Ribulose 1,5-Bisphosphate and Activation of the Carboxylase in the Chloroplast

Received for publication December 15, 1980 and in revised form February 12, 1981

Richard C. Sicher², Alan L. Hatch, David K. Stumpf, and Richard G. Jensen³
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721

ABSTRACT

Ribulose 1,5-bisphosphate in the chloroplast has been suggested to regulate the activity of the ribulose bisphosphate carboxylase/oxygenase. To generate high levels of ribulose bisphosphate, isolated and intact spinach chloroplasts were illuminated in the absence of CO₂. Under these conditions, chloroplasts generate internally up to 300 nanomoles ribulose 1,5-bisphosphate per milligram chlorophyll if O₂ is also absent. This is equivalent to 12 millimolar ribulose bisphosphate, while the enzyme, ribulose bisphosphate carboxylase, offers up to 3.0 millimolar binding sites for the bisphosphate in the chloroplast stroma. During illumination, the ribulose bisphosphate carboxylase is deactivated, due mostly to the absence of CO₂ required for activation. The rate of deactivation of the ribulose bisphosphate carboxylase was not affected by the chloroplast ribulose bisphosphate levels. Upon addition of CO₂, the carboxylase in the chloroplast was completely reactivated. Of interest, addition of 3-phosphoglycerate stopped deactivation of the carboxylase in the chloroplast while ribulose bisphosphate accumulated. With intact chloroplasts in light, no correlation between deactivation of the carboxylase and ribulose bisphosphate levels could be shown.

In contrast, incubation of purified ribulose bisphosphate carboxylase with ribulose bisphosphate irreversibly inhibited activation, especially in the absence of CO₂. Addition of the same amount of ribulose bisphosphate to lysed chloroplasts did cause some deactivation of the carboxylase in the extract, but full activation returned when the ribulose bisphosphate was consumed. The ribulose bisphosphate carboxylase in the chloroplast is not irreversibly inhibited by high levels of ribulose bisphosphate.

Highly active preparations of RuBP⁴ carboxylase/oxygenase (EC 4.1.1.39) are obtained by incubating the enzyme with CO₂ and Mg⁵⁺ prior to the addition of RuBP(1, 3, 7, 11). Preincubation of the enzyme with RuBP inhibited activity during a subsequent assay period and prevented full activation by CO₂ and Mg⁵⁺ (3). This substrate inhibition has been attributed either to the binding of RuBP to an allosteric site (3) or to competitive binding with CO₂ for the active site (15). Studies with purified RuBP carboxylase indicate that there are eight RuBP binding sites per mol enzyme (25). Laing and Christeller (7) proposed that RuBP binds to a single site per protomer, and that during steady state, this binding follows a random mechanism with respect to CO₂ and the release of products.

Evidence about the nature of the RuBP binding site indicates the necessity of an essential lysyl residue based on binding studies with affinity labels, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (13) or N-bromoacetylolethalamine phosphate (18). Studies with pyridoxal phosphate also suggest the presence of a lysyl residue at the active site of RuBP carboxylase (15, 24). An arginyl residue per protomer may also be required for activity (17). Fluorometric studies of the binding of RuBP suggests that the enzyme may have two different binding affinities with the inactive enzyme and only one with the activated enzyme in the presence of CO₂ and Mg⁵⁺ (21).

Recently Paech et al. (14) found that RuBP, while free in solution, spontaneously epimerized to XuBP or underwent degradation to form a diketo compound. Either of these compounds inhibited RuBP carboxylase/oxygenase competitively with respect to RuBP. It was suggested that the inhibition previously attributed to RuBP in purified enzyme studies actually may have been due to the presence of various breakdown products of RuBP in solution. Little or no inhibition occurred if freshly prepared solutions of RuBP were used. In agreement with this observation, CO₂ fixation with either lysed chloroplasts (10) or with a reconstituted chloroplast system (9) is faster if RuBP is generated from ATP and RuBP rather than upon the direct addition of RuBP.

We report the effects of RuBP accumulation in the chloroplast on RuBP carboxylase activity measured immediately upon chloroplast lysis and have been unable to observe irreversible inactivation of the enzyme by high RuBP levels. Under conditions in the chloroplast producing up to 12 mm RuBP as opposed to 3 mm binding sites, a complete reactivation of the enzyme was observed within 10 min after the introduction of CO₂. The addition of RuBP to a chloroplast lysate deactivated the carboxylase but full activity was almost completely restored within 30 min. Irreversible inactivation of the isolated carboxylase by RuBP appears only with the purified enzyme and not with intact or freshly lysed chloroplasts.

MATERIALS AND METHODS

Intact chloroplasts were isolated from newly expanded leaves of 4- to 5-week-old spinach plants (Spinacia oleracea var. Virolav) as described previously (2, 6). Chloroplasts were illuminated in 10 ml stopped serum bottles containing 0.33 M sorbitol, 0.05 M Hepes-NaOH (pH 7.8), 2.0 mm EDTA, 1.0 mm MgCl₂, 1.0 mm MnCl₂, and 0.5 mm K₂HPO₄. These solutions were purged with CO₂-free N₂ or O₂ for 10 min prior to the addition of chloroplasts. All experiments were performed in a water bath at 25 C. Illumination was 800 µE/m²•s as measured with a quantum photometer (Lambda Instrument Co., Lincoln, NE).

The amount of RuBP carboxylase present in chloroplast lysates

---

¹ This work was supported by National Science Foundation grant 77-26284 and by Monsanto Agricultural Products Company to RGJ. This is University of Arizona Agricultural Experiment Station Paper 3399.
² Current address: United States Department of Agriculture/Science and Education Administration, Light and Plant Growth Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705.
³ To whom reprint requests should be sent.
⁴ Abbreviations: RuBP, ribulose 1,5-bisphosphate; Ru5P, ribose 5-phosphate; PGA, 3-phosphoglycerate; FBP, fructose 1,6-bisphosphate; OAA, oxalacetate; XuBP, xylulose 1,5-bisphosphate.
was determined by an adaptation of the method of Goldswaite and Bogorad (4).
Crystalline spinach RuBP carboxylase/oxygenase was kindly provided by Dr. D. P. Bourque of this department.
Chl was determined according to Vernon (22) and RuBP was synthesized from Rib and ATP by previously described procedures (2).
Amounts of chloroplast RuBP were measured by the procedure of Sicher et al. (19).
RuBP carboxylase activity was measured upon chloroplast lysis in hypotonic media (2).

RESULTS

Amount of RuBP Carboxylase in Isolated Spinach Chloroplasts.

Upon separation by sucrose density gradient ultracentrifugation from lysed chloroplasts, RuBP carboxylase comprised about two-thirds of the total soluble protein in isolated spinach chloroplasts.

These preparations had about 5.0 mg RuBP carboxylase/mg Chl, with a specific activity upon activation with CO\textsubscript{2} and Mg\textsuperscript{2+} of 1.1 to 1.3 \mu mol CO\textsubscript{2} fixed/mg protein \cdot min at 25 C (Fig. 1B). Assuming a maximum of eight binding sites per enzyme molecule (25), the calculated concentration of binding sites for RuBP was 75 nmol/mg Chl or 3.0 mm in chloroplasts having a stroma volume of 25 \mu l/mg Chl (20). These observations compare favorably with other reported values for RuBP carboxylase from spinach chloroplasts (12).

Effect of RuBP on Activity of RuBP Carboxylase. Purified spinach RuBP carboxylase was fully activated in less than 5 min when preincubated in Bicine buffer at pH 8.0 with 10 mm NaH\textsubscript{12}CO\textsubscript{3} and 20 mm MgCl\textsubscript{2}. There was a slow loss of activity at 25 C, but after 40 min, about 90% maximal activity remained (Fig. 1A). The enzyme in a preparation of osmotically lysed spinach chloroplasts responded similarly to CO\textsubscript{2} and Mg\textsuperscript{2+} (Fig. 1B). As often observed (3, 7, 14), the purified enzyme was up to 50% deactivated when RuBP (0.43 mm) was added (Fig. 1A). This drop in activation was proportional to the amount of RuBP added. The carboxylase reaction went to completion in about 8 min based on the disappearance of RuBP. The deactivation of the purified enzyme by RuBP appeared irreversible even though conditions of high CO\textsubscript{2} and Mg\textsuperscript{2+} in the reaction medium favored maximal activation once the added RuBP was converted to PGA.

After a like amount of RuBP was added to lysed chloroplasts, activation of the RuBP carboxylase dropped initially but was 90% restored within 15 to 30 min (Fig. 1B). In this experiment, over 300 mol of RuBP were added/mol RuBP binding sites, which amounts to 75 to 100 times more than we have observed in intact chloroplasts.

Effect of CO\textsubscript{2} on RuBP and Activation of RuBP Carboxylase in Chloroplasts. Considerable amounts of RuBP (over 200 nmol/mg Chl) were synthesized when spinach chloroplasts were illuminated for 30 min in a CO\textsubscript{2}-free N\textsubscript{2} atmosphere (Fig. 2). Because no CO\textsubscript{2}...
assimilation occurred, the carbon needed for this synthesis of RuBP came from endogenous chloroplast intermediates and from starch breakdown. After 30 min in the light, only one-third of the original chloroplast RuBP carboxylase activity remained. This decrease in activation was due to the absence of CO₂ and not to chloroplast breakage as the relative intactness of these preparations remained unchanged (2). The addition of CO₂ to the light restored enzyme activity with a drop in RuBP levels. The deactivation appeared completely reversible upon addition of CO₂.

Effects of O₂ on RuBP and Activation of RuBP Carboxylase in Chloroplasts. RuBP carboxylase was similarly deactivated in chloroplast preparations illuminated in a CO₂-free O₂ atmosphere (Fig. 3). The RuBP buildup in 100% O₂ was slower than in 100% N₂, most likely because of RuBP oxygenase activity. It is also possible that RuBP synthesis was reduced in the presence of O₂ due to lowered ribulose 5-phosphate kinase activity (8). Even though more RuBP accumulated in the presence of N₂ the rate of deactivation of the chloroplast RuBP carboxylase was similar under both atmospheres. The process of deactivation did not correlate with chloroplast RuBP amounts.

Effect of Triose-P on Chloroplast RuBP Carboxylase. Chloroplasts illuminated in 100% N₂ in the presence of FBP plus aldolase, to make triose-P, accumulated over 150 nmol RuBP/mg Chl during the first 5 min in the light (Fig. 4). The failure of these chloroplasts to continue to accumulate RuBP may be due to inorganic phosphate depletion caused by the exchange of triose-P for internal P₃ (5). A slow buildup of RuBP occurred in chloroplast samples without FBP, yet the deactivation of the RuBP carboxylase was similar under both conditions. Again, it appeared that the deactivation process of the carboxylase in the absence of CO₂ was independent of the chloroplast RuBP level.

Effect of OAA and PGA on Chloroplast RuBP Carboxylase. Activation of the RuBP carboxylase can be influenced by NADPH (3, 9). In order to turn over the NADPH pool in intact chloroplasts during illumination under conditions of low CO₂, OAA or PGA was added. The dicarboxylate translocator of the chloroplast envelope mediates the uptake of OAA, where it is reduced to malate by NADPH-malate dehydrogenase (5). In Experiment 1, Table I, the presence of OAA stopped the light-dependent accumulation of chloroplast RuBP without affecting the deactivation of the carboxylase. The failure to accumulate RuBP may have been due to O₂ evolution and the RuBP oxygenase.

Table I. Response of Chloroplast RuBP Levels and Carboxylase Activity upon Illumination

<table>
<thead>
<tr>
<th>Additions to Chloroplasts</th>
<th>RuBP</th>
<th>Carboxylase activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg Chl</td>
<td>μmol CO₂/mg Chl-min</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2 mm PPI</td>
<td>37</td>
<td>4.9</td>
</tr>
<tr>
<td>Plus 1.7 mm OAA</td>
<td>55</td>
<td>4.7</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mm PGA</td>
<td>27</td>
<td>4.3</td>
</tr>
<tr>
<td>Plus 6.7 mm DL-glyceraldehyde</td>
<td>36</td>
<td>3.6</td>
</tr>
</tbody>
</table>

FIG. 4. Effect of added triose-P on RuBP carboxylase activity and chloroplast RuBP. Chloroplasts (0.090 mg Chl/ml) were illuminated according to "Materials and Methods" after solutions were flushed with CO₂-free N₂ for 10 min either with (△) or without (○) FBP (5.0 mM) and rabbit muscle aldolase (1.1 IU/ml) added to make triose-P.
Addition of PGA did support a 4-fold accumulation of RuBP and was inhibited by Dl-glyceralddehyde (2) (Experiment 2, Table I). Activation of the RuBP carboxylase in the chloroplast could be stabilized by addition of PGA. The stabilization was variable between chloroplast preparations and may reflect the ability of a given preparation to exchange internal phosphate for external PGA (5).

DISCUSSION

Plant chloroplasts synthesize considerable amounts of RuBP carboxylase. Information on this enzyme and the activity it expresses in chloroplasts are a key to understanding what factors control the rate of photosynthetic CO₂ assimilation. Interestingly, isolated chloroplasts are capable of accumulating considerable amounts of the substrate, RuBP, in the absence of CO₂ or O₂. Levels of RuBP up to 300 nmol/mg Chl have been seen. This accumulation can be assisted by adding external carbon sources such as FBP to the chloroplast suspension or by incubating chloroplasts in the presence of PPI, which probably reduces leakage of sugar phosphates (23). The cause of the deactivation of the enzyme in the chloroplast appeared primarily due to the absence of CO₂. The presence of PGA stabilized activation of the RuBP carboxylase in the absence of CO₂, while RuBP was still accumulating. The presence of O₂ did not stabilize activation. With intact chloroplasts we found no correlation between the rate of RuBP carboxylase inactivation and the internal concentration of RuBP.

The similarities as well as the differences in response between the purified RuBP carboxylase and the carboxylase as it operates in the chloroplast need proper understanding. Both enzymes are activated by CO₂ and Mg²⁺ (11). The purified carboxylase is irreversibly inhibited by RuBP preparations and this occurs even in the presence of high CO₂. However, when RuBP is added to a chloroplast lysate, the carboxylase rapidly reactivates upon the conversion of RuBP to PGA. The chloroplast appears able to protect against inactivation of the carboxylase by high ribulose bisphosphate levels (greater than 250 nmol/mg Chl), a condition often found within leaves of whole plants undergoing photosynthesis at air levels of CO₂ (16). The mechanisms of this protection are currently being investigated.

LITERATURE CITED

13. NORTON IL, MH WELCH, FR HARTMAN 1975 Evidence for essential lysyl residues in ribulosebisphosphate carboxylase by use of the affinity label 3-bromo-1,4-dihydroxy-2-butane 1,4-bisphosphate. J Biol Chem 250: 8062-8068
15. PAECH C, NE TOLBERT 1978 Active site studies of ribulose 1,5-bisphosphate carboxylase/oxygenase with pyridoxal 5'-phosphate. J Biol Chem 253: 7864-7872
17. SCHLOS JF, IL NORTON, CD STRINGER, FC HARTMAN 1978 Inactivation of ribulosebisphosphate carboxylase by modification of arginyl residues with phenylglyoxal. Biochemistry 17: 5626-5631
20. SICHER RC, RG JENSEN 1979 Photosynthesis and ribulose 1,5-bisphosphate levels in intact chloroplasts. Plant Physiol 64: 880-883
21. VATER J, J SALNIKOW 1979 Identification of two binding sites of the d-ribulose 1,5-bisphosphate carboxylase/oxygenase from spinach for d-ribulose 1,5-bisphosphate and effectors of the carboxylation reaction. Arch Biochem Biophys 194: 190-197