Changes of Ribulose Bisphosphate Carboxylase/Oxygenase Content, Ribulose Bisphosphate Concentration, and Photosynthetic Activity during Adaptation of High-CO₂ Grown Cells to Low-CO₂ Conditions in Chlorella pyrenoidosa

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ABSTRACT

Changes of some photosynthetic properties of high-CO₂ grown cells of Chlorella pyrenoidosa during adaptation to low-CO₂ conditions have been investigated. The Kₚ value of photosynthesis of the high-CO₂ grown cells for dissolved inorganic carbon was 3.3 millimolar and decreased to 25 to 30 micromolar within 4 hours after transferring to air. In the presence of saturating CO₂ concentrations the photosynthetic activity of the high-CO₂ grown cells was 1.5 times as high as that of the low-CO₂ grown cells. There was a significant rise of the photosynthetic activity during adaptation of the high-CO₂ cells to air, followed by a steady decrease. The activity of ribulose 1,5-bisphosphate carboxylase/oxygenase in both the high- and low-CO₂ grown cells was close to the photosynthetic activity of the cells. The concentration of ribulose 1,5-bisphosphate (RuBP) was higher in the low-CO₂ adapting and low-CO₂ grown cells than in the high-CO₂ grown cells regardless of the photosynthetic rate. This seems to be due to an increased RuBP regeneration activity during adaptation followed by maintenance of the new higher concentration. The RuBP level always exceeded the concentration of ribulose 1,5-bisphosphate carboxylase/oxygenase RuBP binding sites in both the high- and low-CO₂ grown cells at any dissolved inorganic carbon concentration.

To understand the control of photosynthetic CO₂ fixation, it is important to know the limiting step(s) of photosynthesis under various environmental conditions. Farquhar et al. (6, 7) and von Caemmerer and Farquhar (22) analyzed the regulation of photosynthetic CO₂ fixation in C₃ plant photosynthesis. In their model (7), photosynthetic CO₂ fixation in the presence of saturating light is limited by the activity of Rubisco,¹ when the CO₂ concentration is low, while it is limited by the rate of production of chemical energy on thylakoid membranes or ultimately by the rate of RuBP regeneration from PGA, when the CO₂ concentration is saturating. Their model has been supported by Badger et al. (3), who showed that the concentration of RuBP in bean leaves was much higher than that of the RuBP binding sites of Rubisco at low CO₂ and was decreased to or below the level of the binding sites with increasing atmospheric CO₂ concentration. Perchowitz et al. (17), however, obtained results with wheat leaves that were not consistent with Farquhar's model.

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³ Abbreviations: RuBP, ribulose 1,5-bisphosphate; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; PGA, 3-phosphoglycerate; DIC, dissolved inorganic carbon.

MATERIALS AND METHODS

Organism and Culturing. Chlorella pyrenoidosa Chick (UTEX 252) was grown in Allen's medium buffered with 50 mm glycylglycine buffer at pH 8.0 at a light intensity of 200 to 250 μE m⁻² s⁻¹ and 25°C (25). The culture was bubbled with sterile 1% CO₂ in air or air. The cells, in exponential growth, were collected by centrifugation at 1000g for 3 min at room temperature (20–25°C) and used for experiments immediately.

Measurement of Photosynthetic O₂ Evolution. The O₂ evolution measurement method of Miller et al. (15) was used, unless otherwise stated. Chlorella cells were collected by centrifugation at 10,000g for a few seconds at room temperature, washed with 50 mm glycylglycine buffer (pH 8.0) containing low dissolved O₂, suspended in the same buffer, and bubbled with CO₂-free N₂ gas until the O₂ concentration decreased to less than 5 μM. After the O₂ compensation point, where endogenous DIC in the medium or as a pool in the cells was depleted and photosynthetic O₂ evolution and respiratory O₂ uptake was balanced, was attained at a light intensity of 250 μE m⁻² s⁻¹, photosynthetic O₂ evolution was measured with a Clark-type O₂ electrode (Hansa-
tech Ltd., Kings Lynn, Norfolk, U.K.) at 25°C in a reaction mixture (1 ml) composed of 50 mM glycolglycine buffer (pH 8.0), 250 units of carbonic anhydrase, and various concentrations of sodium bicarbonate. The light intensity was 250 \( \mu \text{E m}^{-2} \text{s}^{-1} \).

**Determination of RuBP and PGA.** *Chlorella* cells, photosynthesizing under illumination, were killed by drawing 9 ml of the cell suspension into an illuminated disposable syringe which contained 1 ml of 4.5 mM HClO4. If the syringe was not illuminated during killing, the measured RuBP content was decreased by 50%. The killed cells were cooled promptly in ice and sonicated at 0°C for 1 min. The sonicate was centrifuged at 10,000g for 10 min to remove denatured cell protein and debris. The supernatant was neutralized to pH 6.0 to 7.0 with 5 and 0.5 M KOH, and stored in ice for 30 min. The resulting KClO4 was removed by centrifugation at 10,000g for 5 min and the supernatant was lyophilized. The lyophilized metabolites were dissolved in 0.5 ml of 50 mM Tris-HCl buffer (pH 8.0), and KClO4 was again removed by centrifugation at 10,000g for 10 s. The supernatant was used for determinations of RuBP and PGA.

RuBP in the extract was determined by measuring acid stable \(^{14}\text{C}\) after the reaction of the extracted RuBP and NaH\(^{14}\text{CO}_3\), the specific radioactivity of which had been determined, for 1 h at 25°C (3) in the presence of fully activated Rubisco purified from spinach to an electrophoretically homogeneous state (25). PGA was determined by measuring the absorbancy change of NADH in the conversion of PGA to glycerol-P in the presence of phosphoglycerate kinase, glyceraldehyde 3-P dehydrogenase (NAD\(^+\)), triose-P isomerase, and glyceral-P dehydrogenase (NAD\(^+\)) (3).

Recovery of authentic RuBP and PGA added to the *Chlorella* cells were 50.7 ± 3.2% \((n = 3)\) and 92.2 ± 2.2% \((n = 3)\), respectively. The concentrations of these metabolites reported here have been corrected for the above recoveries.

**Determination of Rubisco Content and Activity.** The concentration of RuBP binding sites of Rubisco in *Chlorella* cell extracts prepared as reported previously (25) was determined by the \(^{14}\text{C}\) carboxypentitol bisphosphate-polyethylene glycol method (25). Rubisco content was calculated by dividing the quantity of binding sites by the mol wt of one large plus one small subunit. Cell disruption for the assay of the activity and the assay followed the previous methods.

**DIC and Chl Measurement.** DIC was determined by the method of Miller et al. (15). Chl was quantified according to Arnon (1).

**RESULTS**

**RuBP and PGA Content of Low-CO\(_2\) Grown *Chlorella.*** The photosynthetic O\(_2\) evolution and amounts of RuBP and PGA in low-CO\(_2\) grown cells of *Chlorella* are shown in Figure 1 when 1 mM sodium bicarbonate was added to the cells after they had reached the O\(_2\) compensation point. Photosynthetic O\(_2\) evolution showed a lag phase for about 3 min before it reached its maximum activity. RuBP concentration was 120 nmol mg\(^{-1}\) Chl at the O\(_2\) compensation point, decreased to 35 nmol mg\(^{-1}\) Chl 15 s after the addition of bicarbonate, and thereafter was constant at 37 nmol mg\(^{-1}\) Chl. The PGA level was 48 nmol mg\(^{-1}\) Chl at the O\(_2\) compensation point, and addition of bicarbonate caused a sudden increase to 120 nmol mg\(^{-1}\) Chl. This pattern of changes in RuBP and PGA is expected from the operation of the photosynthetic carbon reduction cycle in *Chlorella* photosynthesis and indicates that the method of determining these levels of these metabolites was adequate.

**Changes of Photosynthetic Properties of High-CO\(_2\) Grown Cells of *Chlorella* during Adaptation to Low-CO\(_2\) Conditions.** Except for changes in the \(K_m\) (DIC) (2, 12, 13, 15, 18, 20) and carbonic anhydrase levels (8, 18, 20), changes in other photosynthetic properties have not been reported for high-CO\(_2\) grown algal cells adapting to low-CO\(_2\) conditions. Figure 2 shows changes of photosynthetic activity in the presence of a saturating concentration of DIC (30 mM), \(K_m\) (DIC) values of *Chlorella* cells for photosynthesis, intracellular concentrations of RuBP and PGA, and DIC in the medium, when the high-CO\(_2\) grown cells of *Chlorella* were bubbled with 1% CO\(_2\) in air for 2.5 h in the light, transferred to new 50 mM glycolglycine buffer (pH 8.0), and bubbled with CO\(_2\)-free air for 30 min in the dark, and then bubbled with air and illuminated at 250 \(\mu\)E m\(^{-2}\) s\(^{-1}\). The response of photosynthetic rate to increasing amounts of DIC in high-CO\(_2\) grown and low-CO\(_2\) grown *Chlorella* and in the high-CO\(_2\) grown cells adapted for 2 h to air is shown fully in Figure 3. The high-CO\(_2\) grown cells had a maximum photosynthetic activity of 170 \(\mu\)mol mg\(^{-1}\) Chl h\(^{-1}\). The maximum activity increased to 220 \(\mu\)mol mg\(^{-1}\) Chl h\(^{-1}\) 2 h after transfer to air, and then gradually decreased to 120 \(\mu\)mol mg\(^{-1}\) Chl h\(^{-1}\). The \(K_m\) value of the high-CO\(_2\) grown cells for DIC was 3.3 mM and decreased to 25 to 30 \(\mu\)M, which was the value of the low-CO\(_2\) grown cells, 2 to 3 h after the transfer.

DIC in the incubation medium was 6.74 \(\mu\)M when the culture was bubbled with 1% CO\(_2\) in air, and decreased to 0.18 \(\mu\)M when CO\(_2\)-free air was bubbled through new glycolglycine buffer in the dark for 30 min. Changing the flushing gas to air and illumination of the cell suspension caused a sudden increase of DIC in the incubation medium to 0.5 mM due to dissolution of air CO\(_2\) to the medium and inefficiency of the high-CO\(_2\) grown cells of *Chlorella* to photosynthesize at these low DIC concentrations. As the affinity of the *Chlorella* cells for DIC increased, the DIC in the medium gradually decreased to 80 \(\mu\)M and was constant thereafter.

The concentration of RuBP in the high-CO\(_2\) grown cells in 1% CO\(_2\) was 30 to 60 nmol mg\(^{-1}\) Chl. The concentration decreased to 10 nmol mg\(^{-1}\) Chl in the dark. In the light 30 min after transferring to air, the concentration had increased to 126 nmol

![Fig. 1. Changes of RuBP and PGA levels and photosynthetic activity in low-CO\(_2\) grown *Chlorella* after addition of bicarbonate to cells at O\(_2\) compensation point attained by bubbling with CO\(_2\)-free air. The photosynthetic activity was determined by an O\(_2\) electrode. For determination of the concentrations of RuBP and PGA, the *Chlorella* cells were incubated under the same conditions as for photosynthetic O\(_2\) evolution, except that the incubation volume was 5 ml. Chl concentration was 15 \(\mu\)g ml\(^{-1}\). O\(_2\) concentration was 21%. The photosynthetic rate was 85 \(\mu\)mol mg\(^{-1}\) Chl h\(^{-1}\) after a lag period. The figure shows the results from a single experiment that was representative of three separate experiments.](image-url)
CHLORELLA PHOTOSYNTHESIS AND ADAPTATION TO LOW-CO₂

FIG. 2. Changes of photosynthetic properties and RuBP and PGA in high-CO₂ grown cells transferred to air. The incubation mixture was bubbled with 1% CO₂ in air in the light, CO₂-free air in the dark and air at the rate of 2 L min⁻¹ per 400 ml of the mixture. The light path of the plexiglass incubation vessel was 1.2 cm. The initial Chl density was 15 µg ml⁻¹ and the light intensity was 250 µE m⁻² s⁻¹. Samples were removed at the indicated times for the determination of RuBP, PGA, photosynthetic rate in the presence of 30 mM sodium bicarbonate and 250 units of carbonic anhydrase at 250 µE m⁻² s⁻¹ and 25°C, and the Km (DIC) for photosynthesis.

FIG. 3. Photosynthetic O₂ evolution activities of the high-CO₂ grown, low-CO₂ adapting, and low-CO₂ grown cells. The low-CO₂ adapting cells are the cells adapted for 2 h to air in Figure 2.

mg⁻¹ Chl and then continued to increase to a steady state of 170 nmol mg⁻¹ Chl. PGA level was twice as high in the high-CO₂ grown cells in 1% CO₂ as in the low-CO₂ adapted cells in air.

In Figure 2, the Km value of the high-CO₂ grown cells for DIC decreased from 3.3 mM to 25 to 30 µM during adaptation of the cells to air. The ratio of [DIC] in the medium to Km (DIC) was calculated to be 2.0 for the high-CO₂ grown cells in 1% CO₂ and 3.2 for the low-CO₂ adapting cells in air. This implies that the consumption rate of RuBP by the low-CO₂ adapting cells at 80 µM DIC was higher than that of the high-CO₂ grown cells at 6.74 mM DIC, and that the high-CO₂ grown cells should contain higher RuBP levels than the low-CO₂ adapting cells at these respective DIC concentrations, if the rate of regeneration of RuBP was the same in both cell types.

We have calculated the rate of RuBP consumption by Rubisco, namely the rate of photosynthetic CO₂ fixation, at the time when the cells were sampled by resolving Michaelis-Menten equation with the actual values of [DIC] in the medium, the Km (DIC) values of the cells and the maximum activities of photosynthesis at that time, all obtained from Figure 2. The calculation assumed that the regeneration rate of RuBP was constant throughout the experiments in Figure 2. These calculations (Fig. 4) showed that the RuBP concentration of the low-CO₂ adapting cells at 80 to 90 µM DIC should have been similar to that of the high-CO₂ grown cells at 6.74 mM DIC because the rate of utilization was assumed to be the same. However, this was not the case, as can be seen in Figure 2. The RuBP levels of the low-CO₂ adapting cells were much higher than those of the high-CO₂ grown cells, indicating that the former cells had a higher efficiency of RuBP regeneration and maintained higher levels of RuBP than the latter.

To further check this observation, the RuBP levels in both cell types were plotted against [DIC]/Km of photosynthesis for DIC (Fig. 5). This kind of plotting is useful for comparing the rates of RuBP consumption by the cells having different Km values for DIC; [DIC]/Km for DIC approximates the extent of the attainment of the rate of photosynthesis or RuBP consumption to its maximum. In the high-CO₂ grown cells, the RuBP level was about 100 nmol mg⁻¹ Chl below a ratio of 0.2 of [DIC]/Km and about 200 nmol mg⁻¹ Chl at a ratio of 0.8 of [DIC]/Km. Above this point, the level was constant at about 50 nmol mg⁻¹ Chl. The RuBP concentration of the low-CO₂ adapting cells, on the contrary, was 250 to 350 nmol mg⁻¹ Chl at a ratio of 3 of [DIC]/Km, and about 100 nmol mg⁻¹ Chl when the ratio of [DIC]/Km was over 4.

In Table 1 are shown the concentration of RuBP binding sites of Rubisco, the Rubisco content and activity of Rubisco in the high-CO₂ grown, low-CO₂ adapting, and low-CO₂ grown cells of Chlorella. The level of RuBP binding sites was estimated to be 22 nmol mg⁻¹ Chl in the high-CO₂ grown cells which corresponded to 1.55 mg Rubisco mg⁻¹ Chl. The Rubisco activity of the high-CO₂ grown cells was 174 µmol mg⁻¹ Chl h⁻¹. These values of the low-CO₂ grown cells were 11 nmol mg⁻¹ Chl, 0.77

FIG. 4. Calculation of the expected rates of RuBP utilization in the course of adaptation of high-CO₂ cells to low-CO₂ conditions. Km for DIC, [DIC] and Vₘₐₓ were from Figure 2.
mg mm$^{-1}$ Chl, and 116 $\mu$mol mg$^{-1}$ Chl h$^{-1}$, respectively. Adaptation to air for 4 h caused a slight increase in Rubisco content and activity. *Chlorella* cells adapted to air for 9 h showed intermediate values between the two cell types, even though the $K_m$ (DIC) indicated that adaptation to low-CO$_2$ conditions was completed within 3 h after transfer to a low-CO$_2$ concentration (Fig. 2). There was a difference in specific activity of Rubisco between high-CO$_2$ grown or low-CO$_2$ adapting *Chlorella* and low-CO$_2$ grown *Chlorella*.

Relationship between the RubBP Level and Photosynthetic Activity in High-CO$_2$ and Low-CO$_2$ Grown Cells. From these adaptation experiments, it was suggested that the steady state concentration of RubBP during photosynthesis was quite different between high-CO$_2$ and low-CO$_2$ grown cells of *Chlorella*. In order to confirm this, the RubBP concentrations and the photosynthetic activities were determined in both cell types at various DIC concentrations. Since photosynthetic O$_2$ evolution occurred in the center of the cell, photosynthetic O$_2$ evolution required a half to a few minutes to reach full activity after addition of sodium bicarbonate to the cells at the O$_2$ compensation point (Fig. 1), the cells were killed only after O$_2$ evolution was linear at each individual DIC concentration. The cells were killed by adding HClO$_2$ to a final concentration of 0.45 M directly to the cell suspension in a small vial with a serum cap and the acidified suspension was left at the same light intensity (250 $\mu$E m$^{-2}$ s$^{-1}$) as during previous photosynthesis until the cells turned brown. Throughout the incubation and killing, the suspension was stirred with a magnetic stirrer.

The RuBP levels were plotted against the rates of photosynthetic O$_2$ evolution at various given DIC concentrations up to 30 mm in the high-CO$_2$ grown cells and 1 mm in the low-CO$_2$ grown cells (Fig. 6). This type of plot is very useful for comparing RuBP levels in both cell types at the same RuBP utilization rates. The RuBP concentration, in the low-CO$_2$ grown cells, showed a peak of 180 nmol mm$^{-1}$ Chl (which occurred at the $K_m$ concentration of DIC) and declined to 40 to 120 nmol mm$^{-1}$ Chl at [DIC] which gave rise to the maximum photosynthetic activity. This response for this large variation is known. On the other hand, the RuBP level of the high-CO$_2$ grown cells was 110 nmol mm$^{-1}$ Chl at the O$_2$ compensation point, and decreased to about 40 nmol mm$^{-1}$ Chl with increasing concentrations of DIC.

**DISCUSSION**

The expected changes in the amounts of RuBP and PGA for C$_3$ photosynthesis (24) were obtained in low-CO$_2$ grown *Chlorella* when bicarbonate was supplied to the cells (Fig. 1). But when high-CO$_2$ grown cells were transferred to low-CO$_2$ conditions, the changes in the amount of RubBP were not those that we expected (Figs. 2 and 4). Immediately after the transfer of the high-CO$_2$ grown cells to low-CO$_2$ conditions in the light, the rate of photosynthesis in the cells would be small (Fig. 3). During this period the DIC in the medium increased (due to dissolution of air CO$_2$ to the medium and respiration) and the RuBP concentration increased, an observation consistent with a lack of CO$_2$ in the cells (Fig. 2). After 3 h, as observed by others (18, 20), the cells had adapted to low-CO$_2$ conditions and the $K_m$(DIC) for photosynthesis had decreased from 3.3 mm to 50 $\mu$m. High rates of photosynthesis would now occur in the cells at the [DIC] of the medium (Figs. 2 and 3) but the RubBP level did not decrease.

This unexpected maintenance of high RubBP levels, when the cells were photosynthesizing at high rates, was investigated further in high-CO$_2$ grown cells, in such cells adapting to low-CO$_2$ conditions and in low-CO$_2$ grown cells. It was found that for similar calculated (Fig. 5) or actual (Fig. 6) rates of photosynthesis the steady state level of RubBP was much higher in high-CO$_2$ grown cells than in high-CO$_2$ grown cells. These observed results suggest that the RubBP regeneration rate had increased when high-CO$_2$ grown cells were transferred to low-CO$_2$ conditions (Fig. 2) and that the regulation of RubBP levels had now changed so that higher concentrations of RubBP were maintained in the cells (Figs. 5 and 6). The explanation or mechanism for these changes are not fully apparent at the present time. But the catalytic reaction of Rubisco is a two substrate reaction that proceeds in a random mechanism (10). With this mechanism, a decrease of the concentration of one substrate greatly increases the apparent $K_m$ of the enzyme for the other substrate (5). The elevated level of RubBP in low-CO$_2$ adapted cells may allow Rubisco to function at full activity when the CO$_2$ concentration has been increased by the development of the CO$_2$ concentrating mechanism.

Table 1. Changes of Rubisco Content and Activity during Adaptation of High-CO$_2$ Grown *Chlorella* Cells to Air

<table>
<thead>
<tr>
<th>CHLORELLA CELLS</th>
<th>CONCENTRATION OF RUBBP BINDING SITES</th>
<th>RUBISCO CONTENT</th>
<th>RUBISCO ACTIVITY</th>
<th>RUBISCO SPECIFIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol mm$^{-1}$ Chl</td>
<td>mg mm$^{-1}$ Chl</td>
<td>$\mu$mol mm$^{-1}$ Chl h$^{-1}$</td>
<td>$\mu$mol mm$^{-1}$ Rubisco min$^{-1}$</td>
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<tr>
<td>High-CO$_2$ grown</td>
<td>22</td>
<td>1.55</td>
<td>174.3</td>
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<tr>
<td>Low-CO$_2$ adapting</td>
<td>4-h adapted</td>
<td>28</td>
<td>1.95</td>
<td>190.4</td>
</tr>
<tr>
<td></td>
<td>9-h adapted</td>
<td>13</td>
<td>0.94</td>
<td>145.9</td>
</tr>
<tr>
<td>Low-CO$_2$ grown</td>
<td>11</td>
<td>0.77</td>
<td>116.6</td>
<td>2.52</td>
</tr>
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</table>
obtained with this of killed and reported that the RuBP because amount the levels of in sites the maximum concentration amount the. clear cells also decreased efficiency 15 was than the decrease by supplying CO2 RuBP levels allows development. It is well known the 20).

The simultaneous determination of Rubisco content and RuBP levels allows one to consider limiting steps in Chlorella photosynthesis. The theory of Farquhar et al. (6, 7) proposes that photosynthetic CO2 fixation is limited by the low affinity of Rubisco under low-CO2 conditions and is limited by RuBP concentration under saturating CO2 conditions. In Chlorella, however, the concentration of RuBP never decreased below the number of RuBP binding sites but was always at least double the concentration of the binding sites. This would suggest that under saturating light, Chlorella photosynthesis is not limited by the amount of RuBP at any DIC concentration. At saturating DIC concentration it would seem that Rubisco is fully activated and the maximum photosynthetic rate of Chlorella is determined by the amount of Rubisco in the cells.

von Caemmerer et al. (23) reported that the RuBP level in Chlamydomonas was below the concentration of RuBP binding sites in the presence of high DIC concentrations. The measured levels of RuBP in their experiments, however, may be low because they did not illuminate the syringe in which the cells were killed and we have found that a lack of illumination during this step resulted in extractable RuBP levels of about one-half that obtained with an illuminated syringe. Badger et al. (3) also reported that the RuBP concentration was less than the concentration of RuBP binding sites in bean leaves under saturating CO2 concentrations. However, they compared the RuBP concentration to the concentration of RuBP binding sites reported for spinach (9) but the concentration of binding sites in bean may be lower than in spinach. Certainly, in Chlorella, the concentration of RuBP does not appear to ever limit photosynthesis but additional results are required to determine the frequency and distribution of this situation.

LITERATURE CITED