Measurement and Preservation of the in Vivo Activation of Ribulose 1,5-Bisphosphate Carboxylase in Leaf Extracts

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ABSTRACT

Photosynthetic carbon fixation is regulated in the chloroplast by the amount of ribulose 1,5-bisphosphate carboxylase which is activated. The activated carboxylase was preserved in detached leaves (barley, maize, soybean, spinach, wheat) for 90 min when stored on ice. With leaf extracts stored at 2°C, the amount of activated enzyme, representing that originally in the leaf, as well as the fully activated enzyme, formed by incubation of leaf extracts with Mg2+ and bicarbonate, both slowly declined in activity. However, for each activity this decline was proportional such that the ratio (percent activation) appeared constant. No change was observed in activation of the enzyme during the brief time of leaf homogenization. Optimal conditions (Mg2+, incubation time) for measurement of leaf activation of ribulose bisphosphate carboxylase vary depending on the plant.

3-Phosphonopropionate, a positive effector of the purified ribulose bisphosphate carboxylase and a metabolically inert analog of 2-phosphoglycolate, was used to examine what metabolic effectors might do to enzyme activation during leaf homogenization and preparation of the extract at 2°C. Activation under these conditions was not altered by 3-phosphonopropionate. When 3-phosphonopropionate was brushed on attached leaves or taken up by the transpiration stream of detached leaves, a considerable increase in activation of the carboxylase was measured.

If, instead of allowing the enzyme to fully activate, an aliquot of the crude homogenate is introduced directly to saturating CO2 and Mg2+ in the presence of RuBP without preincubation, a lower rate of CO2 fixation occurs (designated as initial activity). This latter activity indicates the in vivo potential or degree of activation of the enzyme in the leaf (9, 10). While neither the initial nor total activity can be directly equated with the in vivo rate of the enzyme, they always exceed the photosynthetic rate of the intact leaves (10). Due to the manner in which the assays are performed, initial activity is probably a measure of both the E-CO2-Mg2+ forms of the enzyme while total activity is a measure of all three forms as E-CO2-Mg2+.

MATERIALS AND METHODS

Plant Material. Wheat, barley, and maize were grown in pots as previously described (10). Spinach was grown hydroponically in a plant growth chamber. Soybean was grown in a greenhouse. 3-Phosphonopropionic acid was obtained from Alfa Products, Danvers, MA.

Assay of RuBP Carboxylase. Leaf tissue (0.5–1 g) was ground with 1 ml buffer (100 mM Hepes [pH 7.7], 20 mM KCl) in a glass homogenizer maintained at 2°C. The following were included for soybean: 5% PVP (average mol wt 40,000) and for maize: 20 mM Na2SO4 and diatomaceous earth. The homogenate was filtered through two layers of Miracloth. The assay mixture contained 100 mM Hepes [pH 7.7], 20 mM KCl, 30 mM MgCl2, 1 mM dithioerythritol, and 12 mM NaHCO3 (1 Cl/mol). Assays were carried out at 25°C in plastic vials capped with serum stoppers. Total activity was measured by adding 20 μl crude homogenate to 460 μl assay mixture. After attaining full activation at 25°C, 20 μl 15 mM RuBP was added to start the reaction. The reaction was stopped after 30 s by addition of an equal volume of 1 M HCl. Initial activity was measured by adding 20 μl crude homogenate to 480 μl assay mixture as for the total activity determination except that RuBP was already present. The reaction was terminated after 30 s by addition of acid. Percent activation was expressed as 100 times initial activity divided by total activity.

RESULTS AND DISCUSSION

Stability of RuBP Carboxylase Activity Following Homogenization of Leaf Tissue. Initial and total carboxylase activities were measured following storage of wheat leaf tissue for up to 3 h at ice temperatures. Both initial and total activity declined slowly (Fig. 1A) while percent activation did not change (Fig. 1B). The endog-
during min vation when enzyme stored at catalytically active 30
sumed enzymous the time as described. The presence of addition NaHCO3
FIG. 1. Effect of storage on ice of wheat homogenates on (A) initial (C) and total (●) activities and (B) percent activation of RuBP carboxylase. At time zero, leaves from 2-wk-old wheat seedlings were harvested and homogenized as described. The filtrate was stored on ice until assayed at the time indicated.

Table I. Activity of RuBP Carboxylase in Crude Wheat Extracts Measured at 2 and 25°C

<table>
<thead>
<tr>
<th>Extract Source</th>
<th>2°C</th>
<th>25°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Total</td>
<td>Percent Activation</td>
</tr>
<tr>
<td>Flag leaves*</td>
<td>0.59</td>
<td>13.4</td>
<td>23.0</td>
</tr>
<tr>
<td>Seedlingsb</td>
<td>0.14</td>
<td>4.64</td>
<td>11.9</td>
</tr>
</tbody>
</table>

- *Flag leaves were from greenhouse grown plants.
- *Seedlings were grown in a growth chamber.

Endogenous RuBP which was released from the chloroplasts was consumed during this period indicating that the enzyme was still catalytically active even at ice temperatures. When samples of the total activity assay were acidified after 10 min at 25°C, prior to addition of RuBP to the assay, some 14C had already been incorporated. This fixed 14C was 1 to 2% of the 13C incorporated during the usual 30 s assay of total activity. When RuBP and NaH14CO3 were supplied to crude wheat extracts at ice temperatures in assay media, 14C was incorporated into acid stable counts albeit at a very low rate (Table I). The rates at 2°C were linear for 45 min and were proportional to the degree of activation of the enzyme measured at 25°C.

The presence of 300 μM CO2 (122 mM HCO3− [pH 7.7]) or 30 mM Mg2+ during grinding at 2°C had no effect on percent activation when determined immediately (data not shown). However, percent activation did change when crude homogenates were stored at 2°C for 30 min in the presence of 390 μM CO2 with or without 30 mM Mg2+ (Table II). The observed increases were probably due to the slow conversion of the E form of the enzyme to the E-CO2 and then the E-CO2-Mg2+ form during storage on ice. When the tissue was ground in the presence of Mg2+ and stored under N2 to reduce CO2 availability, percent activation increased slightly. Following storage for 30 min at 25°C with 10 mM CO2 over 20% of the total carboxylase activity was lost while percent activation changed slightly suggesting that both active and inactive (but still capable of being activated) enzyme was being lost at about the same rate. These observations suggest that because slow changes in both initial and total activities can occur even at ice temperatures (Fig. 1), it is not advisable to store leaf tissue extracts beyond the time needed to measure CO2 fixation activity.

Preservation of RuBP Carboxylase Activity in Whole Leaves. Activation of the carboxylase could be preserved for up to 90 min when detached leaves were stored on ice. In wheat, no changes in initial or total activity were observed during storage under these conditions. With spinach leaves, although percent activation did not change (Fig. 2), initial and total activity each declined about 25% following 2.5 h storage on ice (data not shown). The preservation of activation reflects the maintenance by low temperature of enzyme form (E, E-CO2 and E-CO2-Mg2+). As discussed above, once the enzymes were homogenized, depending on conditions, the initial and total activities began to change slowly. Thus for best preservation of carboxylase activation, detached leaves usually should be ground just prior to assay of percent activation of the enzyme. As stability of carboxylase activation in the leaf can vary somewhat between plant species, the optimal conditions unique to each tissue must be determined in advance.

Table II. Influence of Storage Conditions on Properties of RuBP Carboxylase

<table>
<thead>
<tr>
<th>Time after grinding (min)</th>
<th>0.5</th>
<th>30</th>
<th>30</th>
<th>30</th>
<th>30</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (C)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>CO2 (μM)</td>
<td>10*</td>
<td>10*</td>
<td>390</td>
<td>390</td>
<td>b</td>
<td>10*</td>
</tr>
<tr>
<td>Mg2+ (mM)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Enzyme Properties</th>
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<th>15</th>
<th>21</th>
<th>37</th>
<th>19</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Activation</td>
<td></td>
<td>12.6</td>
<td>11.5</td>
<td>12.7</td>
<td>11.8</td>
<td>11.8</td>
<td>9.7</td>
</tr>
<tr>
<td>Total Activity</td>
<td></td>
<td>μmol CO2 Fixed</td>
<td>(mg Chl·min)</td>
<td>(mg Chl·min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.6</td>
<td>11.5</td>
<td>12.7</td>
<td>11.8</td>
<td>11.8</td>
<td>9.7</td>
</tr>
</tbody>
</table>

*Estimated assuming buffer was in equilibrium with air levels of CO2.

* Tissue was ground and stored under nitrogen.

FIG. 2. Effect of storage of leaf tissue on ice on percent activation of RuBP carboxylase. At time zero leaves from 2-wk-old wheat (●) or 6-wk-old spinach (○) were harvested, cut up and stored on ice. Samples were removed, homogenized and assayed at the times indicated.
Changes in RuBP Carboxylase Activity during Homogenization of Leaf Tissue. The possibility that during homogenization potential effectors of enzyme activity came in contact with the enzyme and thereby influenced activation was investigated. PPA is an isosteric analog of 2-phosphoglycolate and can act as a positive effector of RuBP carboxylase activity (3) (Table III, Experiment I). In crude leaf extracts, PPA is not hydrolyzed to Pi as is P-glycolate (3) because of the substitution of a C-P bond for the usual C-O-P phosphate ester linkage (2). Other effectors of RuBP carboxylase, such as 6-P-gluconate or NadPH (4), would be metabolized if added to a crude leaf extract so that effects on activation of the carboxylase could be variable. When supplied via the transpiration stream of detached leaves or when brushes on attached leaves, PPA apparently reached the chloroplast as activation of the RuBP carboxylase occurred. When 50 mM PPA was fed to detached spinach leaves in light for 2 h the PPA carboxylase activity increased to approach that of the total (Table III, Experiment 2). This data suggests that PPA can influence the in vivo activation of the carboxylase. Although foliar application of PPA to wheat seedlings increased the activation of the RuBP carboxylase, it depressed the photosynthetic CO2 exchange rate in air (data not shown). High levels of PPA in the tissue may have competed with RuBP for the binding sites on the enzyme and effectively reduced the in vivo catalytic activity as suggested by Badger and Lorimer (1).

The presence of 10 mM PPA during grinding had little or no effect on activation of the carboxylase (Table III, Experiment 3). It is unlikely that any endogenous effector in leaves, when diluted upon grinding would exist at 10 mM. This suggests that, as disruption of the tissue at 2°C causes effectors to come in contact with the enzyme, the degree of activation would not be altered prior to assay.

Determination of Activation and Assay Conditions in Crude Preparations of RuBP Carboxylase. Conditions for fully activating the enzyme as well as for the assay of initial and total activity must be optimized for each species examined. Initial activity of maize, spinach, soybean, and wheat enzyme had a rather broad pH optimum between 7.8 and 8.2 (9) which fell off slowly at either extreme. Total activity was optimal between pH 7.7 and 7.8 and declined above these values (data not shown). For all species pH

7.7 was chosen for both initial and total activity assays and for the grinding buffer.

Mg concentration was also optimized for activation and assay of the enzyme. For wheat carboxylase the optimum was 30 mM. Enzyme from spinach, soybean, and maize responded similarly with optima at 30, 30, and 40 mM respectively. Because the binding of Mg2+ with the E-CO2 form of the enzyme is a fast process (7) measurement of initial activity will indicate the two forms that existed in vivo, E-CO2-Mg2+ and E-CO2, which was rapidly converted to E-CO2-Mg2+ in the reaction mixture. When initial activity assays were performed in the absence of added Mg2+ a lower rate was observed (data not shown). It is doubtful that measurements without Mg2+ represent only the E-CO2-Mg2+ form of the enzyme in vivo since bound Mg2+ may be released in the absence of added Mg2+.

The length of time necessary to completely activate the enzyme, as represented by the highest rates for total activity, is an important consideration. With crude preparations from wheat, spinach, maize and barley full activation was reached in 3 to 10 min at 25°C (Fig. 3) and the enzyme remained activated for at least 12 to 20 min. Soybean carboxylase was an exception. Full activation was reached at 1 min and began to decline after 5 min, losing up to 20% of its peak activity shortly thereafter. This relatively short period of full activation of the soybean RuBP carboxylase probably reflected high levels of phenolic compounds acting to denature the enzyme rather than affecting activation per se.

CONCLUSIONS

The in vivo percent activation of the RuBP carboxylase can be effectively preserved for several hours if intact wheat leaves are stored on ice, although initial and total activities declined slowly with spinach leaves. RuBP carboxylase should be assayed as soon as possible following homogenization of the leaves. Assay criteria need to be optimized for each species examined. These include pH and Mg2+ concentration of the assay media as well as the time necessary for full activation of the enzyme. Proper measurement of the activation of the RuBP carboxylase may lead to better understanding of the role of activation in regulating photosynthesis.
LITERATURE CITED

1. BADGER MR, GH LORIMER 1981 Interaction of sugar phosphates with the catalytic site of ribulose 1,5-bisphosphate carboxylase. Biochemistry 20: 2219–2225


4. HATCH AL, RG JENSEN 1980 Regulation of ribulose 1,5-bisphosphate carboxylase from tobacco: changes in pH response and affinity for CO₂ and Mg²⁺ induced by chloroplast intermediates. Arch Biochem Biophys 205: 587–594


