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Short Communication

Dark/Light Modulation of Ribulose Bisphosphate Carboxylase Activity in Plants from Different Photosynthetic Categories[1]

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ABSTRACT

Ribulose bisphosphate carboxylase/oxygenase (RuBPCase) from several plants had substantially greater activity in extracts from light-exposed leaves than dark leaves, even when the extracts were incubated *in vitro* with saturating HCO_3^- and Mg^{2+} concentrations. This occurred in *Glycine max*, *Lycopersicon esculentum*, *Nicotiana tabacum*, *Panicum bisulcatum*, and *P. hylaeicum* (C_3); *P. maximum* (C_4 phosphoenolpyruvate carboxykinase); *P. milioides* (C_3/C_4); and *Bromelia pinguin* and *Ananas comosus* (Crassulacean acid metabolism). Little or no difference between light and dark leaf extracts of RuBPCase was observed in *Triticum aestivum* (C_3); *P. miliaceum* (C_4 NAD malic enzyme); *Zea mays* and *Sorghum bicolor* (C_4 NADP malic enzyme); *Moricandia arvensis* (C_3/C_4); and *Hydrilla verticillata* (submersed aquatic macrophyte). It is concluded that, in many plants, especially Crassulacean acid metabolism and C_3 species, a large fraction of ribulose-1,5-bisphosphate carboxylase/oxygenase in the dark is in an inactivatable state that cannot respond to CO_2 and Mg^{2+} activation, but which can be converted to an activatable state upon exposure of the leaf to light.

The enzyme RuBPCase[3] (EC 4.1.1.39) occupies a pivotal, potentially rate-limiting role in the PCR and PCO cycles (see review, 9) and, as a consequence, the regulation of this enzyme *in vitro* has been subject to considerable investigation. To achieve the catalytic state required for fixation of CO_2 or O_2, isolated RuBPCase has to be activated with CO_2 and Mg^{2+} (7, 9, 10). In a slow reaction, activator CO_2 binds to a lysine residue on the nonactivated enzyme (E) to form a carbamate (EC). A rapid binding of Mg^{2+} then follows to produce the active enzyme state (ECM). A number of chloroplast metabolites, particularly sugar phosphates, are positive or negative effectors of the enzyme (5, 6, 9), although most of the effects occur at suboptimal levels of CO_2 and Mg^{2+} (5, 9). The substrate RuBP also acts as a potent negative effector (inhibitor) when it binds before activator CO_2 and Mg^{2+} (5, 6, 9), although in the enzyme of some prokaryotes, this effect is minimal (6). Recent evidence suggests that the phosphorylated effectors exert their effect by influencing the relative rates of activation and deactivation (5). When the nonactivated enzyme is bound with a negative effector (EF), such as RuBP, it appears to be incapable of undergoing CO_2/Mg^{2+} activation (5, 6).

Less is known about the *in vivo* regulation of RuBPCase. Light and CO_2 are both essential for full activation *in vivo* (2, 3, 11, 12, 14, 15). Light produces a concurrent proton uptake and Mg^{2+} ion efflux by the thylakoid membranes that raises the pH and the Mg^{2+} concentration in the stromal region occupied by RuBPCase. This has been shown to be a component of the light activation mechanism (3). The explanation is still incomplete, as extrapolations from purified enzyme data indicate that chloroplastic CO_2 levels are too low for activation (9). We have demonstrated recently that RuBPCase extracted from field-grown soybeans during the dark period has only about one-third of the activity of that from light-sampled leaves, even when the RuBPCase is incubated *in vitro* with saturating (*i.e.* activating) levels of CO_2 and Mg^{2+} at alkaline pH (15). This suggests that in soybean leaves in the dark there exists an inactivatable state of RuBPCase with respect to CO_2 and Mg^{2+}. Furthermore, the rate of conversion by light of this inactive form appears to be a major determinant of the amount of fully activated enzyme (ECM), even during daylight hours (15). Other researchers confirm these observations (13).

In the present work, our intent was to determine if this dark-induced, inactivatable state was restricted to soybean, or whether it occurred in other species, especially in different photosynthetic categories. The genus *Panicum* provided the opportunity to study the effect in several species representing various photosynthetic categories, all within the same genus. The results reported here suggest that this inactivatable state is probably widespread among plants, particularly those in the CAM and C_3 categories. However, it is not ubiquitous in that at least one C_3 (wheat), several C_4 plants including a species of *Panicum* (millet), and the SAM plant *Hydrilla* lack the effect.

MATERIALS AND METHODS

Plant Materials, Growth, and Sampling Conditions. Soybean (*Glycine max* [L.] Merr. cv 'Bragg') was grown in acrylic environment-controlled chambers located outdoors and exposed to full summer sun irradiance, as described previously (15). Maize (*Zea mays* L. cv 'McCurdy 84aa'), wheat (*Triticum aestivum* L.

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[3] Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase; PCR, photosynthetic carbon reduction; PCO, photorespiratory carbon oxidation; SAM, submersed aquatic macrophyte; NAD-ME, NAD malic enzyme; NADP-ME, NADP malic enzyme; PEP-CK, phosphoenol-pyruvate carboxykinase.

cv 'Florida 301'), and *Bromelia pinguin* L. were field-grown during the spring; maize also was greenhouse-grown. Tomato (*Lycopersicon esculentum* Mill. cv 'Better Boy'), tobacco (*Nicotiana tabacum* L. cv 'Wisconsin 38'), sorghum (*Sorghum bicolor* [L.] Moench cv 'Funks G-522'), *Moricandia arvensis* [L.] DC., pineapple (*Ananas comosus* [L.] Merr.), and guinea grass (*Panicum maximum* Jacq.) were grown inside a greenhouse during the fall, with day/night temperatures of 30°C and 25°C, respectively. *Panicum bisulcatum*, *P. hylaeicum* Mez., millet (*P. miliaceum* L.), and *P. milioides* Nees ex. Trin. were grown in a growth chamber under a 14-h, 30°C day/22°C night, and a quantum irradiance of 800 μmol/m^2·s (400–700 nm). Hydrilla (*Hydrilla verticillata* [L.f.] Royle), an aquatic angiosperm, was collected from Orange Lake, Florida, and incubated under growth chamber conditions which maintain the plant in the high photorespiration state (4).

To determine the response of RuBPCase activity to leaf conditions of light and dark, leaf samples were taken in the dark 1 h before the start of the light period (before sunrise), and in the middle of the light period (usually at noon under full sun irradiance for field- and greenhouse-grown plants). Immediately after removal from the plant, the leaves were plunged into liquid N_2, a procedure that required less than 1 s, ground to a powder, and then stored continuously at liquid N_2 temperatures until analysis. This procedure was shown to produce no loss of RuBPCase activity or change in activation state over rapidly assayed, fresh material (15), even after storage of the samples for 18 months.

Extraction and Assay of RuBPCase. The extraction and assay of nonactivated and activated RuBPCase was performed in a manner similar to that described previously (15). The activity of HCO_3^-/Mg^{2+}-activated RuBPCase was determined by incubating 0.1 ml of the enzyme supernatant for 5 min at 30°C directly in the assay mixture, which consisted of: 50 mM Tris-HCl, 5 mM DTT, 10 mM $MgCl_2$, 0.1 mM EDTA, and 20 mM $NaH^{14}CO_3$ (0.2 μCi/μmol) at pH 8.5. The reaction was then initiated with 0.1 ml of RuBP, giving a final RuBP concentration of 0.5 mM, and terminated after 45 s with 0.1 ml of 6 N HCl. To ensure that no other carboxylase was active in the crude extracts, the control assays had RuBP omitted. The incorporation of acid-stable ^{14}C was measured by liquid scintillation spectrometry. Each value was the mean of triplicate determinations of two subsamples from a combined pool of more than 10 leaves. The RuBPCase activities were expressed on a Chl basis, determined by the method of Arnon (1).

RESULTS AND DISCUSSION

Table I shows the RuBPCase activities in extracts obtained from soybean and tobacco leaves in the light and dark. Extracts from leaves sampled in the light were from 2-fold to almost 5-fold higher in RuBPCase activity than the dark leaf extracts. This difference was largely maintained even after prior incubation of the enzyme extracts with saturating HCO_3^- and Mg^{2+}. Incubation with HCO_3^- and Mg^{2+} usually resulted in some increase in activity over the nonactivated enzyme, but could not replace the need for exposure of the leaf to light. These data confirm and extend our previous work which indicated that a substantial proportion of the RuBPCase in soybean leaves in the dark was in a form that was not fully activatable with CO_2 and Mg^{2+} (15). For soybean and tobacco RuBPCase, these data appear to challenge the explanation for the *in vivo* role of light, which invokes Mg^{2+} as the major factor (3).

Table I. *Nonactivated and HCO_3^-/Mg^{2+}-Activated RuBPCase Activity in Extracts of Soybean and Tobacco Leaves Sampled in the Dark and the Light*

Mean values ± SD are presented.

Plant	Leaf Sampling Conditions	RuBPCase Activity	
		Nonactivated	Activated
		μmol CO_2/mg Chl·h	
Glycine max	Dark (night)	181.4 ± 6.3	227.9 ± 8.7
	Light (day)	526.8 ± 10.6	602.1 ± 19.7
Nicotiana tabacum	Dark (night)	136.5 ± 4.4	280.0 ± 7.6
	Light (day)	627.9 ± 5.3	622.4 ± 11.7

Table II documents the dark inactivation results for several higher plants encompassing various photosynthetic categories. The HCO_3^-/Mg^{2+}-activated extracts from light-exposed leaves of *P. bisulcatum* and *P. hylaeicum* (both C_3 species) showed 39% and 32% higher activity than the dark leaf extracts. The other C_3 species responded similarly, as the light extracts ranged from 46% to 154% higher in activity (Table II). The exception to this trend among the C_3 species tested was wheat, which exhibited little difference in RuBPCase activity from light- or dark-sampled leaves.

Guinea grass, in the C_4 PEP-CK subgroup, had a similar response to most of the C_3 species, in that the RuBPCase activity was almost 160% greater in the light-exposed leaves. In contrast, millet (C_4 NAD-ME subgroup), as well as maize and sorghum (C_4 NADP-ME subgroup), showed little difference in RuBPCase activity between light and dark leaves. In the case of maize, this was true for both field- and greenhouse-grown plants, although the maximum extractable activity varied as a function of the growth conditions. A similar lack of an effect was found with the SAM plant, *Hydrilla*, in its high photorespiration state (Table II). The results differed for the C_3/C_4 intermediate species (Table II). *Moricandia arvensis* showed only a minor difference (7%) between light- and dark-sampled leaves, whereas for *P. milioides* the difference was 51%.

The greatest effect was found in the CAM category. Both CAM plants that were examined showed an almost absolute *in vivo* requirement for light in order to attain the maximum extractable RuBPCase activity. In the dark, the leaf extracts exhibited very little enzyme activity (Table II). Recent data from Littlejohn and Ku (8) also indicate that RuBPCase in the CAM plant *Opuntia erinacea* has a dark state that is virtually inactive and is not activated *in vitro* by CO_2 and Mg^{2+}.

These data, although obtained with a limited number of plants, indicate that the dark, inactivatable state of RuBPCase is probably widespread, but not ubiquitous, in C_3 species. It is present also in some C_4 species, but the data are yet too limited to determine whether it is restricted to the C_4 PEP-CK subgroup. The phenomenon also occurs in some C_3/C_4 intermediates, and is very marked in CAM plants. This difference in RuBPCase activation which exists, even among species within the genus *Panicum*, may be related to ultrastructural or biochemical differences among chloroplasts, or to species differences in the regulatory properties of the enzyme.

We propose that, for leaves exemplified by soybean and tobacco, the RuBPCase in the dark is in an inactivatable or inhibited form (E_I) with respect to activator CO_2 and Mg^{2+}. When exposed to light *in vivo*, it appears that E_I is converted to the activatable form (E) by some unknown mechanism. In soybean, this conversion is rapid as it occurs within 5 min at high irradiance, and requires a PAR level of 600 to 800 μmol/m^2·s (15). The E state can then be activated with CO_2 and Mg^{2+} to create the catalytic state. Thus, in some species, light appears to have at least two effects in the activation of RuBPCase: (a) to convert E_I to E; and (b) to raise the stromal Mg^{2+} and pH sufficiently to produce the ECM form of the enzyme. The data for field-grown soybeans suggest that for much of the day the E_I

Table II. *The HCO_3^-/Mg^{2+}-Activated RUBPCase Activity in Extracts of Plants from Various Photosynthetic Categories*

Leaves were sampled in the light or dark. Mean values ± SD are presented. The percent change in the light-exposed leaves is calculated as [(light/dark) − 1] 100.

Plant	Photosynthetic Category	HCO_3^-/Mg^{2+}-Activated RuBPCase Activity		Change
		Dark-sampled leaf extract	Light-sampled leaf extract	
		μmol CO_2/mg Chl·h		%
Panicum bisulcatum	C_3	156.2 ± 4.8	217.7 ± 5.3	39.4
Panicum hylaeicum	C_3	121.6 ± 2.8	160.0 ± 4.3	31.6
Glycine max	C_3	235.5 ± 8.1	598.6 ± 14.5	154.2
Lycopersicon esculentum	C_3	268.8 ± 5.9	393.7 ± 8.2	46.5
Nicotiana tabacum	C_3	280.0 ± 7.6	622.4 ± 11.7	122.3
Triticum aestivum	C_3	599.1 ± 13.6	585.6 ± 26.9	−2.3
Panicum maximum	C_4 PEP-CK	90.5 ± 3.1	234.0 ± 5.1	158.6
Panicum miliaceum	C_4 NAD-ME	50.8 ± 2.1	51.7 ± 2.5	1.8
Zea mays (field)	C_4 NADP-ME	362.8 ± 14.2	365.1 ± 7.1	0.6
Zea mays (greenhouse)	C_4 NADP-ME	199.9 ± 4.2	198.8 ± 4.5	−0.6
Sorghum bicolor	C_4 NADP-ME	126.8 ± 5.9	140.1 ± 7.6	10.5
Panicum milioides	C_3/C_4	123.9 ± 5.6	187.5 ± 3.0	51.3
Moricandia arvensis	C_3/C_4	488.6 ± 8.1	521.0 ± 8.0	6.6
Ananas comosus	CAM	0.4 ± 0.1	13.9 ± 0.3	3375
Bromelia pinguin	CAM	3.1 ± 0.8	87.7 ± 13.1	2729
Hydrilla verticillata	SAM	84.5 ± 4.2	78.8 ± 3.9	−6.8

to E conversion is the greater limitation (15).

Our current working hypothesis is that the E_I state may be the physiological counterpart of the EF state reported for purified enzyme in the presence of negative effectors (5). It has been suggested that RuBP might act as an *in vivo* negative effector to influence the degree of activation (14). For purified spinach enzyme, the half-life for release of RuBP from the inactive enzyme (EF state) was 60 min at 24°C (6). Similarly, in soybean, the dark-induced state was quite stable *in vitro*, as it was not altered by Sephadex G-25 gel filtration of the crude, dark extract (13, 16). If RuBP binding was responsible for the *in vivo* E_I state in soybean, then some light-dependent mechanism must substantially alter the tight binding and long half-life.

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