Causes for the Disappearance of Photosynthetic CO₂ Fixation with Isolated Spinach Chloroplasts

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ABSTRACT

When isolated spinach chloroplasts are illuminated, photosynthesis and CO₂ fixation die off within 30 to 90 minutes. Even when air levels of CO₂ are used which maintain high and rate-saturating amounts of ribulose 1,5-bisphosphate inside the plastids, CO₂ fixation declines. The decline begins with a drop in activity of the ribulose 1,5-bisphosphate carboxylase/oxygenase, specifically loss of the enzyme-activator CO₂-Mg²⁺ form. Next, the light reactions cause gradual leakage of the carboxylase and other stromal proteins to the suspending medium. The chloroplast outer envelope appears to reseal and protect the thylakoids since there is little change in the ferricyanide-dependent Hill reaction. In the dark, under otherwise identical conditions, leakage of carboxylase does not occur.

Experiments studying CO₂ fixation and photosynthesis using intact isolated chloroplasts have been performed for many years (4). Most of these experiments utilize high or saturating levels of CO₂ supplied from bicarbonate in the suspending medium. Unlike the intact plant, the rates of photosynthesis with isolated chloroplasts die off and disappear after 20 to 40 min. One of the apparent reasons why this die off occurs is the inability of the isolated chloroplasts to maintain the RuBP levels needed to support CO₂ fixation. Even though the levels of RuBP can be high when spinach chloroplasts are illuminated with air levels of CO₂ (about 10 μM in solution), the die off of photosynthetic CO₂ fixation still occurs after 15 to 30 min (12, 13).

Photosynthesis, and the accompanying die off, were studied at air levels of CO₂ using a closed apparatus under conditions which permitted photosynthesis without depletions of CO₂ below 10 μM. The concentration of RuBP produced in the chloroplasts remained at or above the binding site concentration of the Rubisco and did not appear to limit CO₂ fixation. During the disappearance of CO₂ fixation the total or maximal activity of Rubisco remained high and mostly unchanged. As noted in this paper, the total activity of Rubisco is that activity measured after full activation with saturating Mg²⁺ and bicarbonate. However, along with the die off of CO₂ fixation, the activity of Rubisco inside the plastids also decreased, though not to zero. This activity, called initial activity, is measured directly upon lysis of the chloroplasts into assay medium containing Mg²⁺, bicarbonate, and RuBP.

Measurements of Rubisco activity actually detect two enzyme complexes, the enzyme-activator CO₂ form, called E-C and the enzyme-activator CO₂-Mg²⁺ form, called E-C-M. Only the E-C-M form can bind RuBP preparatory to catalysis with CO₂ or O₂ (7). Measurements of the E-C-M activity indicate that it is lost during the die off of CO₂ fixation and only the E-C form contributes to the initial activity. Directly following the loss of E-C-M, significant amounts of Rubisco as well as other proteins were released from the chloroplast stroma into the suspending medium.

MATERIALS AND METHODS

Preparation of Chloroplasts. Chloroplasts were isolated from 6 to 8 week old spinach plants (Spinacia oleracea, var Viroflay) as previously described (13) with 80 to 90% of the chloroplasts intact as measured by ferricyanide-dependent O₂ evolution (6).

Air Level CO₂ Chamber and CO₂ Fixation. Photosynthetic CO₂ fixation was carried out at air levels of CO₂ by a modification of the procedures described previously (13). Rather than using a gas recirculating system to bubble air through the chloroplasts, the sealed chamber with the sample was shaken by a reciprocating shaker going 28 to 30 rpm with 4 cm oscillation. The chloroplasts (2 ml, 20 μg Chl/ml) were placed in rectangular sample wells having sloping ends which gave a large surface area (about 4.5 cm²). This arrangement facilitated sufficient CO₂ exchange with the gas phase during shaking to maintain 10 μM CO₂ during the entire experiment. The floor of the chamber served as the CO₂ generator-reservoir to maintain the CO₂ levels in the gas phase. The chloroplast suspension solution was 0.33 m sorbitol, 25 mM Hepes (pH 7.8) with NaOH, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, and 0.1 mM K₂HPO₄. Sodium pyrophosphate (2 mM), catalase (600 IU/ml), and carbonic anhydrase (0.02 mg/ml) were added to the sample vials in the sealed chamber prior to an equilibration period. The suspension medium in the sample wells and the gas phase of the closed system were brought into equilibrium with regards to CO₂ by shaking 1 to 2 h prior to the addition of chloroplasts. During shaking the sealed chamber was illuminated with 400 μE (m²·s)⁻¹ by a bank of VHO fluorescent lamps placed beneath a Plexiglas bath of 25°C.

Two identical chambers were used at the same time: one chamber, containing KH¹³CO₃, was used to determine the amount of fixed carbon during the experiment. Samples (50 μl) were removed from the sample wells and placed in 450 μl of 0.5 m HCl. These samples were dried down and the acid stable product counted to determine the amount of carbon fixed/mg Chl. Samples from the second chamber, containing unlabeled KHCO₃, were used in the assays for the initial, total, and E-C-M Rubisco activities as well as for the RuBP levels.

Measurement of the Initial and Total Activities of Rubisco. Measurement of the initial and total activities of Rubisco from spinach chloroplasts containing 30 μg Chl/ml was performed by a modification of the method of Metzger and Ball (14).
lysed chloroplasts was also done as previously reported (13) except that 20 to 50 µl aliquots of chloroplast suspension from the sample wells were lysed into 450 µl assay buffer containing 25 mM Hepes, 20 mM MgCl₂, 0.6 mM RuBP, 1 mM DTE, and 1 mM KH₄CO₃ (13). Total activities were measured by adding RuBP after 5 to 10 min activation. The amount of Rubisco activity outside of the chloroplasts was determined by placing 100 µl of the chloroplast suspension into a 1.5 ml polypropylene tube, centrifuging it in a Beckman Microfuge for 5 to 10 s which reached 7000 g, and assaying the colorless supernatant. This procedure caused little rupture of the chloroplasts as noted by the lack of increase in Rubisco activity measured in the supernatant of dark incubated chloroplasts. Of all the various methods tested to separate chloroplast from the suspending medium and minimize loss of Rubisco activity, such as filtration through Whatman GF/C glass fiber filters, centrifugation proved most reliable.

The measurement of the catalytically active form of Rubisco, E-C-M, was performed as described elsewhere (9). This measurement was made in a similar manner to the initial and total activities except that the Mg²⁺ was replaced with 0.4 mM EDTA in the assay buffer. The sample was diluted at least 25-fold into the assay buffer to insure that any Mg²⁺ brought over into the assay would be completely bound by the EDTA. The RuBP levels were measured as previously described (11).

**Chloroplast Volume and pH Measurements.** The volume of the chloroplast stroma and stroma pH were determined per Heldt (3) with the following modifications. The chloroplasts were centrifuged through a silicone oil layer of Dow Corning 702:Stauffer-Wacker SWS-122, 20 CSTK, ratio 3:7, having a density of 1.046 g/ml into a 20 µl pad of 10% HClO₄. The chloroplasts were illuminated during removal from the closed chamber and during centrifugation. The chloroplast suspension contained H₂O and [¹⁴C]sucrose for the volume measurements and [¹⁴C]5,5-dimethylxazolidin-2,4-dione for the pH measurements.

**RESULTS**

When isolated chloroplasts were illuminated in solution having air levels of CO₂, the fixation of CO₂ ceased within 30 to 45 min (Fig. 1A). Under the conditions of these experiments the CO₂ concentration remained at approximately 10 µM aqueous CO₂ while the RuBP concentration inside the chloroplasts was up to 190 nmol (mg Chl)⁻¹ (Fig. 1B). As this spinach had 11.5 mg Rubisco protein/mg Chl, this level of RuBP was equivalent to the concentration of binding sites for RuBP on Rubisco (about 167 nmol [mg Chl]⁻¹) (8, 10). Similar observations were reported previously by Stumpf and Jensen (13). Although a substantial amount of the RuBP was released from the chloroplasts, the outside concentration remained between 2 to 8 µM RuBP because of the large dilution.

Notice that the total activity of Rubisco in Figure 1C remained constant while the initial activity, which increased up to 5 min illumination, steadily decreased as photosynthesis died off. However, even though there was no photosynthetic CO₂ fixation after 60 min there was still significant Rubisco initial activity (about 25-30 µmol[mg Chl·h]⁻¹) (Fig. 1C).

During the time that photosynthesis died off, measurements of the chloroplast volume and stroma pH were made (Table I). During the course of the experiments the chloroplasts underwent some swelling and contraction. No marked changes in stroma pH was detected which could account for the die off in CO₂ fixation. At 60 min there was often a decrease in the water-permeable space which indicated a decrease in the chloroplast volume. These volume decreases were not consistent between experiments.

Accompanying the die off of photosynthesis, Rubisco activity appeared outside of the chloroplasts. This leakage was deter-

![Graph](attachment:image.png)

**FIG. 1.** Chloroplasts at air levels of CO₂. A. Photosynthetic CO₂ fixation; B, RuBP levels inside the chloroplasts after separation of plastids from suspending medium; C, total and initial activities of Rubisco.

**Table I. Volume of the Chloroplast Stroma and pH at Air Levels of CO₂**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time</th>
<th>H₂O Space</th>
<th>Sucrose Space</th>
<th>Chloroplast Volume</th>
<th>pH*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 min</td>
<td>78.3</td>
<td>34.9</td>
<td>43.7</td>
<td>7.87</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>51.5</td>
<td>23.9</td>
<td>27.6</td>
<td>8.28</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>81.6</td>
<td>41.7</td>
<td>39.9</td>
<td>7.44</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>93.2</td>
<td>61.0</td>
<td>32.2</td>
<td>7.84</td>
</tr>
<tr>
<td>2</td>
<td>5 min</td>
<td>77.9</td>
<td>39.0</td>
<td>38.9</td>
<td>7.79</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>84.7</td>
<td>49.9</td>
<td>34.8</td>
<td>7.94</td>
</tr>
<tr>
<td></td>
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<td>97.6</td>
<td>55.9</td>
<td>41.7</td>
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<tr>
<td></td>
<td>60</td>
<td>79.9</td>
<td>54.6</td>
<td>25.3</td>
<td>8.19</td>
</tr>
</tbody>
</table>

*Estimated per Heldt (3) using [¹⁴C]dimethylxazolidin-2,3-dione.

mined by removing the chloroplasts by centrifugation. Note in the experiment shown in Figure 2 that over 60% of the total carboxylase activity was outside the chloroplasts by 60 min. In Figure 3 the initial and total activities either from the inside or outside of the chloroplasts are shown. The solid lines depict the total activity; the dashed lines depict the initial activity. Note that the initial activity located outside goes to a constant value as expected when E-C broke down to the inactive form of Rubisco in the low Mg²⁺ of the suspending medium. Also, the initial activity remaining in the chloroplast does not go to zero even though CO₂ fixation has stopped and much of the Rubisco is now outside of the chloroplasts.

As Rubisco leaked out following the die off of photosynthesis, the chloroplasts still appeared intact as determined by the Hill reaction using ferricyanide (Table II). Some chloroplast breakage was observed only after 60 min, but it was not proportional to the leakage of Rubisco into the suspending medium.

Most of the Rubisco was activated as the E-C and E-C-M
forms at the start of the experiment. As previously described, when measurements of initial activity are made, both the E-C and the E-C-M forms are measured. The amount of the E-C-M form inside the chloroplast has been estimated during the die off (Fig. 4). While Rubisco activity in terms of the E-C-M form was not the same as the photosynthetic rate, the two were proportional. Note that as the E-C-M form of Rubisco inside the chloroplast goes to zero, the rate of photosynthetic CO₂ fixation also goes to zero.

The results depicted in Figures 1 to 4 were from experiments having air levels (10 μM CO₂) in solution. The die off of photosynthesis seen here with isolated chloroplasts occurred even faster when saturating CO₂ was used. As photosynthesis died off with 10 mm bicarbonate, the leakage of Rubisco activity increased rapidly to 80% into the suspending medium by 90 min light

(Fig. 5). However, chloroplasts under the same conditions, but without light, did not lose Rubisco. This suggests that processes related to the light-trapping reactions were involved in the loss of Rubisco from the chloroplasts. Experiments with high CO₂ demonstrated again that the die off of photosynthetic CO₂ fixation and the E-C-M activity go together, but they both decayed
(c) Rubisco must be active and capable of reacting CO₂ and RuBP to form PGA. The enigma of photosynthesis with isolated chloroplasts, especially when they are illuminated with air levels of CO₂, is that the process stops even when CO₂ and RuBP are present. Rubisco must have become inactive, yet previous measurements had suggested that a significant amount of activity was still present (13). Measurements of the E-C-M complex indicate that this form disappeared as photosynthesis died off. The presence of the E-C-M complex accounts for the activity.

Care must be taken in concluding that activity of the E-C-M form is quantitatively the same as the rate of light-dependent CO₂ fixation with isolated chloroplasts (Fig. 4). Photosynthesis was measured with air levels of CO₂ while the E-C-M activity used saturating amounts of bicarbonate. Although both approach zero at approximately the same rate, the absolute rates of each still differ significantly. If both rates are compared at the same CO₂ concentration then the photosynthetic rate is 5- to 8-fold greater than the E-C-M rate (Table IV). The reason for this difference is unknown at present. It appears that the E-C-M complex may be a small complex that takes up CO₂ from the chloroplasts to occur. Even in the presence of a detergent to speed up the rate of lysis the measured rate of E-C-M has been 0.12 to 0.16 of the rate of CO₂ fixation (Table IV). If the measured E-C-M activity were similar to the rate of photosynthesis, then the E-C-M form would account for 0.31 to 0.46 of the initial activity with the rest due to the E-C form. Upon lysis of isolated chloroplasts the E-C-M form has usually been less than 10% of the initial activity. With purified spinach Rubisco, the activity of the E-C-M form is the same as the rate of CO₂ fixation. When using purified Rubisco, RuBP is used to stabilize the E-C-M form before it dissociates in the assay medium (5, 9).

Loss of the E-C-M complex suggests that the thylakoid system loses the ability during photosynthesis to maintain conditions in the stroma such as Mg²⁺ needed for activation and stabilization of the E-C-M complex. Addition of Mg²⁺ to the suspending medium in excess of the Mg²⁺-EDTA complex results in an even greater loss of CO₂ fixation (13). As yet we have been unable to add compounds to chloroplast suspensions which significantly reduce the die off.

During CO₂ fixation with isolated chloroplasts, there are only small changes in the total activity of Rubisco. Recent studies with Rubisco activities in extracts from intact plant leaves indicates that this value can be greatly altered within min in vivo following changes in environmental conditions (2, 14). The mechanisms used by the plant to inhibit or activate the total Rubisco activity are currently being investigated. At 60 to 90 min illumination of isolated chloroplasts Rubisco total activity often declines 5 to 20%.

The leakage of Rubisco out of chloroplasts with resealing of the outer envelope has been previously reported. In 1975, Lilley et al. (6) noted the presence of an intermediate class of chloroplasts which had resealed following release of stromal protein and were unable to utilize ferricyanide as a Hill oxidant. As they indicated, the presence of this intermediate type of plastids would cause overestimation of the proportion of intact chloroplasts and reduce their photosynthetic activity on a Chl basis.

Certainly accompanying the loss of stromal proteins from the chloroplast is the loss of other compounds such as RuBP, Mg²⁺, Pi, etc. Addition of these compounds to the suspending media did not reduce the die off of photosynthesis. What is significant is that the ratio of RuBP in the chloroplast to the binding sites of Rubisco remaining in the chloroplast increased from 0.3 at 5 min to greater than 1 as the die off occurred after 15 min (Figs. 1 and 2). Even though leakage does occur, the levels of RuBP are not limiting and are not the cause for the die off of photosynthesis.

Under conditions where RuBP levels are not limiting, the
sequence of events occurring in the chloroplast during the die off of photosynthetic CO₂ fixation appears as follows: The first event is the loss of activity of Rubisco, specifically loss of the E-C-M complex. Then, as CO₂ fixation continues to die, the light reactions in the chloroplast cause the outer envelope to open and release Rubisco as well as other stromal proteins. SDS-PAGE of the chloroplast suspending medium during illumination showed no preferential loss of protein (data not shown). The outer envelope must reseal so that the reactions at the thylakoid are protected, the Hill reaction with added ferricyanide cannot occur, and the chloroplast appears intact. It is not known what is produced in the stroma which attacks the outer envelope but it could be activated O₂ such as hydrogen peroxide and/or superoxide, produced by PSI (1).

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LITERATURE CITED