Photosynthetic activities of a synchronously grown aerobic N₂-fixing unicellular cyanobacterium, *Synechococcus* sp. Miami BG 043511

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Photosynthetic activities of a synchronously grown aerobic N₂-fixing unicellular cyanobacterium, *Synechococcus* sp. strain Miami BG 043511, were examined. Cells 1 h after the onset of synchronous growth exhibited an O₂ evolution rate of 400–500 µmol (mg Chl *a*)⁻¹ h⁻¹ at saturating light intensity, while it decreased to 40–80 µmol (mg Chl *a*)⁻¹ h⁻¹ in 12 h cells. Changes in the relative fluorescence intensity (excitation wavelength 430 nm, emission 685 nm) were not as large as the changes in photosynthetic O₂ evolution activity. Photosynthetic electron transport activities of both photosystem II and photosystem I in 12 h cells were lower than those in 1 h cells. Photosystem II activity with 2,5-dimethyl-*p*-benzoquinone, and photosystem I activity with diaminodurene and methyl viologen in 12 h cells were about 64 and 46%, respectively, of those in 1 h cells. However, the reduction in photosynthetic electron transport activities was not sufficient to account for the reduction in photosynthetic O₂ evolution activity in 12 h cells, which was about 14% of that in 1 h cells. Based on these observations, the reduction in photosynthetic O₂ evolution activity in 12 h cells was ascribed to the combined effect of a reduction in both the photochemical and the biochemical steps of photosynthesis.

Introduction

Biological N₂ fixation is an extremely O₂-sensitive process (Gallon, 1981; Robson & Postgate, 1980; Stewart, 1980). Despite this, N₂-fixing cyanobacteria can carry out both oxygenic photosynthesis and anoxygenic N₂ fixation. In heterocystous strains, these reactions are spatially separated (Stewart, 1980). Some of the unicellular cyanobacteria also fix N₂ under photoautotrophic conditions (Gallon, 1981; Huang & Chow, 1986; Mitsui *et al.*, 1985; Singh, 1973; Wyatt & Silvey, 1969). In *Gloeocapsa* spp. N₂ fixation and photosynthesis appear in a temporally separated manner during growth (Gallon *et al.*, 1974). The temporal appearance of nitrogenase activity was also demonstrated in cultures grown under light-dark cycles (Mullineaux *et al.*, 1981).

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Abbreviations: Chl *a*, chlorophyll *a*; DAD, diaminodurene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethyl-*p*-benzoquinone; MV, methyl viologen; PC, C-phycocyanin; PSI, photosystem I; PSII, photosystem II.

In our previous studies with synchronous cultures of *Synechococcus* sp. strain Miami BG 043511, it was shown that the cells grew by temporally separating the phases of photosynthesis and N₂ fixation within a cell cycle (Mitsui *et al.*, 1986, 1987). During the cell cycle, a drastic decrease in photosynthetic O₂ evolution activity was observed at the phase of N₂ fixation, even under continuous illumination. To our knowledge, however, a study on the photosynthetic properties of synchronously grown N₂-fixing cyanobacteria has not hitherto been reported. In non-N₂-fixing *Anacystis nidulans* (*Synechococcus* sp.), a peak of photosynthetic O₂ evolution appeared in the middle of the first cell cycle, although the change in photosynthetic O₂ evolution was much less intense during the second cell cycle (Csatorday & Horvath, 1977). In contrast, the distinct oscillation of photosynthetic O₂ evolution by N₂-fixing *Synechococcus* sp. strain BG 043511 was observed for at least three consecutive cell cycles under continuous illumination (Mitsui *et al.*, 1987). The drastic decrease in photosynthetic O₂ evolution activity is likely to have a considerable influence on the cells' ability to carry out N₂ fixation.

This paper is concerned with the cause of the change in photosynthetic O₂ evolution during a cell cycle under

N₂-fixing conditions. Changes in the activities of O₂ exchange and in the intensities of relative fluorescence of PSII have been measured during a cell cycle, and permeaplast preparations have been used to compare the photosynthetic electron transport activities of cells during the phases of high and low photosynthetic O₂ evolution.

Methods

Growth conditions. *Synechococcus* sp. Miami BG 043511 was grown in a medium free of combined nitrogen as described previously (Leon *et al.*, 1986), except that Tris (50 mg l⁻¹) was omitted and Na₂CO₃ (1 g l⁻¹) was replaced with NaHCO₃ (2.5 g l⁻¹). To obtain synchronous growth, 1.5 litre cultures, grown in 2 litre culture cylinders (8 cm in diameter and 50 cm in height), were exposed to dark-light-dark periods of 16 h each under continuous aeration. Synchronous growth commenced when the cultures were returned to continuous light, as reported previously (Mitsui *et al.*, 1986, 1987; Mitsui & Cao, 1988). Cultures were gassed with approximately 4% (v/v) CO₂ in air. A needle valve, attached to the CO₂ flowmeter, was used to control the CO₂ enrichment in the air to bring the pH of the culture medium to 7.6. Light intensity and temperature for growth were 150 µmol photons m⁻² s⁻¹ and 30 °C, respectively.

Measurements of photosynthetic and respiratory O₂ exchange. O₂ exchange was monitored using a Clark-type electrode in a reaction vessel as described previously (Kumazawa & Mitsui, 1985). The culture sample (about 2 ml) was removed from the culture cylinder and placed into the reaction vessel (1.8 ml) without any treatment. O₂ uptake in the dark and evolution in the light were determined at 30 °C. Illumination was provided by a slide projector equipped with a 150 W tungsten lamp. Light intensities were as indicated in the text and the legends to the table and figures.

Measurement of photosynthetic electron transport activity. To measure photosynthetic electron transport activities, cells were treated with lysozyme to obtain permeaplasts. Cells were harvested and washed once with 1 M-sorbitol containing EDTA (4 mM) and HEPES (40 mM, pH 7.5). They were then resuspended in 1 M-sorbitol containing lysozyme (0.1%, w/v) and HEPES (40 mM, pH 7.5), and incubated at 30 °C for the times indicated in Fig. 3 and the legend to Table 1. After the lysozyme treatment, the cells were collected by centrifugation and washed once with 1 M-sorbitol containing MgCl₂ (10 mM) and HEPES (40 mM, pH 7.5) and resuspended in this same medium. The concentration of the cells corresponded to 60–100 µg Chl *a* ml⁻¹. The basic reaction medium contained cells equivalent to 3–5 µg Chl *a* ml⁻¹ (for the DAD-MV assay – see below – one-half of this Chl *a* concentration was used), 0.2 M-NaCl and 40 mM-HEPES (pH 7.5). Also present in the respective assays were: 1 mM-MV for H₂O-MV; 1 mM-MV, 1 mM-DAD, 3.6 mM-ascorbate and 5 µM-DCMU for DAD-MV; or 0.8 mM-DMQ and 1 mM-potassium ferricyanide for H₂O-DMQ. Since preincubation of cells under hypotonic conditions, by deferring the addition of NaCl for several minutes before the assay, led to higher activities in the H₂O-MV and DAD-MV assays (data not shown), these activities were measured after hypotonic treatment. This hypotonic treatment did not lead to the immediate release of PC. Illumination was provided by a slide projector equipped with a 150 W tungsten lamp which was filtered through an orange (550 nm) cutoff filter. Light intensity at the surface of the reaction vessel was 2500 µmol photons m⁻² s⁻¹. The temperature of the reaction vessel was maintained at 30 °C and O₂ exchange was monitored as described above.

Measurement of fluorescence intensity and Chl *a* and PC contents. Fluorescence intensity was measured using a Shimadzu RF-510 spectrofluorometer. Cells were taken from the culture cylinder and diluted with culture medium to an OD₆₇₅ of approximately 0.02 (1 cm light path). The cell suspension was kept in the dark at room temperature (approximately 25 °C) for 5 min. Fluorescence intensity at 685 nm before and after the addition of DCMU (5 µM) was recorded for 7–8 min after the cells were excited at 430 nm. Relative fluorescence intensity was calculated by dividing the fluorescence intensity by the Chl *a* concentration of the respective samples. In order to increase the sensitivity, the photomultiplier tube of the spectrofluorometer was replaced with an R-928 tube (Hamamatsu Photonics). In addition, a 390 nm cutoff filter was inserted between the exciting light source and the sample. Chl *a* was extracted with methanol and determined spectrophotometrically by the method of Holm-Hansen & Riemann (1978). PC was extracted with 0.05 M-potassium phosphate buffer (pH 6.7) by repeated freezing and thawing and determined spectrophotometrically as described by Siegelman & Kycia (1978).

Results

Changes in the activity of photosynthetic O₂ evolution during synchronous growth are shown in Fig. 1(a). The culture, which was exposed to continuous light (150 µmol photons m⁻² s⁻¹) after synchronization, was sampled periodically and assayed under the same light intensity as used for growth. Increase in cell numbers occurred approximately between 6 h and 9 h and between 27 h and 31 h (data not shown). After the onset of synchronous growth, rates of about 250 µmol O₂ (mg Chl *a*)⁻¹ h⁻¹ were observed between 0 and 7 h, decreasing to approximately 15 µmol (mg Chl *a*)⁻¹ h⁻¹ between 11 h and 15 h. Since the size of the O₂-measuring reaction vessel (1.8 ml) was quite different to that of the culture cylinders (see Methods), and the light intensities measured were those at the outer surface of each vessel, the rates of photosynthetic O₂ evolution may not accurately represent those in the culture vessels. In fact, the level of dissolved O₂ between 11 h and 15 h was about 80% of air saturation, even though the culture was bubbled with 4% CO₂ in air. This observation indicates that there was no net O₂ production, but net O₂ uptake in the culture vessel between 11 h and 15 h. Rates of respiration were about 50–100 µmol (mg Chl *a*)⁻¹ h⁻¹ during the phase of high photosynthetic O₂ evolution and about 160 µmol (mg Chl *a*)⁻¹ h⁻¹ during that of low photosynthetic O₂ evolution.

Both Chl *a* and PC contents increased during the phase of high photosynthetic O₂ evolution (Fig. 1(b)). The decrease in PC, observed between 7 h and 11 h, coincided with the decrease in photosynthetic O₂ evolution activity. Re-initiation of the increase in PC per unit culture volume started at 13 h. An increase in photosynthetic O₂ evolution activity started from 15 h.

Changes in relative fluorescence intensities during synchronous growth are shown in Fig. 1(c). The

Fig. 1. Changes in the rate of O₂ exchange (a), Chl *a* and PC contents (b), relative fluorescence intensity (c), and the ratio of relative fluorescence ($-\text{DCMU}/+\text{DCMU}$) (d) during synchronous growth of *Synechococcus* sp. strain Miami BG 043511. In (a), open and filled symbols indicate, respectively, rates of O₂ evolution in the light and O₂ uptake in the dark. Light intensity for the measurement of O₂ evolution was $150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. In (b), open and filled symbols indicate, respectively, the changes in Chl *a* and PC contents. In (c), filled symbols indicate samples which contained $5 \mu\text{M}$ -DCMU. Relative fluorescence intensity is expressed per unit Chl *a* (see Methods).

fluorescence intensities in the absence of DCMU were about 20–40% of those in the presence of DCMU. The relative fluorescence intensities of control cells were constant between 0 and 9 h and between 17 h and 31 h. However, they increased slightly between 11 h and 15 h and at 33 h. Measurements in the presence of DCMU indicated that the relative fluorescence intensities tended to decrease between 9 h and 13 h and between 31 h and 33 h. Thus, the phase of low photosynthetic O₂ evolution coincided with the enhanced relative fluorescence intensity in the absence of DCMU and with the decreased relative fluorescence intensity in the presence of DCMU. In Fig. 1(d), change in the ratio of relative fluorescence intensity, i.e. (relative fluorescence in the absence of DCMU)/(that in the presence of DCMU), is shown. Interestingly, higher ratios were observed during the phase of low photosynthetic O₂ evolution activity.

Fig. 2. Effect of light intensity on the O₂ exchange of *Synechococcus* sp. strain Miami BG 043511. Rates of O₂ evolution in 1 h cells (a) and 12 h cells (b) were measured at different light intensities. Filled symbols indicates samples which contained $5 \mu\text{M}$ -DCMU.

Rates of O₂ exchange as a function of light intensity were measured using cells at the phases of high (1 h cells) and low (12 h cells) photosynthetic O₂ evolution (Fig. 2). At saturating light intensities, net O₂ evolution rates of approximately 400 and $40 \mu\text{mol (mg Chl } a)^{-1} \text{ h}^{-1}$ were observed for 1 h and 12 h cells, respectively. The rates of respiratory O₂ uptake in the dark of 1 h and 12 h cells were approximately 20 and $130 \mu\text{mol (mg Chl } a)^{-1} \text{ h}^{-1}$, respectively. The effect of the addition of $5 \mu\text{M}$ -DCMU, a concentration sufficient to completely inhibit the MV-catalysed Mehler reaction of permeoplast preparation of this strain (data not shown), is also shown in Fig. 2. The addition of DCMU caused a significant reduction in the rate of O₂ exchange in the light. In 1 h cells, O₂ exchange in the presence of DCMU was inhibited at a low light intensity ($33 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), and the inhibited rate did not change even when the light intensity was increased. This is in agreement with the observation by Sandmann & Malkin (1984) that respiration in unicellular cyanobacteria is inhibited by illumination. By inhibiting PSII activity with DCMU, they estimated the rate of respiratory O₂ uptake in the light. Assuming that the rate of O₂ exchange in the presence of DCMU represents respiratory O₂ uptake in the light, the gross rate of photosynthetic O₂ evolution can be calculated from the difference between the O₂ exchange rate in the absence and presence of DCMU. Thus, gross photosynthetic O₂ evolution in 12 h cells was about 10% of that in 1 h cells at saturating light intensities.

The photosynthetic electron transport activities of the 1 h (high O₂ evolution) and 12 h (low O₂ evolution) cells were also measured. As shown with other cyanobacteria

Fig. 3. Rates of photosynthetic electron transport activities in *Synechococcus* sp. strain Miami BG 043511 as a function of the duration of lysozyme treatment with and without subsequent hypotonic treatment. Assays were performed on 1 h cells (a, b) and 12 h cells (c, d) which had been incubated with lysozyme for the times indicated (see text for full details). (a, c) H₂O-MV assay; (b, d) DAD-MV assay. Filled symbols indicate samples which were incubated under hypotonic conditions (see text) for several minutes before the assay. Data points at 0 min incubation time indicate those samples treated with lysozyme but not incubated at 30 °C.

(Papageorgiou & Lagoyanni, 1985) *Synechococcus* sp. strain Miami BG 043511 is impermeable to electron carrier compounds, such as MV or DAD, without treatment of the cells with lysozyme to obtain permeoplasts. Fig. 3 shows the photosynthetic electron transport activities of 1 h and 12 h cells as a function of the incubation time with lysozyme. As described in Methods, the assay was also carried out with hypotonic treatment. It should be pointed out that data points at 0 min incubation time indicate those samples treated with lysozyme but not incubated at 30 °C.

The highest activities of H₂O-MV and DAD-MV were observed with 1 h and 12 h cells when they were incubated with lysozyme at 30 °C for 0 and 20 min, respectively (Fig. 3). Activities, as a rate of O₂ exchange, of H₂O-MV and DAD-MV were about 480 and 1900 μmol (mg Chl *a*)⁻¹ h⁻¹ with 1 h cells, and 430 and 1100 μmol (mg Chl *a*)⁻¹ h⁻¹ with 12 h cells, respectively. The rates of net photosynthetic O₂ evolution (at 1000 μmol photons m⁻² s⁻¹) of 1 h and 12 h cells were 390 and 38 μmol O₂ (mg Chl *a*)⁻¹ h⁻¹, respectively, when the cells were assayed without any treatment. Thus, the rates of photosynthetic electron transport activities in 12 h cells were much higher than the rate expected from the photosynthetic O₂ evolution activity of intact cells.

The electron transport activities of 1 h and 12 h cells fluctuated from one culture to another (Table 1,

Table 1. Comparison of the photosynthetic electron transport activities in 1 h and 12 h cells of *Synechococcus* sp. Miami BG 043511

Light intensity for the H₂O-CO₂ assay (intact cells) was 1000 μmol photons m⁻² s⁻¹. Cells used for the H₂O-MV, H₂O-DMQ and DAD-MV assays were incubated with lysozyme at 30 °C for 0, 0 and 20 min, respectively. In the case of H₂O-MV and DAD-MV, cells were preincubated under hypotonic conditions as in Fig. 3. Numbers in parentheses are standard deviations (*n* = 5 for H₂O-MV and DAD-MV; *n* = 3 for H₂O-DMQ).

Condition	Expt no.	O ₂ exchange μmol O ₂ (mg Chl <i>a</i>) ⁻¹ h ⁻¹		(b)/(a) (%)
		1 h cells (a)	12 h cells (b)	
H ₂ O-CO ₂ (O ₂ evolution)	1	464	56	12
	2	514	82	16
H ₂ O-MV (O ₂ uptake)	1	332 (±29.5)	301 (±29.4)	91
	2	439 (±12.7)	353 (±19.2)	81
H ₂ O-DMQ (O ₂ evolution)	1	209 (±8.3)	127 (±1.9)	61
	2	133 (±9.6)	90 (±6.1)	67
DAD-MV (O ₂ uptake)	1	2300 (±160)	1070 (±138)	46
	2	2490 (±233)	1010 (±41.5)	46

experiments 1 and 2). However, the trend in the changes in respective activities can be compared. Among the results of two independent cultures, net photosynthetic O₂ evolution activities in 12 h cells were about 14% of those in 1 h cells. Electron transport activities of PSII + PSI in 12 h cells, as measured with H₂O-MV, were about 86% of those in 1 h cells. On the other hand, activities of PSII (H₂O-DMQ) in 12 h cells were about 64% of those in 1 h cells. Activities of PSI (DAD-MV) in 12 h cells were 46% of those in 1 h cells.

Discussion

There are non-heterocystous cyanobacteria which are capable of synthesizing nitrogenase only under anaerobic conditions (Rippka & Waterbury, 1977). One such strain is *Oscillatoria* sp. Miami BG7. Since strain BG7 cannot fix N₂ under aerobic conditions, the cells become nitrogen starved, which is accompanied by a decrease in Chl *a* and an increase in carbohydrate (glycogen) contents to the levels of 0.1–0.2% and about 60% (w/w) of the dry weight, respectively (Kumazawa & Mitsui, 1981; Mitsui *et al.*, 1983). The decrease in PC observed in the present study, which coincided with the decrease in photosynthetic O₂ evolution activity (Fig. 1a, b), indicates that strain BG 043511 also becomes nitrogen limited during the transition period between the phases

of photosynthesis and N_2 fixation. One of the major differences between strains BG7 and BG 043511 is that the latter strain is capable of reducing its photosynthetic O_2 evolution activity even though there are substantial amounts of photosynthetic pigments in the cells (Fig. 1b).

The level of dissolved O_2 in the culture medium during the phase of low photosynthetic O_2 evolution was about 80% of air-saturation, even though the culture was gassed with 4% CO_2 in air. Respiratory activity may therefore contribute to the cells' ability to grow diazotrophically at the light intensity employed for culture growth. However, the estimated gross photosynthetic O_2 evolution at saturating light intensities in 12 h (low O_2 evolution) cells was only 10% of that in 1 h (high O_2 evolution) cells. Thus, the lower net photosynthetic O_2 evolution at saturating light intensities cannot be ascribed solely to the enhanced respiratory activity. These observations indicate that photosynthetic O_2 evolution capability in 12 h cells was reduced substantially by mechanisms other than respiratory O_2 uptake.

It has been shown that *in vivo* fluorescence of Chl *a* at 685 nm is maximized when an inhibitor of PSII, such as DCMU, is added (Murata & Takamiya, 1967). If the reduction in PSII donor side activity is the cause of the change in photosynthetic O_2 evolution activity, a substantial decrease in the fluorescence intensity of the DCMU-added cells would be expected during the phase of low photosynthetic activity. However, the change in relative fluorescence intensity during a cell cycle in the presence of DCMU indicated that the decrease in PSII activity was not as large as that in photosynthetic O_2 evolution in 12 h cells (Fig. 1c).

The activity of PSII + PSI measured in the presence of MV in 12 h cells was higher than those using other assay conditions (Table 1). This phenomenon was also observed in repeated experiments (data not shown). However, the reason for this is not clear at present. In 12 h cells the activities of PSII in the presence of DMQ and of PSI in the presence of DAD-MV were about 64% and 46%, respectively, of those in 1 h cells. If one considers that the reduced supply of electrons at PSII in 12 h cells was further reduced at PSI, the overall reduction could be 29% ($0.64 \times 0.46 = 0.29$, or 29%). Thus, the minimum estimate for the overall PSII + PSI activity would be 29%. However, the decrease in the activity of photosynthetic net O_2 evolution (average about 14%) was greater still (Table 1, H_2O - CO_2 by intact cells). These observations indicate that both PSII and PSI activities were lower in 12 h cells than in 1 h cells, although the decrease in photosynthetic electron transport activity in 12 h cells is not sufficient to account for the drastic decrease in photosynthetic O_2 evolution (Table 1). The increase in the ratio of relative fluores-

cence, i.e. (in the absence of DCMU)/(in the presence of DCMU), during the phase of low photosynthetic O_2 evolution (N_2 fixation) shown in Fig. 1(d) could be due to a decrease in the activity of PSI or a biochemical step of photosynthesis (CO_2 fixation; dark reaction). Therefore, we interpret the results of this study as indicating that the decrease in photosynthetic O_2 evolution activity in 12 h cells was due to reductions in both the photochemical and the biochemical reactions of photosynthesis.

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