Photoregulation of Fructose and Glucose Respiration in the Intact Chloroplasts of *Chlamydomonas reinhardtii* F-60 and Spinach

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The photoregulation of chloroplastic respiration was studied by monitoring in darkness and in light the release of \(^{14}\)CO\(_2\) from whole chloroplasts of *Chlamydomonas reinhardtii* F-60 and spinach (*Spinacia oleracea* L.) supplied externally with \([^{14}\text{C}]\)glucose and \([^{14}\text{C}]\)fructose, respectively. CO\(_2\) release was inhibited more than 90% in both chloroplasts by a light intensity of 4 W m\(^{-2}\). Oxidants, oxaloacetate in *Chlamydomonas*, nitrite in spinach, and phenazine methosulfate in both chloroplasts, reversed the inhibition. The onset of the photoinhibitory effect on CO\(_2\) release was relatively rapid compared to the restoration of CO\(_2\) release following illumination. In both darkened chloroplasts, dithiothreitol inhibited release. Of the four enzymes (fructokinase, phosphoglucose isomerase, glucose-6-P dehydrogenase, and gluconate-6-P dehydrogenase) in the pathway catalyzing the release of CO\(_2\) from fructose, only glucose-6-P dehydrogenase was deactivated by light and by dithiothreitol.

There is considerable evidence that within the chloroplast the upper reactions of glycolysis (Glc-6-P to glyceraldehyde-3-P) and the oxidative pentose-P pathway are responsible for carbohydrate degradation (Heber et al., 1967; Krause and Bassham, 1969; Stitt and ap Rees, 1980; Klein, 1986; Gibbs et al., 1990) and coexist with the enzymes of the photosynthetic carbon reduction cycle. It is now widely accepted that light/dark modulation of chloroplastic enzymic activity is an important process that directs metabolism in the light to synthesize carbohydrates and other storage products and to degrade these compounds (principally starch) in the dark (Buchanan, 1980; Anderson, 1986; Scheibe, 1991).

Of the two pathways involved in carbohydrate dissimilation, light appears to regulate mainly the oxidative pentose-P pathway (Anderson et al., 1974; Anderson and Duggan, 1976). Reactivation of Glc-6-P dehydrogenase is possible achieved in the dark by a reversal of the Fd-thioredoxin system coupled to O\(_2\) (Scheibe and Anderson, 1981; Schurman, 1983). An additional effect of light on metabolites in intact chloroplasts is a striking shift in the NADPH:NADP ratio from 0.1 to 0.3 in the dark up to 2.0 to 2.5 during illumination (Lendzian and Bassham, 1975). It has been suggested that high ratios of NADPH:NADP inhibit the activity of Glc-6-P dehydrogenase (Wildner, 1975; Lendzian and Bassham, 1975) and of the glycolytic enzyme phosphofructokinase (Cseke et al., 1982) within the illuminated chloroplast.

The occurrence and regulatory properties of both the oxidative pentose-P and glycolytic pathways have been demonstrated in the soluble fraction of lysed spinach (*Spinacia oleracea* L.) chloroplasts (Kaiser and Bassham, 1979). The broken preparation is a useful tool to evaluate the regulatory properties of the two pathways; nevertheless, experimental conditions are limited mainly to changes in substrate concentrations. Also, the physiological significance of some of the findings may be questioned, because the level of intermediary metabolites in the extract are unlike those in the intact chloroplast. This is the result of an approximately 500-fold volume difference between the extract and the stromal compartment of the intact chloroplast. Therefore, an investigation of the regulatory properties of carbohydrate degradation in the intact chloroplast seemed warranted.

In this report, we turn our attention to the photoregulation of carbohydrate degradation in the intact chloroplast. This was accomplished by monitoring in darkness and in light the release of \(^{14}\)CO\(_2\) from intact chloroplasts of *Chlamydomonas reinhardtii* F-60 and spinach externally supplied with \(^{14}\)C-sugars. Our experimental approach is based on two findings: (a) the oxidative pentose-P pathway appears to be solely responsible for the release of CO\(_2\) from Glc and Fru supplied externally to whole chloroplasts (Stitt and ap Rees, 1980; Ahluwalia et al., 1989; Chen and Gibbs, 1991) and (b) gluconate-6-P dehydrogenase, the second enzyme and the one that catalyzes the decarboxylative step of the oxidative pentose-P pathway, is not responsive to light (Lendzian and Ziegler, 1970).

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Abbreviations: OAA, oxaloacetate; PMS, phenazine methosulfate.
MATERIALS AND METHODS

Plant Material and Chloroplast Isolation

Spinach (Spinacia oleracea L.) var Longstanding Bloomsdale (purchased from Agway, Inc., Waltham, MA) was grown and used to prepare intact chloroplasts as described earlier (Kow et al., 1977). Chlamydomonas reinhardtii 137C (+) and 137C (−) and the mutant strain F-60 (obtained from Professor R.K. Togasaki, Indiana University, Bloomington, IN) were grown under fluorescent lights on acetate-supplemented medium as described previously (Willeford and Gibbs, 1989). The procedure for isolating chloroplasts from F-60 followed those of Klein et al. (1983).

14CO2 Measurements

Rates of CO2 evolution were determined by adding intact chloroplasts of F-60 to a reaction mixture containing 50 mM Tris-HCl (pH 8.2), 120 mM mannitol, 2 mM EDTA, 1 mM MgCl2, 1 mM MnCl2, 2 mM MgATP, and [U-14C]Glc. In the case of spinach, intact chloroplasts were added to a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl2, 1 mM MnCl2, 2 mM MgATP, and [U-14C]Fru.

The reaction mixtures were incubated in a Gilson respirometer bath maintained at approximately 25°C for 10 min in the main compartments of 15-mL Warburg flasks carrying the chloroplast suspension in a sidearm and 0.3 mL of 2 M triethanolamine in the center well. The vessels were sealed with serum stoppers, and the reactions were initiated by tipping in the chloroplasts from the sidearm to bring the final reaction mixture to 1.0 mL.

When needed, light was provided by General Electric 75-W reflector flood bulbs in the Gilson respirometer. Different light intensities were obtained by a dimming device and by shading the Warburg flasks with a varying number of stainless steel screens. Darkness was achieved by wrapping the Warburg flasks with two layers of aluminum foil.

Following termination of the reaction by addition of perchloric acid to a final concentration of 5% (v/v), the flasks were shaken slowly for an additional 1 h to allow the released 14CO2 to be absorbed totally by the triethanolamine. The triethanolamine was transferred into a scintillation via1 container containing SML biodegradable counting scintillant (Amersham). Radioactivity was determined in a Beckman L5-150 liquid scintillation counter.

Photosynthetic reassimilation of respired 14CO2 by the spinach chloroplasts was prevented by inclusion of Dl-glyceraldehyde in the reaction medium (Stokes and Walker, 1972). Inasmuch as F-60 cannot reassimilate respired CO2 because of the lack of ribulose-5-P kinase (Moll and Levine, 1970), glyceraldehyde was not included in the reaction mixtures containing Chlamydomonas chloroplasts.

Enzyme Assays and Chl Determination

Intact chloroplasts (with a Chl concentration of about 15 

μg mL−1) were used directly in the assay mixtures. Triton X-100 at a final concentration of 0.1% was added to each assay to lyse the chloroplasts completely and make the enzymes accessible to added cofactors and substrates. The reaction mixture for Glc-6-P dehydrogenase was 25 mM Tris-HCl (pH 8.2), 7 mM MgCl2, 0.33 mM NADP, and 5 mM Glc-6-P. For gluconate-6-P dehydrogenase, the Glc-6-P was replaced by 5 mM gluconate-6-P. Fructokinase and phosphoglucose isomerase were assayed by the methods of Turner and Copeland (1981). The rate of formation of NADPH was measured with a Gilford recording spectrophotometer (model 250). The specific activities of the enzymes were determined on a per mg Chl basis, so that, if necessary, a correction could be made for extraplastidic contamination (Klein, 1986). Chl was determined by the method of Arnon (1949).

Reagents

Radioactive sugars were purchased from ICN Radiochemicals and DuPont NEN. Unlabeled materials were purchased from Sigma.

RESULTS AND DISCUSSION

In an earlier communication concerning spinach chloroplasts (Ahluwalia et al., 1989), Glc was used as the respirable substrate. In this study (details will be dealt with in a forthcoming report), Fru was the sugar externally added to the spinach chloroplasts, because 14CO2 is evolved from Fru at a higher rate than from Glc. An enzymic profile of fructokinase and glucokinase in isolated spinach chloroplasts is consistent with this observation (Schnarrenberger, 1990).

CO2 evolution from [U-14C]Glc in F-60 and from [U-14C]Fru in spinach chloroplasts was strongly inhibited by light...
even at extremely low intensity (Fig. 1). One explanation for our results is a light-induced increase in the rate of glycolysis, which would limit carbon entering the oxidative pentose-P pathway. Phosphofructokinase, the regulatory enzyme of glycolysis, is believed not to be stimulated by light (Kelly and Latzko, 1977) but is inhibited by NADPH (Cseke et al., 1982), a chloroplast reductant that is prevalent in the light. In addition, we found (data not shown) that light did not increase the rate of CO₂ evolution in the presence of glycolate-2-P, an inhibitor of phosphofructokinase. In contrast, this inhibitor enhances almost 2-fold CO₂ evolution from *Chlamydomonas* and spinach chloroplasts externally supplied with sugars in the dark (Ahluwalia et al., 1989; Chen and Gibbs, 1991).

The time course of CO₂ evolution and the effect of a light intensity of 0.5 W m⁻² (approximately laboratory background illumination) on the rate of CO₂ evolution in the spinach chloroplast is presented in Figure 2. The dark rate of CO₂ evolution as reported earlier for the *Chlamydomonas* chloroplast (Chen and Gibbs, 1991) is approximately linear after a brief lag up to 30 min. At 0.5 W m⁻², light deactivates the system about 50% within 2.5 min, and the full effect is seen after 20 min.

If accumulation of light-driven reductants results in the inhibition of sugar respiration in the *Chlamydomonas* and spinach chloroplasts, it is reasonable to expect that supplementation of the reaction mixtures with oxidants can reverse the effect. To this end, OAA was added to the *Chlamydomonas* and nitrite to the spinach chloroplast suspension (Table 1). Nitrite was not suitable for the algal chloroplast because F-60 lacks nitrite reductase. In illuminated *Chlamydomonas* chloroplasts, 5 mM OAA enhanced CO₂ evolution by 6-fold but did not reverse completely the inhibition of Glc respiration by light. In the illuminated spinach chloroplast, 100 μM nitrite enhanced CO₂ evolution about 15-fold and essentially restored the dark rate. It is worth noting that OAA functioned poorly as an oxidant in both the dark and illuminated spinach chloroplast. As an example, 10 mM OAA enhanced the dark rate of CO₂ about 15% and had no effect on the inhibition resulting from an intensity of 0.5 W m⁻² (data not shown).

The operation of a malate/OAA shuttle coupled to stromal malate dehydrogenase can account for the effect of OAA. Using NADPH as a cofactor, OAA can be reduced to malate, thereby maintaining an NADPH:NADP ratio that allows the Glc-6-P dehydrogenase reaction to continue. Additionally, by this sequence, OAA would modulate the Fd/thioredoxin system, preventing deactivation of Glc-6-P dehydrogenase. In the spinach chloroplast, photogenerated-reduced Fd acts as the electron donor for the reduction of nitrite to NH₃ via stromal nitrite reductase, preventing an accumulation of NADPH and the thioredoxin-mediated photodeactivation of Glc-6-P dehydrogenase.

In Table 1, we compare the effects of naturally occurring oxidants, OAA and nitrite, with that of PMS on reversing the light inhibition with increasing intensity. At 4 W m⁻² PMS, but not OAA, totally overcame the inhibition of CO₂ evolution from Glc by F-60 chloroplasts. At the higher light intensity (50 W m⁻²), PMS restored the rate up to 40% of the rate in the dark. Similarly, PMS was more effective than nitrite in the spinach chloroplast. Clearly, at the higher light intensities, the photo-driven electron transport rate exceeds even the accepting capacity of PMS, resulting in an inhibition of the oxidative pentose-P pathway. Inasmuch as photosynthetic fixation of CO₂ by these chloroplasts is saturated at approximately 600 W m⁻², protection against inhibition of CO₂ evolution by a physiological acceptor such as nitrite is restricted to low-light intensities.

The presence of DCMU, an inhibitor of photosynthetic

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**Table 1. Effect of OAA, nitrite, and light on ¹⁴CO₂ release from [U-¹⁴C]Glc in Chlamydomonas and [U-¹⁴C]Fru in spinach chloroplasts**

<table>
<thead>
<tr>
<th></th>
<th>Chlamydomonas</th>
<th>Spinach</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OAA</td>
<td>Nitrite</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>Light</td>
</tr>
<tr>
<td>[μM]</td>
<td>[μM]</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>288</td>
<td>29</td>
</tr>
<tr>
<td>0.5</td>
<td>417</td>
<td>58</td>
</tr>
<tr>
<td>2.5</td>
<td>404</td>
<td>106</td>
</tr>
<tr>
<td>5.0</td>
<td>428</td>
<td>177</td>
</tr>
<tr>
<td>10.0</td>
<td>486</td>
<td>172</td>
</tr>
</tbody>
</table>

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**Figure 2.** Effect of light on the time course of ¹⁴CO₂ release from [U-¹⁴C]Fru in spinach chloroplasts. Included in the reaction mixture described in "Materials and Methods" were 525 μM [U-¹⁴C]Glc (3.3 mCi mmol⁻¹), 23.5 μg of Chl, and OAA. The light intensity was 4 W m⁻². Spinach: Included in the reaction mixtures described in "Materials and Methods" were 500 μM [U-¹⁴C]Fru (2.0 mCi mmol⁻¹), 85 μg of Chl, 5 mM di-glyceraldehyde, and potassium nitrite. The light intensity was 0.5 W m⁻². Light intensity was controlled by shading the flasks with screens. Experiments were carried out at 25°C and were terminated after 30 min with perchloric acid.

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electron transport, would be expected to neutralize the photoinhibition of chloroplastic respiration. In spinach, 10 μM DCMU fully restored CO₂ evolution at an intensity of 0.5 W m⁻² and restored up to 50% at 4 W m⁻². However, inclusion of 10 to 50 μM DCMU in the *Chlamydomonas* reaction mixture did not change the strong inhibitory effect of 0.5 and 4 W m⁻². One possible explanation for the ineffectiveness of DCMU is the presence of an NADPH-plastoquinone oxidoreductase in the *Chlamydomonas* thylakoids, which allows the functioning of a PSI-driven cyclic electron transport through NADPH, resulting in a fairly stable NADPH:NADP ratio (Maione and Gibbs, 1986).

In both illuminated chloroplasts, inhibition was not due to the accumulation of H₂O₂, because addition of catalase, which is usually added to isolated, photosynthesizing chloroplast preparations, did not restore the rate of CO₂ evolution (Chen, 1991).

The kinetics of dark restoration of CO₂ evolution from [U⁻¹⁴C]Fru were measured in the spinach chloroplasts following exposure to 10 min of illumination at 0.5 W m⁻². In contrast to deactivation of the system (Fig. 1), reversal of inactivation occurred slowly, and after 20 min in the dark, less than 50% of the original rate was obtained (Fig. 3). Additionally, nitrite, which protected against inactivation of CO₂ release in the illuminated chloroplast (Table I), had no effect on the recovery process.

**Figure 3.** Reversibility of light inhibition of ¹⁴CO₂ evolution from [U⁻¹⁴C]Fru in spinach chloroplasts. Included in the reaction mixtures described in "Materials and Methods" were 500 μM [U⁻¹⁴C]Glc (3.3 mCi mmol⁻¹), 36.5 μg of Chl, 10 mM OAA, and 10 mM PMS as indicated. Spinach: Included in the reaction mixture were 500 μM [U⁻¹⁴C]Fru (2.0 mCi mmol⁻¹), 2 μg of Chl, 10 mM DTT, and 100 mM KNO₂ as indicated. Experiments were carried out at 25°C for 30 min and were terminated with perchloric acid.

**Table II.** Effect of electron acceptors on ¹⁴CO₂ release from [U⁻¹⁴C]-Glc and [U⁻¹⁴C]-Fru in *Chlamydomonas* and spinach chloroplasts at different light intensities

<table>
<thead>
<tr>
<th>Light Intensity</th>
<th>Chlamydomonas</th>
<th>Light Intensity</th>
<th>Spinach</th>
</tr>
</thead>
<tbody>
<tr>
<td>W m⁻²</td>
<td>CO₂ evolved</td>
<td>W m⁻²</td>
<td>CO₂ evolved</td>
</tr>
<tr>
<td>0</td>
<td>308</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>OAA</td>
<td>329</td>
<td>KNO₂</td>
<td>85</td>
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<tr>
<td>PMS</td>
<td>415</td>
<td>PMS</td>
<td>158</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>OAA</td>
<td>68</td>
<td>KNO₂</td>
<td>58</td>
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<td>446</td>
<td>PMS</td>
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<td>32</td>
</tr>
<tr>
<td>PMS</td>
<td>155</td>
<td>PMS</td>
<td>126</td>
</tr>
</tbody>
</table>

41 mmol mg⁻¹ of Chl h⁻¹. A, Nitrite absent during and after illumination; O, nitrite present during illumination.
Table IV. Effect of DTT and light intensity on Glc-6-P dehydrogenase in spinach chloroplasts

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DTT, 0.1 mm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DTT, 2.5 mm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DTT, 5.0 mm</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Zero time (none)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dark, 30 min (none)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5 W m⁻², 10 min</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>600 W m⁻², 10 min</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>-</td>
</tr>
</tbody>
</table>

Fructokinase, Glc-6-P isomerase, and gluconate-6-P dehydrogenase did not respond to either DTT or illumination (data not shown).

CONCLUSION

Of the four enzymic reactions involved in the release of CO₂ from simple sugars supplied externally to illuminated intact chloroplasts, clearly the Glc-6-P dehydrogenase reaction is the light/dark modulated step. To account for the photoregulation of this dehydrogenase, Anderson and Duggan (1976) have proposed a coordinated regulatory mechanism. The first response to light is an increasing level of NADPH, followed by a secondary and slower reaction now regarded to be catalyzed by the Fd/thioredoxin pathway (Buchanan, 1980; Anderson, 1986; Scheibe, 1991). Our findings are consistent with this proposal. Thus, the striking inhibition of CO₂ release at the extremely low-light intensity of 0.5 W m⁻² was apparently the result of the first response, because the enzymic activity of Glc-6-P dehydrogenase was not affected.

Finally, in whole spinach chloroplasts, the ratio of NADPH:NAD⁺ has been shown to return immediately to the dark level when the light is turned off (Lendzian and Bassham, 1975). Activation of light-deactivated Glc-6-P dehydrogenase is also rapid (Anderson and Avron, 1976; Yuan and Anderson, 1987). However, restoration of CO₂ release following illumination is comparatively slow. The present data indicate the possible involvement of an additional factor in the intact chloroplast distinct from the NADPH:NAD⁺ ratio and reactivation of the dehydrogenase reaction that regulates the restorative period.

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