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

Richard C. Sicher
United States Department of Agriculture/ARS-PPHI, Light and Plant Growth Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland 20705

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 NaHCO₃ to the incubation medium prevented dark-inactivation of the carboxylase. There was no permanent CO₂-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 CO₂ and Mg²⁺. In contrast to the enzyme in protoplasts, the carboxylase in intact barley chloroplasts was not fully reactivated by light at atmospheric CO₂ levels. The lag phase in carbon assimilation was not lengthened by dark-adapting protoplasts to low CO₂ concentrations. Photosynthetic light-activation of the carboxylase was not involved in photosynthetic induction. Irradiance response curves for reactivation of the carboxylase and for CO₂ 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 CO₂ levels.

MATERIALS AND METHODS

Protoplast and Chloroplast Isolation. Intact mesophyll protoplasts were isolated from 7- to 9-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 (Veget Biochemicals, Tucson, AZ), 0.5% (w/v) Macerase (Calbiochem-Behring), and 1 mM CaCl₂. Subsequent filtration and centrifugation steps were performed at 0°C as described by Edwards et al. (2) except that solutions contained 1 mM MgCl₂ instead of CaCl₂.

For chloroplast preparation, intact protoplasts were suspended in medium containing 0.33 m sorbitol, 50 mM Mes-NaOH (pH 6.5), 10 mM Na₃P₂O₇ 5 mM MgCl₂, 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 (200g, 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 MgCl₂, and 1 mM MnCl₂. Chloroplasts and protoplasts were used immediately upon isolation.

Photosynthesis Measurements. Light-dependent ¹⁴CO₂ 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 MgCl₂, 4.8 mM NaH¹⁴CO₃ (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 µE/m²·min PAR) was provided by a bank of eight, 1,500-w cool-white fluorescent bulbs (F48T12; General Electric) and irradiance was decreased to 90% of the initial light intensity.

1 Abbreviation: RuP₂, ribulose-1,5-bisphosphate.
where indicated with stainless steel wire screens (Newark Wire Cloth Co., Newark, NJ).

**RuP2 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 mM sorbitol, 50 mM Hepes-NaOH (pH 7.6), 1 mM MgCl₂ and NaHCO₃ as indicated. For experiments at atmospheric levels of CO₂, solutions were prepared with CO₂-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 RuP₂ 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 MgCl₂, 9.6 mM NaH¹⁴CO₃ (0.33 Ci/mol), and 0.6 mM RuP₂ (1, 12). Assays were terminated after 30 s and acid-stable radioactivity was determined by liquid scintillation counting (14). There was no lag in ¹⁴CO₂ fixation if protoplasts were broken by passage through a 26 gauge syringe needle prior to mixing with the sorbitol-free assay medium.

**RESULTS AND DISCUSSION**

Light-Dependent Activation of RuP₂ Carboxylase in Intact Barley Protoplasts. In situ activation of RuP₂ carboxylase in intact barley protoplasts was not increased during a 20-min dark incu-
Fig. 4. Rates of 14CO2 fixation and reactivation of RuP2 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, RuP2 carboxylase activities were determined (see under "Materials and Methods"). B, Conditions were as in A except that samples were made 5 mM with NaH14CO3 (0.33 Ci/mol) immediately before illumination. Rates of 14CO2 fixation were determined between 5 and 10 min of light.

Fig. 5. Effect of light-activation of RuP2 carboxylase on the lag in 14CO2 fixation by intact barley protoplasts. Amounts of 14CO2 fixed (○, △) by intact barley protoplasts (0.015 mg Chl) and activation of RuP2 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 NaH14CO3 (0.33 Ci/mol) prior to illumination. Maximum rates of 14CO2 fixation (μmol CO2/mg Chl-h) are shown in parentheses.

bation period with 5 mM NaHCO3 (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 RuP2 carboxylase activation in isolated barley protoplasts incubated with high CO2.

Carboxylase activity decreased about 50% when intact barley protoplasts were dark-incubated 20 min in sorbitol medium equilibrated with air-levels of CO2 (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 CO2 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 CO2 concentrations. Added NaHCO3 did not increase enzyme activation in the light, suggesting that air-levels of CO2 were saturating for light-activation of the protoplast carboxylase. These results show that light-activation of the barley protoplast carboxylase is reversible at low CO2 and that failure to observe a dark enzyme inactivation in Figure 1A was due to a CO2-dependent activation of the enzyme by added NaHCO3.

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 NaHCO3 and MgCl2 at pH 8.1 (data not shown). There was a rapid activation (f0.5 of 30 s) of the carboxylase in ruptured protoplasts by CO2 and Mg2+ if the enzyme was previously inactivated in situ by a 20-min dark incubation at air-levels of CO2. This finding indicated that preincubation conditions were optimal for enzyme activation. Inasmuch as there were no significant CO2-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 RuP2 Carboxylase in Intact Barley Chloroplasts. Initial RuP2 carboxylase activity in isolated intact barley chloroplasts decreased about 50% following 10 min of dark incubation without added NaHCO3 at 25°C and pH 7.8 (Fig. 2). Similar results have been published for the spinach chloroplast enzyme which was also almost completely deactivated after 30 or 40 min in the dark without CO2 (1, 15). There was a partial light-activation of the barley chloroplast carboxylase at air-levels of CO2; however, addition of NaHCO3 was necessary to attain complete enzyme activation. Both barley (R. C. Sichert unpublished) and spinach (1) chloroplast carboxylase activities decreased with prolonged illumination in the absence of CO2. 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 CO2.

Reactivation of Dark-Inactivated RuP2 Carboxylase in Barley Protoplasts by CO2 and Light. Following a 20 min dark incubation in air-equilibrated sorbitol medium, about 2 mM NaHCO3 (57 μM CO2 [pH 7.6]) were required to half-maximally reactivate the carboxylase in intact barley protoplasts (Fig. 3). In similar experiments, Bahr and Jensen (1) found that 122 μM CO2 (pH 7.8) half-maximally reactivated the carboxylase in intact spinach chloro-
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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$^2$/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). Perchlorowicz 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 $\mu$mol/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 dependent on 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.

LITERATURE CITED


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