Nitrogenase derepression, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110

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The regulation of nitrogenase derepression, plus the catalytic activity and protein concentration of glutamine synthetase (GS), nitrate reductase (NR), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phycoerythrin (PE) were studied in the filamentous non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110. Both nitrogen limitation and microaerobic incubation were essential for the derepression of nitrogenase. Oxygen caused irreversible inactivation of nitrogenase, as well as repression of its synthesis. A temporal separation of N₂ fixation and net photosynthetic O₂ evolution was observed under a N₂/CO₂ (95:5, v/v) atmosphere. Repeated peaks of nitrogenase and growth were observed. Immunogold localization showed that in N₂-fixing cultures, all cells, including those undergoing division, contained nitrogenase, and that the nitrogenase antigen was uniformly distributed throughout the cells without any preferential association with cellular structures. Rubisco was mainly located in carboxysomes of both N₂-fixing and NO₃⁻-grown cells. Both N₂-fixing and NO₃⁻-grown cells showed similar levels of PE, which was associated with the thylakoid membranes. GS antigen was distributed throughout the cells and the relative amounts of this enzyme, as well as its activity, were 20% higher in N₂-fixing than in NO₃⁻-grown cultures. NO₃⁻ uptake and NR systems were found to be NO₃⁻-inducible, with very low activities in N₂-fixing cultures. The latter may be important in avoiding competition for Mo between nitrogenase and NR.

Introduction

Cyanobacteria are O₂-evolving photosynthetic prokaryotes, many of which are also capable of autotrophic growth using N₂ as the sole nitrogen source (Stewart, 1980; Gallon, 1989). Cellular integration of N₂ fixation in cyanobacteria requires strategies for protection of nitrogenase from atmospheric and photosynthetically produced O₂, provision of ATP and reductant, and efficient assimilation of N₂-derived ammonium. In some cyanobacteria this is achieved by development of specialized cells called heterocysts, resulting in spatial separation of photosynthesis (located in vegetative cells)

and N₂ fixation (located in heterocysts); fixed carbon moves from vegetative cells to heterocysts and fixed nitrogen from heterocysts to vegetative cells (Stewart, 1980; Wolk, 1982; Bergman *et al.*, 1986). Several other metabolic changes conducive to nitrogenase functioning occur during heterocyst development, including: (1) loss of photosynthetic O₂ evolution, phycobiliproteins and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); (2) increases in respiratory activity and in the activity of enzymes of the oxidative pentose phosphate pathway; (3) loss of nitrate uptake and nitrate reductase (NR) systems; (4) increased levels of glutamine synthetase (GS) necessary for assimilation of N₂-derived ammonia (Wolk, 1982; Kumar *et al.*, 1985; Rai & Bergman, 1986; Renström-Kellner *et al.*, 1990).

The strategies for O₂ protection during aerobic N₂-fixation by non-heterocystous cyanobacteria have been studied in detail, with the conclusion of a temporal separation of N₂-fixation and photosynthesis (see Gallon, 1989). However, very few studies have been conducted on non-heterocystous cyanobacteria which fix

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Abbreviations: ATCC, American Type Culture Collection; GOGAT, glutamate synthase; GS, glutamine synthetase; NR, nitrate reductase; PCC, Pasteur Culture Collection; PE, phycoerythrin; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TEM, transmission electron microscopy; UTEX, culture collection of algae at University of Texas (formerly Indiana University Culture Collection).

N_2 only under microaerobic or anaerobic conditions. The reasons for the lack of aerobic N_2 -fixation in such cyanobacteria are not fully understood. Under microaerobic to anaerobic conditions, a temporal separation of net O_2 evolution and nitrogenase activity has been observed in *Plectonema boryanum* (Weare & Benemann, 1974) and *Phormidium faveolarum* (Weisshaar & Böger, 1983). However, repeated peaks of alternating nitrogenase activity and oxygen evolution or growth have not been demonstrated. On the other hand, there are conflicting reports of N_2 fixation and concomitant growth in *Plectonema boryanum* (Rogerson, 1980; Pearson & Howsley, 1980; Giani & Krumbein, 1986). Furthermore, virtually no information is available regarding levels of phycoerythrin (PE), GS, nitrate uptake, NR and Rubisco under diazotrophic growth conditions in these cyanobacteria.

Plectonema boryanum PCC 73110 is a filamentous non-heterocystous cyanobacterium which fixes N_2 under microaerobic to anaerobic conditions (Stewart & Lex, 1970). In the present investigation, we used this strain to study the derepression, subcellular localization and regulation of nitrogenase, and changes in GS, Rubisco, PE, nitrate uptake and NR when nitrate-grown cultures adapted to diazotrophic growth.

Methods

Organism and growth conditions. *Plectonema boryanum* PCC 73110 (ATCC 29407 and UTEX 594) and *Gloeotheca* PCC 6909 (ATCC 27152 and UTEX 795) were grown in batch cultures using BG-11 medium (Rippka *et al.*, 1979) at 25 °C and a photon fluence rate of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$. *Oscillatoria limosa* (Stal & Bergman, 1990) was grown on artificial sea water medium ASN_3 (Rippka *et al.*, 1979) at 20 °C and a photon fluence rate of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$. N_2 -fixing cultures of *Gloeotheca* and *O. limosa* were obtained by transferring the cultures to nitrogen-free media (' N_2 -medium'; BG-11₀ and NO_3 -free ASN_3 , respectively). Derepression of nitrogenase in *P. boryanum* was achieved as described below.

Nitrogenase derepression. Aerated batch cultures of *P. boryanum* grown on BG-11 medium were harvested by centrifugation during the exponential phase. The cells were washed and resuspended in BG-11₀ medium to a cell density of 200 $\mu\text{g ml}^{-1}$ (3 $\mu\text{g chlorophyll } a \text{ ml}^{-1}$). These cultures were subdivided into 20 ml batches and transferred to 100 ml capacity serum-stoppered Erlenmeyer flasks. These were sparged with the desired gas mixture at specified times and maintained at 25 °C and a photon fluence rate of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$. These flasks were directly used for acetylene reduction assay, each assay lasting 30 min; the cultures were not transferred to another vessel, in order to avoid air contamination.

Enzyme assays. Nitrogenase activity (EC 1.18.6.1) was estimated *in vivo* by gas chromatography using the acetylene reduction assay (Stewart *et al.*, 1967). GS biosynthetic activities (EC 6.3.1.2) were measured in cell extracts as described by Sampaio *et al.* (1979). Ferredoxin-dependent NR activity was measured in cells made permeable with toluene, using methyl viologen as electron donor, by

Fig. 1. SDS-PAGE and immunoblotting of cell extracts from N_2 -fixing *P. boryanum*. NO_3 -grown cells were transferred to N_2 -medium and nitrogenase induced as described in Fig. 2(b). Immunoblots of GS (lane 1), nitrogenase Fe-protein (lane 2), Rubisco (lane 3) and PE (lane 4) are shown. Lane 5, molecular mass markers.

following the formation of NO_2^- from NO_3^- as described by Manzano *et al.* (1976). NO_3^- was estimated colorimetrically as described by Snell & Snell (1949).

NO_3^- uptake. This was measured by estimating the rate of disappearance of NO_3^- from the medium (final concentration 100 μM). NO_3^- was estimated by the difference in absorbance between 202 and 250 nm as described by Calero *et al.* (1980).

Protein and chlorophyll *a*. Protein concentration was measured according to Bradford (1976) and chlorophyll *a* according to MacKinney (1941).

O_2 exchange. O_2 evolution and consumption were measured using a polarographic Clark-type oxygen electrode installed in a 3 ml Plexiglass container with magnetic stirring. The measurements were done at 25 °C and at a photon fluence rate of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Antibodies. Rabbit anti-*Rhodospirillum rubrum* nitrogenase Fe-protein, anti-*Anabaena* PCC 7120 GS, anti-*Phormidium persicinum* PE and anti-*Sinapis alba* Rubisco were gifts from Dr S. Nordlund (University of Stockholm, Sweden), Professor R. Haselkorn (University of Chicago, USA), Dr D. Guard-Friar (New York State Department of Health, USA) and Dr R. Oelmüller (University of Freiburg, Germany), respectively. The specificity of these antibodies was tested against crude extracts of N_2 -fixing *P. boryanum* cells by Western blotting (Fig. 1). All antibodies were found to be mono-specific, recognizing a single polypeptide of known subunit molecular mass relating to their respective antigens (nitrogenase 36 kDa; GS 53 kDa; Rubisco 56 kDa; PE 20 kDa) (see also Bergman & Rai, 1989). Secondary antibodies (goat anti-rabbit IgG conjugated to colloidal gold size 5 or 10 nm, and conjugated to horseradish peroxidase) were obtained from Amersham and Bio-Rad, respectively.

Western blotting. *P. boryanum* cells were harvested by centrifugation (3000 g, 5 min) and the pellet resuspended in sample buffer (1:1, v/v) consisting of 10 mM-Tris/HCl (pH 8), 1 mM-EDTA, 2.5% SDS, 5%

β -mercaptoethanol and 0.01% bromophenol blue. These samples were boiled for 5 min and centrifuged at 15000 g for 5 min. Samples (1 μ l) of the supernatant were used for SDS-PAGE and subsequent immunoblotting as described by Braun-Howland *et al.* (1988), using antibodies against nitrogenase Fe-protein, GS, PE and Rubisco at a dilution of 1:500 (v/v).

Immunogold labelling. Preparation of samples for transmission electron microscopy (TEM) and the protocols for immunolabelling were essentially the same as described before (Bergman *et al.*, 1985) except that the secondary antibody (goat anti-rabbit IgG) was conjugated to size 5 or 10 nm colloidal gold and that different primary antibodies were used as required (see figure legends). Five grids containing ultrathin sections of NO_3^- -grown and N_2 -fixing *P. boryanum* samples were immunolabelled. For quantification, 10–15 photographs were taken, of which one set of representative photographs is presented. In control experiments, the primary antibody was omitted.

TEM and quantification of the immunolabel. TEM was performed using a Zeiss EM 10 transmission electron microscope operated at 60 kV. Relative levels of various antigens were estimated by counting gold particles in various cell types using TEM photographic prints. These were then converted to number of gold particles per μm^2 cell area taking into account the magnification of the prints used and the area counted. In total, 100 μm^2 cell area was counted in each case and the values presented are means ($\pm$ SE) from 10–15 counts. A similar exercise was done to calculate background labelling by counting gold particles per unit area outside the cells. Background labelling was also calculated in control experiments where primary antibody was omitted during immunolabelling.

Chemicals and gases. All the supplies for electron microscopy were obtained from Agar Aids, for electrophoresis from Pharmacia and for immunoblotting from Bio-Rad. All other chemicals were from Sigma. Gases were obtained from AGA Special Gases, Stockholm.

Results

Derepression of nitrogenase

No nitrogenase activity or protein could be detected in NO_3^- - or NH_4^+ -grown *P. boryanum* cultured either under air or with N_2/CO_2 (95:5, v/v) sparging (data not shown). Upon transfer to N_2 -medium and periodic sparging with N_2/CO_2 , development of acetylene-reducing activity started after 30 h (Fig. 2a). The activity continued to increase during the next 40 h, after which it steadily declined. The pattern and specific activity were similar to those noted by Stewart & Lex (1970). However, when the cells were nitrogen starved for 24 h under aerobic conditions prior to the periodic N_2/CO_2 sparging, acetylene-reducing activity developed much faster (within 2 h) and reached a peak by 6–7 h (Fig. 2b), with maximal specific activity similar to that in Fig. 2(a). To see if the faster development of nitrogenase activity was due to the activation of pre-existing nitrogenase protein developed during the 24 h nitrogen starvation, development of acetylene-reducing activity was followed in cells where chloramphenicol or rifampicin was added at the end of nitrogen starvation and just before the start of

Fig. 2. Nitrogenase induction in *P. boryanum*. (a) NO_3^- -grown cells were transferred to N_2 -medium as detailed in Methods and maintained at 25 °C with a photon fluence rate of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Starting at zero time and at 6 h intervals they were sparged for 15 min with N_2/CO_2 (95:5, v/v) at a rate of 1000 ml h^{-1} . Nitrogenase activity was measured in 30 min assays under a N_2/CO_2 gas phase and under the light and temperature conditions mentioned above. In this and other figures the nitrogenase activity quoted is the mean of that during the 30 min before the points shown. (b) Same as (a) except that prior to the start of N_2/CO_2 sparging (zero time) the cultures were nitrogen starved for 24 h by incubating them in N_2 -medium with continuous air sparging (2000 ml h^{-1}). Symbols in (b): $\blacktriangle$, control; $\square$, plus chloramphenicol (100 $\mu\text{g ml}^{-1}$); $\blacksquare$, plus rifampicin (100 $\mu\text{g ml}^{-1}$). Chloramphenicol and rifampicin were added at the start of N_2/CO_2 sparging (i.e. after the aerobic nitrogen-starvation period). This and other experiments (Figs 3a, 4, 5, 6) were all done in duplicate. The values presented are means of two measurements from each replicate.

N_2/CO_2 sparging. As seen in Fig. 2(b), acetylene-reducing activity did not appear under such conditions. These results indicate that nitrogenase protein was absent during the aerobic nitrogen starvation period and that the derepression on N_2/CO_2 sparging was due to fresh synthesis of nitrogenase. An absence of nitrogenase protein in aerobic cultures was also observed by immunoblotting cell extracts of *P. boryanum* which had been nitrogen starved for 30 h under aerobic conditions (Fig. 3b, lane 1). These results show that nitrogenase

Fig. 3. (a) Loss of nitrogenase activity on exposure to air ($\square$) and its regain upon transfer to a N_2/CO_2 atmosphere ($\circ$, $\bullet$) in *P. boryanum*. Nitrogenase derepression was achieved as in Fig. 2(b) and after appearance of the nitrogenase peak, the cells were sparged with air for 5 min at the rate of 2000 ml h^{-1} . Nitrogenase activity was measured at 30 min intervals under aerobic conditions. At the time indicated by the arrow, the gas phase was changed to N_2/CO_2 and nitrogenase activity measured at 30 min intervals under N_2/CO_2 in the absence ($\circ$) and presence ($\bullet$) of chloramphenicol or rifampicin (both $100\text{ }\mu\text{g ml}^{-1}$). (b) Detection of nitrogenase Fe-protein by immunoblotting in cell extracts of *P. boryanum*. Lane 1, extract from cells maintained in aerobic N_2 -medium for 30 min; lane 2, extract from cells with peak nitrogenase activity; lane 3, extract from cells which were exposed to air for 2 h after appearance of the nitrogenase peak; lane 4, extract of cells which had been exposed to air for 4 h after appearance of nitrogenase activity; lane 5, molecular mass markers.

derepression required both low cellular nitrogen and microaerobic to anaerobic conditions, and that the longer time required for nitrogenase derepression in Fig. 2(a) was due to the time required for the depletion of

Fig. 4. (a) Nitrogenase activity ($\blacksquare$) and protein content ($\circ$) of *P. boryanum*. (b) Nitrogenase activity ($\blacksquare$), protein content ($\circ$) and net O_2 evolution rate ($\square$) during the first phase of N_2 fixation in (a). Culture conditions and other details as in Fig. 2(b) control.

intracellular nitrogen reserves under non-optimal growth conditions.

Re-exposure of N_2 -fixing *P. boryanum* cells to air caused a rapid decline in acetylene-reducing activity, which became undetectable after 90 min of exposure (Fig. 3a). To see whether the decline in nitrogenase activity was due to inactivation of the enzyme or to protein degradation/modification, immunoblots of cell extracts were done using *P. boryanum* cells exposed to air for increasing periods of time after the appearance of peak nitrogenase activity (Fig. 3b). After 2 h of exposure to air, when nitrogenase activity had become undetectable, nitrogenase Fe-protein was still detectable (lane 3), although the cross-reaction was less intense than that in *P. boryanum* cells under N_2/CO_2 (lane 2). In both cases, only a single polypeptide of 36 kDa was detected corresponding to nitrogenase Fe-protein. These data indicate that loss of nitrogenase activity on exposure to air was due to nitrogenase inactivation followed by degradation. Such inactivation did not involve modification of Fe-protein to a higher molecular mass form as

Fig. 5. Effects of air, darkness, NH_4^+ and NO_3^- on nitrogenase activity in *P. boryanum*. Nitrogenase activity was derepressed as in Fig. 2(b). After the appearance of the nitrogenase peak, the conditions were changed (zero time) and the response of nitrogenase activity was monitored during the next 6 h. ●, Cultures transferred to darkness; ■, NH_4Cl added to a final concentration of 2 mM; □, KNO_3 added to a final concentration of 10 mM; ○, cultures transferred to aerobic atmosphere. The NH_4Cl and KNO_3 solutions were sparged with N_2/CO_2 for 30 min before addition. The solutions were also buffered with 10 mM-HEPES/NaOH (pH 7.5). One hundred percent activity represents $18 \text{ nmol C}_2\text{H}_4 \text{ formed min}^{-1} (\text{mg protein})^{-1}$.

noted in other cyanobacteria (Ernst *et al.*, 1990; Reich & Böger, 1989; Smith *et al.*, 1987; Stal & Bergman, 1990). To see if the inactivation of the nitrogenase was reversible, *P. boryanum* cells were transferred back to a N_2/CO_2 atmosphere after 90 min of exposure to air (Fig. 3a). Acetylene-reducing activity reappeared after 90 min and reached a peak in 3 h. Such reappearance of nitrogenase activity was sensitive to chloramphenicol and rifampicin. Thus, inactivation of nitrogenase under air was concluded to be irreversible.

Growth, N_2 fixation and O_2 evolution

When nitrogenase was derepressed as in Fig. 2(b), and the cultures maintained under similar conditions over a

prolonged period, repeated peaks of acetylene-reducing activity were observed (Fig. 4a). An increase in protein content, which was taken as indicative of growth, followed. A detailed analysis of one such peak of nitrogenase activity and growth phase (Fig. 4b) showed that during the appearance of nitrogenase the rate of net O_2 evolution declined rapidly, becoming undetectable by the time nitrogenase activity reached its peak. No growth occurred during this period. Net O_2 evolution was detectable again after 7 h. This coincided with growth and with a decline in nitrogenase activity. These data indicate that under the conditions used here, *P. boryanum* can grow photoautotrophically using N_2 as nitrogen source, in repeated cycles of N_2 fixation and growth, and under such conditions there is a temporal separation of net O_2 evolution and nitrogenase activity. During the maximal N_2 -fixing period, O_2 evolution may be balanced by respiratory O_2 consumption, resulting in the absence of net O_2 exchange.

Effects of NH_4^+ and NO_3^- and darkness on nitrogenase activity

After nitrogenase derepression as in Fig. 2(b), the effects of darkness, NH_4^+ and NO_3^- were studied during the 6 h stable period when maximal nitrogenase activity was expressed. Transfer of N_2 -fixing cells into darkness resulted in a rapid decline in acetylene-reducing activity, which became undetectable within 2 h (Fig. 5). This decline was similar to that observed in air. ATP and/or reductant were probably the essential factors supplied by light reactions. Addition of 2 mM- NH_4Cl resulted in a slower decline of acetylene-reducing activity, perhaps because NH_4^+ may have acted by repressing nitrogenase synthesis rather than inhibiting its activity. NO_3^- did not affect nitrogenase activity during the initial 2 h of incubation, but a slow decline in activity was seen thereafter. The difference in the effects of NO_3^- and NH_4^+ may be due to slower rates of nitrate uptake and/or metabolism in N_2 -fixing cultures (see below).

Table 1. NO_3^- uptake and NR activities in *P. boryanum*, *Gloeotheca* 6909 and *O. limosa* grown on N_2 , NO_3^- or NH_4^+

NO_3^- uptake activities are expressed as $\text{nmol NO}_3^- \text{ taken up min}^{-1} (\text{mg protein})^{-1}$ and NR activities as $\text{nmol NO}_2^- \text{ formed min}^{-1} (\text{mg protein})^{-1}$. NO_3^- -medium represents BG-11 in the case of *Gloeotheca* 6909 and *P. boryanum*, and ASN_3 in the case of *O. limosa*. N_2 -medium represents the respective nitrogen-free medium (BG-11₀ or NO_3^- -free ASN_3). NH_4^+ -medium represents N_2 -media supplemented with NH_4Cl (final concentration 2 mM) and buffered with 10 mM-HEPES/NaOH (pH 7.5). The values presented are means $\pm$ SE of three measurements from a single experiment done in triplicate. ND, Not detectable.

Growth medium	Nitrate uptake			Nitrate reductase		
	<i>Plectonema</i>	<i>Gloeotheca</i>	<i>Oscillatoria</i>	<i>Plectonema</i>	<i>Gloeotheca</i>	<i>Oscillatoria</i>
N_2 -medium	0.6 ± 0.1	0.2 ± 0.05	0.2 ± 0.04	0.5 ± 0.1	0.2 ± 0.04	0.3 ± 0.1
NO_3^- -medium	7.5 ± 0.3	4.9 ± 0.25	6.0 ± 0.30	7.8 ± 0.4	4.3 ± 0.10	4.7 ± 0.2
NH_4^+ -medium	ND	ND	ND	ND	ND	ND

Fig. 6. Induction of NO_3^- uptake ($\square$, $\bullet$) and NR ($\circ$, $\blacksquare$) activities on transfer of N_2 -fixing *P. boryanum* cells to NO_3^- -medium (BG-11₀) without ($\square$, $\blacksquare$) or with ($\circ$, $\bullet$) chloramphenicol ($100 \mu\text{g ml}^{-1}$). Nitrogenase was derepressed as in Fig. 2(b). After the appearance of the nitrogenase peak, the cells were harvested by centrifugation and resuspended in NO_3^- -medium (zero time), then maintained under aerobic growth conditions. At time intervals samples were withdrawn and NO_3^- uptake rate and NR activities measured.

NO_3^- uptake and NR activities

NO_3^- -grown cultures of *P. boryanum* showed NO_3^- uptake and NR activities (Table 1) similar to those reported earlier (Ida & Mikami, 1983). In contrast, the NO_3^- uptake and NR activities of N_2 -fixing cultures were only 10% of those in NO_3^- -grown cells (activity measured after appearance of peak nitrogenase activity). These activities did not change during the period of nitrogenase decline that also corresponded to net O_2 evolution and growth (data not shown). NH_4^+ -grown cells had no detectable levels of NO_3^- uptake or NR. Essentially similar results were found in the case of the non-heterocystous cyanobacteria *Gloeotheca* 6909 and *O. limosa*, which fix N_2 aerobically (Table 1). When N_2 -fixing cells of *P. boryanum* were transferred to NO_3^- -medium, NO_3^- uptake and NR activities increased, reaching a maximum within 10–12 h (Fig. 6). The increase was sensitive to chloramphenicol. These results imply that the NO_3^- uptake and NR systems in *P. boryanum* are substrate-inducible and NH_4^+ -repressible.

Nitrogenase localization

Immunogold labelling of nitrogenase Fe-protein in NO_3^- -grown cells (Fig. 7a) and NH_4^+ -grown cells (data not shown) showed label intensity similar to background (4–6 gold particles per μm^2 cell area). N_2 -fixing

P. boryanum cells showed nitrogenase antigen uniformly distributed throughout the cell without preferential association with any cellular structure (Fig. 8a). All the cells in all the filaments examined had a similar pattern and intensity of labelling. Cells undergoing division also had nitrogenase label. The density of label was 95 ± 12 gold particles per μm^2 cell area. These results confirm the lack of nitrogenase in NO_3^- - and NH_4^+ -grown cells and imply no spatial separation or subcellular compartmentalization of nitrogenase in *P. boryanum*. Similar results have been reported in *P. boryanum* 581 using antibodies against nitrogenase Mo-Fe protein (Smoker *et al.*, 1989).

GS activity and cellular localization of GS antigen

Potential changes in GS activity and protein concentration during derepression of nitrogenase were examined in NO_3^- -grown and in N_2 -fixing *P. boryanum* cells. GS biosynthetic activity in the two cultures was 45 ± 2.8 and 56 ± 3.1 nmol product formed min^{-1} (mg protein) $^{-1}$, respectively. Immunolabelling experiments showed that the GS antigen was distributed throughout the cell both in NO_3^- -grown and in N_2 -fixing cells (Figs 7b, 8b). The relative densities of the gold label were 22 ± 2.5 and 27 ± 2.6 gold particles per μm^2 cell area, respectively. Thus, a 20% increase in GS activity and protein label occurred on nitrogenase derepression. This is in contrast to the report of Nagatani & Haselkorn (1978), who found no increase in GS activity during nitrogenase derepression under an argon atmosphere. The differences may have arisen due to different conditions used for nitrogenase derepression, including the fact that we used N_2/CO_2 (95:5, v/v) for nitrogenase derepression.

Rubisco localization

Rubisco was localized in NO_3^- -grown and in N_2 -fixing cells of *P. boryanum* (Figs 7c, 8c). In the latter case, the cells were processed for immunolabelling at the beginning of the appearance of nitrogenase, when the nitrogen stress, and therefore the difference in Rubisco, is likely to be highest. Rubisco was present in both NO_3^- -grown and N_2 -fixing cells. In both cases, an intense labelling was found in carboxysomes and a lower intensity in the cytoplasm. The overall level of Rubisco in N_2 -fixing cells was 20% lower than that in NO_3^- -grown cells (43 ± 3.4 and 54 ± 4 gold particles per cell, respectively). Smoker *et al.* (1990) noted a much higher (over 50%) reduction

Fig. 7. Immunogold localization of nitrogenase (a), GS (b), Rubisco (c) and PE (d) in NO_3^- -grown cells of *P. boryanum*. Rabbit anti-*R. rubrum* nitrogenase Fe-protein (a), anti-*Anabaena* 7120 GS (b), anti-*S. alba* Rubisco (c) and anti-*P. persicinum* PE (d) were used as primary antibodies at a dilution of 1:100. Goat anti-rabbit IgG conjugated to 5 nm (a, c, d) or 10 nm (b) colloidal gold was used as secondary antibody at a dilution of 1:20. Cb, carboxysome. Bar, 1 μm (all parts of the figure are at the same magnification).

in Rubisco levels on nitrogenase derepression in *P. boryanum* 581. However, the cells had been stressed for nitrogen for 40 h in an argon atmosphere lacking N_2 . These results show that unlike the situation in heterocysts, derepression of nitrogenase in *P. boryanum* does not lead to a total loss of Rubisco.

Localization of PE

During nitrogenase derepression in *P. boryanum*, a transient decrease in phycocyanin has been noted (Stewart & Lex, 1970; Weare & Benemann, 1974). To see if PE concentrations also change during nitrogenase derepression, immunogold labelling of PE was examined in NO_3^- -grown and in N_2 -fixing cells. In the latter case, cells for immunolabelling were taken at the beginning of the appearance of nitrogenase activity, when the differences are likely to be maximal. An intense PE labelling associated with thylakoid membranes was found both in NO_3^- -grown and in N_2 -fixing cells (Figs 7d, 8d). In both cases, the amounts of PE were similar (447 ± 15 and 435 ± 18 gold particles per cell, respectively), indicating little or no degradation of PE in *P. boryanum* under the conditions of nitrogenase derepression used here.

Discussion

Under a N_2/CO_2 atmosphere *P. boryanum* PCC 73110 synthesized nitrogenase and showed nitrogenase activity in the absence of combined nitrogen (Figs 2, 4). These activities are among the highest reported by other workers using this strain (Stewart & Lex, 1970; Weare & Benemann, 1974; Nagatani & Haselkorn, 1978; Rogerson, 1980; Pearson & Howsley, 1980; Giani & Krumbein, 1986). Since the cultures used were non-synchronous, development of nitrogenase in all the cells (including those undergoing cell division) and the uniform distribution throughout the cells (Fig. 8) argue against the possibility of nitrogenase being expressed during a particular phase of the cell cycle or being compartmentalized within the cell (Mitsui *et al.*, 1986; Giani & Krumbein, 1986). Similar patterns of labelling have been found in *O. limosa* (Stal & Bergman, 1990), *P. boryanum* UTEX 581 (Smoker *et al.* 1989) and *Gloeotheca* PCC 6909 (A. N. Rai & B. Bergman, unpublished results).

O_2 removal or nitrogen limitation alone did not lead to derepression of nitrogenase. This was indicated by the lack of nitrogenase derepression under aerobic con-

ditions irrespective of the nitrogen status of the cell, and by the fact that even on N_2/CO_2 sparging nitrogenase derepression occurred only under nitrogen-limited conditions (Figs 2, 3, 7a). These results are consistent with, and provide evidence for, the suggestion that nitrogenase derepression in *P. boryanum* requires both O_2 removal and nitrogen limitation (Nagatani & Haselkorn, 1978). Absence of nitrogenase derepression in the presence of combined nitrogen is consistent with earlier observations in cyanobacteria including *P. boryanum* (Stewart, 1980; Gallon, 1989; Stewart & Lex, 1970). However, the fact that O_2 removal or lowering of O_2 tension was necessary for nitrogenase derepression, despite a temporal separation of net O_2 evolution and nitrogenase activity, and that nitrogenase activity declined sharply on exposure to air or with the onset of net O_2 evolution endogenously (Fig. 4), suggests that the O_2 -scavenging capacity in *P. boryanum* is much more limited than in other non-heterocystous cyanobacteria. Indeed, Weare & Benemann (1974) found respiration to be of only limited significance in O_2 -protection in *P. boryanum*.

Our studies showing irreversible inactivation of nitrogenase by O_2 in *P. boryanum* (Fig. 3) are consistent with the results of Weare & Benemann (1974). Nitrogenase Fe-protein has been shown to be modified to a higher molecular mass form by O_2 in some heterocystous cyanobacteria, which results in reversible inactivation of the protein but renders it insensitive to O_2 damage (Smith *et al.*, 1987; Reich & Böger, 1989; Ernst *et al.*, 1990). A similar form has also been noted in *O. limosa* (Stal & Bergman, 1990). The fact that such a modification was not found in *P. boryanum* (Fig. 3b) may explain why inactivation of nitrogenase in this strain was irreversible and resulted in degradation of the enzyme.

The temporal separation of N_2 fixation and net O_2 evolution noted here (Fig. 4) also confirms the findings of Weare & Benemann (1974). In addition, the results show that *P. boryanum* can grow photoautotrophically with repeated cycles of N_2 fixation and growth. Since nitrogenase was found to be irreversibly inactivated and degraded on exposure to air, and regain of nitrogenase activity required fresh synthesis of nitrogenase (Figs 3, 5), it is possible that repeated phases of N_2 fixation required fresh nitrogenase synthesis and that during the following microaerobic phase nitrogenase was degraded. Giani & Krumbein (1986) have demonstrated N_2 fixation and concomitant photoautotrophic growth in *P. boryanum* at lower light intensities with continuous N_2/CO_2 flushing. Thus, depending on the culture conditions, *P. boryanum* seems capable of photoauto-

Fig. 8. Immunogold localization of nitrogenase (a), GS (b), Rubisco (c) and PE (d) in N_2 -fixing *P. boryanum* cells. Nitrogenase derepression was achieved as in Fig. 2(b). The cells used for immunolabelling were sampled either at the beginning (c, d) or at the peak (a, b) of nitrogenase activity. Other details as in Fig. 7.

trophic growth either with repeated phases of N_2 fixation (when sparged with N_2/CO_2 periodically) or concomitantly with continuous N_2 fixation (when sparged with N_2/CO_2 continuously to remove any net O_2 evolved, keeping the culture microaerobic). Although the precise mechanism of temporal separation is not clear, it is possible that this is achieved by changes in rates of photosynthesis, with respiration balancing photosynthetic O_2 evolution during N_2 fixation while increased rates of photosynthesis result in net O_2 evolution during the growth that follows. This may occur due to transient changes in phycobiliprotein levels. Although we found no significant changes in PE levels (Figs 7, 8), phycocyanin levels are known to change and repeated degradation and synthesis of phycocyanin has been noted earlier (Stewart & Lex, 1970; Weare & Benemann, 1974; Giani & Krumbein, 1986).

Mo may be a limiting factor in cyanobacterial cells (Bagchi *et al.*, 1985). Nitrogenase and NR are both molybdoenzymes and require reduced ferredoxin as electron donor (Guerrero & Lara, 1987). Indeed Nagatani & Haselkorn (1978) have shown that in absence of Mo, the nitrogenase proteins synthesized are inactive. Occurrence of NR and nitrogenase in the same cell is likely to lead to competition for Mo and reduced ferredoxin. Heterocystous cyanobacteria avoid such competition by spatial separation of nitrogenase and NR (Kumar *et al.*, 1985; Rai & Bergman, 1986). Such spatial separation is not possible in non-heterocystous cyanobacteria since nitrogenase is present in all the cells. A temporal separation of NR and nitrogenase activity was not found in the non-heterocystous cyanobacteria tested (Table 1). However, the fact that nitrate uptake and NR were found to be NO_3^- -inducible, with very low activities in N_2 -fixing cultures (Fig. 6; Table 1) means that these cyanobacteria are able to effectively minimize competition between NR and nitrogenase under diazotrophic growth conditions. It is noteworthy that N_2 -fixing *P. boryanum* cells retain the capacity to develop nitrate uptake and NR (Fig. 6), unlike heterocysts, where these systems are lost (Rai & Bergman, 1986).

As in heterocystous cyanobacteria, the GS-GOGAT pathway has been shown to be the route of primary NH_4^+ assimilation in *P. boryanum* (Meeks *et al.* 1978). An increase in GS activity and protein level has been noted when vegetative cells differentiate into heterocysts (see Wolk, 1982; Bergman *et al.*, 1985). This has been shown to be linked to nitrogenase expression and to be necessary for assimilation of N_2 -derived NH_4^+ (Renström-Kellner *et al.*, 1990). Our results showing an increase of GS with derepression of nitrogenase (Figs 7, 8) are consistent with the above view. These findings also explain why NO_3^- is less inhibitory and acts more slowly than NH_4^+ . As mentioned above, NO_3^- uptake and NR

levels are very low in N_2 -fixing cultures and availability of NO_3^- requires 3–4 h for induction of NO_3^- uptake and NR activity (Table 1, Fig. 6), while NH_4^+ assimilation remains active throughout. These effects resemble the effects of NO_3^- and NH_4^+ on nitrogenase activity and protein in heterocysts (Renström-Kellner *et al.*, 1990). Thus, during N_2 fixation *P. boryanum* cells functionally resemble heterocysts. However, unlike heterocysts, N_2 -fixing *P. boryanum* cells retain PE and Rubisco (Figs 7, 8), they retain the capacity to develop NO_3^- uptake and NR activity on nitrate availability (Fig. 6), and their PC levels change only transiently (Stewart & Lex, 1970; Weare & Benemann, 1974; Giani & Krumbein, 1986).

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