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Reversible Light-Activation of Ribulose Bisphosphate Carboxylase/Oxygenase in Isolated Barley Protoplasts and Chloroplasts

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ABSTRACT

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase displayed near-maximal activity in isolated, intact barley (*Hordeum vulgare* L. cv. Pennrad) mesophyll protoplasts. The carboxylase deactivated 40 to 50% *in situ* when protoplasts were dark-incubated 20 minutes in air-equilibrated solutions. Enzyme activity was fully restored after 1 to 2 minutes of light. Addition of 5 millimolar $NaHCO_3$ to the incubation medium prevented dark-inactivation of the carboxylase. There was no permanent CO_2-dependent activation of the protoplast carboxylase either in light or dark. Activation of the carboxylase from ruptured protoplasts was not increased significantly by *in vitro* preincubation with CO_2 and Mg^{2+}. In contrast to the enzyme in protoplasts, the carboxylase in intact barley chloroplasts was not fully reactivated by light at atmospheric CO_2 levels. The lag phase in carbon assimilation was not lengthened by dark-adapting protoplasts to low CO_2 demonstrating that light-activation of the carboxylase was not involved in photosynthetic induction. Irradiance response curves for reactivation of the the carboxylase and for CO_2 fixation by isolated barley protoplasts were similar. The above results show that there was a fully reversible light-activation of the carboxylase in isolated barley protoplasts at physiologically significant CO_2 levels.

The enzyme RuP_2[1] carboxylase/oxygenase catalyzes the initial reactions of both the photorespiratory and reductive pentose phosphate pathways (5). The enzyme binds CO_2 in a rate-determining step and subsequently reacts with Mg^{2+} to form the catalytically active enzyme-CO_2-Mg^{2+} complex (9). Formation of the active ternary complex is readily reversible, pH-dependent, and occurs at a discrete activator site on the molecule (8, 9).

The role of this activation process *in vivo* is still uncertain. The enzyme in freshly prepared, intact spinach chloroplasts was about half-maximally activated when assayed by a rapid lysis procedure (1) and was further activated or inactivated *in situ* by altering the CO_2 level in solution. Illumination also activated the carboxylase in the intact chloroplast as a result of light-dependent increases of stromal pH and Mg^{2+} (1, 3). In contrast to these findings with spinach chloroplasts, Robinson *et al.* (12) reported that there were no significant light or CO_2-dependent changes in the activation of RuP_2 carboxylase in isolated, intact wheat or barley protoplasts. These authors demonstrated that the carboxylase *in vivo* remained activated after illumination and suggested that the low carboxylase activities observed in spinach chloroplasts were the result of enzyme deactivation during the isolation procedure.

Perchorowicz *et al.* (11) and Machler and Nosberger (10) independently observed an activation of RuP_2 carboxylase in attached wheat leaves when seedlings were shifted from low to high irradiances. These results with enzyme extracts prepared from wheat leaves in equilibrium with air-levels of CO_2 suggested that previous findings with protoplasts should be reexamined under a variety of incubation conditions. This report describes a modulation of RuP_2 carboxylase activity in isolated barley protoplasts by CO_2 and light.

MATERIALS AND METHODS

Protoplast and Chloroplast Isolation. Intact mesophyll protoplasts were isolated from 7- to 9-d-old barley (*Hordeum vulgare* L. cv. Pennrad) seedlings that were grown in a greenhouse in plastic pots containing vermiculite and were watered daily with complete nutrient solution (16). Daylengths were extended to 14 h with high pressure Na lamps. Protoplasts were prepared daily from leaves of plants that had been in light for 1 to 2 h (2). Leaf pieces (0.5 mm) were digested in the dark for 1.5 h at 30°C in medium (pH 5.5) containing 0.5 M sorbitol, 1% (w/v) Cellulase (Vega Biochemicals, Tucson, AZ), 0.5% (w/v) Macerase (Calbiochem-Behring), and 1 mM $CaCl_2$. Subsequent filtration and centrifugation steps were performed at 0°C as described by Edwards *et al.* (2) except that solutions contained 1 mM $MgCl_2$ instead of $CaCl_2$.

For chloroplast preparation, intact protoplasts were suspended in medium containing 0.33 M sorbitol, 50 mM MeS-NaOH (pH 6.5), 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, 2 mM sodium isoascorbate, and 0.1% (w/v) BSA at 0°C and were ruptured by one passage through a 20 μm nylon net (4). The chloroplast suspension was collected by centrifugation (200*g*, 45 s) and the resulting pellet was resuspended in 0.33 M sorbitol, 50 mM Hepes-NaOH (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. Chloroplasts and protoplasts were used immediately upon isolation.

Photosynthesis Measurements. Light-dependent $^{14}CO_2$ fixation by barley mesophyll protoplasts was measured at 25°C in 0.6 ml medium containing 0.4 M sorbitol, 50 mM Hepes-NaOH (pH 7.6), 1 mM $MgCl_2$, 4.8 mM $NaH^{14}CO_3$ (0.33 Ci/mol), and protoplasts (0.02–0.03 mg Chl/ml). Aliquots (0.05 ml) were removed at indicated times and acid stable counts were determined by liquid scintillation counting (14). Illumination (600 $\mu E/m^2 \cdot min$ PAR) was provided by a bank of eight, 1,500-w cool-white fluorescent bulbs (F48T12; General Electric[2]) and irradiance was decreased

[1] Abbreviation: RuP_2, ribulose-1,5-bisphosphate.

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

where indicated with stainless steel wire screens (Newark Wire Cloth Co., Newark, NJ).

RuP_2 Carboxylase Activity Determinations. For enzyme activity measurements intact protoplasts (0.02–0.05 mg Chl/ml) were incubated in 0.5 to 1.0 ml medium containing 0.4 M sorbitol, 50 mM Hepes-NaOH (pH 7.6), 1 mM $MgCl_2$, and $NaHCO_3$ as indicated. For experiments at atmospheric levels of CO_2, solutions were prepared with CO_2-free NaOH and were purged with air for 30 min prior to use. Incubations were performed at 25°C in 5 ml plastic sample cups sealed with rubber serum-bottle stoppers. The air space (1 ml maximum volume) above the protoplast suspension was flushed with humidified air at flow rates of 40 to 60 ml/min. Samples were illuminated as described for photosynthesis measurements and protoplasts were kept from settling by gently shaking the sample vials at 2 or 3 min intervals. Barley chloroplasts were incubated in sealed sample cups as previously described (15).

To assay RuP_2 carboxylase activity, a 0.025-ml sample of the protoplast or chloroplast suspension was injected into 0.25 ml medium containing 50 mM Bicine-NaOH (pH 8.1), 10 mM $MgCl_2$, 9.6 mM $NaH^{14}CO_3$ (0.33 Ci/mol), and 0.6 mM RuP_2 (1, 12). Assays were terminated after 30 s and acid-stable radioactivity was determined by liquid scintillation counting (14). There was no lag in $^{14}CO_2$ fixation if protoplasts were broken by passage through a 26 gauge syringe needle prior to mixing with the sorbitol-free assay medium.

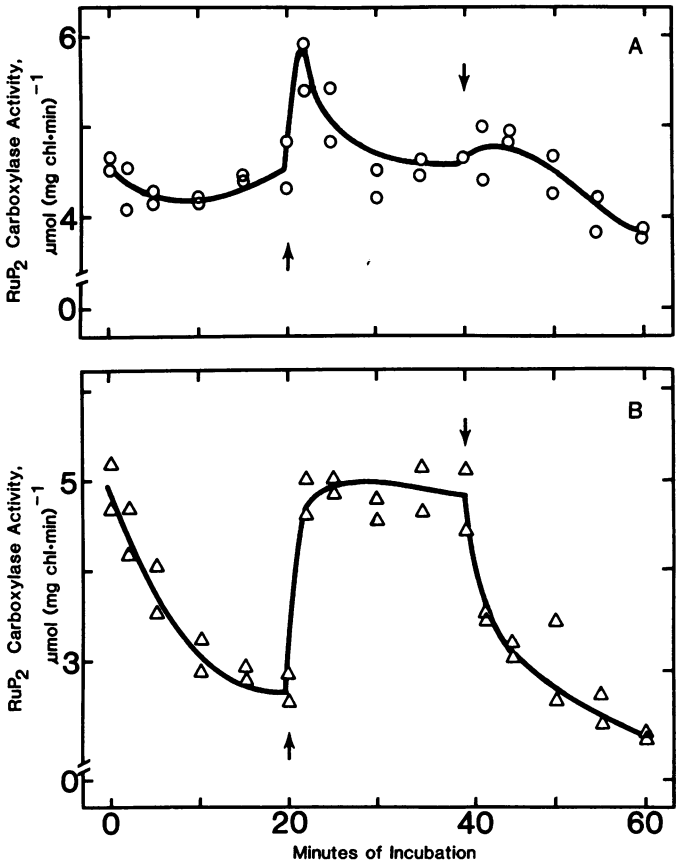

FIG. 1. Effects of light and CO_2 on the *in situ* activation of RuP_2 carboxylase in intact barley protoplasts. A, Protoplasts (0.048 mg Chl) were incubated in 1.0 ml medium containing 0.4 M sorbitol, 50 mM Hepes-NaOH (pH 7.6), 1 mM $MgCl_2$, and 5 mM $NaHCO_3$. Samples (0.025 ml) were removed at indicated times and assayed for RuP_2 carboxylase activity (see under "Materials and Methods"). Arrows (↑[on], ↓[off]) indicate when illumination was provided. Data are given for experiments performed on consecutive days. B, Conditions were as in A except that $NaHCO_3$ was not added to the incubation medium.

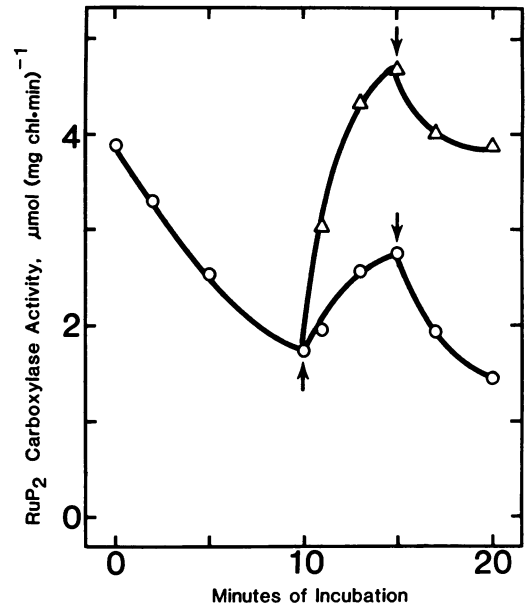

FIG. 2. Effects of CO_2 and light on the *in situ* activation of RuP_2 carboxylase in isolated barley chloroplasts. A 0.05-ml sample of intact barley chloroplasts (0.012 mg Chl) was added to 0.05 ml medium containing 0.33 M sorbitol, 50 mM Hepes-NaOH (pH 7.6), 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 1.8 mM sodium isoascorbate. Solutions were preequilibrated with air for 30 min prior to the addition of chloroplasts. After 10 min of dark incubation at air-levels of CO_2, samples were illuminated for 5 min either with (△) or without (○) 6.9 mM $NaHCO_3$. Arrows (↑[on], ↓[off]) indicate when illumination was provided.

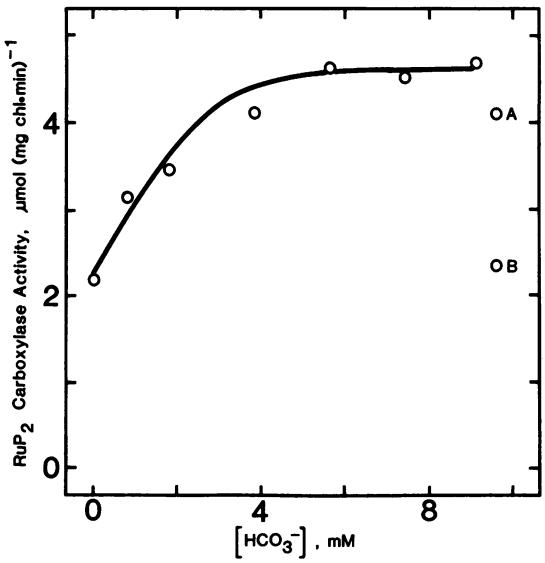

FIG. 3. Dark reactivation of barley protoplast RuP_2 carboxylase by CO_2. Intact barley protoplasts (0.019 mg Chl) were dark-incubated 20 min in 0.5 ml medium containing 0.4 M sorbitol, 50 mM Hepes-NaOH (pH 7.6), and 1 mM $MgCl_2$ prior to the addition of $NaHCO_3$. Samples were removed after an additional 10 min incubation with $NaHCO_3$, and RuP_2 carboxylase activity was determined as described in "Materials and Methods." Barley protoplast RuP_2 carboxylase activities when initially isolated (A) and after 20 min of dark inactivation (B) are shown.

RESULTS AND DISCUSSION

Light-Dependent Activation of RuP_2 Carboxylase in Intact Barley Protoplasts. *In situ* activation of RuP_2 carboxylase in intact barley protoplasts was not increased during a 20-min dark incu-

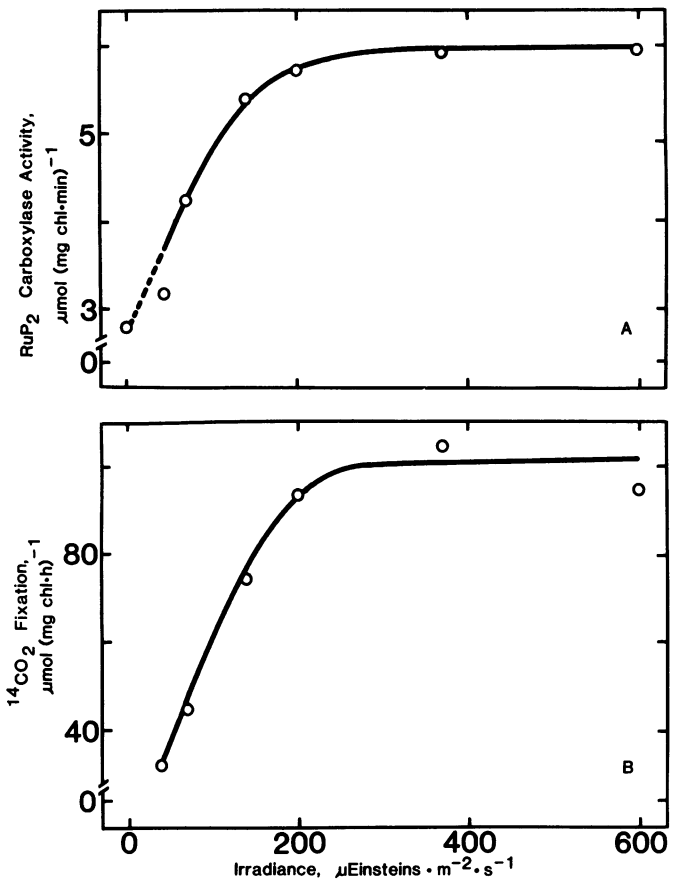

FIG. 4. Rates of $^{14}CO_2$ fixation and reactivation of RuP_2 carboxylase in intact barley protoplasts as a function of irradiance. A, Intact barley protoplasts (0.014 mg/Chl) were dark preincubated 20 min in 0.48 ml medium as in Figure 3. After an additional 10 min of illumination, RuP_2 carboxylase activities were determined (see under "Materials and Methods"). B, Conditions were as in A except that samples were made 5 mM with $NaH^{14}CO_3$ (0.33 Ci/mol) immediately before illumination. Rates of $^{14}CO_2$ fixation were determined between 5 and 10 min of light.

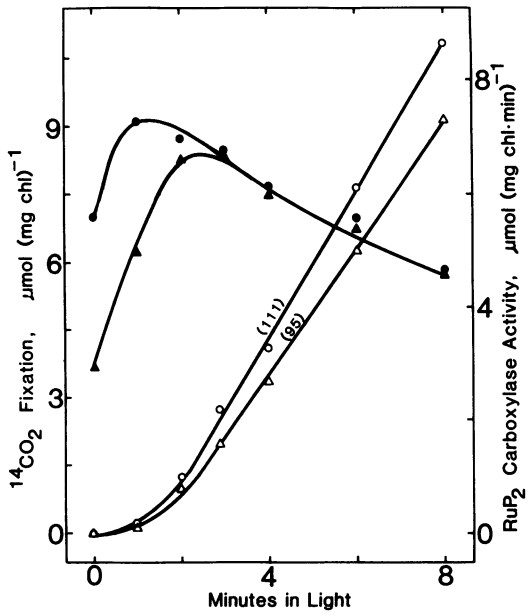

FIG. 5. Effect of light-activation of RuP_2 carboxylase on the lag in $^{14}CO_2$ fixation by intact barley protoplasts. Amounts of $^{14}CO_2$ fixed (○, △) by intact barley protoplasts (0.015 mg Chl) and activation of RuP_2 carboxylase (●, ▲) were measured (see under "Materials and Methods") either before (○, ●) or after △, ▲) samples were dark-incubated 20 min as described in Figure 3. Samples were made 4.8 mM with $NaH^{14}CO_3$ (0.33 Ci/mol) prior to illumination. Maximum rates of $^{14}CO_2$ fixation (μmol CO_2/mg Chl·h) are shown in parentheses.

bation period with 5 mM $NaHCO_3$ (Fig. 1A). Results are presented from experiments performed on consecutive days and demonstrated that carboxylase activity in isolated protoplasts was similar from preparation to preparation. There was a transient (2–5 min) 1.2-fold activation of the carboxylase upon illumination and then enzyme activity decreased about 20% during a final 20-min dark period. These results confirm previous findings (12) that there are no major light-dark changes in RuP_2 carboxylase activation in isolated barley protoplasts incubated with high CO_2.

Carboxylase activity decreased about 50% when intact barley protoplasts were dark-incubated 20 min in sorbitol medium equilibrated with air-levels of CO_2 (Fig. 1B). The enzyme was fully reactivated after 2 min of illumination and then deactivated about 50% in 20 min when illumination was discontinued. Lilley and Walker (7) calculated with CO_2 concentration in sorbitol medium in equilibrium with air at 20°C and pH 7.6 to be about 11 μM. Activation of the carboxylase in illuminated protoplasts remained high for 20 min regardless of low external CO_2 concentrations. Added $NaHCO_3$ did not increase enzyme activation in the light, suggesting that air-levels of CO_2 were saturating for light-activation of the protoplast carboxylase. These results show that light-activation of the barley protoplast carboxylase is reversible at low CO_2 and that failure to observe a dark enzyme inactivation in Figure 1A was due to a CO_2-dependent activation of the enzyme by added $NaHCO_3$.

In agreement with the findings of Robinson *et al.* (12), carboxylase activity in isolated barley protoplasts was 80 to 100% of the maximum value observed after 5 to 10 min of *in vitro* preincubation with 10 mM $NaHCO_3$ and $MgCl_2$ at pH 8.1 (data not shown). There was a rapid activation ($t_{0.5}$ of 30 s) of the carboxylase in ruptured protoplasts by CO_2 and Mg^{2+} if the enzyme was previously inactivated *in situ* by a 20-min dark incubation at air-levels of CO_2. This finding indicated that preincubation conditions were optimal for enzyme activation. Inasmuch as there were no significant CO_2-dependent increases in enzyme activation either *in situ* or *in vitro*, it was concluded that the carboxylase in freshly prepared barley protoplasts was near-maximally activated.

Light-Dependent Activation of RuP_2 Carboxylase in Intact Barley Chloroplasts. Initial RuP_2 carboxylase activity in isolated, intact barley chloroplasts decreased about 50% following 10 min of dark incubation without added $NaHCO_3$ at 25°C and pH 7.8 (Fig. 2). Similar results have been published for the spinach chloroplast enzyme which was almost fully deactivated after 30 or 40 min in the dark without CO_2 (1, 15). There was a partial light-activation of the barley chloroplast carboxylase at air-levels of CO_2; however, addition of $NaHCO_3$ was necessary to attain complete enzyme reactivation. Both barley (R. C. Sicher unpublished) and spinach (1) chloroplast carboxylase activities decreased with prolonged illumination in the absence of CO_2. Compared to protoplasts, the enzyme in barley chloroplasts deactivated faster and to a greater extent in the dark and achieved less reactivation in light at air-levels of CO_2.

Reactivation of Dark-Inactivated RuP_2 Carboxylase in Barley Protoplasts by CO_2 and Light. Following a 20 min dark incubation in air-equilibrated sorbitol medium, about 2 mM $NaHCO_3$ (57 μM CO_2 [pH 7.6]) was required to half-maximally reactivate the carboxylase in intact barley protoplasts (Fig. 3). In similar experiments, Bahr and Jensen (1) found that 122 μM CO_2 (pH 7.8) half-maximally reactivated the carboxylase in intact spinach chloro-

plasts. Results of Figure 4 demonstrate that deactivation of RuP_2 carboxylase in intact protoplasts will occur only at low CO_2 concentrations and not under conditions usually employed for photosynthesis measurements.

The irradiance response of $^{14}CO_2$ fixation by isolated barley protoplasts with saturating $NaHCO_3$ is shown in Figure 4B. Maximum rates of carbon assimilation were observed above 200 $\mu E/m^{-3}\ s^{-1}$. Light-activation of RuP_2 carboxylase in intact barley protoplasts exhibited a similar response to increasing irradiance at air-levels of CO_2 (Fig. 4A). Perchorowicz *et al.* (11) also observed a correlation between the irradiance dependence of photosynthetic CO_2 exchange and light-activation of RuP_2 carboxylase in wheat leaves. The rate of *in vitro* $^{14}CO_2$ fixation by RuP_2 carboxylase was almost 180 μmol/mg Chl·h after *in situ* deactivation (Fig. 4A). This enzyme activity in the dark exceeded the light-saturated rate of carbon assimilation by intact barley protoplasts.

Photosynthetic Induction and Light-Activation of RuP_2 Carboxylase in Barley Protoplasts. Upon illumination, isolated protoplasts and chloroplasts exhibit a lag of several minutes before maximum rates of $^{14}CO_2$ fixation are attained (13). Walker and his colleagues (6, 13) concluded that the initial lag in photosynthetic carbon assimilation was not attributable to light-activation of the carboxylase, which did not increase significantly in isolated wheat chloroplasts or protoplasts during the induction period. However, these studies were conducted with samples in which the carboxylase was predominantly in the activated form. Results of Figure 5 show that the lag in carbon assimilation was independent of the initial activation level of the carboxylase in isolated barley protoplasts. These findings confirm previous results (6, 13) suggesting that light-activation of RuP carboxylase was not involved in photosynthetic induction.

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