Photosynthesis and Ribulose 1,5-Bisphosphate Levels in Intact Chloroplasts

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ABSTRACT
The response of ribulose 1,5-bisphosphate levels and CO₂ fixation rates in isolated, intact spinach chloroplasts to pyrophosphate, triose phosphates, di-1-glyceraldehyde, O₂, catalase, and irradiance during photosynthesis has been studied. Within 1 minute in the light, a rapid accumulation of ribulose bisphosphate was measured in most preparations of intact chloroplasts, and this subsequently dropped as CO₂ fixation increased. Pyrophosphate, triose phosphates, and catalase increased CO₂ fixation and also the levels of ribulose bisphosphate. CO₂ fixation was inhibited by di-1-glyceraldehyde and O₂ with corresponding decreases in ribulose bisphosphate. When the rate of photosynthesis decreased at limiting irradiances (low light), the level of ribulose bisphosphate in the chloroplast did not always decrease, suggesting that ribulose bisphosphate was not limiting CO₂ fixation under these conditions. When triose phosphates (fructose bisphosphate plus aldonase) were added to suspensions of chloroplasts at low irradiances, ribulose bisphosphate increased while CO₂ fixation decreased. These observations provide considerable evidence that high ribulose bisphosphate levels clearly are not solely sufficient to permit rapid rates of CO₂ fixation, but that factors other than ribulose bisphosphate concentration are overriding the control of photosynthesis.

Isolated chloroplasts are capable of using carbon reserves to produce considerable ribulose bisphosphate. Upon illumination in the absence of CO₂ and O₂, intact chloroplasts produced up to 13 millimolar ribulose bisphosphate.

RuBP⁵ carboxylase-oxygenase occupies a central role in the biochemical regulation of photosynthetic CO₂ fixation in green plants (5). If the level of the substrate for carboxylation, RuBP, does not saturate CO₂ fixation, then the processes involved in its synthesis, such as electron transport, photophosphorylation, or the activity of enzymes of the reductive pentose-P pathway, are limiting for photosynthesis. Contrarily, if RuBP levels are saturating, then CO₂ fixation will be limited either by CO₂ availability or by the activity of RuBP carboxylase. Measurements of the RuBP level can be of value in determining where photosynthesis is rate-limited.

CO₂ acts as a positive effector of the RuBP carboxylase. It both activates and serves as a substrate for the enzyme in the chloroplast (1, 12). O₂ competes with CO₂ as a substrate for the RuBP carboxylase. The inhibition of photosynthesis by atmospheric O₂ in isolated chloroplasts has been attributed to a depletion of RuBP due to O₂-stimulated P-glycolate formation (15). A second expla-

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² Abbreviations: RuBP: ribulose 1,5-bisphosphate; FBP: fructose 1,6-bisphosphate; R5P: ribose 5-phosphate.

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RESULTS
Chloroplast RuBP and CO₂ Fixation. Metabolic studies with both algae (20) and intact isolated chloroplasts (14) indicated that RuBP carboxylase is active in the light and inactive in the dark. Chloroplasts in the light accumulate RuBP, which, in the 1st min of darkness, decreases to a constant but significant level as carboxylation is inactivated (4, 16).

Present studies confirmed these observations (Fig. 1). In the light there was a brief (15-30 s) initial lag followed by a period of linear CO₂ fixation. During this time the chloroplast RuBP pool increased. When the light was turned off, CO₂ fixation ceased and the RuBP level decreased. During the 1st min of a second light period, CO₂ fixation resumed and the RuBP level rose from below 10 to over 130 nmol/mg Chl. Before the dark period, the rate of CO₂ fixation was 94 μmol/mg Chl-h. This compares to a maximum rate of 64 μmol/mg Chl-h during the second light period, suggesting that RuBP availability was not limiting the rate of CO₂ fixation during that time.

Effects of PPI on Chloroplast RuBP Levels and CO₂ Fixation. The effect of PPI on the rate of CO₂ fixation by isolated spinach chloroplasts is shown in Figure 2. During the 1st min of illumination there was an increase in chloroplast RuBP, which, in the absence of PPI, subsequently dropped to a steady-state level of 8 to 10 nmol/mg Chl. Addition of 1.25 mM PPI stimulated the rate of CO₂ fixation up to 10 min, but between 10 and 20 min both
rates of CO2 fixation appeared about equal. Between 4 and 20 min, chloroplasts with PPI accumulated RuBP. This build-up occurred even as the rate of CO2 fixation declined, suggesting that RuBP was not limiting CO2 fixation during this period. By 30 min CO2 fixation apparently failed to sustain the continual accumulation of RuBP.

Current evidence suggests that PPI interferes with the action of the phosphate translocator (2, 26). By preventing the export of sugar phosphates, PPI causes intermediates of the reductive pentose-P pathway to accumulate in the chloroplast, which could result in enhanced rates of CO2 fixation (2, 18). In Figure 2 this may have been the case between 1 and 10 min.

In the light, isolated intact chloroplasts can accumulate large amounts of RuBP, especially in the absence of CO2 and O2. With 2 mM PPI, chloroplasts illuminated for 40 min in a N2 atmosphere have been observed to accumulate over 320 nmol RuBP/mg Chl. The same chloroplasts without PPI generated over 100 nmol RuBP/mg Chl. The internal stroma volume of these chloroplasts was 25 μl/mg Chl, so that RuBP inside the chloroplast was 13 and 4 mM, respectively. The effect of PPI was not duplicated with the same amount of Pi. Since CO2 was absent and there is not sufficient carbon in the total intermediates of the pathway (5), the carbon required for 13 mM RuBP probably came from starch breakdown.

Effects of O2 and Catalase on Chloroplast RuBP and CO2-Fixation. As previously observed (10), the uptake of CO2 by isolated spinach chloroplasts was stimulated by the addition of catalase to the medium. In our hands the level of chloroplast RuBP was generally more than doubled when catalase (>1000 IU/ml) was included during photosynthesis, and this corresponded to a 2- to 4-fold increase in the rate of CO2 fixation (data not shown). Isolated chloroplasts can reduce O2 to the superoxide radical during photosynthesis and this radical is dismutated to H2O2 by superoxide dismutase, a chloroplast enzyme (9). The inhibition of photosynthesis by H2O2 could be caused by the oxidation of sulphydryl groups of chloroplast enzymes such as NADP-glyceraldehyde 3-P dehydrogenase (9) and FBP phosphatase (23) by H2O2.

The inhibiting effects of O2 on CO2 fixation have been extensively investigated (7, 15, 19, 22). This inhibition occurs by O2 lowering chloroplast RuBP levels as well as by competing with CO2 as a substrate for the RuBP carboxylase-oxygenase (15). With 70 to 80 μM CO2, 100% O2 inhibited chloroplast CO2 fixation more than 70% compared to fixation at air levels of O2 (Table 1A). Added catalase and triose-P (FBP plus aldolase) stimulated the rate of CO2 fixation 4- to 6-fold (Table 1B). The photosynthetic rate with 100% O2 was still half that with 21% O2. RuBP amounts were increased by the presence of catalase and triose-P; however, the level was less with 100% O2. This stimulation of photosynthesis suggests that the RuBP levels produced in chloroplasts without catalase and triose-P were limiting. Our experience suggests that levels of chlororop RuBP greater than 15 to 20 nmol/mg Chl are most likely saturating for CO2 fixation. Thus, the difference in CO2 fixation between 21 and 100% O2 in Table 1B was probably due to the direct competitive inhibition of RuBP carboxylase-oxygenase by O2 (7).

These observations are consistent with the findings of Gibbs and co-workers (3, 22) who demonstrated that catalytic amounts of FBP and ri5P reduced O2 inhibition of CO2 fixation. Recently, Collatz (8), using Chlamydomonas reinhardtii and separated spinach leaf cells, reported that upon lowering O2 from 21 to 3% there were corresponding increases in photosynthesis and levels of RuBP.

Effects of dl-Glyceraldehyde and Sugar Phosphates on Chloroplast RuBP and CO2 Fixation. CO2 fixation by isolated chloroplasts was completely inhibited by 15 mM dl-glyceraldehyde,

Table 1. Effect of O2, Catalase and FBP-Aldolase on Rates of CO2 Fixation and Levels of RuBP in Spinach Chloroplasts

<table>
<thead>
<tr>
<th>Experiment</th>
<th>CO2 Fixation</th>
<th>RuBP</th>
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<tbody>
<tr>
<td></td>
<td>μmol/mg Chl-h</td>
<td>nmol/mg Chl</td>
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<tr>
<td>A.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21% O2</td>
<td>29.5</td>
<td>11.3</td>
</tr>
<tr>
<td>100% O2</td>
<td>7.9</td>
<td>8.5</td>
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<tr>
<td>plus FBP-aldolase and catalase</td>
<td></td>
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<tr>
<td>B.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21% O2</td>
<td>117.6</td>
<td>33.8</td>
</tr>
<tr>
<td>100% O2</td>
<td>57.6</td>
<td>24.3</td>
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with a corresponding drop in RuBP to almost zero during the first 2 min of illumination (Fig. 3). Chloroplasts treated with DL-glyceraldehyde are unable to generate RuBP, most likely because the transketolase reaction of the photosynthetic carbon reduction pathway is inhibited (25). However, chloroplasts with 1 mM DL-glyceraldehyde had only 2 to 3 mmol less RuBP/mg Chl than chloroplasts without the inhibitor, yet CO₂ fixation was reduced more than 40%.

We have noted that 1 mM DL-glyceraldehyde does not affect catalysis by the purified RuBP carboxylase, but with illuminated intact chloroplasts it lowers the activation of the enzyme (1). DL-Glyceraldehyde may also be inhibiting the formation of more optimal Mg²⁺ or pH conditions in the chloroplast stroma (21, 27). Obviously, electron transport and photophosphorylation producing NADPH and ATP are not limiting when the RuBP levels are sufficient. The role of electron transport in maintaining the proper pH and Mg²⁺ gradient across the thylakoid membranes must be considered as a major factor in the regulation of activity of the RuBP carboxylase in the chloroplast.

Chloroplasts when incubated with FBP plus aldolase (to generate triose-P) or Ri5P produced more RuBP during the first 4 min in the light. There was no increase in the rate of CO₂ fixation (Fig. 4). Apparently for these chloroplasts, 22 to 24 nmol RuBP/mg Chl saturated CO₂ fixation during this period.

Effects of Irradiance on CO₂ Fixation and Chloroplast RuBP. At a limiting irradiance (25 μE/m²·s) the rate of CO₂ fixation between 1 and 4 min in the light was 18.9 μmol/mg Chl-h (Fig. 5). At saturating irradiance (800 μE/m²·s) the same chloroplast preparation fixed CO₂ at 98.3 μmol/mg Chl-h during this period. Although the kinetics of the light-on RuBP formation were somewhat modified at the lower irradiance, the chloroplast RuBP pool size was almost identical during the 4- to 10-min period. In this experiment, the availability of RuBP was apparently not the cause of the limited rate of CO₂ fixation at the lower irradiance. This is not always the case as we have also noted with other chloroplast preparations and lower irradiances that the RuBP level can be correspondingly low and possibly limiting for CO₂ fixation (data not shown). However, with added FBP and aldolase, RuBP levels increased while CO₂ fixation decreased at lower irradiances (Fig. 6). Clearly, the drop in the rate of CO₂ fixation under these conditions must be due to a drop in activity of the RuBP carboxylase (1).

**FIG. 3.** Effect of DL-glyceraldehyde on CO₂ fixation and levels of chloroplast RuBP. CO₂ fixation (— — —) was determined with solution C plus PPI (1.0 mM), NaH¹⁴CO₃ (10 mM, 0.5 μCi/μmol), chloroplasts (0.082 mg Chl/ml or B, 0.065 mg Chl/ml) and (A) FBP (1.0 mM) and rabbit muscle aldolase (1.25 IU/ml) or (B) Ri5P (1.0 mM). Chloroplast RuBP (— — —) was determined from similar solutions having NaH¹⁴CO₃ (24).
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Fig. 6. CO₂ fixation and chloroplast RuBP levels as a function of irradiance in the presence of FBP. Rates of CO₂ fixation (△) were determined after 10-min illumination with solution C plus NaH¹⁴CO₃ (10.4 mM, 0.5 μCi/μmol), FBP (1.0 mM), aldolase (1.25 IU/ml), and chloroplasts (0.077 mM Chl/ml). Samples for RuBP (♂) were taken from similar reaction mixtures at 10 min (24).

Lower irradiances which result in lowered rates of CO₂ fixation can result in lowered chloroplast RuBP levels. This would be expected because CO₂ fixation is needed to provide carbon for RuBP production. If chloroplasts have sufficient carbon reserves to supply RuBP by conversion of triose-P or starch breakdown, RuBP levels in the chloroplast do increase at lower irradiances (Fig. 6). These observations that RuBP can be high when CO₂ fixation is decreasing strongly support the proposal that the RuBP carboxylase is a key metabolic regulator of photosynthesis (12).

Our results indicate that chloroplasts are capable of supplying RuBP faster than it is consumed by the RuBP carboxylase, especially if sufficient sources of fixed carbon are available (i.e., starch, triose-P, etc.). The intact leaf also possesses the photosynthetic glycolate pathway for recycling most of the carbon lost by the RuBP oxygenase back to the chloroplast. Therefore, the sensitivity to atmospheric O₂ as seen by RuBP depletion (Table I) in isolated chloroplasts might not be expected in the intact leaf (7). If RuBP were supplied sufficiently in a leaf, inhibition by O₂ might still occur and be explained by the competitive action of O₂ with CO₂ at the catalytic site of the enzyme (7).

When chloroplast CO₂ fixation is maximal (between 1 and 10 min), most of our measurements indicated that chloroplast RuBP levels below 15 to 20 nmol/mg Chl limited CO₂ fixation. Kirk and Heber (15) similarly found that 7 nmol/mg Chl in chloroplasts limited CO₂ fixation. The internal volume of our chloroplasts, measured as the difference between the [³H]H₂O-permeable space and the [¹⁴C]sucrose-impermeable space (11) was about 25 μl/mg Chl. With this volume, 15 nmol RuBP/mg Chl is equivalent to 0.6 mmRuBP. With 6 μg RuBP carboxylase/mg Chl having eight binding sites per mole of enzyme, there are 3 to 4 mm binding sites for RuBP in the chloroplast (12). Perhaps only half of the RuBP-binding sites are available as suggested by the effects of tonic strength on the purified enzyme (28). As the dissociation constant for the enzyme-RuBP complex is less than 30 μM (12), the presence of a large excess of carboxylase would bind almost all of the RuBP in the chloroplast. We find that intact leaves from these spinach plants usually contain 80 to 100 nmol RuBP/mg Chl, suggesting that the low levels found in intact chloroplasts may be an artifact of isolation.

The activities of Ru5P kinase, triose-P dehydrogenase, and FBP and sedoheptulose bisphosphate phosphatase are enhanced in the light (6, 16, 23). However, the evidence that high RuBP levels can exist in chloroplasts at limiting irradiances suggests that the activities of these enzymes may not limit photosynthetic CO₂ fixation. Light-driven photo phosphorylation and electron transport are important to produce RuBP, but when RuBP is saturating, these parameters are not limiting photosynthesis. Other factors such as the rate of electron transport in maintaining the proper pH and Mg⁺⁺ gradients across the thylakoid membranes must be considered as a major factor in regulating RuBP carboxylase activity and photosynthesis in the chloroplast (1, 21, 27). When RuBP levels saturate CO₂ fixation, the activity of RuBP carboxylase and its affinity for CO₂ must be the restraining point for photosynthesis.

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LITERATURE CITED