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Regulation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activity in Response to Reduced Light Intensity in C_4 Plants[1]

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The light-dependent regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity was studied in 16 species of C_4 plants representing all three biochemical subtypes and a variety of taxonomic groups. Rubisco regulation was assessed by measuring (a) the ratio of initial to total Rubisco activity, which reflects primarily the carbamylation state of the enzyme, and (b) total Rubisco activity per mol of Rubisco catalytic sites, which declines when 2-carboxyarabinitol 1-phosphate (CA1P) binds to carbamylated Rubisco. In all species examined, the activity ratio of Rubisco declined with a reduction in light intensity, although substantial variation was apparent between species in the degree of Rubisco deactivation. No relationship existed between the degree of Rubisco deactivation and C_4 subtype. Dicots generally deactivated Rubisco to a greater degree than monocots. The total activity of Rubisco per catalytic site was generally independent of light intensity, indicating that CA1P and other inhibitors are not major contributors to the light-dependent regulation of Rubisco activity in C_4 plants. The light response of the activity ratio of Rubisco was measured in detail in *Amaranthus retroflexus, Brachiaria texana,* and *Zea mays*. In *A. retroflexus* and *B. texana*, the activity ratio declined dramatically below a light intensity of 400 to 500 μmol of photons m^{-2} s^{-1}. In *Z. mays*, the activity ratio of Rubisco was relatively insensitive to light intensity compared with the other species. In *A. retroflexus*, the pool size of ribulose bisphosphate (RuBP) declined with reduced light intensity except between 50 and 500 μmol m^{-2} s^{-1}, when the activity ratio of Rubisco was light dependent. In *Z. mays*, by contrast, the pool size of RuBP was light dependent only below 350 μmol m^{-2} s^{-1}. These results indicate that, in response to changes in light intensity, most C_4 species regulate Rubisco by reversible carbamylation of catalytic sites, as commonly observed in C_3 plants. In a few species, notably *Z. mays*, Rubisco is not extensively regulated in response to changes in light intensity, possibly because the activity of the CO_2 pump may become limiting for photosynthesis at subsaturating light intensity.

In C_3 plants, the activity of Rubisco is postulated to be regulated either by reversible carbamylation of a Lys residue in the catalytic site, enabling catalysis, or by the binding of inhibitors such as CA1P to carbamylated catalytic sites, disabling catalysis (Seemann et al., 1990; Portis, 1992). Regulation of Rubisco activity by reversible carbamylation occurs in response to changes in light intensity as well as the concentration of CO_2 and O_2, whereas inhibition of Rubisco activity by CA1P occurs only in response to varying PPFD (Sharkey et al., 1986; Sage et al., 1990; Seemann et al., 1990). At physiological levels of CO_2 in C_3 plants (5–10 μM), full carbamylation of Rubisco requires Rubisco activase (Salvucci, 1989; Portis, 1990). In the absence of Rubisco activase, only 20 to 40% of Rubisco catalytic sites are carbamylated under physiological conditions, leading to a significant inhibition of photosynthesis (Salvucci, 1989; Portis, 1990). However, full carbamylation of Rubisco can occur if CO_2 levels are increased well above ambient in the absence of RuBP (above 100 μM; Andrews and Lorimer, 1987).

In C_4 plants, the CO_2-concentrating mechanism is estimated to increase the CO_2 concentration in the bundle sheath 10- to 100-fold above that in the mesophyll tissue (Furbank and Hatch, 1987; Jenkins et al., 1989). This high CO_2 partial pressure may promote full carbamylation of Rubisco, even in the absence of Rubisco activase, raising the possibility that reversible carbamylation is not an important regulatory mechanism in C_4 plants. Results of a previous study of the regulation of Rubisco activity in the C_4 plant *Zea mays* support this hypothesis, because the degree of Rubisco deactivation in darkness is much less in *Z. mays* than in C_3 plants (Usuda, 1990). However, in the presence of RuBP and absence of Rubisco activase, carbamylation of Rubisco from C_3 plants is inhibited, even at high concentrations of CO_2 (Portis et al., 1986; Robinson et al., 1988). Consequently, Rubisco activase may be required by C_4 plants to fully carbamylate Rubisco. C_4 plants are known to contain Rubisco activase (Salvucci et al., 1987). Rubisco activity in C_4 plants may also be inhibited by light-dependent binding of CA1P, because CA1P occurs in darkened leaves of some C_4 species (Moore et al., 1991), and Rubisco activity in darkened leaves of a few C_4 species is inhibited by a tight binding inhibitor, putatively CA1P (Vu et al., 1984; Servaites et al., 1986).

To understand better the regulation of Rubisco in C_4 plants, we studied the light-dependent regulation of Rubisco activity in 16 C_4 species representing each biochemical subtype

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Abbreviations: CA1P, 2-carboxyarabinitol 1-phosphate; CABP, 2-carboxyarabinitol 1,5-bisphosphate; NAD(P)-ME, NAD(P)-malic enzyme; PCK, phospho*enol*pyruvate carboxykinase; PGA, 3-phosphoglyceric acid; RuBP, ribulose 1,5-bisphosphate.

(NADP-ME, NAD-ME, and PCK) and a variety of taxonomic groups.

MATERIALS AND METHODS

Plant Material

The effect of light intensity on the activity of Rubisco was examined in 16 species of C_4 plants, one C_3-C_4 intermediate species, and four C_3 species. Plants were grown in either an outdoor garden, a greenhouse, or natural stands in the field (see Table I for species list and associated growth conditions). Plants were selected to represent each C_4 subtype, including both monocots and dicots. The C_3-C_4 intermediate and C_3 plants were included in the study for comparative purposes. Five species from the genus *Panicum*, three C_4 species representing each subtype, one C_3-C_4 intermediate species, and one C_3 species, were also included. Complete light responses of Rubisco regulation were determined for three species, *Amaranthus retroflexus* L. (NAD-ME type), *Zea mays* L. (NADP-ME type), and *Brachiaria texana* (Buckley) S.T. Blake (PCK type).

Plants in the greenhouse were grown in a 6-L pot containing a peat:perlite loam mixture (Fafard mix No. 3; Conrad Fafard Inc., Springfield, MA) and were fertilized daily with a Johnson-Hoagland solution (Epstein, 1972) modified to contain 20 μM iron as Sequestrene 138 (Ciba-Geigy Corp., Greensboro, NC). Plants grown in an outdoor garden were irrigated and fertilized with the modified Johnson-Hoagland solution as necessary to avoid signs of water and nutrient stress. Plants from natural stands received no care.

Light treatments were established by shading the plants with plastic netting of neutral density and/or cloth fabric. After a minimum of 60 min at a given light intensity, approximately 4 cm^2 of leaves were sampled with a portable, hand-operated freeze-clamp device. The copper clamping heads were prechilled in liquid nitrogen. Samples were stored in liquid nitrogen until assayed. Samples were collected between 10 AM and 4 PM at temperatures ranging between 22 and 35°C. Temperature differences between treatments within a species were less than 6°C. We observed no effect of sampling temperature on the activity ratio of Rubisco measured at 25°C (data not shown).

Rubisco Assay

For each sample, the activity of Rubisco was assayed at 25°C immediately after extraction and again after a 10- to 16-min incubation in an "activation buffer" (20 mM $NaHCO_3$ and 28 mM $MgCl_2$ at pH 8.0 and room temperature), which fully carbamylates the enzyme. The ratio of activity in the initial extract to the activity of fully carbamylated enzyme reflects the apparent carbamylation state of Rubisco (Butz and Sharkey, 1989) and is often termed the "activation state" of Rubisco. However, because processes other than carbamylation may affect the activity of Rubisco, we prefer to call the ratio of activities in the initial extract (initial activity) to that of the fully carbamylated aliquot (total activity) the "activity ratio" of Rubisco. Binding of CA1P reduces the total activity of Rubisco, and measurements of total activity per Rubisco content are used to estimate the extent of CA1P inhibition (Seemann et al., 1985; Kobza and Seemann, 1988; Seemann et al., 1990).

Leaves were extracted at 0 to 4°C in a pH 8.0 buffer containing 100 mM Bicine, 1 mM DTT, 1 mM Na-EDTA, 3.3% (w/v) insoluble PVP, 0.14% (w/v) BSA, 20 mM $MgCl_2$, and 0.15 μM $NaHCO_3$. This extraction buffer contains more $NaHCO_3$ and $MgCl_2$ than used previously (Sharkey et al., 1986; Sage et al., 1990) because it better maintained the activity of Rubisco in initial extracts (data not presented). A 100-μL aliquot of the extract was immediately assayed (within 150 s of extraction) by adding it to 400 μL of assay buffer (100 mM Bicine [pH 8.2], 1 mM Na-EDTA, 0.1–0.3 units mL^{-1} of ribulose-5-P kinase, 1 unit mL^{-1} phosphoribuloisomerase, 2.0 mM ATP, 1.6 mM ribose-5-P, 28 mM $MgCl_2$, and 19 mM [^{14}C]$NaHCO_3$; specific radioactivity of 20.2 Bq $nmol^{-1}$), which was modified from that used by Seemann and Sharkey (1986). RuBP was generated in situ from ribose-5-P using ATP, ribulose-5-P kinase, and phosphoribuloisomerase. After a 30-s assay, the reaction was terminated with 2 N HCl, and the acid-stable radioactivity was determined by liquid scintillation spectroscopy.

Immediately after the initial activity was assayed, a 900-μL aliquot of crude extract was treated with 100 μL of an activation buffer (100 mM $MgCl_2$, 200 mM $NaHCO_3$, 100 mM Bicine [pH 8.2]) to activate Rubisco. After the aliquot was incubated 10 to 16 min at room temperature, Rubisco activity was assayed as above to give the activity of fully carbamylated Rubisco. Rubisco content in 60 to 90 μL of the activated extract was then assayed by determining the amount of [^{14}C]CABP bound to Rubisco, as described previously (Seemann and Sharkey, 1986).

The carbamylation state of Rubisco was also assessed using a CABP-trapping assay modified from Butz and Sharkey (1989). Samples were extracted in the same extraction buffer described above for activity assays. Immediately after the extract was centrifuged, a 60-μL aliquot was added to 400-μL microcentrifuge tubes containing 100 μL of the extraction buffer and 2 μL of 2 mM [^{14}C]CABP (with some carboxyribitol bisphosphate contaminant). After 3 to 4 min, 100 μL of a 2 mM [^{12}C]CABP solution and 25 μL of a 200 mM $NaHCO_3$ solution were added to the mixture. This mixture was incubated 3 to 4 h at 37°C in the presence of antibodies to Rubisco and then filtered on a Gelman (Ann Arbor, MI) GS-450 metricel membrane filter. A second aliquot was activated as for the total activity assays, treated with 2 μL of [^{14}C]CABP, and incubated at 37°C in the presence of Rubisco antibodies for 3 to 4 h. Radioactivity bound to the filters was counted by liquid scintillation spectroscopy. The ratio of recovered radioactivity in the initial extract to that in the activated extract reflects the carbamylation ratio of Rubisco in C_3 plants as described by Butz and Sharkey (1989).

Metabolite Assays

Metabolites were extracted by grinding leaf half-discs in 3.5% (v/v) $HClO_4$ at liquid N_2 temperatures as described by Seemann and Sharkey (1986). Following thawing, the acidic mixture was adjusted to pH 6 to 7 with 1.6 N KOH and refrozen until assayed. The amount of RuBP in *A. retroflexus* and *Z. mays* was measured by determining $^{14}CO_2$ incorpo-

rated into acid-stable products in the presence of Rubisco (Seemann and Sharkey, 1986). In *B. texana*, recovery of RuBP during extraction was too low to allow for accurate estimation of RuBP content. In *A. retroflexus*, the photosynthetic metabolites PGA, triose phosphates, Glc-6-P, Fru-6-P, and Fru-1,6-bisP were determined spectrophotometrically using a coupled-enzyme assay as previously described (Seemann and Sharkey, 1986).

All chemicals were purchased from Sigma Chemical Co. except for [^{14}C]$NaHCO_3$, which was purchased from ICN (Irvine, CA), and [^{14}C]CABP, which we generated from RuBP and [^{14}C]NaCN using a procedure similar to that described by Butz and Sharkey (1989).

Gas Exchange Measurements

The light response of photosynthesis was measured in *A. retroflexus*, *Z. mays*, and *B. texana*. For *A. retroflexus* and *Z. mays*, a Li-Cor 6200 photosynthesis apparatus (Li-Cor Inc., Lincoln, NE) was used to measure photosynthesis in garden-grown plants at a range of light intensities. In *B. texana*, a null-balance gas exchange system similar to that described by Sharkey et al. (1986) was used to determine the light response of photosynthesis in greenhouse-grown plants.

RESULTS

Light-Dependent Regulation of Rubisco in *A. retroflexus* (NAD-ME Type)

In field-grown *A. retroflexus*, photosynthesis rates in full sunlight were high, typically above 40 μmol of CO_2 m^{-2} s^{-1} and declined with reductions in light intensity below 2000 μmol m^{-2} s^{-1} (Fig. 1A). Similarly, the activity ratio of Rubisco was dependent on light intensity (Fig. 1B), particularly below 500 μmol of photons m^{-2} s^{-1}. However, the change in the activity of Rubisco between a PPFD of 2000 and 500 μmol m^{-2} s^{-1} was much less than the change in photosynthesis (Fig. 1A). In the dark, the activity ratio of Rubisco was higher relative to that found at low light. No differences in the total activity per CABP-binding sites was observed at any light intensity (data not shown), indicating that CA1P or other inhibitors are not involved in the regulation of Rubisco in this species.

RuBP pool size also declined with decreasing light intensity (Fig. 1B), as previously observed in C_3 plants and *Amaranthus edulis* (Perchorowicz et al., 1981; von Caemmerer and Edmondson, 1986; Leegood and von Caemmerer, 1988). At saturating PPFD, RuBP pools were near 6 mol of RuBP mol^{-1} of CABP-binding sites, approximately double that of C_3 plants at light saturation (Seemann and Sharkey, 1986; von Caemmerer and Edmondson, 1986). RuBP levels declined 50% between 2000 and 700 μmol m^{-2} s^{-1}, which was similar in magnitude to the decline in photosynthesis over the same range of PPFD. RuBP pools stabilized somewhat at 2 to 3 mol of RuBP mol^{-1} of CABP-binding sites between 500 and 50 μmol of photons m^{-2} s^{-1}. This range of PPFD corresponds to that at which the activity ratio of Rubisco was most light dependent.

The pool size of PGA in *A. retroflexus* increased with increasing PPFD between darkness and 500 μmol photons m^{-2} s^{-1} (Fig. 2). Above 500 μmol m^{-2} s^{-1}, PGA pools were independent of PPFD and were 5- to 10-fold larger than typically observed in C_3 plants (Seemann and Sharkey, 1986; von Caemmerer and Edmondson, 1986). As previously reported for *A. edulis* (Leegood and von Caemmerer, 1988), triose phosphates, Glc-6-P, and Fru-6-P increased with increasing PPFD but showed no evidence of saturation. Only Fru-1,6-bisP was independent of PPFD. When expressed on the basis of catalytic sites, these metabolites were also higher (3- to 10-fold) than observed in C_3 plants under similar conditions (Seemann and Sharkey, 1986; Sage et al., 1988). The Glc-6-P:Fru-6-P ratio in *A. retroflexus* was near 3 at low PPFD and decreased to near 2 at high PPFD, which was similar to what Leegood and von Caemmerer (1988) observed in *A. edulis*.

Light-Dependent Regulation of Rubisco in *Z. mays* (NADP-ME Type) and *B. texana* (PCK Type)

To complement the results obtained with *A. retroflexus*, the light response of the activity ratio of Rubisco was measured for plants from the other two C_4 subtypes. In *Z. mays*, the light response of photosynthesis was nearly identical with that in *A. retroflexus* (Fig. 3A). However, in contrast to *A. retroflexus*, *Z. mays* had a reduced light dependence of the activity ratio of Rubisco (Fig. 3B). The pool size of RuBP was dependent on light intensity only below a PPFD of 350 μmol m^{-2} s^{-1} (Fig. 3B), which was similar to what Usuda (1990)

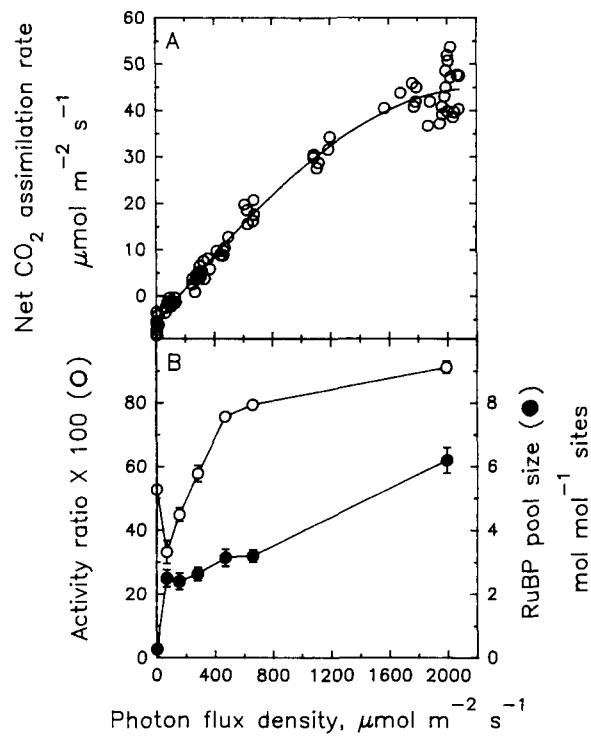

Figure 1. The relationship between PPFD and (A) the rate of net CO_2 assimilation or (B) the activity ratio of Rubisco (O) and the pool size of RuBP (●, in mol of RuBP mol^{-1} of CABP-binding sites) in leaves of the C_4 plant *A. retroflexus*. Activity ratios and RuBP pool sizes are means ± SE, where $n = 5$ to 6.

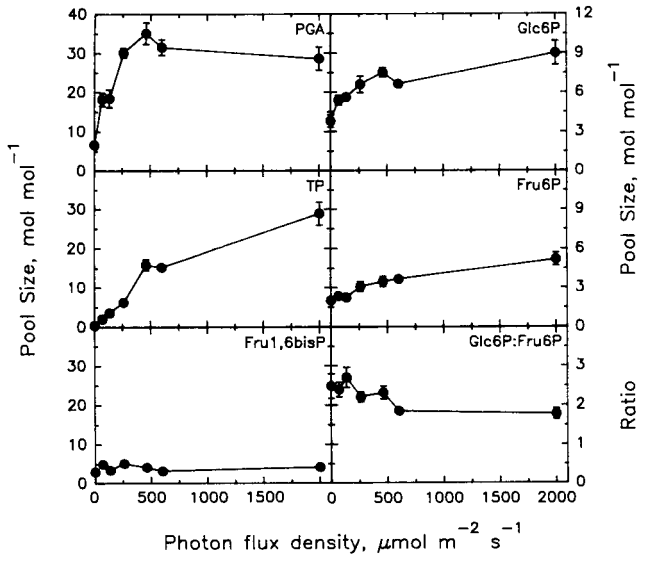

Figure 2. The relationship between PPFD and pool size (mol mol^{-1} of CABP-binding sites) of PGA, triose phosphates (TP), Fru-1,6-bisP, Glc-6-P, Fru-6-P, and the ratio of Glc-6-P to Fru-6-P in leaves of *A. retroflexus*. Means ± SE. n = 4 to 6.

reported for *Z. mays*. Consequently, the light responses of RuBP pool size and the activity ratio of Rubisco were not correlated with changes in photosynthesis above a PPFD of 350 μmol m^{-2} s^{-1}.

In *B. texana*, the photosynthetic capacity was relatively low, resulting in a low light saturation point (Fig. 4A). The activity ratio of Rubisco exhibited a pronounced light response below a PPFD of 400 μmol m^{-2} s^{-1} (Fig. 4B), which is similar in nature to the response observed in *A. retroflexus*. (The high activity ratios of *B. texana* appear to result from degradation of total Rubisco activity during activation, which we were unable to prevent.) Full Rubisco activation occurred at a lower PPFD in *B. texana* than in *A. retroflexus*. This difference may reflect differences in photosynthetic capacity, with the species having a lower light saturation point of photosynthesis also exhibiting a lower light saturation point of Rubisco activation.

As with *A. retroflexus*, no reduction in total activity per CABP-binding sites was observed at any light intensity with either *Z. mays* or *B. texana*. *Z. mays* can have moderate levels of CA1P in darkness (0.2 mol of CA1P mol^{-1} of Rubisco sites; Moore et al., 1991), but results here and elsewhere (Servaites et al., 1986) indicate that this level of CA1P does not inhibit Rubisco activity.

CABP-Trapping Assay and in Vitro Effects on Carbamylation

To examine whether the contrasting pattern of apparent carbamylation in *A. retroflexus* and *Z. mays* may have been an artifact, we used the CABP-trapping assay to measure the carbamylation ratio in leaves collected from field-grown plants exposed to high (1500 ± 200 μmol m^{-2} s^{-1}) and low (70 ± 10 μmol m^{-2} s^{-1}) PPFD. At high PPFD, the estimated carbamylation ratio (×100) was 84.8 ± 1.2 in *A. retroflexus* and 71.4 ± 2.3 in *Z. mays*. At low PPFD, the carbamylation ratio was 53.0 ± 0.7 for *A. retroflexus* and 74.3 ± 5.3 for *Z. mays*. These results are consistent with the activity ratio measurements in that the estimated carbamylation ratio in *A. retroflexus* declined about 40% following shading, whereas in *Z. mays*, shading had no effect on the carbamylation ratio.

Artifacts associated with extraction and assay do not appear to explain the reduced light response of the activity ratio of Rubisco in *Z. mays*. Upon extraction, the initial activity of Rubisco in the extraction buffer decayed with time at 4°C in both high- and low-light samples from *Z. mays*, causing a decline in the estimated activity ratio (Fig. 5). This indicates that deactivated Rubisco from maize would not spontaneously activate during extraction, and we were able to detect modest declines in the activity ratio that did occur in vitro. In *A. retroflexus*, a slight deactivation of Rubisco was observed in high-intensity light samples, whereas low-intensity light samples exhibited little change in activity following extraction.

Survey of Light-Dependent Regulation of Rubisco in C_4 Plants

Because distinctly different responses of Rubisco regulation were observed between *A. retroflexus* and *Z. mays* following shading, numerous C_4 species were surveyed to identify any relationship between the regulation of Rubisco and C_4 sub-

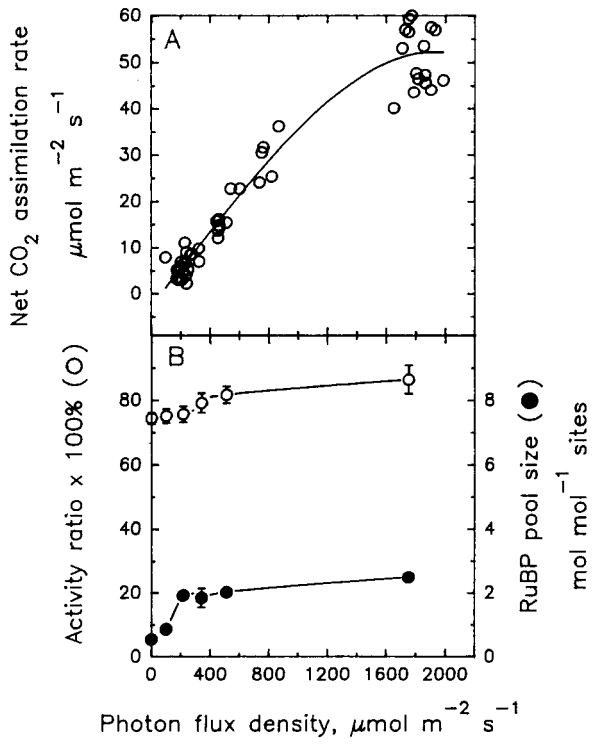

Figure 3. The relationship between PPFD and (A) the rate of net CO_2 assimilation or (B) the activity ratio of Rubisco (O) and the pool size of RuBP (●, in mol of RuBP mol^{-1} of CABP-binding sites) in leaves of the C_4 plant *Z. mays*. Activity ratios and RuBP pool sizes are means ± SE, where n = 5 to 8.

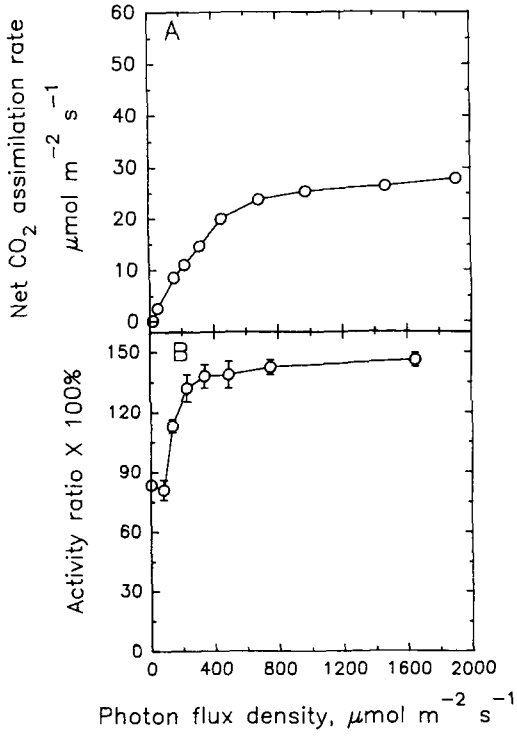

Figure 4. The relationship between PPFD and (A) the rate of net CO_2 assimilation or (B) the activity ratio of Rubisco in leaves of the C_4 plant *B. texana*. The light response of photosynthesis in A was determined on a single leaf and is representative of three independent measurements on separate leaves. Activity ratios are means ± SE, where $n = 4$ to 6.

type or taxonomic affiliation. For the survey, leaf samples were collected at high (>1200 μmol m^{-2} s^{-1}) or low PPFD (60 to 100 μmol m^{-2} s^{-1}). All species exhibited a significant decline in the activity ratio of Rubisco following exposure to low-intensity light (Table I). However, considerable variation existed among the species, with some deactivating Rubisco by less than 25% and others deactivating Rubisco by more than 70%. No relationship was observed between the degree of Rubisco deactivation and C_4 subtype. For example, in NADP-ME species exposed to low light, the activity ratio declined slightly in *Z. mays* and *Cyperus irea* but declined markedly in *Portulaca grandiflora* and *Flaveria trinervia*. There appeared to be a relationship between taxonomic status and degree of Rubisco deactivation in low light. Monocots tended to deactivate Rubisco less than dicots when shaded. With the exception of *Panicum antidotale* and *Panicum miliaceum*, the C_4 grasses and *C. irea* reduced the activity ratio less than 45%, whereas the dicots reduced the activity ratio by 60 to 74%. Both *Portulaca* species exhibited extensive reduction in activity ratio upon shading (65–71%), despite belonging to different C_4 subtypes. Moreover, regardless of C_4 subtype, the *Panicum* species generally exhibited substantial deactivation of Rubisco by 40 to 78%, which is similar to the range encountered in C_3 plants. The C_3-C_4 intermediate included in the study (*Panicum milioides*) also had an activity ratio at low PPFD that was typical of C_3 species.

Activity ratios greater than 1 were observed in a number of plants at high PPFD, notably the *Panicum* and *Brachiaria* species (Table I). We believe this occurs because of a loss of total activity during the activation step.

Total activity per CABP-binding site significantly declined ($P < 0.05$) in low light in only three of the 16 C_4 species surveyed (Table II). In *C. irea*, the reduction was 11%, in *Panicum maximum* it was 13%, and in *Zoysia japonica* it was 28%. Molar activities of Rubisco from C_4 plants were frequently greater than in C_3 plants, supporting the hypothesis of Seemann et al. (1984) that C_4 Rubisco generally has a higher specific activity. However, differences were not as pronounced as reported by Seemann et al. (1984).

DISCUSSION

In C_4 plants, the catalytic capacity of Rubisco in vivo is regulated downward following transfer of plants to shaded conditions. In most C_4 plants, reversible carbamylation appears to be the primary means for the regulated reduction in Rubisco capacity, because the activity ratio declined upon shading or darkening leaves while the total molar activity was largely insensitive to PPFD. The total molar activity declined significantly in low PPFD in only three of the 16 C_4 species studied, and in two of these, *C. irea* and *P. maximum*, the decline was only 11 to 13%. In the third species, *Z. japonica*, the decline in total activity of Rubisco was 28%, indicating that CA1P and/or other inhibitors may be an important contributor to the light-dependent regulation of Rubisco activity in this species. CA1P also appears to be important in completely darkened *P. maximum* leaves, in which substantial amounts of CA1P (0.44 mol of CA1P mol^{-1} of Rubisco catalytic sites) have been identified and the inhibition of total Rubisco activity is pronounced (Vu et al., 1984; Moore et al., 1991). In low PPFD, the contribution of CA1P to Rubisco regulation in *P. maximum* appears modest, how-

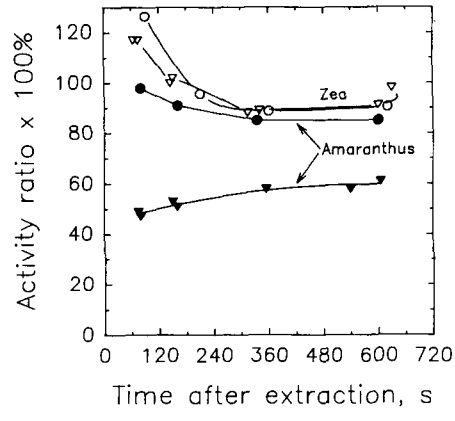

Figure 5. The change in the activity ratio of Rubisco in *Z. mays* (open symbols) and *A. retroflexus* (closed symbols) following extraction. Samples collected at either high (1500 μmol of photons m^{-2} s^{-1}; circles) or low (70 μmol m^{-2} s^{-1}; triangles) PPFD were homogenized beginning at time zero, rapidly centrifuged, and allowed to incubate on ice for the indicated period. Each point is the mean of three samples.

Table I. *Activity ratio of Rubisco × 100% in C_4, C_3–C_4 intermediate, and C_3 plants at high (>1200 μmol of photons m^{-2} s^{-1}) or low PPFD (80 ± 20 μmol m^{-2} s^{-1})*

Means ± SE. n = 4 to 7. Numbers in parentheses refer to growth conditions: (1), outdoor garden cultivation; (2), greenhouse cultivation; (3), naturally occurring stands. Asterisks indicate significant differences ($P < 0.05$) between species at high- and low-intensity light.

Species	Light Intensity		Decrease
	High	Low	
			%
NADP-ME			
Zea mays (1)	112 ± 2	96 ± 2	14*
Digitaria sanguinalis (1)	109 ± 4	72 ± 3	34*
Sorghum bicolor (2)	118 ± 7	81 ± 6	31*
Paspalum dilatatum (3)	107 ± 1	73 ± 2	32*
Cyperus irea (3)	93 ± 4	73 ± 2	22*
Flaveria trinervia (2)	107 ± 1	43 ± 3	60*
Portulaca grandiflora (2)	107 ± 5	31 ± 3	71*
Panicum antidotale (2)	150 ± 5	56 ± 3	63*
NAD-ME			
Cynodon dactylon (3)	104 ± 3	65 ± 2	38*
Atriplex pentandra (2)	90 ± 3	23 ± 2	74*
Portulaca oleracea (3)	105 ± 2	37 ± 3	65*
Amaranthus retroflexus (1)	102 ± 4	40 ± 4	61*
Panicum miliaceum (2)	201 ± 3	45 ± 2	78*
PCK			
Zoysia japonica (1)	85 ± 1	66 ± 4	22*
Brachiaria texana (2)	146 ± 9	81 ± 12	45*
Panicum maximum (2)	99 ± 3	59 ± 7	40*
C_3–C_4 intermediate			
Panicum milioides (2)	131 ± 4	57 ± 5	56*
C_3 Plants			
Spinacia oleracea (1)	87 ± 2	53 ± 4	39*
Pueraria lobata (kudzu) (3)	93 ± 2	51 ± 5	45*
Chenopodium album (2)	88 ± 3	36 ± 1	59*
Panicum boliviense (2)	145 ± 5	51 ± 1	65*

ever, causing a 13% decline in total molar activity relative to high-light controls.

In most of the C_4 species studied, the decline in the activity ratio of Rubisco in response to shading was greater than 30%, as is commonly observed in C_3 plants. However, in three of the 16 C_4 species (*Z. mays*, *C. irea*, and *Z. japonica*), the reduction in activity ratio was less than 25%, indicating reduced regulation of Rubisco by reversible carbamylation. In *Z. japonica*, and to a lesser extent in *C. irea*, the limited decline in the activity ratio of Rubisco was associated with a significant decline in total molar activity. This indicates that some of the downward regulation of Rubisco activity results from inhibitor binding, which may partly explain why decarbamylation appears to be less pronounced in these species. Reversible carbamylation is often less important in C_3 plants with extensive CA1P inhibition of Rubisco (Kobza and Seemann, 1988; Seemann et al., 1990).

In *Z. mays*, it is not clear what, if anything, is responsible for further regulation in Rubisco capacity. Although CA1P accumulates in the dark in leaves of *Z. mays* (Moore et al., 1991), no inhibition of total Rubisco activity is observed either in darkness or low PPFD (Table II; Servaites et al., 1986), demonstrating that CA1P plays little role in the regulation of Rubisco in this species. Artifacts associated with the assay of activity ratio do not appear to explain the lack of regulation. In a CABP-trapping assay, we detected no difference in carbamylation ratio between high and low PPFD in *Z. mays* but did in *A. retroflexus*. Moreover, initial Rubisco activity in *Z. mays* declined following extraction, indicating that spontaneous activation of Rubisco from low-light samples did not occur (Fig. 5). If RuBP regeneration is limiting photosynthesis at subsaturating PPFD in *Z. mays*, a lack of Rubisco regulation in response to light reduction should be accompanied by a decrease in RuBP levels. In this and earlier studies with *Z. mays* (Usuda, 1990), little decline in RuBP pools was observed with reduced PPFD between 2000 and about 400 μmol of photons m^{-2} s^{-1}. Leegood and von Caemmerer (1989) ob-

Table II. *Total molar activity of Rubisco in C_4, C_3–C_4 intermediate, and C_3 plants at high (>1200 μmol of photons m^{-2} s^{-1}) or low PPFD (80 ± 20 μmol m^{-2} s^{-1})*

Means ± SE. n = 4 to 7. Asterisks indicate significant differences ($P < 0.05$) between species at high- and low-intensity light.

Species	Light Intensity		Change
	High	Low	
	mol of CO_2 mol^{-1} of Rubisco s^{-1}		%
NADP-ME			
Z. mays	35.8 ± 1.8	34.8 ± 6.4	−3
D. sanguinalis	35.3 ± 2.0	33.7 ± 2.3	−5
S. bicolor	43.8 ± 1.0	42.3 ± 0.5	−3
P. dilatatum	45.6 ± 1.5	47.2 ± 1.5	+4
C. irea	45.6 ± 1.1	40.8 ± 1.2	−11*
F. trinervia	33.4 ± 0.7	34.2 ± 1.2	+2
P. grandiflora	44.7 ± 3.4	43.4 ± 2.3	−3
P. antidotale	28.7 ± 1.4	33.8 ± 1.0	+18*
NAD-ME			
C. dactylon	29.7 ± 2.1	27.2 ± 1.1	−8
A. pentandra	35.3 ± 0.8	34.8 ± 0.8	−1
P. oleracea	48.0 ± 3.6	48.9 ± 2.7	+2
A. retroflexus	29.8 ± 2.3	30.2 ± 1.0	+1
P. miliaceum	11.5 ± 0.9	12.0 ± 0.6	+4
PCK Type			
Z. japonica	30.4 ± 1.1	21.9 ± 0.8	−28*
B. texana	21.8 ± 3.0	22.3 ± 3.0	+2
P. maximum	46.8 ± 2.3	40.6 ± 1.6	−13*
C_3–C_4 Intermediate			
P. milioides	13.3 ± 0.3	15.2 ± 1.7	+14
C_3 Plants			
S. oleracea	28.6 ± 2.1	30.4 ± 2.4	+6
P. lobata (kudzu)	21.4 ± 1.3	16.4 ± 0.8	−23*
C. album	23.6 ± 0.3	23.4 ± 1.0	−1
P. boliviense	10.2 ± 0.4	10.6 ± 0.7	+4

served a modest dependence of RuBP pool size on PPFD above 300 μmol m^{-2} s^{-1} in *Z. mays*, but the response was much less than the change in photosynthesis over the same PPFD. If RuBP pools are relatively stable and Rubisco is not deactivated, then a factor other than RuBP regeneration should be responsible for the reduction in CO_2 assimilation with declining PPFD. In C_4 plants, this factor could be the activity of the C_4 pump, which, if limiting, could reduce the supply of CO_2 to the bundle sheath. The concentration of CO_2 in the bundle sheath of C_4 plants has been estimated to decline as PPFD is reduced (Furbank and Hatch, 1987), but the extent to which this contributes to the limitation of photosynthesis is not clear. Below a PPFD of 300 to 400 μmol m^{-2} s^{-1}, photosynthesis in *Z. mays* appears to be limited by RuBP regeneration because RuBP pools exhibit a pronounced decline, similar in magnitude to the decline in photosynthesis over the same range of PPFD.

RuBP regeneration appears to be an important limitation in *A. retroflexus* at all subsaturating PPFDs. RuBP pools decline to a similar degree as photosynthesis between a PPFD of 2000 and 500 μmol m^{-2} s^{-1}, when the activity ratio of Rubisco exhibits only a slight decline. Although the pool size of RuBP was well above the concentration of Rubisco catalytic sites in this range of PPFD, concentrations of PGA and other photosynthetic intermediates in C_4 plants may be large enough at higher light intensities to compete effectively for catalytic sites of Rubisco, increasing the K_m for RuBP and increasing the concentration at which RuBP is saturating (Foyer et al., 1987; Leegood and von Caemmerer, 1989). Consistent with this hypothesis, the pool size of some major photosynthetic intermediates (PGA, triose phosphates, Fru-6-P, and Glc-6-P) at high-intensity light were found to be 3- to 10-fold greater on a CABP-binding site basis in *A. retroflexus* than in C_3 plants under similar conditions. A large proportion of the pools of these metabolites are likely to be present in the bundle sheath, as opposed to the mesophyll. For example, in *Z. mays*, about 72% of the PGA and nearly 50% of the hexose monophosphates are in the bundle sheath under high-intensity light conditions (Leegood, 1985; Stitt and Heldt, 1985).

One purpose of the regulation of Rubisco capacity appears to be the maintenance of RuBP pools above levels that saturate Rubisco, thus shifting some of the immediate control over photosynthesis from RuBP regeneration to Rubisco (von Caemmerer and Edmondson, 1986; Sage, 1990). When the capacity of RuBP regeneration is limiting photosynthesis, deactivation of Rubisco can cause RuBP pools to become independent of PPFD (von Caemmerer and Edmondson, 1986; Sage et al., 1990). In C_3 plants, RuBP pools are estimated to be saturating above approximately 2 mol of RuBP mol^{-1} of CABP-binding sites, and deactivation of Rubisco capacity commonly occurs when RuBP pools would otherwise decrease below this value (Seemann and Sharkey, 1986; von Caemmerer and Edmondson, 1986; Sage et al., 1990). In *A. retroflexus*, a similar pattern is observed. The reduction in the activity ratio of Rubisco becomes pronounced below a PPFD of 500 μmol m^{-2} s^{-1}, when RuBP pool size nears 2 mol mol^{-1}. In *Z. mays*, by contrast, the relative lack of Rubisco deactivation allows RuBP pools to decline below 2 mol mol^{-1} at a higher PPFD than occurs in either *A. retroflexus* or C_3 plants.

The results from our study imply that Rubisco activase has a similar role in plants differing in photosynthetic pathway. Based on results with Rubisco from C_3 plants (Jordan and Chollet, 1983; Portis et al., 1986; Robinson and Portis, 1988), RuBP pool sizes in C_4 plants such as *A. retroflexus* appear high enough that carbamylation will be inhibited without Rubisco activase, even at the high CO_2 concentrations predicted to occur in the bundle sheath. Therefore, the ability of Rubisco activase to remove RuBP and other potential inhibitors from Rubisco catalytic sites is probably necessary to maintain high levels of Rubisco activation in C_4 plants. Because the activity of Rubisco activase is dependent on the energy status of the leaf (Robinson and Portis, 1988; Campbell and Ogren, 1990), conditions causing low thylakoid energization would lead to reduced activation of Rubisco, unless RuBP levels decline to near zero. In accordance with this hypothesis, when RuBP pools remain above 2 mol mol^{-1} in *A. retroflexus*, the activity ratio of Rubisco declines with reductions in light intensity. However, in darkness, when RuBP pools are near zero and activase presumably has little activity, the activity ratio of Rubisco increases. This may reflect spontaneous carbamylation of some Rubisco catalytic sites, which can occur at air levels of CO_2 in the absence of RuBP (Portis et al., 1986).

In summary, the pattern of Rubisco regulation in *A. retroflexus* indicates that the capacity for RuBP regeneration limits the rate of photosynthesis at subsaturating light intensities, similar to observations with C_3 species. This pattern may predominate in C_4 species, because most surveyed here exhibited pronounced reductions in the activity ratio of Rubisco at low light. In *Z. mays*, the limited effect of light intensity between 2000 and 400 μmol of photons m^{-2} s^{-1} on the activity ratio of Rubisco and pool size of RuBP indicate that something other than RuBP regeneration, perhaps C_4 pump activity, can exert significant control over photosynthesis at subsaturating PPFDs. Although not very widespread, this pattern indicates important variation in the pattern of limitation and regulation of C_4 photosynthesis in response to environmental change.

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LITERATURE CITED

Andrews TJ, Lorimer G (1987) Rubisco: structure, mechanisms, and improvement. *In* MD Hatch, NK Boardman, eds, The Biochemistry of Plants, Vol 10: Photosynthesis. Academic Press, New York, pp 132–218

Butz ND, Sharkey TD (1989) Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylation ratios. Plant Physiol **89:** 735–739

Campbell WJ, Ogren WL (1990) Electron transport through photosystem I stimulates light activation of ribulose bisphosphate carboxylase/oxygenase (Rubisco) by Rubisco activase. Plant Physiol **94:** 479–484

Epstein E (1972) Mineral Nutrition of Plants: Principles and Perspectives. John Wiley & Sons, New York

Foyer C, Furbank RT, Walker DA (1987) Interactions between ribulose-1,5-bisphosphate carboxylase and stromal metabolites. I. Modulation of enzyme activity by Benson-Calvin cycle intermediates. Biochim Biophys Acta **894:** 157–164

Furbank RT, Hatch MD (1987) Mechanism of C_4 photosynthesis. The size and composition of the inorganic carbon pool in the bundle sheath cells. Plant Physiol **85:** 958–964

Jenkins CLD, Furbank RT, Hatch MD (1989) Inorganic carbon diffusion between C_4 mesophyll and bundle sheath cells. Plant Physiol **91:** 1356–1363

Jordan DB, Chollet R (1983) Inhibition of ribulose-1,5-bisphosphate carboxylase by substrate ribulose 1,5-bisphosphate. J Biol Chem **258:** 13752–13758

Kobza J, Seemann JR (1988) Mechanisms for the light-dependent regulation of ribulose-1,5-bisphosphate carboxylase activity and photosynthesis in intact leaves. Proc Natl Acad Sci USA **85:** 3815–3819

Leegood RC (1985) The intercellular compartmentation of metabolites in leaves of *Zea mays* L. Planta **164:** 163–171

Leegood RC, von Caemmerer S (1988) The relationship between contents of photosynthetic metabolites and the rate of photosynthetic carbon assimilation in leaves of *Amaranthus edulis* L. Planta **174:** 253–262

Leegood RC, von Caemmerer S (1989) Some relationships between contents of photosynthetic intermediates and the rate of photosynthetic carbon assimilation in leaves of *Zea mays* L. Planta **178:** 258–266

Moore Bd, Kobza J, Seemann JR (1991) Measurement of 2-carboxyarabinitol 1-phosphate in plant leaves by isotope dilution. Plant Physiol **96:** 208–213

Perchorowicz JT, Raynes JA, Jensen RG (1981) Light limitation of photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings. Proc Natl Acad Sci USA **78:** 2985–2989

Portis AR Jr (1990) Rubisco activase. Biochim Biophys Acta **1015:** 15–28

Portis AR Jr (1992) Regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. Annu Rev Plant Physiol Plant Mol Biol **43:** 415–437

Portis AR Jr, Salvucci ME, Ogren WL (1986) Activation of ribulose bisphosphate carboxylase/oxygenase at physiological CO_2 and ribulose bisphosphate concentrations by Rubisco activase. Plant Physiol **82:** 967–971

Robinson SP, Portis AR (1988) Involvement of stromal ATP in the light activation of ribulose 1,5-bisphosphate carboxylase/oxygenase in intact chloroplasts. Plant Physiol **86:** 293–298

Robinson SP, Streusand VJ, Chatfield JM, Portis, AR Jr (1988) Purification and assay of Rubisco activase from leaves. Plant Physiol **88:** 1008–1014

Sage RF (1990) A model describing the regulation of ribulose-1,5-bisphosphate carboxylase, electron transport, and triose phosphate use in response to light intensity and CO_2 in C_3 plants. Plant Physiol **94:** 1728–1734

Sage RF, Sharkey TD, Seemann JR (1988) The in-vivo response of the ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated CO_2 in *Phaseolus vulgaris* L. Planta **174:** 407–416

Sage RF, Sharkey TD, Seemann JR (1990) Regulation of ribulose-1,5-bisphosphate carboxylase activity in response to light intensity and CO_2 in the C_3 annuals *Chenopodium album* L. and *Phaseolus vulgaris* L. Plant Physiol **94:** 1735–1742

Salvucci ME (1989) Regulation of Rubisco activity *in vivo*. Physiol Plant **77:** 164–171

Salvucci ME, Werneke JM, Ogren WL, Portis AR Jr (1987) Purification and species distribution of Rubisco activase. Plant Physiol **84:** 930–936

Seemann JR, Badger MR, Berry JA (1984) Variations in specific activity of ribulose-1,5-bisphosphate carboxylase between species utilizing differing photosynthetic pathways. Plant Physiol **74:** 791–794

Seemann JR, Berry JA, Freas SM, Krump MA (1985) Regulation of ribulose bisphosphate carboxylase activity *in vivo* by a light modulated inhibitor of catalysis. Proc Natl Acad Sci USA **82:** 8024–8028

Seemann JR, Kobza J, Moore Bd (1990) Metabolism of 2-carboxyarabinitol 1-phosphate and regulation of ribulose-1,5-bisphosphate carboxylase activity. Photosyn Res **23:** 119–130

Seemann JR, Sharkey TD (1986) Salinity and nitrogen effects on photosynthesis, ribulose-1,5-bisphosphate carboxylase and metabolite pool sizes in *Phaseolus vulgaris* L. Plant Physiol **82:** 555–560

Servaites JC, Perry MAJ, Gutteridge S, Keys AJ (1986) Species variation in the predawn inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol **82:** 1161–1163

Sharkey TD, Seemann JR, Berry JA (1986) Regulation of ribulose-1,5-bisphosphate carboxylase in response to changing pressure of O_2 and light in *Phaseolus vulgaris*. Plant Physiol **81:** 788–791

Stitt M, Heldt HW (1985) Generation and maintenance of concentration gradients between the mesophyll and bundle sheath in maize leaves. Biochim Biophys Acta **808:** 400–414

Usuda H (1990) Light and C_4 photosynthesis: how can the stromal system sense differences in light intensity to adjust its activities to overall flux? Bot Mag Tokyo Special Issue **2:** 159–173

von Caemmerer S, Edmondson DL (1986) The relation between steady-state gas exchange, in vivo ribulose bisphosphate carboxylase activity and some carbon reduction cycle intermediates in *Raphanus sativus*. Aust J Plant Physiol **13:** 669–688

Vu JCV, Allen LH, Bowes G (1984) Dark/light modulation of ribulose bisphosphate carboxylase activity in plants from different photosynthetic categories. Plant Physiol **76:** 843–845