Ribulose Bisphosphate Carboxylase/Oxygenase Content Determined with $[^{14}C]$Carboxypentitol Bisphosphate in Plants and Algae

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ABSTRACT

As is the case with spinach ribulose bisphosphate carboxylase/oxygenase (Rubisco), $[^{14}C]$carboxyarabinitol bisphosphate (CABP) bound to purified Chlorella Rubisco with a molar ratio of unity to large subunit of the enzyme. The concentration of binding sites in extracts of photosynthetic organisms was determined by reacting the extracts with $[^{14}C]$carboxypentitol bisphosphate (CPBP) and precipitating the resultant Rubisco-$[^{14}C]$CABP complex with a combination of polyethylene glycol-4000 and MgCl$_2$. Plots of the relationship between concentrations of $[^{14}C]$CPBP in the reaction mixture and the precipitated $[^{14}C]$CPBP gave a straight line and the concentration of binding sites was estimated by extrapolation to zero $[^{14}C]$CPBP since the dissociation constant of CABP with Rubisco is 10$^{-11}$ molar. Spinach, pea, and soybean leaves contained 6.4 to 6.8 milligrams Rubisco per milligram chlorophyll, corresponding to 92 to 97 ribulose bisphosphate-binding sites per milligram chlorophyll. The Rubisco content of sunflower and wheat leaves was 5.3 to 5.5 milligrams per milligram chlorophyll. The concentrations in C$_3$ plants were not uniform and corn and Panicum miliaceum leaves contained 3 and 7 milligrams Rubisco per milligram chlorophyll. The Rubisco content of green algae was one-fifth to one-sixth that of C$_3$ plant leaves and was affected by the CO$_2$ concentration during growth. The content of Euglena and blue-green algae is also reported.

In the last decade it has been possible to show that there are sufficient amounts of all of the enzymes of photosynthetic carbon reduction cycle in leaves to sustain the maximum rates of photosynthesis that have been observed (23). Under most conditions, then, the rate of photosynthesis is controlled not by the amount of any enzyme but by other factors. Of considerable interest, at the moment, is the control that can be exerted at the carboxylation reaction. Rubisco$^2$ is a complex enzyme that must be activated (17, 19), and the amount of active enzyme in the leaf may control photosynthesis (21). Alternatively, Farquhar et al. (12) and von Caemmerer and Farquhar (27) postulated that CO$_2$ assimilation was limited by the enzymic rate of Rubisco at limiting intracellular concentrations and by the RuBP regeneration rate at saturating intercellular concentrations. Some support for this model was presented by several workers (8, 14, 28) but other results showed that, in a region where RuBP should be limiting, the concentrations of RuBP in the leaves exceeded the amount of active sites of Rubisco (5, 21).

It is well recognized that the interpretation of experiments relating the control of photosynthesis at the level of RuBP requires a knowledge of the number of active Rubisco sites in the leaf (5, 8, 21, 28). With the exception of a few prokaryotes (4), all photosynthetic organisms (1) contain Rubisco with eight large and eight small subunits and eight active sites per mol of enzyme. The amount of Rubisco in photosynthetic organisms has been determined by immunological techniques (9, 16, 21), but the purification of the protein and the preparation of antibody are time-consuming processes. CPBP binds to Rubisco (9, 20, 22, 26), and CABP, a structural analog of the transition state intermediate of the carboxylation reaction, binds with a $K_D$ of 10$^{-14}$ m (22). Rather than precipitating the $[^{14}C]$CPBP-Rubisco complex with antibody (9), we describe in this paper a modification of the method using PEG-4000 (18) to precipitate the complex. The concentration of binding sites and hence the amount of Rubisco can be determined in a few hours.

MATERIALS AND METHODS

Plants and Algae. Pea (Pisum sativum L. cv Alaska), soybean (Glycine max L. Merr. cv Harosoy), spinach (Spinacia oleracea L. cv Longstanding Bloomdale), wheat (Triticum aestivum L. cv Frederick), sunflower (Helianthus annuus L. cv Mennonite), corn (Zea mays L. Illinois Foundation Hybrid FR9XF37), and Panicum miliaceum Nees ex. Trin. were cultivated in a greenhouse. The plants were watered with a commercial fertilizer daily. Expanding green leaves of these plants grown for 4 to 12 weeks were used for experiments. Selaginella martensii had been cultured in the green house of the Department of Biology, Queen’s University.

Chlorella pyenoidosa Chick (UTEX 252) was cultivated in the light (200–250 $\mu$E m$^{-2}$ s$^{-1}$) in Allen’s medium (2) buffered with 50 mM glycol-glycine buffer (pH 8.0). Ankistrodesmus braunii (UTEX 750), Selenastrum minutum isolated from Lake Ontario by Ivor Elrfi (Department of Biology, Queen’s University), Anabaena flos-aquae from Dr. D.H. Turpin (Department of Biology, Queen’s University), and Anacystis nidulans (UTEX 625) were grown in Gorham’s (15) medium (pH 8.0). The green and blue-green algae were illuminated at 100 to 150 and 50 to 100 $\mu$E m$^{-2}$ s$^{-1}$, respectively. Euglena gracilis Z was grown in Cramer and Myer’s medium (pH 6.8) (11) and with a light intensity of 100 to 150 $\mu$E m$^{-2}$ s$^{-1}$. These cultures were bubbled with air or 1% CO$_2$ in air throughout the growth period.

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3 Abbreviations: Rubisco, ribulose bisphosphate carboxylase/oxygenase; RuBP, ribulose bisphosphate; CPBP, 2-carboxypentitol bisphosphate; CABP, 2-carboxyarabinitol bisphosphate; PMSF, phenylmethylsulfonyl fluoride.
Purification of Rubisco from *Chlorella pyrenoidosa*. *Chlorella* Rubisco was purified according to McCurry et al. (18) with some modifications. All procedures were conducted at 0 to 4°C. The 1% CO₂-grown cells (wet weight 30 g) suspended in 25 mM Tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol and 1 mM EDTA were disintegrated by sonication. The cell extract, after centrifugation at 10,000 g for 10 min was subjected to (NH₄)₂SO₄ precipitation. The protein fraction that precipitated between 30 and 60% (NH₄)₂SO₄ was dialyzed against the above Tris buffer overnight and applied onto a column (4 x 40 cm) of DEAE-cellulose that had been equilibrated with the buffer. After extensive washing of the column with the buffer, Rubisco was eluted with a 0 to 0.5 M NaHCO₃ linear gradient in the buffer. The eluted Rubisco fractions, determined by the method of Lorimer et al. (17), were collected and the enzyme was precipitated with 60% (NH₄)₂SO₄. The precipitated Rubisco was dissolved in a small volume of the Tris buffer and layered on a column (1.5 x 100 cm) of Sepharose 4B equilibrated with the same buffer. The elution rate was 2 ml/h. The active fractions were collected and Rubisco was recovered with 60% (NH₄)₂SO₄. These column chromatographies were repeated. The Rubisco appeared pure SDS-polyacrylamide electrophoretically (29).

Preparation of 2-[^4]C]Carboxypentitol Bisphosphate. CPBP was prepared according to the method of Siegel and Lane (26) and purified by chromatography (20). The prepared CPBP is an epimer mixture of CABP and 2-carboxyribitol bisphosphate (20, 22, 26). The specific radioactivity of the synthesized [^4]C]CPBP was 1250 dpm/nmol.

Estimation of RuBP Binding Sites and Rubisco in Leaf and Algal Extracts. Plant leaves were homogenized with a mortar and pestle at room temperature (20–22°C) in a homogenizing buffer containing 50 mM Bicine-NaOH buffer (pH 8.2), 20 mM NaHCO₃, 20 mM MgCl₂, 10 mM DTT, 1 mM EDTA, 1 mM cysteine-HCl, 0.5 mM PMSF, and 1% PVP at the ratio of 5 ml of buffer to 0.5 g fresh weight of the leaves. The homogenate was centrifuged at 10,000 g for 3 min at room temperature in order to collect all of membraneous and soluble components liberated from leaf tissue. Fifty ml Hepes-NaOH buffer (pH 7.6) was substituted for the Bicine buffer and 1% PVP was omitted in the cell disruption of green and blue-green algae and *Euglena*. *Chlorella* cells were disintegrated twice in a precooled French pressure cell at 10,000 p.s.i. at 4°C, and other algae and *Euglena* by sonication at 0°C. After dilution of the extract with the homogenizing buffer (PVP), it was preincubated for 5 min at 25°C in centrifuge tubes capped with Parafilm. The reaction of Rubisco and [^4]C]CPBP was started by adding 32 to 196 μM [^4]C]CPBP. After 1 h, the reaction mixture was cooled promptly in ice and 4 ml of precooled 30% PEG-4000 (18) containing either 50 mM Bicine-NaOH buffer (pH 8.2) for plants or 50 mM Hepes-NaOH buffer (pH 7.6) for algae and *Euglena*, and both 20 mM NaHCO₃ and 20 mM MgCl₂ were added to the mixture and stirred vigorously with a glass rod until diffraction of light passing through the PEG solution vanished. The PEG solution was placed in ice for 30 min to allow complete precipitation of proteins. After centrifugation of the solution at 25,000g for 15 min at 0°C, the resultant pellet and the centrifuge tube were extensively washed with 20% PEG-4000 containing the above solutions three times. The washed pellet was dissolved in 1 ml of 1% Triton using a Vortex mixer, and an aliquot of the Triton solution was used for determining radioactivity with a scillation counter. The amount of Rubisco was estimated by multiplying the mol number of the determined RuBP-binding sites by 70,000, the mol wt of one large and one small subunit of Rubisco (1).

Determination of Chi and Protein. Chi of *Chlorella* was determined with 100% methanol (25) and that of other algae and plants by the method of Arnon (3). Colorimetric determination of purified *Chlorella* Rubisco was conducted according to the method of Bradford (7) with the Rubisco that had been dialyzed against sufficient volume of distilled H₂O twice, lyophilized, and dissolved in dilute alkali.

RESULTS

CABP-Binding of *Chlorella* Rubisco. Although binding of CABP to spinach Rubisco and its kinetics have been studied (22), the CABP-binding capacity of algal Rubisco is unknown. Figure 1 is an elution profile of [^4]C]CPBP-Chlorella Rubisco complex formed by incubating [^4]C]CPBP and purified *Chlorella* Rubisco from a Sephade G-75 column. The radioactivity was recovered in both protein and small mol wt fractions. The ratio of mol of [^4]C]CABP recovered in the protein fractions to that of the protomer of the large subunit of the Rubisco was close to unity, indicating that 1 mol of the protomer bound 1 mol of CABP.

Evaluation of the Method to Determine the Amount of RuBP-Binding Sites and Rubisco Content with [^4]C]CPBP. Since plant and algal Rubisco binds 8 mol of CPBP per mol of Rubisco, determination of the RubBP-binding sites with [^4]C]CPBP is useful to estimate the amount of Rubisco. In the present method, homogenizing and reaction buffers contained 1 mM cysteine-HCl and 0.5 mM PMSF, which inhibit alkaline phosphatase (10) and protease (24), respectively. [^4]C]CABP-Rubisco complex was made insoluble with 24% PEG-4000 in combination with 20 mM MgCl₂ (18), and unreacted [^4]C]CPBP was washed out of the complex with 20% PEG-4000. After washing the complex with the PEG solution three times, no radioactivity was detected in supernatant after sedimentation of the complex. The complex was also separated from unreacted [^4]C]CPBP by gel chromatography of Sephadex G-75 as described in Figure 1. Although both methods gave the same value, we adopted the PEG method since it is simpler and faster.

FIG. 1. Elution profile of [^4]C]CPBP-Chlorella Rubisco complex from a Sephade G-75 column. Purified *Chlorella* Rubisco (0.6 mg) was reacted with 0.1 mM [^4]C]CPBP (1250 dpm/nmol) in the presence of 50 mM Bicine-NaOH buffer (pH 8.2), 20 mM NaHCO₃, 20 mM MgCl₂, and 1 mM DTT for 1 h at 25°C. After the reaction, an aliquot of the reaction mixture was applied onto a Sephade G-75 column (1.9 x 44 cm) equilibrated with the above reaction buffer. The inserted figure shows the mol ratio of [^4]C]CABP and Rubisco large subunit protomer (•), Amounts of Rubisco; (O), radioactivity of [^4]C]CPBP.

Since the prepared CPBP is an epimer mixture of CABP and carboxyribitol bisphosphate (20) and Kₑₐ of CABP is 10⁷ times smaller than that of carboxyribitol bisphosphate (22), CPBP was considered to be the compound that bound to Rubisco specifically but CPBP was the compound(s) that was used for experiments.
the separation of the complex by gel chromatography was very
time-consuming and could not completely remove $[^{14}C]$CPBP
bound to protein nonspecifically.

Figure 2 shows the amount of protein-bound $[^{14}C]$CPBP re-
covered when soybean leaf extract was reacted with various
concentrations of $[^{14}C]$CPBP in the presence or absence of 50
mM RuBP. The amount increased linearly with increasing
$[^{14}C]$CPBP in the reaction mixture, and the reaction of
$[^{14}C]$CPBP with Rubisco and the bulk protein was not affected
by RuBP. Since $K_d$ of the binding of CABP on the RuBP-binding
site of Rubisco is as low as $10^{-7}$ M (22), the increase in binding
of $[^{14}C]$CPBP with increasing concentrations of $[^{14}C]$CPBP in
the reaction mixture from 32 to 196 μM was due to nonspecific
binding of $[^{14}C]$CPBP to the bulk protein. The intercept of
the line with the ordinate, where the nonspecific binding is elimi-
nated, represents the amount of specific binding of $[^{14}C]$CPBP
to Rubisco. In this experiment, the RuBP-binding site of Rubisco
in soybean leaves was 94 nmol mg$^{-1}$ Chl and this corresponded
to 6.6 mg Rubisco mg$^{-1}$ Chl. The specific plus nonspecific
binding of $[^{14}C]$CPBP to protein increased with increasing
amounts of extract in the reaction mixture up to 50 μg Chl (Fig.
3). The linearity indicates that both specific and nonspecific
bindings depend on the amount of protein in the reaction
mixture.

In the present method using $[^{14}C]$CPBP, Rubisco in leaf or
algal extract was incubated at 25°C for 1 h since the entity that
binds to Rubisco with very low $K_d$ is CABP and epimerization
of carboxyribitol bisphosphate to CABP is a very slow reaction
(22). Attachment of $[^{14}C]$CPBP to proteins by covalent bonds
through its phosphate groups was possible during the long time
reaction, since crude leaf or algal extract was used in the present
method. To find if this had occurred, the isolated $[^{14}C]$CPBP-
protein complex was denatured with 5% SDS and subjected to
gel filtration on Sephadex G-25 (data not shown). $[^{14}C]$CPBP
that had originally been bound to the protein and the protein
were separated from each other, indicating that there was no
binding through covalent bonds.

Estimation of Rubisco Content in Plants and Algae. The
Rubisco concentration in plants and algae was determined using
the $[^{14}C]$CPBP-PEG method described above. The amount of protein-

bound $[^{14}C]$CPBP increased linearly with increasing concentra-
tions of $[^{14}C]$CPBP in the reaction mixture (Fig. 4). The
intercepts of the lines on the ordinate was determined by the
least squares method with correlation coefficients close to unity.
Two lines with different slopes were obtained in the estimation
for wheat, but this seems to have been due to using different
manufacturing lots of PEG-4000. Of importance is that the
intercepts on the ordinates were constant. Table I summarizes
the numbers of RubP-binding sites of Rubisco and the Rubisco
concentration in plants and algae. In such C3 plants as spinach,
pea, and soybean, there were 92 to 97 nmol of binding sites mg$^{-1}$
Chl, corresponding to 6.4 to 6.8 mg Rubisco mg$^{-1}$ Chl. The
enzyme content in wheat and sunflower was lower, 5.3 to 5.6
mg mg$^{-1}$ Chl. The Rubisco level of Selaginella was about half
that of spinach. While the amount in corn (a C4 plant) was 3.0
mg mg$^{-1}$ Chl, another C4 plant, Panicum miliaceum, contained
7.0 mg Rubisco mg$^{-1}$ Chl.

The Rubisco levels in green algae were only one-third those of
C3 plants, and were affected by CO2 concentration during growth.
Chlorella grown on 1% CO2 contained 1.5 mg Rubisco mg$^{-1}$ Chl
which was 2-fold greater than in the air-grown cells. On the other
hand, both 1% CO2- and air-grown Euglena contained similar
amounts of Rubisco (1.4 mg mg$^{-1}$ Chl). The Rubisco content of

![Fig. 2. Relationship between concentrations of $[^{14}C]$CPBP in
the reaction mixture and amounts of protein-bound $[^{14}C]$CPBP with soybean
leaf extract. (O), Control; (●), in the presence of 50 μM RuBP added 5
min before addition of $[^{14}C]$CPBP to the reaction mixture.](image)

![Fig. 3. Dependence of the amount of protein-bound $[^{14}C]$CPBP on
the amount of soybean leaf extract in the reaction mixture.](image)

![Fig. 4. Estimation of RuBP-binding sites of Rubisco of leaves of
several plants and a blue-green alga. r is the correlation coefficient of
the line by the least squares method.](image)
Table 1. Rubisco Contents in Photosynthetic Organisms

Rubisco contents in leaves and cells in these organisms were determined as described in “Materials and Methods.” Correlation coefficients obtained in least squares method were always over 0.992.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Amount of RuBP-Binding Site (nmol mg⁻¹ Chl)</th>
<th>Rubisco Content (mg mg⁻¹ Chl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₃ plants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td>97</td>
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</tr>
<tr>
<td>Wheat</td>
<td>76</td>
<td>5.3</td>
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<tr>
<td>Pea</td>
<td>92</td>
<td>6.4</td>
</tr>
<tr>
<td>Soybean</td>
<td>94</td>
<td>6.5</td>
</tr>
<tr>
<td>Sunflower</td>
<td>80</td>
<td>5.6</td>
</tr>
<tr>
<td>Selaginella martensi</td>
<td>53</td>
<td>3.7</td>
</tr>
<tr>
<td>C₄ plants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>43</td>
<td>3.0</td>
</tr>
<tr>
<td>Panicum miliaceum</td>
<td>101</td>
<td>7.0</td>
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<td>Green algae</td>
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<td>Chlorella pyrenoidosa</td>
<td>1% CO₂-grown</td>
<td>22</td>
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<td></td>
<td>Air-grown</td>
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<td>Selenastrum minutum</td>
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<td></td>
<td>Air-grown</td>
<td>9</td>
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<td>Euglenoid</td>
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<td>20</td>
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<tr>
<td></td>
<td>Air-grown</td>
<td>20</td>
</tr>
<tr>
<td>Blue-green algae</td>
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<tr>
<td>Anabaena flos-aquae</td>
<td>1% CO₂-grown</td>
<td>44</td>
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<tr>
<td></td>
<td>Air-grown</td>
<td>28</td>
</tr>
<tr>
<td>Anacystis nidulans</td>
<td>Air-grown</td>
<td>38</td>
</tr>
</tbody>
</table>

blue-green algae was intermediate between those of C₃ plants and green algae. In the case of A. flos-aquae, the level depended on the CO₂ concentration on which the cells had been grown.

**DISCUSSION**

Specific binding of CABP to Rubisco and nonspecific binding of CPBP to bulk proteins increased linearly with the protein concentration (Fig. 3). The nonspecific binding depends on the type of leaf extract, and the characteristics of the PEG-6000 but within any given conditions it increased linearly with concentration of [¹⁴C]CPBP (Fig. 4). Because of this relationship, the total binding could be corrected for nonspecific binding by the extrapolation of the line to zero concentration of [¹⁴C]CPBP. We therefore interpret the binding to represent specific binding of CABP to Rubisco and this is consistent with the Kₑ of 10⁻¹⁰ M (22) for this reaction. RuBP (Fig. 2) and carboxyribulose bisphosphate, which bind to Rubisco with Kₑ of 10⁻⁸ M (22), did not interfere with this reaction.

Using the method outlined and purified Rubisco from Chlorella, it was shown that [¹⁴C]CABP formed a complex of unit mol ratio with Rubisco large subunits and that this complex could be eluted from a Sephadex G-75 column without dissociation (Fig. 1). This indicates that CABP binds to the algal Rubisco with a low Kₑ similar to that observed in the spinach enzyme.

The Rubisco concentrations of C₃ plants were similar to those reported earlier (16) but the value for wheat was lower than that reported by Perchowericz et al. (21).

In the C₄ plants, corn and Panicum miliaceum, the Rubisco levels were 3 and 7 mg·mg⁻¹ Chl, respectively. These values are considerably greater than those reported for C₄ plants by Ku et al. (16), and the reason for these differences is not known. Certainly, the estimation of Rubisco content after separation of the enzyme on sucrose density gradients (6) would probably result in a great underestimation (16). It may also be that the antibody to tobacco Rubisco does not quantitatively precipitate the enzyme from C₄ plants (16).

The Rubisco content of green algae and Euglena was considerably less than that of C₃ plants but similar to that reported for Chlamydomonas (28). The Rubisco content of blue-green algae, on the basis of Chl, was higher than that in green algae. In both green and blue-green algae, the Rubisco content was affected by the CO₂ concentration during growth, with cells grown on high CO₂ concentrations having the higher enzyme levels. In contrast, the Rubisco content of Euglena was not affected by CO₂ concentration during growth, as observed with Chlamydomonas (28).

The accurate determination of Rubisco content in plants and algae in a few hours at the same time makes it possible to discuss the functional proportion of the enzyme in photosynthetic CO₂ fixation among these organisms. In C₄ plants, such as spinach, pea, and soybean, the determined Rubisco content may constitute the photosynthetic rate at 800 to 900 μmol CO₂ fixed mg⁻¹ Chl h⁻¹, provided that the specific activity of the enzyme is 2.2 μmol mg⁻¹Rubisco min⁻¹ (18). Comparison of the predicted CO₂ fixation rate with the actual photosynthetic rate of leaves indicates that Rubisco participates in photosynthesis with 25 to 30% of its full activity even in the presence of saturating CO₂ and light in these plants. In contrast, green algae and Euglena contain much less Rubisco than these C₄ plants even though these microorganisms fix CO₂ at similar rates. Nearly full activity of Rubisco must be needed during photosynthesis in these organisms.

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