Regulation of Energy Balance in Photosystems in Response to Changes in CO₂ Concentrations and Light Intensities during Growth in Extremely-High-CO₂-Tolerant Green Microalgae

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Abstract

Regulation of energy balance in photosystems in response to extremely-high-CO₂ (40%) and low-CO₂ (0.04%) stress was studied in extremely-high-CO₂-tolerant green microalgae, Chlorococcum littorale and Chlorella sp. UK001. To investigate the energy input process, we assessed an F₇₁₄/F₆₈₅-ratio in a 77K fluorescence emission spectrum induced by 440-nm excitation in intact cells, which represents a ratio of fluorescence intensities derived from light-harvesting chlorophyll complexes in PSI and PSII. The F₇₁₄/F₆₈₅-ratio increased in several days after transferring C. littorale cells from air to 40% CO₂, from 3% to 40% CO₂ and from 3% to air. In all cases, the increase in the F₇₁₄/F₆₈₅-ratio was observed in high cell density culture, but no or a little increase was apparent in sparse cell density culture, when these cultures were illuminated at 250 μmol photon m⁻² s⁻¹. Even in the sparse culture, however, a similar increase in the F₇₁₄/F₆₈₅-ratio was observed when C. littorale cells were transferred from 3% to 40% CO₂ to 20 μmol photon m⁻² s⁻¹. The cell density did not affect the F₇₁₄/F₆₈₅-ratio when CO₂ concentration was kept at 3%. The activity of PSI electron (e⁻) transport was much higher in 40% CO₂-grown cells than in 3% CO₂-grown cells irrespective of the cell density during the culture, whereas the difference in PSI activity between them was small. The PSI activity at high cell density was higher also in air-grown cells than that in 3% CO₂-grown cells. In both dense and sparse culture, 40% CO₂-grown cells and air-grown cells showed higher relative quantum yield of PSI in the presence of DCMU than 3% CO₂-grown cells, suggesting an increase in cyclic electron flow around PSI. Likewise, the increase in the F₇₁₄/F₆₈₅-ratio in response to the transfer to 40% CO₂ was observed also in another extremely-high-CO₂-tolerant alga, Chlorella sp. UK001. The possible role of the increases in the F₇₁₄/F₆₈₅-ratio, PSI/PSII activity ratio and cyclic e⁻ transport activity in extremely-high-CO₂ acclimation is discussed in comparison with low-CO₂ acclimation.

Key words: Acclimation — Chlorella sp. UK001 — Chlorococcum littorale — Chlorophyll fluorescence — CO₂ concentration.

Introduction

In response to changes in environmental conditions, photosynthetic organisms regulate the balance between the light energy input, electron transport and metabolism either to maintain maximal levels of energy conversion or prevent over-reduction and subsequent damage. This regulation is achieved by at least two mechanisms, the state transition and the adjustment of the relative quantities (stoichiometry) of PSI and PSII, being often accompanied with change in the cyclic electron transport activity (for reviews, Melis 1991, Fujita et al. 1994, Allen 1995, Bendall and Manasse 1995, Huner et al. 1998). The state transition is a regulatory mechanism that alters excitation energy distribution between PSII and PSI by transferring light-harvesting antenna complexes (LHC) from PSII to PSI (and vice versa) via phosphorylation-dephosphorylation of LHCII. The adjustment of PSI : PSII stoichiometry accompanies change in PSII reaction center ratio and depends on de novo synthesis. The former mechanism can be observed within several min or less and therefore is called short-term adaptation, whereas the latter is called long-term adaptation because change in PSI/PSII stoichiometry ratio usually occurs on the order of h to days. Many studies have demonstrated that light quality and intensity, temperature, salt stress and their combinations affect photosynthetic characteristics such as the PSI/PSII reaction center ratio, electron transport capacities of two photosystems, cyclic electron transport activity and 77K fluorescence emission spectrum in cyanobacteria and green microalgae (e.g. Schubert and Hagemann 1990, Jeanjean et al. 1993, Murakami and Fujita 1993, Maxwell et al. 1994, Hibino et al. 1996, Murakami et al. 1997a, Murakami et al. 1997b, Miskiewicz et al. 2000).

The concentration of CO₂ present in air (0.04%) is the limiting condition for photosynthesis (Aizawa and Miyachi 1986). Bürger et al. (1988) and Miyachi et al. (1996) found in...
some cyanobacteria and unicellular green algae, by measuring 77K fluorescence emission spectra, that the ratio of PSI/PSII fluorescence (F₇₁₀₋₇₄₀/F₆₇₀₋₇₀₀) was higher in air-grown cells than in the cells grown under 4.4% CO₂. They also found that the quantum requirement for photosynthetic oxygen evolution was higher in the cells grown under 4.4% CO₂ than in air-grown cells. Besides air level of CO₂ concentration, it was reported in a cyanobacterium, *Anacystis nidulans*, that the cells grown under low CO₂ supply conditions (0.2% CO₂) showed higher PSI/PSII reaction center ratio than those grown at 3% CO₂ (Manodori and Melis 1984). The higher PSI/PSII fluorescence and reaction center ratio under limited-CO₂ conditions are thought to reflect the requirement of the excess energy from PSI for cyclic electron flow to drive the inorganic carbon pump as suggested by Ogawa and Ogren (1985) and Ogawa et al. (1985) (for a recent review, Kaplan and Reinhold 1999). Form of inorganic carbon source (CO₂ or HCO₃⁻) was also reported to affect the PSI/PSII stoichiometry in *Synechocystis* PCC 6714 (Murakami et al. 1997b).

In contrast to low-CO₂ acclimation, less attention has been paid to the mechanism underlying the photosynthetic acclimation of algae to extremely high concentrations of CO₂ (>20%), since studies on the *Chlorella* species have shown that CO₂ above 5% was not advantageous for growth (Nielsen 1955). However, we had isolated a marine green microalga, *Chlorococcum littorale*, which could grow rapidly under extremely high CO₂ concentrations (Kodama et al. 1993) during the study for application of industrial exhaust usually containing 10–20% CO₂ to algal cultivation. The finding of this alga prompted us to study the mechanism underlying the acclimation to extremely high-CO₂ concentrations.

When air-grown cells of *C. littorale* were transferred to 20–40% CO₂, one of the unique features was that active photosynthesis and hence growth were enhanced after a lag period of 1–2 d at 20% CO₂, and of 3–6 d at 40% CO₂ (Pesheva et al. 1994). During the lag period, transient increases in the PSI/PSII electron transport activity ratio and the PSI/PSII fluorescence ratio (F₇₁₀/F₆₈₅) in 77K fluorescence emission spectra were observed (Pesheva et al. 1994, Iwasaki et al. 1998). The transient increase in the PSI/PSII fluorescence ratio accompanied by inhibition of photosynthetic O₂ evolution was induced by 40% CO₂ within several min (Demidov et al. 2000). Based on these and other results, we have proposed that the transient state 2 transition regulated the energy balance during the lag period. Likewise, we recently found that the rapid inhibition of photosynthesis by 40% CO₂ leading to the lag period could be ascribed to inactivation of the Calvin-Benson cycle due to rapid acidification of the stroma by intracellular carbonic anhydrase (CA), which had been induced in air-grown cells (Satoh et al. 2001). CA catalyzes the reaction, CO₂+H₂O→HCO₃⁻+H⁺, and therefore enhances proton production (acidification) under excess CO₂ concentrations. We also found in the previous study that *C. littorale* cells that had been grown under 3–5% CO₂ had no CA activity and therefore could grow without any lag period upon the acclimation to 40% CO₂. We are interested to see whether some regulation of energy balance is required even after the cells resume photoautotrophic growth, after acclimation to extremely high (40%) or low CO₂ concentrations.

In the present study, we therefore investigated the changes in energy balance during and after acclimation of *C. littorale* cells to 40% CO₂ either from air or 3% CO₂. The same changes were studied also with another extremely-high-CO₂-tolerant alga, *Chlorella* sp. UK001 (Murakami et al. 1998), during and after acclimation from air to 40% CO₂. It has been known that this alga could grow without a lag period even when the cells are transferred from air to 40% CO₂ (Satoh et al. 2001). We also investigated the changes in energy balance during and after the acclimation from 3% CO₂ to air in *C. littorale* cells. Similar studies on the changes in energy balance during low-CO₂ acclimation have been carried out with some cyanobacteria and green algae (Bürger et al. 1988, Miyachi et al. 1996).

**Results**

Fig. 1A (closed circles) shows that the cell concentration during growth under air conditions remained at a low level of around 5 (mg Chl) liter⁻¹, indicating that the growth rate was proportional to the dilution rate. When these air-grown *C. littorale* cells were transferred to 40% CO₂, the cells grew with increased velocity until day 8, after a lag period from day 1 to day 3. After day 8, the cell concentration became constant at around 35 (mg Chl) liter⁻¹, indicating that the velocity of growth decreased again to the level proportional to the dilution rate. In another experiment (open circles), the cell concentration was kept mostly below 10 (mg Chl) liter⁻¹. The quantum efficiency of PSII of the air-grown *C. littorale* cells was about 0.4 (Fig. 1B). The value decreased within 30 min after the transfer to 40% CO₂, started to increase on day 2, and finally remained constant at around 0.6.

Time-course changes in the 77K fluorescence emission spectra during these acclimation processes are shown in Fig. 1C. These spectra normally showed peaks at around 685 nm, 698 nm and 714 nm. The peak at the shortest wavelength was mainly due to the light-harvesting antenna chlorophyll of PSII, whereas the peak at the longest wavelength mainly originated from the light-harvesting chlorophyll complex of PSI (Murata and Satoh 1986). A change in the ratio of the fluorescence intensity at 714 and 685 nm (F₇₁₀/F₆₈₅-ratio, Fig. 1D) therefore represents a relative alteration of the antenna sizes of PSI and PSII. The transient changes in the growth, photosynthetic activity and 77K fluorescence emission spectra during the lag period were the same as those that had been observed previously (Pesheva et al. 1994, Iwasaki et al. 1996, Iwasaki et al. 1998, Demidov et al. 2000).

In the present study, we extended the evaluation period until the cell concentration became constant under 40% CO₂ in the continuous culture (Fig. 1A, closed circles). This led to the finding that the PSI peak again became more marked and
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finally dominated (solid spectra in Fig. 1C and closed circles in Fig. 1D), in accordance with the increase in the cell concentration (Fig. 1A, closed circles). In contrast, no such increase in the PSI peak was apparent (gray spectra in Fig. 1C and open circles in Fig. 1D) when the cell concentration was kept sparse (Fig. 1A, open circles).

Similar experiments were carried out by transferring 3% CO₂-grown C. littorale cells to 40% CO₂ (Fig. 2). In one experiment (closed circles), the cell concentration remained constant at about 40 (mg Chl) liter⁻¹ throughout the experimental
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Fig. 2  Time-course changes in the cell concentration (A), Φ₀ (B), 77K fluorescence emission spectra (C) and F₇₁₄/F₆₈₅-ratio (D) during the acclimation of 3% CO₂-grown C. littorale cells to 40% CO₂. In one experiment (closed circles and solid-line spectra), C. littorale cells were grown under 3% CO₂ by continuous culture with a dilution rate of 0.0125 h⁻¹, and then transferred to 40% CO₂ without changing the dilution rate. In another experiment (open circles and gray-line spectra), 3% CO₂-grown cells which had been kept in the continuous culture were diluted to about 2 (mg Chl) liter⁻¹ on day −1, further grown under 3% CO₂ for 24 h without continuous dilution and subsequently transferred to 40% CO₂ after diluting the cell concentration again to about 2 (mg Chl) liter⁻¹. After that, the cell concentration was kept mostly below 10 (mg Chl) liter⁻¹ by diluting the culture once a day. Culture vessel and light conditions were the same as described in Fig. 1. The CO₂ concentration was changed to 40% at the time indicated by the vertical dotted lines.
and hence no increase in the F$_{\text{77K}}$ fluorescence emission spectra (Fig. 2C, gray spectra) – 240% CO$_2$ in the sparse culture (below 10 (mg Chl) liter$^{-1}$).

The changes in 77K fluorescence emission spectra during acclimation of C. littorale cells from 3 to 40% CO$_2$ were therefore investigated at both 20 and 250 µmol photon m$^{-2}$ s$^{-1}$ (Fig. 3). To minimize the mutual shading of cells, the cells were kept thin and sparse in a flat oblong culture vessel (inner thickness of 2.8 cm). It was demonstrated that the PSI peak increased even in the sparse culture when 3% CO$_2$-grown cells of C. littorale were acclimated to 40% CO$_2$ at 20 µmol photon m$^{-2}$ s$^{-1}$, but not at 250 µmol photon m$^{-2}$ s$^{-1}$. In this experiment, however, the PSII peak was still dominant, unlike the 77K fluorescence emission spectra observed in the C. littorale cells acclimated to 40% CO$_2$ in the dense culture (Fig. 1, 2). The cells in the dense culture with a 5-liter cylindrical vessel (Fig. 1, 2) were seesawing between 250 and 20 µmol photon m$^{-2}$ s$^{-1}$, whereas the cells were kept at 20 µmol photon m$^{-2}$ s$^{-1}$ in a flat oblong culture vessel (Fig. 3). These situations would be one of the reasons which caused difference in relative level of PSII peak between the experiments carried out with a 5-liter cylindrical (Fig. 1, 2) and a flat vessel (Fig. 3).

The effect of the cell concentration on 77K fluorescence spectra under 3% CO$_2$ was further confirmed in Fig. 4. Although the cell concentration finally increased to about 40 (mg Chl) liter$^{-1}$ (Fig. 4A), no change in the 77K fluorescence emission spectra was apparent (Fig. 4B), and the F$_{714}$/F$_{685}$-ratio remained constant at 0.7–0.8 (Fig. 4C) throughout the experimental period.

When air-grown Chlorella sp. UK001 cells were transferred to 40% CO$_2$ by a continuous culture, the cells grew steadily with increased velocity until day 3, before the growth rate decreased to a level proportional to the dilution rate (Fig. 5A), confirming that this alga could acclimate to 40% CO$_2$ without a lag period (Satoh et al. 2001). This result is supported by no marked decrease occurring in the quantum efficiency of PSII in this alga (Fig. 5B). The increase in the PSI peak in the 77K fluorescence emission spectra (Fig. 5C), and hence an increase in F$_{714}$/F$_{685}$-ratio (Fig. 5D), was apparent after transferring the air-grown Chlorella sp. UK001 cells to 40% CO$_2$, although the PSI peak was dominant even in air-grown cells in this alga. Unlike the observation in C. littorale (Fig. 1), acclimation of Chlorella sp. UK001 from air to 40% CO$_2$ involved no rapidly-induced transient increase in the F$_{714}$/F$_{685}$-ratio (Fig. 5).

In each experiment shown in Fig. 1 and 2, the light intensity at the surface of the vessel was about 250 µmol photon m$^{-2}$ s$^{-1}$. When the same intensity of light was applied to the cylindrical apparatus with a diameter of ca. 15 mm, photosynthetic O$_2$ evolution measured by Clark-type O$_2$ electrode was nearly saturated (unpublished data). When the vessel contained dense culture (around 40 (mg Chl) liter$^{-1}$), the light intensity was less than 20 µmol photon m$^{-2}$ s$^{-1}$ at the center of the vessel due to mutual shading of algal cells. On the other hand, only slight mutual shading was expected in sparse culture (below 10 (mg Chl) liter$^{-1}$).

Fig. 3 Effects of light intensity during growth on time-course changes in 77K fluorescence emission spectra upon acclimation of 3% CO$_2$-grown C. littorale cells to 40% CO$_2$. The 3% CO$_2$-grown cells that had been grown in continuous culture were transferred to a new flat oblong culture vessel at a density of 5 (mg Chl) liter$^{-1}$ on day 0 in the presence of 50 mM MES-NaOH (pH 5.5) buffer and subjected to 40% CO$_2$ at 20 (solid-line spectra) and 250 (gray-line spectra) µmol photon m$^{-2}$ s$^{-1}$. Throughout the experiments, the cell concentration was kept below 10 (mg Chl) liter$^{-1}$ by diluting these batch cultures once a day.

period, indicating that the cells grew with a constant velocity equivalent to the dilution rate even after their transfer to 40% CO$_2$. In another experiment (open circles), the cell concentration increased immediately after diluting the cell suspension irrespective of the CO$_2$ concentration applied during algal growth, confirming that the cells grew without any inhibition at 40% CO$_2$. After their transfer to 40% CO$_2$, no decrease was apparent in the quantum efficiency of PSII (Fig. 2B), indicating that there had been no inhibition of photosynthesis by 40% CO$_2$.

During the growth period under 3% CO$_2$, no remarkable difference in the 77K fluorescence emission spectra was apparent between the dense culture (Fig. 2C, solid spectra) and the sparse culture (Fig. 2C, gray spectra). In these spectra, the PSI peak was larger than the PSI peak, giving values for the F$_{714}$/F$_{685}$-ratio of 0.6–0.7 (Fig. 2D). After transferring 3% CO$_2$-grown cells to 40% CO$_2$ in the dense culture, the PSI peak became dominant with the smaller PSII peak (Fig. 2C, solid spectra), resulting in an increase in the F$_{714}$/F$_{685}$-ratio up to around 1.4 (Fig. 2D, closed circles). In contrast, when the cells were transferred to 40% CO$_2$ in the sparse culture, no change in the 77K fluorescence emission spectra (Fig. 2C, gray spectra) and hence no increase in the F$_{714}$/F$_{685}$-ratio (Fig. 2D, open circles) was apparent.

In each experiment shown in Fig. 1 and 2, the light intensity at the surface of the vessel was about 250 µmol photon m$^{-2}$ s$^{-1}$. When the same intensity of light was applied to the cylin-
Energy balance in algae under excess and low CO₂ concentration in each culture was controlled as shown in Fig. 6A. After the transfer to air, the quantum efficiency of PSII decreased from about 0.6 to 0.4 in both the dense (Fig. 6B, closed circles) and the sparse (Fig. 6B, open circles) culture, indicating that photosynthesis was limited under both conditions. In the sparse culture, the PSI peak became more marked after the transfer to air, although the PSII peak remained dominant (Fig. 6C, gray spectra), and the $F_{714}/F_{685}$-ratio changed from 0.6 to 0.8–0.9 (Fig. 6D, open circles). On the other hand, in the dense culture, the PSI peak became dominant (Fig. 6C, solid spectra), and the $F_{714}/F_{685}$-ratio correspondingly increased from around 0.6 to around 1.4 (Fig. 6D, closed circles).

In 3% CO₂-grown C. littorale cells, although both PSI and PSII electron transport activities were higher in the cells grown in the sparse culture than those grown in the dense culture, the PSI/PSII activity ratio was around 1 in either case (Table 1). In both the sparse and the dense culture, 40% CO₂-grown cells of C. littorale showed much higher PSI activity and lower PSII activity than those of the 3% CO₂-grown cells. This resulted in the increased PSI/PSII activity ratio in 40% CO₂-grown cells, irrespective of the cell concentrations during the growth. Differences in the PSI and PSII activities and likewise in the PSI/
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PSII activity ratio between 3\% CO\textsubscript{2}- and air-grown cells were relatively small in the sparse culture. The marked increase in the PSI activity with a little increase in the PSII activity was observed in air-grown cells grown in the dense culture. Both air- and 40\% CO\textsubscript{2}-grown \textit{C. littorale} cells showed higher relative quantum yield of PSI in the presence of DCMU than 3\% CO\textsubscript{2}-grown cells at any red light intensity given during measurements (Fig. 7). In all cases of air-, 3\% CO\textsubscript{2}- and 40\% CO\textsubscript{2}-grown cells, difference in the relative quantum yield of PSI between the cells grown in the dense and in the sparse culture was not considerable.

Fig. 5 Time-course changes in the cell concentration (A), \( \Phi_{II} \) (B), 77K fluorescence emission spectra (C) and \( F_{714}/F_{685} \)-ratio (D) during the acclimation of air-grown \textit{Chlorella} sp. UK001 cells to 40\% CO\textsubscript{2} in a continuous culture with a dilution rate of 0.0125 h\textsuperscript{-1} under 250 μmol photon m\textsuperscript{-2} s\textsuperscript{-1} at the surface of the 5-liter cylindrical vessel. The CO\textsubscript{2} concentration was changed to 40\% at the time indicated by the vertical dotted lines.
Fig. 6  Time-course changes in the cell concentration (A), $\Phi_{II}$ (B), 77K fluorescence emission spectra (C) and $F_{714}/F_{685}$-ratio (D) during the acclimation of 3% CO$_2$-grown C. littorale cells to air (0.04% CO$_2$). Under 3% CO$_2$ conditions (until day –1), the cell concentration was kept at around 50 (mg Chl) liter$^{-1}$ by continuous dilution in a 5-liter cylindrical vessel. On day –1, a new 5-liter culture vessel was inoculated with a portion of the cell suspension of the dense culture at a density of 2 (mg Chl) liter$^{-1}$, further grown under 3% CO$_2$ in a batch culture and transferred to air on day 0 (open circles and gray-line spectra). The remainder of the dense culture was transferred to air in batch culture (closed circles and solid-line spectra). Under air conditions, the cell concentration was maintained by daily dilution at around 60 (mg Chl) liter$^{-1}$ and 10 (mg Chl) liter$^{-1}$ for the dense and the sparse culture, respectively. Light intensity was 250 μmol photon m$^{-2}$ s$^{-1}$ at the surface of the respective vessel. The CO$_2$ concentration was changed to that of air at the time indicated by the vertical dotted lines.
In the present study, we found an increased PSI/PSII fluorescence ratio in *C. littorale* cells growing under 40% CO$_2$, irrespective of whether the cells were acclimated from air (Fig. 1) or 3% CO$_2$ (Fig. 2), i.e. irrespective of whether the cells were acclimated to 40% CO$_2$ after, or without a lag period. This increase under 40% CO$_2$ took several days to complete and was observed at high cell concentrations, but not at sparse cell density when cells were illuminated at 250 μmol photon m$^{-2}$ s$^{-1}$ (Fig. 1, 2). In continuous culture in which fresh nutrients are constantly supplied to the algal cells, it is most probable that the increase in the PSI/PSII fluorescence ratio induced by 40% CO$_2$ in the dense culture is caused by the limitation of light energy due to mutual shading of algal cells. This inference was supported by the result obtained with a flat culture vessel (Fig. 3) that the increase in the PSI/PSII fluorescence ratio was induced by elevating CO$_2$ concentrations from 3 to 40% at a limited light intensity (20 μmol photon m$^{-2}$ s$^{-1}$) in the sparse culture.

The limitation of light energy due to mutual shading at high cell density, however, did not increase the PSI/PSII fluorescence ratio when CO$_2$ concentration was kept at 3% CO$_2$ (Fig. 4). In another extremely-high-CO$_2$-tolerant alga, *Chlorella* sp. UK001, the increase in the F$_{714}$/F$_{685}$ ratio in response to 40% CO$_2$ stress without a lag period was also apparent after transferring air-grown cells to 40% CO$_2$ (Fig. 5). These results suggest that a relative increase in PSI antenna size is specifically required for extremely-high-CO$_2$-tolerant algae to grow under extremely-high-CO$_2$ concentrations at limited light intensity. The fact that increase in the PSI/PSII fluorescence ratio at 40% CO$_2$ under light-limited conditions occurred after the order of days (Fig. 1–3) suggests PSI : PSII stoichiometry adjustment in this alteration of antenna sizes of PSI and PSII. Unfortunately, it was impossible to investigate the effects of inhibitors of protein synthesis on the changes in the 77K fluorescence spectra, since treatments with those inhibitors for such long periods were lethal for *C. littorale*. Quantitative and qualitative changes in PSI and PSII reaction centers and antenna pigments in response to 40% CO$_2$ stress both at sufficient and limited light conditions remain for future investigation.

We further investigated potential electron transport capacities of PSI and PSII in *C. littorale* using artificial electron acceptors and donors (Table 1). It was found that the electron transport capacity of PSI in *C. littorale* cells was enhanced by growing under extremely-high CO$_2$ conditions irrespective of the cell density and hence of light conditions. It is likely that the enhanced PSI activity would be used to increase cyclic electron transport. This was supported by the finding that relative quantum yield of PSI in the presence of DCMU, which can represent cyclic electron transport efficiency, was higher in 40% CO$_2$-grown *C. littorale* cells than those acclimated to 3% CO$_2$.

**Table 1** Effects of CO$_2$ and cell concentrations during growth on electron transport activities of PSI and PSII in *C. littorale*

<table>
<thead>
<tr>
<th>CO$_2$</th>
<th>Cell density $^a$</th>
<th>PSI activity (μmol O$_2$ (mg Chl)$^{-1}$ h$^{-1}$)</th>
<th>PSII activity (μmol O$_2$ (mg Chl)$^{-1}$ h$^{-1}$)</th>
<th>PSI/PSII activity ratio</th>
</tr>
</thead>
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<tr>
<td>3%</td>
<td>sparse</td>
<td>185±9</td>
<td>174±10</td>
<td>1.1</td>
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<tr>
<td></td>
<td>dense</td>
<td>97±14</td>
<td>100±6</td>
<td>1.0</td>
</tr>
<tr>
<td>40%</td>
<td>sparse</td>
<td>441±23</td>
<td>122±0</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>dense</td>
<td>320±22</td>
<td>78±5</td>
<td>4.1</td>
</tr>
<tr>
<td>0.04%</td>
<td>sparse</td>
<td>193±27</td>
<td>140±4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>dense</td>
<td>315±7</td>
<td>134±9</td>
<td>2.3</td>
</tr>
</tbody>
</table>

$^a$ The sparse and the dense represent the culture whose cell concentration was kept below 10 and around 50 (mg Chl) liter$^{-1}$, respectively.

**Discussion**

![Fig. 7](image) The relative quantum yield of PSI in the presence of DCMU at various actinic red light intensities in *C. littorale* cells acclimated to 40% CO$_2$ (triangles), 3% CO$_2$ (circles) and air (squares) in the dense culture (around 50 (mg Chl) liter$^{-1}$; open symbols) and in the sparse culture (below 10 (mg Chl) liter$^{-1}$; closed symbols). Pulsed measuring beam (about 1 μmol photon m$^{-2}$ s$^{-1}$) was produced by ED 800T unit. Actinic red light (a peak wavelength of 650 nm, half-band width of 25 nm) was generated by 102 L LED lamp. A saturating multipleturnover flashlight (50 ms) was applied with a xenon discharge lamp (XMT 103).
CO₂ (Fig. 7). Actual electron flow through PSI cyclic electron transport would be enhanced under light-limited conditions by the above-mentioned alteration of light energy input toward PSI in cells growing under 40% CO₂.

With respect to the regulation of energy balance required to grow under air levels of CO₂ (low-CO₂ stress), it has already been observed that the PSI/PSII fluorescence ratio increased when the cells of cyanobacteria and green algae that had been grown at about 5% CO₂ were acclimated to 0.04% CO₂ (Bürger et al. 1988, Miyachi et al. 1996, see Introduction). We, therefore, attempted to compare the energy balance required for extremely-high-CO₂ acclimation with that required for low-CO₂ acclimation in *C. littorale* cells. It was found that the increase in the PSI/PSII fluorescence ratio during low-CO₂ acclimation was more marked in the dense culture than in the sparse culture (Fig. 6). Air-grown cells of *C. littorale* in the dense culture, showed higher potential electron capacity of PSI and hence PSI/PSII activity ratio than those in 3% CO₂-grown cells, whereas those differences were not remarkable between air- and 3%-CO₂ grown cells in the sparse culture (Table 1). The fact that the relative quantum efficiency of PSI measured in the presence of DCMU was higher in air-grown cells than 3% CO₂-grown cells irrespective of the cell density during the culture (Fig. 7) suggests the enhanced PSI cyclic electron flow in cells growing under air levels of CO₂. These results indicate that regulation of energy balance in photosystems in response to excess-CO₂ stress is similar to that in response to low-CO₂ stress under light-limited conditions.

In connection with the physiological function of the alteration of light energy input toward PSI and the enhanced PSI cyclic electron flow in extremely-high-CO₂ acclimation, we have proposed that maintenance of the intracellular pH value supported by ATP generation by cyclic photophosphorylation is important for *C. littorale* to acquire tolerance for photosynthesis and growth under extremely-high-CO₂ stress (Pronina et al. 1993, Iwasaki et al. 1998, Kurano et al. 1998, Sasaki et al. 1999). In accordance with the proposal, the results obtained in this study further suggest that, under light-limited conditions, the increase in light energy input from LHCl to the enhanced potential capacity of PSI cyclic electron flow would function to generate extra ATP as an energy source of H⁺-transport in extremely-high-CO₂-tolerant algae growing actively under extremely-high-CO₂ conditions. At a sufficient light intensity, such extra ATP generation by the enhanced PSI cyclic electron flow may be unnecessary.

### Materials and Methods

**Organisms and growth conditions**

The marine green alga, *C. littorale*, was maintained at Marine Biotechnology Institute, and *Chlorella* sp. UK001 was a kind gift from Dr. Masakazu Murakami and Dr. Toshiya Muranaka (Biotechnology Laboratory, Sumitomo Chemical Co., Ltd.) through the New Energy and Industrial Technology Development Organization (NEDO). The inorganic culture medium for *C. littorale* was the same as that described previously (Iwasaki et al. 1996), and the same culture medium was used for *Chlorella* sp. UK001, except for being prepared in distilled water instead of sea water. Each of these algae was grown autotrophically and continuously in a 5-liter cylindrical culture vessel (14 cm in diameter; Kodama et al. 1993) containing ca. 4 liters of the respective culture medium under a light intensity of 250 μmol photon m⁻² s⁻¹ as described previously (Iwasaki et al. 1996). Dilution rate defined as medium flow rate (0.05 liters h⁻¹) divided by culture volume (4 liters) was kept constant at 0.0125 h⁻¹. The temperature was maintained at 25 and 35°C for *C. littorale* and *Chlorella*. sp. UK001, respectively. Throughout the cultivation, the pH value of each medium was kept at 5.5 by titrating with either NaOH or H₂SO₄ by an automatic pH-stat. Each culture vessel was continuously bubbled with air, with air that had been enriched with 3% CO₂, or with air that had been enriched with 40% CO₂ to obtain air-grown cells, 3% CO₂-grown cells and 40% CO₂-grown cells, respectively.

In order to study the possible effects of the light intensity, the three CO₂ concentrations were used in both high (>40 (mg Chl) liter⁻¹) and sparse (<10 (mg Chl) liter⁻¹) cell density cultures with 5-liter cylindrical culture vessels. For an experiment to study effects of the light intensity, a flat oblong culture vessel (inner thickness of 2.8 cm, a total capacity of 600 ml; Tamiya et al. 1953) was used.

**Measurement of algal growth**

The algal growth was evaluated by measuring the concentration of total chlorophyll which was determined according to Moran (1982).

**Direct evaluation of the quantum efficiency of PSII**

To measure the chlorophyll fluorescence from algal cells growing in a culture vessel, the fiber optic of a PAM Chlorophyll Fluorometer equipped with a 101 ED emitter-detector unit (Walz, Effeltrich, Germany) was directly attached to the glass wall of the vessel as described previously (Demidov et al. 2000). Weakly modulated light (ML; 0.9 μmol photon m⁻² s⁻¹ at 100 kHz) and actinic light (AL; 250 μmol photon m⁻² s⁻¹ from fluorescent lamps) was provided for continuously recording the actual fluorescence intensity (Fs). Pulses of saturating white light (SL; 10,000 μmol photon m⁻² s⁻¹, 800 ms duration) were further given at intervals of 60 s to measure the maximal fluorescence in light (Fm). According to Genty et al. (1989), the quantum efficiency of PSII (ΦPSII) was calculated as (Fm-Fs)/Fm (see van Kooten and Snel 1990 for the nomenclature).

**Measurement of 77K fluorescence emission spectrum**

A portion of the cell suspension was harvested from the culture, diluted with a fresh culture medium containing 50 mM MES-NaOH (pH 5.5) to around 2 μg Chl ml⁻¹ if necessary, placed in a glass measuring tube (standard NMR sampling tube, 0.5 cm o.d.×18 cm, Kusano-kagakukikai, Japan), and kept in liquid nitrogen until being measured. The emission spectrum of chlorophyll fluorescence induced by 440-nm excitation from the cells in liquid nitrogen (77K) was measured in triplicate with a spectrophotometer (model FP-750, JASCO, Japan) as described by Iwasaki et al. (1998).

**Measurements of PSII and PSI electron transport activities**

Algal cells equivalent to 200 μg Chl were collected by centrifugation and resuspended with 10 ml of 50 mM Tris-HCl buffer solution (pH 7.8). A portion (2.5 ml) of the resultant intact cell suspension was diluted with equal volume of the same buffer solution and subjected to measurement of PSII activity. The remainder (5 ml) was used for measurement of PSI activity after disrupting the cells by passing the intact cell suspension three times through a French pressure cell (Ohtake Seisakusho, Tokyo, Japan) at 147 MPa at 4°C. The PSI activity was measured by Hill reaction in the presence of 300 μM freshly
sublimed benzoquinone. The PSI activity was measured by oxygen uptake as the result of the photo-reduction of methylviologen, as described previously (Pesheva et al. 1994). These oxygen exchanges were measured in a vessel with a Clark-type O₂ electrode (Rank Bros., London, U.K.) at 25°C under illumination by a tungsten lamp at 1,000 μmol photon m⁻² s⁻¹.

**Evaluation of the relative quantum yield of PSI**

Algal cell suspension equivalent to 50 μg Chl was harvested from the culture and treated with DCMU (final 5 μM). The treated cells were collected on a glass microfiber filter by filtration and used for measurement of the P700 redox change. The redox state of P700 was monitored in terms of changes in the absorbance at 830 nm by using a PAM Chlorophyll Fluorometer equipped with an emitter-detector unit (ED 800T, Walz) according to the method of Schreiber et al. (1988), as described previously (Demidov et al. 2000). The relative quantum yield of PSI was calculated from the ratio of ΔA/P₇₀₀/ΔA₄₄₀ according to the definitions by Klughammer and Schreiber (1994), as described previously (Demidov et al. 2000).

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**References**


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