytic activity (1). Recent work indicates that this "activation" reaction may be controlled by another chloroplast protein, RbuP_2 carboxylase activase (2, 3). When a portion of the catalytic sites of the enzyme is not activated, the apparent k_{cat} is correspondingly lower than the intrinsic maximum k_{cat} of the fully activated enzyme. The intrinsic maximum k_{cat} of the enzyme may be restored by incubating the enzyme in saturating concentrations of CO_2 and Mg^{2+}. The second mechanism involves control of the concentration of an inhibitor of RbuP_2 carboxylase activity, 2-carboxyarabinitol 1-phosphate (CA*P*), which binds very tightly to the carbamoylated catalytic site of the enzyme, rendering it inactive (4–7). In contrast to the deactivated enzyme, it is not possible to reestablish maximum catalytic activity of RbuP_2 carboxylase with CA*P* bound to it by incubating the enzyme in the presence of high levels of CO_2 and Mg^{2+}. When CA*P* is present, the apparent k_{cat} is decreased (relative to the maximum intrinsic k_{cat}) in proportion to the fraction of sites occupied by this compound (4).

The significance of these multiple mechanisms for regulating the activity of RbuP_2 carboxylase is not clear, since CA*P* is not present in all species (4, 8, 9). We report here a study of three species (*Spinacea oleracea*, *Beta vulgaris*, and *Phaseolus vulgaris*) chosen because they have very different patterns of regulation of RbuP_2 carboxylase activity in response to changing photosynthetic photon flux density (PPFD). Our results suggest that C_3 plant species can be categorized by the light-dependent mechanisms they use to regulate RbuP_2 carboxylase activity. In one group, regulation of RbuP_2 carboxylase activity in response to changing PPFD is accomplished by CA*P*, and activation may not be important in these species (e.g., *Phaseolus*) for light regulation of the enzyme. In other species, RbuP_2 carboxylase activity is regulated by both activation and levels of CA*P* (e.g., *Beta*). In some species that do not synthesize CA*P* (e.g., *Spinacea*) another inhibitor (possibly RbuP_2) appears to play a role similar to CA*P* and works in concert with changes in activation. These results also suggest the general importance of inhibitors of RbuP_2 carboxylase activity in regulating whole leaf photosynthesis over a range of PPFD.

MATERIALS AND METHODS

Plant Material. *P. vulgaris* and *B. vulgaris* were grown from seed in a greenhouse under conditions described elsewhere (10). *S. oleracea* was grown from seed in a growth room with a 12-hr photoperiod and 25°C/15°C (day/night).

Gas Exchange. Attached leaves of plants that had been in the dark overnight were placed in the cuvette of the gas-exchange system while still in the dark. Leaves were then allowed to equilibrate to steady state for 20–30 min either in the dark or at a specific PPFD before the whole leaf net photosynthetic CO_2 assimilation rate (*A*) was measured. A new plant from the dark was used for each PPFD. Each point in Figs. 1–4 represents the average ± SEM of four to six freeze clampings. The exchange of CO_2 and H_2O between leaf and ambient atmosphere was monitored in a gas-

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Abbreviations: CA*P*, 2-carboxyarabinitol 1-phosphate; PPFD, photosynthetic photon flux density; RbuP_2, ribulose 1,5-bisphosphate.

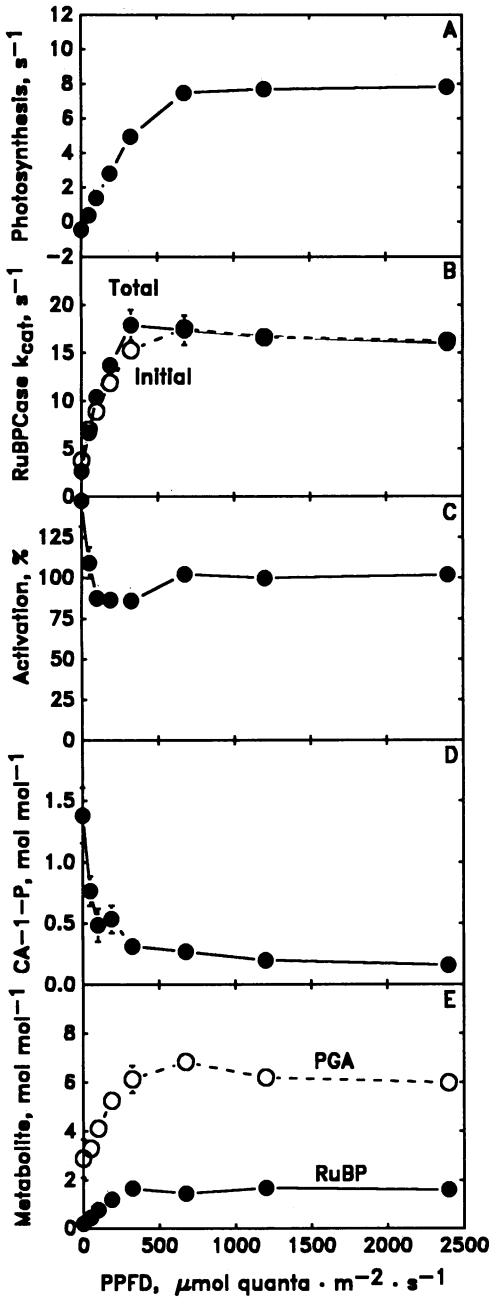

FIG. 1. Responses of *P. vulgaris* to PPFD. (*A*) Intact leaf CO_2 assimilation rate normalized to the RbuP_2 carboxylase (RuBPCase) content of the same leaf (mol of CO_2 per mol of RbuP_2 carboxylase per s). (*B*) *In vivo* specific activity (○, initial k_{cat}) and fully activated specific activity (●, total k_{cat}) of RbuP_2 carboxylase (mol of CO_2 fixed per mol of RbuP_2 carboxylase per s) of freeze-clamped leaves in *A*. (*C*) RbuP_2 carboxylase percent activation *in vivo* [(initial/total activity) × 100] of freeze clamped leaves in *A*. (*D*) Pool size of CA*P* (mol of CA*P* per mol of RbuP_2 carboxylase catalytic sites) of leaves freeze clamped in *A*. (*E*) Pool sizes of RbuP_2 (●) and 3-phospho-D-glycerate (PGA) (○) (mol of metabolite per mol of RbuP_2 catalytic sites) in leaves freeze clamped in *A*. All points are mean ± SEM (n = 4–6). Where no standard error bars are shown, the SEM was smaller than the size of the point.

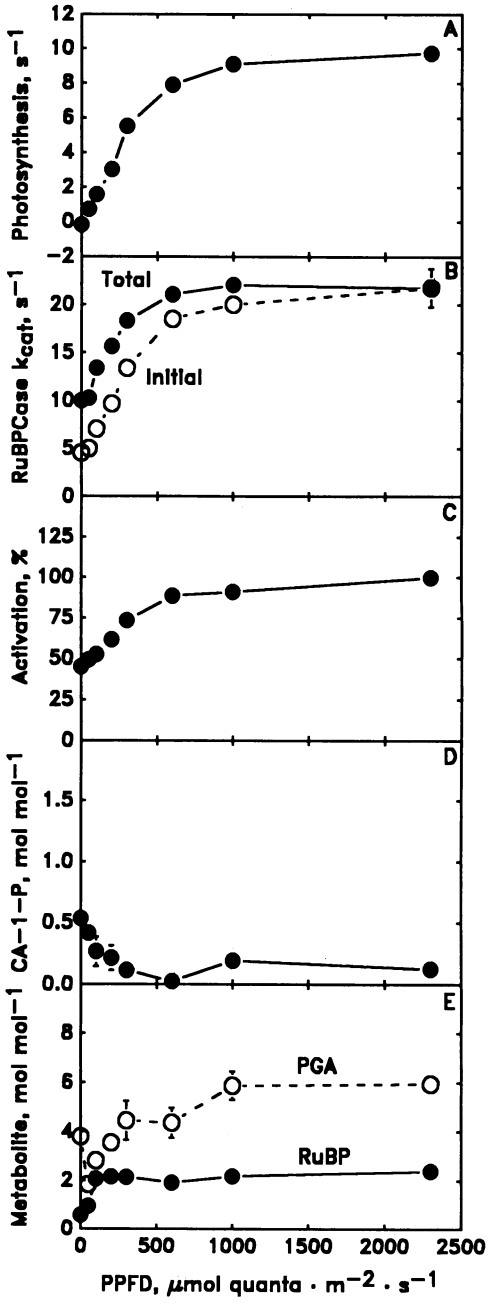

FIG. 2. Responses of *B. vulgaris* to PPFD. *A–E* are the same, respectively, as in Fig. 1.

exchange system described elsewhere (10). External CO_2 and O_2 partial pressures were maintained at 290 μbar and 180 mbar (1 bar = 100 kPa), respectively, and leaf temperature was maintained at 23–24°C. Evaporation, conductance, photosynthesis, and internal CO_2 concentrations were calculated according to ref. 11. *A* was normalized to the RbuP_2 carboxylase content present in the same leaf (mol of CO_2 per mol of RbuP_2 carboxylase per s). Leaf metabolism was terminated in the cuvette by clamping the leaf between two copper heads cooled to the temperature of liquid N_2 (see ref. 10 for details), producing an 8-cm^2 leaf disc split into two equal halves. The leaf halves were stored in liquid N_2 until analysis.

RbuP_2 Carboxylase and Metabolite Measurements. RbuP_2 carboxylase was rapidly extracted from one half of the leaf disc in an ice-cold CO_2-free medium as described (4, 12). The "initial" activity was measured immediately after extraction, while the "total" activity was measured on an aliquot of the extract made 10 mM and 20 mM with HCO_3^- and $MgCl_2$, respectively (10% dilution), and allowed to incubate on ice for 10 min to fully activate RbuP_2 carboxylase. Activity was assayed for 30 s at 25°C as described (12). The concentration of RbuP_2 carboxylase in the leaf sample was determined from the enzyme extract by the binding of 2-[^{14}C]carboxyarabinitol 1,5-bisphosphate as described (4). The k_{cat} (mol of CO_2 fixed per mol of RbuP_2 carboxylase per s) was based on the enzyme content present in the leaf sample and either the

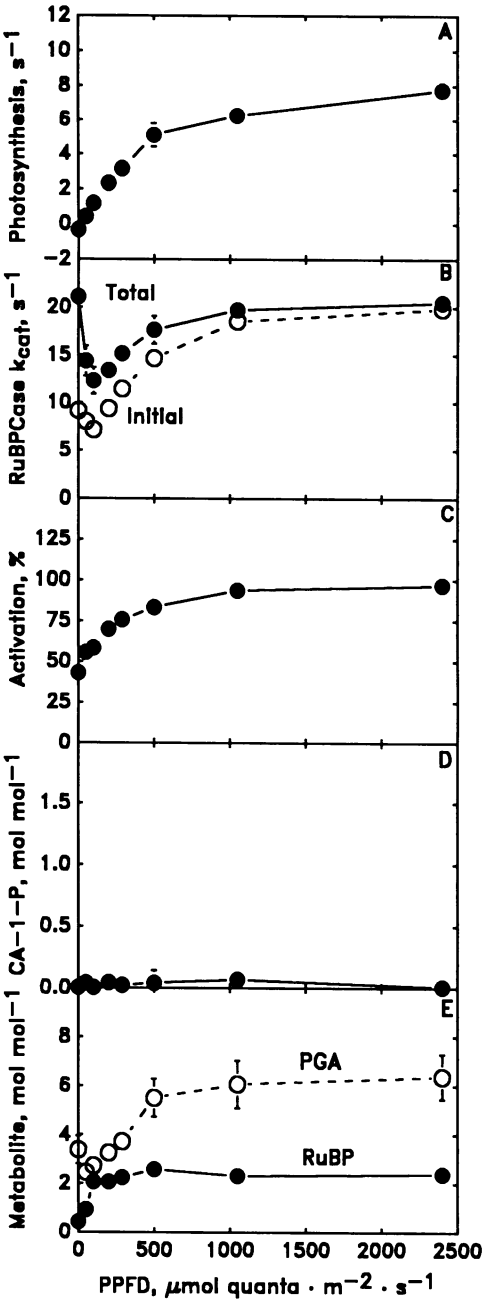

FIG. 3. Responses of *S. oleracea* to PPFD. *A–E* are the same, respectively, as in Fig. 1.

initial or total activity; hence, "initial k_{cat}" and "total k_{cat}." The total k_{cat} is the fully carbamoylated 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. The percentage activation is [initial k_{cat}/total k_{cat}] ($\times 100$) and represents the apparent carbamoylation state of catalytic sites which do not have an inhibitor bound to them.

Metabolites were extracted from the second half of the leaf disc in 3.5% (vol/vol) $HClO_4$ and RbuP_2 and 3-phospho-D-glycerate pool sizes were determined as described (12). The level of CA*P* was estimated by determining the reduction in total activity of fully carbamoylated purified spinach RbuP_2 carboxylase by the inhibitor present in the metabolite extracts as described (4). Pool sizes of all metabolites are expressed on the basis of the RbuP_2 carboxylase catalytic site content (mol of metabolite per mol of RbuP_2 carboxylase catalytic sites) in the same leaf.

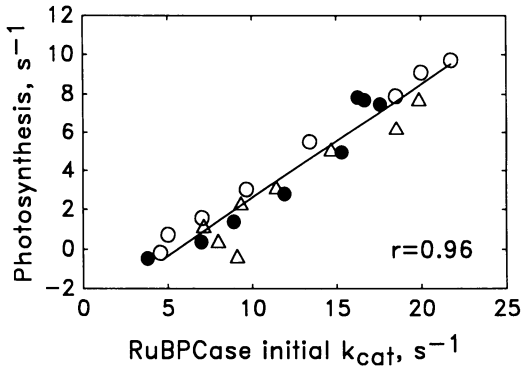

FIG. 4. The relationship between photosynthesis (from Figs. 1*A*, 2*A*, and 3*A*) and the initial k_{cat} of RbuP_2 carboxylase (RuBPCase) (from Figs. 1*B*, 2*B*, and 3*B*). ●, *P. vulgaris*; ○, *B. vulgaris*, △, *S. oleracea*.

RESULTS

Photosynthesis. The response of steady-state net CO_2 assimilation (*A*) to PPFD was similar in all three species examined, *P. vulgaris* (bean), *B. vulgaris* (sugar beet), and *S. oleracea* (spinach). *A* increased in a near linear fashion as PPFD increased at low light levels and reached a light-saturated rate at between 500 and 1000 μmol quanta·m^{-2}·s^{-1} (Figs. 1*A*, 2*A*, and 3*A*). Leaf intercellular CO_2 partial pressure averaged 300 μbar in the dark and 235 μbar at high light.

Photosynthesis vs. Initial k_{cat}. The response of the initial k_{cat} of RbuP_2 carboxylase to increased PPFD was similar in all three species (Figs. 1*B*, 2*B*, and 3*B*). This response closely paralleled the response of *A* to PPFD. If *A* is plotted as a function of the initial k_{cat} of RbuP_2 carboxylase for all three species, the data lie on a common regression line with a correlation coefficient of 0.96 (Fig. 4).

Control of k_{cat} by Enzyme Activation. The percentage activation of RbuP_2 carboxylase is indicated by the increase in catalytic activity after incubation of the leaf extract at saturating CO_2 and Mg^{2+} concentrations. In *Beta* and *Spinacea*, the percentage activation of RbuP_2 carboxylase was 43% and 46% in the dark, respectively, and increased with increased PPFD, approaching saturation (95–100%) between 500 and 600 μmol quanta·m^{-2}·s^{-1} (Figs. 2*C* and 3*C*). In *Phaseolus*, the percentage activation was >100% in the dark but declined to a relatively constant level (95%) over the remaining PPFD range (Fig. 1*C*). The high percentage activation observed in the dark and very low PPFD in this species was an artifact caused by increased binding by free CA*P*, which was present in the enzyme extract during preincubation with high Mg^{2+} and CO_2 concentrations.

Control of k_{cat} by Tight Binding Inhibitors. For *Phaseolus* and *Beta*, the k_{cat} in the dark was 15% and 46%, respectively, of its high light maximum and increased with increased PPFD, reaching a maximum at 325 and 600 μmol quanta·m^{-2}·s^{-1}, respectively (Figs. 1*B* and 2*B*). The total k_{cat} in *Spinacea* was at a maximum in both the dark and high light but declined to a minimum at a PPFD of 100 μmol quanta·m^{-2}·s^{-1} (Fig. 3*B*). At light levels >100 μmol quanta·m^{-2}·s^{-1}, total k_{cat} increased with increasing PPFD, reaching a maximum at 1200 μmol quanta·m^{-2}·s^{-1}.

Inhibitor Concentrations. The steady-state level of CA*P* in dark *Phaseolus* leaves was 1.4 ± 0.2 mol of CA*P* per mol of RbuP_2 carboxylase catalytic sites (Fig. 1*D*), similar to dark levels measured by Seemann *et al.* (4) in this species. As PPFD increased, the steady-state level of CA*P* declined until 300–500 μmol quanta·m^{-2}·s^{-1}. A similar CA*P* pool size is reached regardless of whether the PPFD is increased or decreased to a particular level (unpublished data). The apparent CA*P* content at high light of ≈0.2 mol·mol^{-1} is

likely to represent nonspecific inhibition in the CA*P* assay (see also ref. 4). Comparison of the data in Fig. 1 *B* and *D* demonstrates that the observed level of CA*P* was generally equal to or greater than the level required to produce the inhibition in the corresponding total k_{cat} of *Phaseolus* at any given PPFD (assuming that 1 mol of RbuP_2 carboxylase binding sites will bind stoichiometrically 1 mol of CA*P*). In *Beta*, the inhibitor level was 0.5 ± 0.05 mol·mol^{-1} in the dark and also declined as PPFD increased (Fig. 2*D*), reaching a minimum value at 300 μmol quanta·m^{-2}·s^{-1}. We have not purified this inhibitory compound from *Beta*, but its binding and inhibitory characteristics appear similar to CA*P* from *Phaseolus*. However, unlike *Phaseolus*, the measured level of inhibitor did not totally account for the reduction of the total k_{cat} observed at low PPFD (e.g., at 100 μmol quanta·m^{-2}·s^{-1}, the actual inhibition of the total k_{cat} was 39% but the expected inhibition calculated from the CA*P* pool size is 27%). This discrepancy is discussed below. Despite the reduction in the total k_{cat} of RbuP_2 carboxylase from *Spinacea* at low PPFD, no CA*P* could be detected (Fig. 3*D*). Apparently some inhibitor other than CA*P* was bound to the enzyme at low PPFD, reducing the total k_{cat}. The binding of this compound *in vitro* was apparently temperature sensitive, since the reduced total k_{cat} in *Spinacea* at low PPFD was only observed when the enzyme was activated at ice temperatures, in contrast to activation at room temperature.

Metabolite Concentrations. Steady-state levels of RbuP_2 were low in the dark and higher in the light in all three species (Figs. 1*E*, 2*E*, and 3*E*). In *Phaseolus*, the increase in the level of RbuP_2 with increased PPFD tended to parallel changes in total k_{cat} of RbuP_2 carboxylase, reaching maximum steady-state levels (1.8 mol·mol^{-1}) at 325 μmol quanta·m^{-2}·s^{-1}. In both *Beta* and *Spinacea*, RbuP_2 reached maximum levels (2.0–2.4 mol·mol^{-1}) at the significantly lower PPFD of 100 μmol quanta·m^{-2}·s^{-1}.

The steady-state level of 3-phospho-D-glycerate in *Phaseolus* increased with PPFD, reaching a maximum level at 325 μmol quanta·m^{-2}·s^{-1} (Fig. 1*E*) as was observed for RbuP_2 in this species. In *Beta* and *Spinacea*, the levels of 3-phospho-D-glycerate initially declined between 0 and 50 μmol quanta·m^{-2}·s^{-1} but above this light level 3-phospho-D-glycerate increased with increased PPFD, reaching a maximum between 500 and 1000 μmol quanta·m^{-2}·s^{-1} (Figs. 2*E* and 3*E*).

DISCUSSION

The rate of net CO_2 assimilation expressed on a RbuP_2 carboxylase basis was highly correlated with the apparent k_{cat} of these sites in initial extracts prepared from samples of all three species freeze clamped during steady-state photosynthesis (Fig. 4). This result confirms that mechanisms that control the apparent k_{cat} (as opposed to the level of RbuP_2) account for most of the modulation of RbuP_2 carboxylase activity observed *in vivo* in these studies. Despite the strong correlation between *A* and initial k_{cat} (Fig. 4) the mechanisms used by the species in this study for the light regulation of RbuP_2 carboxylase activity were different. *Phaseolus*, as reported previously (4), appears to accomplish nearly all of the regulation by control of the level of a tight binding inhibitor, CA*P*. *Beta* possesses CA*P*, but only about one-half of the light-dependent RbuP_2 carboxylase regulation in this species is accomplished by this compound. Changes in the Mg^{2+}- and CO_2-dependent activation of enzyme sites accounted for the remainder of the regulation in this species. *Spinacea* had no detectable CA*P* under any condition and appeared to adjust the Mg^{2+}- and CO_2-dependent activation level to a similar extent as *Beta*. However, the mechanism of regulation of RbuP_2 carboxylase activity in *Spinacea* is apparently more complex than anticipated. Evidence indicates that a compound other than CA*P* may bind *in vivo* to RbuP_2 carboxylase. Enzyme extracted from leaves of *Spinacea* freeze clamped at low PPFD could not be restored to full activity by incubation with saturating concentrations of CO_2 and Mg^{2+} at ice temperature. In contrast, enzyme extracted from leaves freeze clamped in the dark or at saturating PPFD could achieve full activity under these conditions (Fig. 3*B*) (see also ref. 13). No CA*P* could be recovered from these extracts and the inhibitory substance was apparently unstable when incubated in crude extracts with Mg^{2+} and CO_2 at room temperature, while CA*P* is stable indefinitely under such conditions.

We have not definitively established the identity of this inhibitory compound in *Spinacea*, but several lines of reasoning lead us to suggest that it is RbuP_2. First, it is well established that RbuP_2 may bind tightly to the decarbamoylated form of RbuP_2 carboxylase *in vitro* (K_d = 20 nM) (14). Second, RbuP_2 levels exceed the level of total catalytic sites at PPFD > 100 μmol quanta·m^{-2}·s^{-1}, and a significant fraction of these sites appear to be decarbamoylated at these low PPFDs. RbuP_2 would thus be expected to form a binary complex with these inactive enzyme sites. Third, the lability of the inhibitor is consistent with the compound being RbuP_2. RbuP_2, if released from the binary complex, should be consumed by the catalytically active RbuP_2 carboxylase also present in the extracts. Increased temperature should speed the dissociation of the binary complex, leading to loss of the inhibitor, as was observed (see also ref. 14). This RbuP_2, which would be present in the deproteinated leaf extracts used for metabolite and CA*P* analysis, would also be consumed in the assay used to detect CA*P* since the extracts are preincubated with activated RbuP_2 carboxylase. Fourth, the apparent k_{cat} declined with increasing PPFD in going from dark to 100 μmol quanta·m^{-2}·s^{-1} (Fig. 3*B*). The decline in k_{cat} correlates with an increase in RbuP_2 from undetectable in the dark to levels in excess of the catalytic site concentration at 100 μmol quanta·m^{-2}·s^{-1} and is consistent with the proposal that RbuP_2 is a negative effector of RbuP_2 carboxylase activation at normal stromal pH (14). However, the possibility that the inhibitor in *Spinacea* may be some other compound that does not bind as tightly as CA*P* or binds only to the deactivated sites cannot be entirely eliminated at this point. Regardless of the identity of the inhibitor present in *Spinacea*, it appears that catalytic sites of RbuP_2 carboxylase in this species are covered *in vivo* by a compound that binds tightly to the catalytic sites of RbuP_2 carboxylase, resulting in the loss of activity of these sites. In this respect, the mechanism in *Spinacea* has some similarity to the mechanism used by *Phaseolus*. With *Beta*, the level of CA*P* was apparently not sufficient to account for all of the covered sites (e.g., those not activated by CO_2 and Mg^{2+}), indicating that the inhibitor in *Spinacea* may also occur (in addition to CA*P*) in this species.

A first step in the increase of RbuP_2 carboxylase activity *in vivo* after an increase in PPFD in any of these species must involve dissociation of an enzyme–inhibitor complex. The recently described RbuP_2 carboxylase activase (2, 3) may accomplish this in the *Spinacea* system, assuming that the inhibitor is RbuP_2. Presumably, some additional enzymic activity is required in the *Phaseolus* system to modify CA*P* so that it is released from and does not reoccupy the activated catalytic sites. While no enzyme has yet been identified that brings about this step, it is reasonable to assume that one exists. There has been a tendency to assume that these mechanisms (RbuP_2 carboxylase activase and CA*P* metabolism) might be mutually exclusive, but this may not be the case. RbuP_2 carboxylase activase has been detected in *Phaseolus* (15), and, if the current hypothesis regarding the function of this enzyme is correct, it follows that RbuP_2 carboxylase activase is required to maintain the RbuP_2 carboxylase of this species fully activated. However, in

contrast to other species, the lack of change in activation state with changing PPFD in *Phaseolus* suggests that RbuP_2 carboxylase activase does not contribute to light-dependent changes in RbuP_2 carboxylase activity, although it may when photosynthesis is feedback limited in this species (10). The studies with *Beta* are particularly important in demonstrating that both mechanisms may operate in parallel. A similar pattern of regulation was observed in the deep-shade species *Alocasia macrorrhiza* (13) and *Nicotiana rustica* (16). Evidence also suggests that this phenomenon occurs in monocots (C_3 and C_4) (9).

The reason for these apparently redundant mechanisms of regulating the k_{cat} of RbuP_2 carboxylase is not clear at the present time. It has been postulated that at atmospheric levels of CO_2 and levels of PPFD that are below that required to saturate A, the rate of regeneration of RbuP_2 can limit the carboxylation reaction (11, 17). However, the level of RbuP_2 in all three species reached a constant maximum that was well in excess of the catalytic site concentration at a significantly lower PPFD than was required to saturate A, as has been observed in other studies (18, 19). This was a consequence of the fact that the apparent k_{cat} of RbuP_2 carboxylase was reduced at lower PPFD by the mechanism(s) of deactivation and/or inhibitor binding. The rate-limiting RbuP_2 pool size has been shown to be in the range of 1.5–2.0 mol·mol^{-1} (12, 20). Therefore, the effective RbuP_2 pool size of all three species at most levels of PPFD were close to rate saturating for carboxylation and photosynthesis. We conclude that one consequence of the regulation of RbuP_2 carboxylase activity in response to light is to maintain RbuP_2 at a saturating level even though the capacity of the regeneration system may be limiting at low PPFD. This regulation may be important in controlling the free concentrations of photosynthetic carbon reduction cycle intermediates and P_i, which appear to be important elements in regulating the balance between the capacities for photosynthetic carbon acquisition and usage via starch and sucrose synthesis (21).

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