Nitrogen fixation by *Plectonema boryanum* has a photosystem II independent component

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INTRODUCTION

Certain cyanobacteria are unique in their ability to conduct two mutually incompatible functions: O₂-evolving photosynthesis and O₂-labile nitrogen fixation. Some diazotrophic cyanobacteria compartmentalize nitrogen fixation in morphologically differentiated cells called heterocysts which lack photosystem II and ribulose biphosphate carboxylase and have greatly reduced phycocyanin (reviewed by Haselkorn, 1978). Among non-heterocystous cyanobacteria, temporal separation of nitrogen fixation from photosynthesis has been proposed as a major mechanism in *Gloeocapsa* (Gallon *et al.*, 1974; Mitsui *et al.*, 1986), *Oscillatoria* (Stal & Krumbein, 1985) and *Synechocystis* (Mitsui *et al.*, 1987) to protect nitrogenase from O₂ evolved during photosynthesis. The molecular basis of the temporal separation of photosynthesis and nitrogen fixation is not known.

*Plectonema boryanum* is a filamentous, non-heterocystous cyanobacterium that fixes nitrogen only under microaerobic conditions (Stewart *et al.*, 1970). When grown microaerobically under nitrogen-starvation conditions in continuous light it shows alternating cycles of photosynthesis and nitrogen fixation (Rai *et al.*, 1992; Misra & Tuli, 1993). Studies on the relationship between photosynthesis and nitrogen fixation have remained rather neglected for such microaerobically diazotrophic cyanobacteria, apparently due to difficulties in developing culture conditions that allow their sustained growth under nitrogen-starvation conditions (Giani & Krumbein, 1986). This report describes studies with rapidly growing nitrogen-fixing cultures of *P. boryanum*. Under such conditions, the period of maximum nitrogen fixation was accompanied by a substantial inhibition of photosystem II activity due to a shift in coupling between the two photosystems. During this period, both CO₂ fixation and nitrogen fixation were partly independent of photosystem II.

METHODS

Organism and growth conditions. *P. boryanum* UTEX 594 is from the University of Texas Collection. Under nitrogen-sufficient conditions, it was grown in BG11 medium (Rippka *et al.*, 1979) at 25 ± 2 °C bubbled with filtered air under continuous illumination as described by Vachhani *et al.* (1993). To establish a nitrogen-fixing culture, the BG11-grown culture was washed twice with BG11ₕ medium (containing no combined nitrogen) and inoculated aseptically into 15 l of BG11ₕ in a fermenter vessel (Microferm Fermentor, New Brunswick Scientific) at OD₅₂₀ 0.1. The culture was stirred continuously at 150 r.p.m. and bubbled with N₂ (Solar 2, Indian Oxygen) at 700 ml min⁻¹ and CO₂ at 5 ml min⁻¹ through independent inlets. The culture was illuminated with white fluorescent tube lights all placed on one side, covering one-half of the fermenter vessel circumference. The temperature of the culture was maintained by circulating water at 25 ± 2 °C through the cooling jacket. The incident light at the surface of the tube lights was 8400 lx (model ANA 999 lux meter, Tokyo Photoelectric Co.). Establishment of cultures of *P. boryanum* with sustained, photautotrophic, diazotrophic growth has been reported (Misra & Tuli, 1993).

Keywords: nitrogen fixation, photosystem II, cyanobacterium, *Plectonema boryanum*
Measurement of dissolved O₂. On-line dissolved O₂ (steady-state dO₂) was continuously monitored with a Clark-type oxygen electrode installed in the fermenter vessel. Light-dependent O₂ evolution by the cyanobacterium was measured using an oxygraph (Gilson Medical Electronics) on an aliquot drawn from the fermenter and resuspended to OD₇₅₀ 3.0 in BG11 containing 50 mM HEPES/NaOH pH 7.5. The surface of the water-jacketed cell of the oxygraph received 5400 lx of water-cooled tungsten light which provided saturating light intensity for O₂ evolution. The culture was concentrated in air and equilibrated in the dark for 5 min before measuring light-dependent O₂ evolution. Photosystem II activity was measured as light-dependent O₂ evolution in the presence of 3 mM MgCl₂ and 1.5 μM gramicidin with or without 500 μM DCMU (2,6-dichloro-p-benzoquinone). O₂ evolution was completely inhibited immediately under all culture conditions following the addition of DCMU [3(3,4-dichlorophenyl)-1,1-dimethylurea] at 10 μM. Photosystem I activity was measured as light-dependent O₂ uptake, driven by 50 μM methyl viologen in a reaction mixture containing 50 mM HEPES/NaOH pH 7.5, 50 μM DCPIP (2,6-dichlorophenol indophenol), 500 μM sodium ascorbate, 10 μM NaCN, 50 μM NaN₃ and 10 μM DCMU.

Photosynthetic CO₂ fixation. This was determined on an aliquot of the cyanobacterial culture resuspended as above. Acid-stable ¹⁴CO₂ incorporation was determined in the presence of 5 μCi NaH¹⁴CO₃ ml⁻¹ (951 mCi mmol⁻¹; 35.2 GBq mmol⁻¹), and 3 mM unlabelled NaHCO₃ at 30 °C following exposure to 5500 lx (saturating light intensity for CO₂ fixation) tungsten light. The culture was equilibrated in the dark for 5 min before measuring CO₂ fixation. Aliquots were drawn after a 5 min exposure to light. ¹⁴C incorporation was linear up to at least 12 min of exposure. Photosystem II independent CO₂ fixation was monitored in the presence of 10 μM DCMU.

Pigments. Phycocyanin content was estimated in the supernatant of sonicated cell suspensions after precipitating the membranes with 1% (w/v) streptomycin sulfate (Tandeau de Marsac & Houmard, 1988). Chlorophyll was extracted in methanol and estimated as described by MacKinney (1941).

Measurement of thermoluminescence. Aliquots of 0.6 ml samples suspended to OD₇₅₀ 3.0 (as described above) were immobilized on 2.5 cm diameter Whatman GF/C filter paper discs and placed in stainless steel planchets. The samples were dark-adapted for 3 min before freezing to 77 K. Glow curves were recorded for samples frozen as such or frozen after the addition of methyl viologen (50 μM) (Rutherford et al., 1984).

Fluorescence measurements. For 77 K fluorescence measurements, the cyanobacterial culture was sampled in the photosynthetic and diazotrophic phases. The culture was resuspended to a chlorophyll concentration of 5 μg ml⁻¹ in BG11₆ containing 50 mM HEPES/NaOH pH 7.5, transferred to capillary tubes, dark-adapted for 30 min, frozen in liquid N₂ and used for recording fluorescence spectra using a Perkin Elmer LS-5 instrument (Allen et al., 1985).

Nitrogenase activity. An acetylene reduction assay was carried out by a method similar to that of David & Fay (1977) except that it was conducted anaerobically. A 2 ml sample of P. boryanum culture was drawn anaerobically from the fermenter and injected into a 5 ml vial containing an N₂ atmosphere. The assay was initiated by replacing 0.5 ml of the gas phase with acetylene. The vials were rotated in the presence of 2000 lx light from tungsten lamps for 30 min before the assay was terminated by injecting 0.5 ml 10% (w/v) TCA. Ethylene was resolved on Porapak T and detected by flame ionization.

RESULTS AND DISCUSSION

In the presence of continuous light (8400 lx) P. boryanum grew microaerobically under nitrogen-starvation conditions (Fig. 1a) by conducting nitrogen fixation and photosynthesis in alternate cycles (Fig. 1b). The doubling time under such conditions of growth was 57 ± 4 h. Dissolved O₂ in the culture medium declined from 35–60 μM during the peak of a preceding photosynthetic phase to 13–17 μM before nitrogenase activity began to appear. The decline in dO₂ was accompanied by a parallel sharp decline in phycocyanin (Fig. 1a) and a several-fold decline in light-dependent O₂ evolution (Table 1). Light-dependent O₂ evolution by samples taken during the diazotrophic phase was stimulated 1.5–2-fold following the addition of DCQ (Table 1) in the oxygraph cell. The DCQ-mediated stimulation was not seen when the culture was in the photosynthetic phase. The results suggested a possible alteration in the photosystem II complex during the diazotrophic phase, resulting in inefficient electron flow to quinone acceptors. Alternatively, oxidation of the reduced plastoquinone pool may be blocked during the nitrogen-fixation phase, leading to inhibition of electron flow to plastoquinones. To distinguish between these alternatives, thermoluminescence glow curves and fluorescence emission were examined for cultures growing in the photosynthetic and diazotrophic phases.

Thermoluminescence data on the periodicity of oscillations following flash excitation (H. S. Misra & T. S. Desai, unpublished) showed no functional differences in the donor side (S states) of the water-oxidizing complex in cultures growing in the diazotrophic and photosynthetic phases.

![Fig. 1. Metabolic pattern in P. boryanum UTEX 594 growing in continuous light under nitrogen-fixing conditions. Growth (■), ratio of phycocyanin to chlorophyll a (○), steady-state dissolved oxygen (△) and acetylene reduction activity (●) are shown for a typical experiment during the first 4 d growth following inoculation. Chlorophyll a increased gradually with growth of the culture; the values were 0.58, 0.58, 0.59, 0.59, 0.72 and 0.79 μg ml⁻¹ at 0, 26, 40, 50, 62 and 72 h respectively. The corresponding values for phycocyanin at these time points were 2.69, 0.64, 1.83, 1.12, 2.88 and 1.77 μg ml⁻¹, respectively.](image-url)
Table 1. Stimulation of O₂ evolution by DCQ during the diazotrophic phase

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>O₂ evolution [nmol O₂ (mg Chl a)⁻¹ min⁻¹]</th>
<th>Nitrogenase activity [nmol C₂H₄ (µg Chl a)⁻¹ min⁻¹]</th>
<th>CO₂ fixation [nmol CO₂ (mg Chl a)⁻¹ min⁻¹]</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>- DCQ</td>
<td>+ DCQ</td>
<td></td>
</tr>
<tr>
<td>Diazotrophic</td>
<td>286 ± 40</td>
<td>592 ± 85</td>
<td>3·5 ± 1·3</td>
</tr>
<tr>
<td></td>
<td>1242 ± 334</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photosynthetic</td>
<td>2665 ± 802</td>
<td>2620 ± 520</td>
<td>2685 ± 591</td>
</tr>
</tbody>
</table>

All values ± standard deviation are means of five to seven aliquots drawn from cultures in three independent experiments. The steady-state dO₂ in the fermenter at the time of drawing the aliquots was 15–18 µM during the diazotrophic phase and 40–80 µM during the photosynthetic phase of cultures growing under nitrogen-fixing conditions. Comparative values for oxygen evolution in a nitrate-grown culture were 3340 ± 442 nmol O₂ (mg Chl a)⁻¹ min⁻¹.

Fig. 2. Thermoluminescence glow curves for P. boryanum UTEX 594 sampled during the photosynthetic phase (a) and the diazotrophic phase (b) of growth under nitrogen-fixing conditions. The glow curve for the diazotrophic phase was recorded at double the sensitivity of that for the photosynthetic phase. The lower traces are glow curves for the corresponding samples to which methyl viologen (MV) was added.

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Phases. The thermoluminescence glow curves showed striking differences, implying differences in the nature of plastoquinone pools in the two phases. The major DCMU-sensitive band appeared at 25 °C in the photosynthetic phase but at 10°C in the diazotrophic phase (Fig. 2). A shift to a lower temperature can result from a fall in redox potential between Qₐ and Qₐ⁻ (i.e. the acceptor side of photosystem II) during the diazotrophic phase (H. S. Misra & T. S. Desai, unpublished). Halogenated benzoquinones like DCQ are known to overcome such impairment (Graan & Ort, 1986). Thus the intracellular nitrogen status (or the ratio of carbon to nitrogen) appeared to regulate the redox levels of secondary quinone acceptor Qₐ, leading to the impairment of electron transfer from the O₂-evolution complex. The function was restored once sufficient intracellular nitrogen had accumulated by the end of the diazotrophic phase. This marked the beginning of the photosynthetic phase, leading to 8- to 9-fold higher light-dependent O₂ evolution and 2- to 2·5-fold higher CO₂ fixation (Table 1).

The 77 K fluorescence emission spectra showed distinct changes in emission by phycocyanin (F₆₄₅) and photosystem I (F₅₇₃), as the growth phase changed from photosynthetic to diazotrophic. In the photosynthetic phase, F₆₄₅ was higher and F₅₇₃ was lower than the corresponding values in the diazotrophic phase. Thus, in the diazotrophic phase there were spectral changes (Fig. 3) indicative of lower phycocyanin and altered excitation energy distribution, apparently in favour of photosystem I. In Synechococcus sp. PCC 6301, redox-controlled phosphorylation of certain polypeptides has been suggested to induce electrostatic decoupling of the phycobilisomes from photosystem II and their closer association with photosystem I (Allen et al., 1985).

Though depression in photosystem II preceded the phase of nitrogenase activity, light was an absolute requirement for sustained nitrogen fixation. In the presence of DCMU at levels that completely inhibited light-dependent O₂ evolution within seconds (data not given), nitrogenase activity was not affected during the 5 h period of observation, provided the culture was incubated in the presence of light (Fig. 4). Incubation of the culture in the dark resulted in a gradual decline of nitrogenase activity, reaching 20% of the initial level after 4 h (or longer, in certain experiments) of incubation plus a 30 min assay in
the dark. However, at all time points through this period, the culture showed restoration of nitrogenase activity (despite the presence of DCMU) when the 30 min assay was conducted in the light (Fig. 4). The results imply light dependence of nitrogenase activity in a manner that is independent of photosystem II. The sources of reducing power and ATP may accumulate during the photosynthetic phase to support nitrogenase activity during the diazotrophic phase when photosystem II is depressed.

![Fig. 3. Normalized 77 K fluorescence emission spectra of *P. boryanum* UTEX 594 culture at excitation wavelength 435 nm. The fluorescence yield (arbitrary units) is plotted for the culture sampled in the photosynthetic phase (○) and the diazotrophic phase (△) of growth.](image)

![Fig. 4. Photosystem II independent light requirement for nitrogen fixation in *P. boryanum* UTEX 594. Three 500 ml aliquots of an actively nitrogen-fixing culture in the diazotrophic phase were drawn from the fermenter vessel. These were bubbled with a mixture of N₂ and CO₂ (as described in Methods) and incubated as follows: in the light in the absence of DCMU (○); in the light in the presence of 10 μM DCMU (●); in the dark in the presence of 10 μM DCMU (△). Subsamples were removed anaerobically at the times indicated and nitrogenase assays performed (in triplicate) using the same conditions of light and DCMU as the sampled culture. A second set of subsamples from the culture incubated in the dark with 10 μM DCMU was assayed (in triplicate) in the light with 10 μM DCMU (△).](image)

### Table 2. Light-dependent, photosystem II independent CO₂ fixation by *P. boryanum* during the diazotrophic phase

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>CO₂ fixation [nmol CO₂ (mg Chl a)⁻¹ min⁻¹]</th>
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<tbody>
<tr>
<td></td>
<td>Light</td>
</tr>
<tr>
<td>Nitrate-grown culture</td>
<td>2705 ± 322</td>
</tr>
<tr>
<td>N₂-fixing culture</td>
<td>1342 ± 202</td>
</tr>
<tr>
<td>Diazotrophic phase</td>
<td>2742 ± 466</td>
</tr>
</tbody>
</table>

Smoker & Barnum (1990) reported an eight- to tenfold increase in glycogen reserves in cultures of *P. boryanum* incubated aerobically under nitrogen starvation conditions compared with cultures grown in the presence of combined nitrogen. Nitrogen-starved cultures utilized glycogen reserves when a microaerophilic environment was provided to allow the onset of nitrogen fixation. Studies are in progress on the level of glycogen in different phases of growth of *P. boryanum* UTEX 594 under the growth conditions used by us and the enhancement of nitrogenase activity by exogenous carbon sources.

While nitrogen-fixing cultures showed a substantial level of photosystem II independent nitrogenase activity, some light-dependent CO₂ fixation was also supported despite inhibition of photosystem II by DCMU (Table 2). In a microaerobically grown nitrogen-fixing culture, DCMU inhibited CO₂ fixation by approximately 50% during the diazotrophic phase and by about 90% during the photosynthetic phase, during the 2 h period of observation, following the addition of DCMU. On the other hand, DCMU inhibition of CO₂ fixation, in a culture grown aerobically with excess combined nitrogen (nitrate-grown culture), was nearly complete (Table 2). The results suggest a partial shift in the source of reductant from photosystem II to an endogenous donor in cultures grown under nitrogen-starvation conditions. The shift is most marked during the diazotrophic phase of growth of a culture grown under nitrogen-fixing conditions.

Light dependence, but photosystem II independence, of nitrogen fixation and the 77 K fluorescence emission changes in the F₇₅₀ component imply the involvement of photosystem I in *P. boryanum* during the diazotrophic phase, for both nitrogenase activity and CO₂ fixation. In a nitrogen-fixing culture, photosystem I activity (estimated as methyl-viologen-dependent O₂ evolution in the
light in the presence of DCMU and reduced DCMU was about 30–40% higher during the diazotrophic phase than in the photosynthetic phase: 163 ± 54 nmol O₂ (mg Chl a)⁻¹ min⁻¹; means significantly different at 2.04% (Student’s t-test). The photosystem I activity of a culture grown aerobically with nitrate as a source of abundantly available combined nitrogen was 111 ± 22 nmol O₂ (mg Chl a)⁻¹ min⁻¹, comparable to that of the nitrogen-fixing culture in the photosynthetic phase.

Our results imply that the decline in photosynthetic O₂ evolution during nitrogen-fixing growth of _P. boryanum_ was due to degradation of light-harvesting pigments and a block in electron flow from photosystem II. This allowed the cyanobacterium to reduce intracellular O₂ evolution to levels non-detrimental to nitrogenase. A substantial level of nitrogenase activity was photosystem I dependent (DCMU resistant) but light requiring. Possibly, the endogenous redoxant accumulated during the photosynthetic phase provided electrons to photosystem I. The accumulated substrates were utilized for nitrogenase function and partly for CO₂ fixation.

Continued fixation of CO₂ in spite of a substantial decline in light-dependent O₂ evolution during the diazotrophic phase resulted in a major deviation of the stoichiometry of the nitrogen-fixing culture in the photosynthetic phase. Thus, the photosynthetic phase provided electrons to photosystem I independent of CO₂ fixation by _P. boryanum_ (Murai & Katoh, 1975; Matthijs _et al._, 1984), _Chlamydomonas_ (Godde, 1982) and _greening potato tubers_ (Janavek _et al._, 1991). Light-dependent utilization of reduced carbon sources by certain cyanobacteria, including _Agnemelliella_ (Lambert & Stevens, 1986), _Synechocystis_ (Jansson _et al._, 1987), _Plectonema_ (Smoker & Barnum, 1990) and _Oscillatoria_ (Gallon _et al._, 1991), has been reported. However, in these studies, photosystem II independent photoheterotrophic growth was demonstrated under conditions that inhibited photosynthesis, i.e., the inclusion of DCMU or the depletion of CO₂. Our study suggests that photosystem II independent pathways of light-dependent electron transfer may have a functional significance under certain stress conditions that lead to depression of photosystem II as under nitrogen-starvation conditions in a microaerobically nitrogen-fixing cyanobacterium, like _P. boryanum_.

Once sufficient nitrogen had been fixed, the cyanobacterium enhanced its ability to harvest light by raising the level of phycoerythrins, reactivated its electron transfer, and therefore entered the photosynthetic phase. Thus oscillations in intracellular nitrogen (or the ratio of carbon to nitrogen) lead to alternating phases of photosynthesis and nitrogen fixation. The molecular and genetic basis that triggers such a metabolic rhythm leading to cyclic temporal separation of the two mutually incompatible physiological processes is not known. _P. boryanum_ provides an attractive system for such studies since a mobilizable plasmid vector has recently become available (Vachhani _et al._, 1993) for this cyanobacterium.

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REFERENCES


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