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Immunochemical evidence that nitrogenase is restricted to the heterocysts in *Anabaena cylindrica*

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Abstract. The question of whether the vegetative cells of *Anabaena cylindrica* synthesize nitrogenase under anaerobic conditions was studied by immunoferritin labelling of the Fe-Mo protein (Component I). Differentiating cultures, incubated under an argon atmosphere, were treated with DCMU 12 h following initiation of induction. DCMU inhibited photosynthetic O_2 production, thus insuring strict anaerobic conditions, but had no effect on nitrogenase induction. Fe-Mo protein levels, as determined by rocket immunoelectrophoresis, increased 5-fold within 24 h of DCMU treatment. Immunoferritin labelling of aldehyde fixed, ultrathin cryosections of anaerobically induced filaments showed that the Fe-Mo protein was restricted to the heterocyst. Ferritin labelling was shown to be specific by the following criteria: (a) substituting preimmune goat serum for the anti-Fe-Mo protein IgG prevented ferritin labelling; (b) ferritin-conjugated, non-homologous rabbit anti-goat IgG did not bind; (c) incubation of anti-Fe-Mo protein IgG treated sections with rabbit anti-goat IgG prior to the treatment with the ferritin label also prevented labelling. The results provide direct immunochemical evidence that nitrogenase is restricted to the heterocysts even under strictly anaerobic conditions.

Key words: Heterocysts – Immunoferritin labelling – Cyanobacteria – *Anabaena* – Nitrogenase

In recent years it has become clear that under aerobic conditions, the heterocysts are the principle loci of nitrogen fixation in species of cyanobacteria that possess these cells (Fay et al. 1968; Weare and Benemann 1973; Peterson and Wolk 1978; Haselkorn 1978). Several features of heterocyst structure and biochemistry suggest that these cells provide an anaerobic site in these O_2-evolving photosynthetic prokaryotes for the O_2-labile nitrogenase enzyme. However, the discovery that the non-heterocystous, filamentous form, *Plectonema boryanum*, was capable of nitrogen fixation under low O_2 tensions (Stewart and Lex 1970) indicated that heterocysts may be dispensable for N_2 fixation by heterocystous algae. Furthermore, under microaerophilic conditions, the vegetative cells of heterocystous forms develop a reducing environment (Thomas and David 1972) and thus, could provide a suitable O_2-free environment for nitrogenase function.

Nitrogenase activity in species of *Anabaena* cultured under low pO_2 (van Gorkom and Donze 1971) and strictly anaerobic conditions (Rippka and Stanier 1978) is very sensitive to inhibition by atmospheric levels of O_2. When *Anabaena* cultures were starved for nitrogen under low pO_2, introduction of air resulted in the restoration of phycocyanin pigments (which are degraded during N-starvation) first in the cells adjacent to the heterocysts. However, when N_2 was added under anaerobic conditions, the gradient in phycocyanin levels was rare and less distinct (van Gorkom and Donze 1971). In a similar study, introduction of N_2 to an anaerobically induced, N-limited, *Anabaena* culture restored cyanophycin levels (a N-storage product) but no gradient originating from the heterocysts was observed (Rippka and Stanier 1978). These observations were interpreted as evidence that under anaerobic conditions, the vegetative cells also fix nitrogen. However, this approach is very indirect and, in view of evidence that O_2 may be an important regulatory factor in ammonia assimilation in legumes (Bergerson and Turner 1978; Rao et al. 1978), other interpretations are possible. The most direct evidence in support of the idea that vegetative cells can synthesize nitrogenase comes from the observation that a spontaneous mutant (Strain 7118, Kratz and Myers 1955) which had lost the capacity to form heterocysts was able to synthesize nitrogenase under anaerobic (but not aerobic) conditions (Rippka and Stanier 1978).

Other studies to localize nitrogenase have been indirect and equivocal. These include the recovery of nitrogenase activity in isolated heterocysts (see Haselkorn 1978 for review), track autoradiography of filaments exposed in $^{13}N_2$ (Wolk et al. 1974) and correlation of nitrogenase activity loss with filament breakage (Stewart et al. 1969; Weare and Benemann 1973). Thus, the question of whether the vegetative cells are capable of expressing the nif genes remains open (see Stanier and Cohen-Bazire 1977; Haselkorn 1978). In the present study, an immunoferritin labelling technique was used to localize nitrogenase in filaments of *Anabaena cylindrica* induced to synthesize nitrogenase under strict anaerobic conditions. The results provide direct evidence that even under strict anaerobiosis, nitrogenase synthesis is restricted to the heterocysts.

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Materials and methods

Culture conditions. Growth and induction of heterocysts in axenic cultures of *Anabaena cylindrica* (UTEX 629) was as described previously (Murry and Benemann 1979). Cells were magnetically stirred and sparged with an atmosphere of air/CO_2 or argon/CO_2 (CO_2 was 0.3%, v/v). Light intensity was 2×10^4 erg. $cm^{-2} \cdot s^{-1}$ and the temperature 28–29° C. To provide anaerobic conditions for nitrogenase synthesis in vegetative cells, 2×10^{-5} M dichlorophenyldimethylurea (DCMU) was added to argon/CO_2-sparged cultures 12 h after removing NH_4^+. Dissolved O_2 was measured using a galvanic dissolved O_2 probe with a D. O. Analyzer model DO-40 (New Brunswick Sci. Co).

Determination of nitrogenase synthesis. In vivo nitrogenase activity was measured by acetylene reduction under an argon atmosphere at a light intensity of 2×10^4 ergs $\cdot$ $cm^{-2} \cdot s^{-1}$, as described previously (Murry and Benemann 1979). O_2 was added as indicated in the text. The Fe-Mo protein of nitrogenase was measured by rocket immunoelectrophoresis as described earlier (Murry et al. 1983).

Cell preparation. Filaments were harvested by centrifugation and resuspended in cold (4° C) phosphate buffer (0.05 M, pH 7.5) containing 2% (w/v) paraformaldehyde. After 35 min, 25% glutaraldehyde (Sigma, St. Louis, MO, USA) was added to a final concentration of 0.1% (v/v) and the filaments were incubated for an additional 20 min. The filaments were washed four times in phosphate buffer and infused with phosphate-buffered sucrose (2 M) and gelatin (1.5%, w/v) for 1 h at 4° C (Tokuyasu 1973). The filaments were suspended in a copper stud, immersed in liquid N_2-cooled freon and stored in liquid N_2.

Ultrathin frozen sections were made at −70 to −90° C with a Porter-Blum MT-2 ultramicrotome equipped with a cryokit (Ivan Sorvall, Inc., Newton, CT, USA). Sections were retrieved from the dry knife edge on a drop of phosphate-buffered sucrose (1.5 M) and gelatin (1.5 %) and transferred to Formvar-coated, 200-mesh nickel grids. Sucrose and gelatin were washed from the sections by floating and grids in several changes of phosphate buffer.

Antisera. The Fe-Mo protein of nitrogenase from *Anabaena cylindrica* (629) was purified to homogeneity (Hallenbeck et al. 1979) and used to induce antibody formation in a Toginberg goat. The serum was purified by ammonium sulfate precipitation and frozen until use in small aliquots at −80° C. The protein concentration of the IgG preparation was 23.7 mg/ml. The antisera was shown to be monospecific to the Fe-Mo protein by the appearance of a single precipitin line in immunodiffusion and immunoelectrophoretic analysis (Murry et al. 1983). Ferritin conjugated rabbit anti-goat (heavy and light chains) was purchased from Cappel Laboratories Inc. (Cochranville, PA, USA) and stored concentrated (17.5 mg protein/ml) at 4° C. Preparations remained active for several months. Normal (preimmune) rabbit serum and rabbit anti-goat IgG were purchased from Antibodies, Inc. (Davis, CA, USA). The antibodiy preparations were diluted just before use in 0.05 M Sorenson's phosphate buffer (pH 7.6). Protein was determined using the Lowry procedure (Lowry et al. 1951).

Immunoferritin labelling. Labelling was carried out at room temperature in covered petri dishes with moistened filter paper on the top surface to prevent desiccation. Care was taken to leave a small film of liquid on the sections between each step. Freshly prepared sections were incubated for 10 to 20 min on droplets (30 to 50 µl) of normal rabbit serum (diluted 1:30) to minimize non-specific binding of the primary antiserum. The solution was drawn off the grid with absorbant paper and the grid placed directly on droplets of goat anti-Fe-Mo protein IgG (diluted 1:25 to 1:50) containing 10% (v/v) normal rabbit serum. Incubation was for 15 to 30 min. The grids were washed in 3 changes of glycine-phosphate buffered saline (0.05 M phosphate buffer, pH 7.6, 0.9% NaCl, 10 mM glycine). The grids were again incubated in normal rabbit serum and transferred to the ferritin conjugate (diluted 1:500 to 1:1000). After an incubation period of 5 to 15 min, the grids were washed in 3 changes of glycine-phosphate buffer to remove any unreacted ferritin followed by a final wash in distilled water. The grids were observed with a Zeiss 9S electron microscope at an accelerating voltage of 60 kV.

Results

Nitrogenase induction. Induction of nitrogenase activity and Fe-Mo protein in a differentiating culture of *A. cylindrica* treated with DCMU is shown in Fig. 1. The kinetics of induction were essentially the same as in the control (without DCMU, data not shown), however, after 12 h of DCMU treatment acetylene reduction activity began to level off. In the control, activity remained linear for the duration of the experiment. DCMU inhibited photosynthetic capacity by over 95% and dissolved O_2 in the culture media declined to less than 5% of air saturation.

A period of nitrogen starvation prior to DCMU addition was necessary to augment cellular glycogen reserves (Allen and Smith 1969; Rippka and Stanier 1978) to support nitrogenase synthesis and activity in the absence of photosynthetic carbon fixation. When DCMU was added within 4 to 6 h after removing NH_4^+, heterocyst differentiation was arrested, the cultures remained green, and no nitrogenase activity could be detected.

Nitrogenase activity was very sensitive to inhibition by O_2 in the anaerobically induced (i.e., DCMU-treated) culture. Atmospheric levels of O_2 inhibited short-term (30 min) acetylene reduction by 70% and activity was reduced greatly at 50% O_2. Nitrogenase activity was very resistant to O_2 in the air-induced culture: maximum activity occurred at atmospheric O_2 levels and 50% O_2 inhibited acetylene reduction by 22%.

Immunoferritin labelling of the Fe-Mo protein. The distribution of the ferritin label on cryosections of a heterocyst (Fig. 3) and vegetative cell (Fig. 4) of aerobically grown *A. cylindrica* treated with goat anti-Fe-Mo protein IgG and ferritin-conjugated rabbit anti-goat IgG was compared. The ferritin label heavily stained the entire heterocyst, while few ferritin molecules are seen on the vegetative cell section. As discussed below, these represent non-specific ferritin staining.

Several control experiments show that ferritin-labelling of the heterocysts was specific. When preimmune goat serum was substituted for anti-Fe-Mo protein IgG, no specific ferritin labelling was observed. Furthermore, sections incubated with goat anti-Fe-Mo protein IgG followed by rabbit anti-goat IgG prior to applying the ferritin-conjugate prevents labelling. Finally, no ferritin labelling was observed

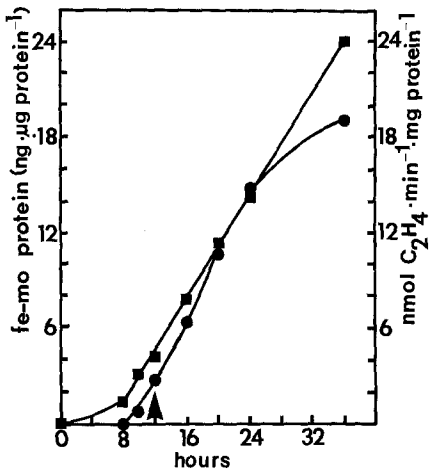

Fig. 1. Time course of nitrogenase induction in *A. cylindrica* under anaerobic conditions. NH_4^+ was removed at h 0 and the cultures incubated in the light under argon/CO_2. 2×10^{-5} M DCMU was added at h 12 (arrow). Fe-Mo protein (■), nitrogenase activity (●) measured under argon

when sections were incubated with ferritin-conjugated, non-homologous rabbit anti-goat IgG preparations (data not shown). Earlier we presented evidence that the primary antiserum was specific to the Fe-Mo protein, or more specifically to an O_2-denatured form, since the purified antigen was exposed to O_2 when introduced into the goat (Murry et al. 1983).

Preservation of tissue antigenicity required use of very light aldehyde fixation and cryomicrotomy to prepare sections. Consequently, details of ultrastructure, particularly the well defined membrane structure observed in plastic embedded sections (see Lang 1965) were not well preserved. However, several morphological features readily allow distinction between the heterocysts and vegetative cells. The vegetative cells but not the heterocysts contain granular inclusions (Fig. 2), perhaps corresponding to carboxysomes (polyhedral bodies) or polyphosphate bodies, both of which disappear in differentiating heterocysts (Lang 1965; Stewart and Codd 1975). The membrane structure of the two cell types also differs greatly in cryosections. The vegetative cell photosynthetic lamellae appear as long continuous layered structures which are concentrated in the cell periphery. The cytoplasm of the heterocysts appeared more dense than the cytoplasm of vegetative cells. The contorted lamellae seen in conventionally fixed heterocysts could not usually be discerned in cryosections (Fig. 2). Sections of the thick laminated heterocyst cell wall, although separated from the cytoplasm, were generally in close proximity to the heterocyst (Fig. 2).

The necessity of using only light aldehyde fixation to preserve antigenicity, presents the possibility that some of the antigen may remain soluble. Table 1 shows that the Fe-Mo protein can not be detected by rocket immunoelectrophoresis after 35 min of paraformaldehyde treatment even when cellular protein concentrations more than 100 times that of the unfixed control culture were used. Although paraformaldehyde fixation alone was sufficient to immobilize the Fe-Mo antigen, ultrastructural preservation was very poor. Thus, it was necessary to follow paraformaldehyde fixation with low concentrations of glutaraldehyde to retain cellular structure.

Immunoferritin labelling of anaerobically induced cells. The specific immunoferritin labelling of the Fe-Mo protein in filaments induced under argon and treated with DCMU for 24 h was also restricted to the heterocyst (Figs. 5 and 6). Whether the sections were incubated in normal rabbit serum or anti-Fe-Mo protein IgG prior to the ferritin staining, a variable but substantial number of ferritin molecules were observed on both our vegetative cell sections and on the surrounding formvar support film. The staining intensity increased with longer incubation periods in the ferritin label and with higher ferritin conjugate (but not anti-Fe-Mo protein IgG) concentrations.

To verify that our light ferritin staining observed on vegetative cells represents non-specific staining rather than a specific antigen-antibody reaction, fifteen electron micrographs were taken at random of ferritin-labelled vegetative cells cultured aerobically and anaerobically. For each micrograph, three to five 2.5 cm^2 blocks were picked at random from the vegetative cells and also from the surrounding formvar support film. The ferritin numbers were counted and the ratio of ferritin numbers per unit area of formvar versus vegetative cell section was determined. The data summarized in Table 2 show that this ratio is close to unity. Furthermore, the Student *t*-test showed that there was no significant difference (at the 95% confidence level) in this ratio between aerobically and anaerobically induced vegetative cells. In contrast, the density of the ferritin label on heterocysts induced under air and anaerobically, was 2 to 3 orders of magnitude greater than labelling of the formvar or vegetative cells.

Discussion

The restriction of specific immunoferritin labelling of the Fe-Mo protein to the heterocysts in anaerobically induced *A. cylindrica* provides direct evidence that the vegetative cells of this organism are unable to express the nif genes. The induction conditions used here (i.e., nitrogen starvation followed by DCMU treatment) have been used to induce nitrogenase activity in non-heterocystous cyanobacteria (Rippka and Waterbury 1977). The high levels of carbon reserves at the time of DCMU addition allowed continued enzyme synthesis and activity for more than 24 h. Thus, when the cells were harvested, nearly 80% of the Fe-Mo protein had been synthesized under strictly anaerobic conditions. Yet no immunoferritin staining of the vegetative cells could be detected statistically. Since under conditions of nitrogen starvation the vegetative cells comprise about 85% of the total cell population, it is possible that Fe-Mo protein is present in these cells in small amounts and escapes detection by the immunoferritin label. However, it is clear that the heterocysts are heavily labelled, whether they were formed under aerobic or anaerobic conditions.

Nitrogenase activity in anaerobically induced cultures were relatively sensitive to inhibition by O_2. However, in comparison with non-heterocystous, filamentous species such as *Plectonema boryanum* where 5% exogenous O_2 abolishes activity (Weare and Benemann 1973), nitrogenase activity in anaerobically induced *A. cylindrica* was markedly more resistant to O_2 inhibition. O_2 sensitivity of nitrogenase in microaerophilic and anaerobic *Anabaena* spp. has been observed in earlier studies (van Gorkom and Donze 1971; Kulasooriya et al. 1972; Rippka and Stanier 1978). O_2 markedly influenced the development of the multilayered

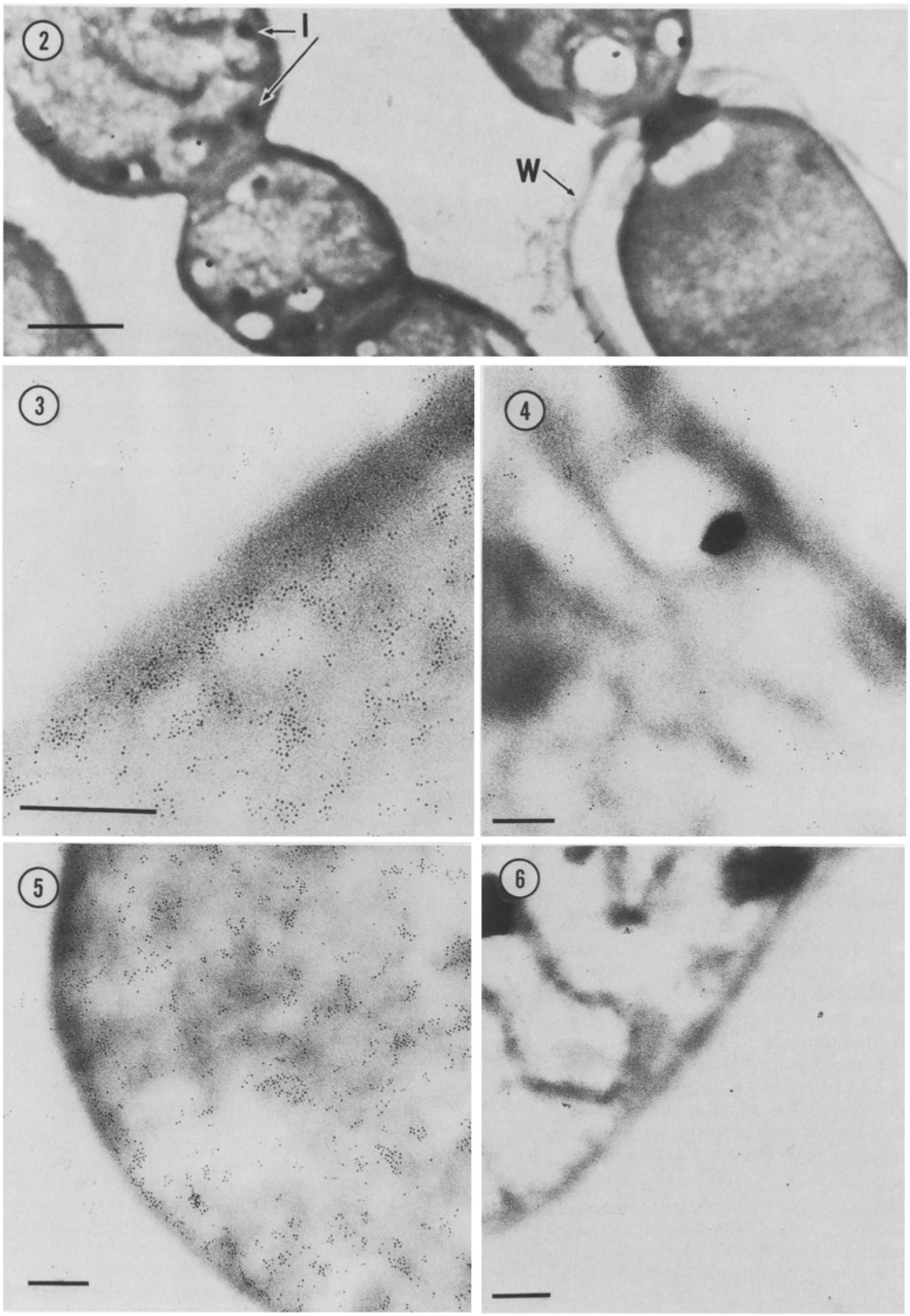

Fig. 2. Unstained ultrathin cryosection of *A. cylindrica*. *W*, heterocyst cell wall; *I*, cytoplasmic inclusions. Bar = 250 μM

Fig. 3. Distribution of ferritin label on ultrathin cryosection of heterocyst of aerobically grown *A. cylindrica*. Bar corresponds to 25 μm in this and following figures

Fig. 4. Ferritin distribution on vegetative cell of aerobically grown *A. cylindrica*

Figs. 5 and 6. Distribution of ferritin label on heterocyst (Fig. 5) and vegetative cell (Fig. 6) of anaerobically induced *A. cylindrica*. Filaments were harvested 24 h after DCMU was added

Table 1. Effect of aldehyde fixation on immunoprecipitable Fe-Mo protein

Fixation period		Protein concentration (μg/μl)	Fe-Mo protein	
Paraformaldehyde (2%)	Glutaraldehyde (0.1%)		μg protein applied	Cone peak height (mm)
–	–	2.86	2.68	4.3
35 min	–	2.7	54	0
35 min	20 min	1.99	39.8	0
35 min	60 min	2.12	42.4	0

Air-grown *A. cylindrica* was fixed in paraformaldehyde or paraformaldehyde followed by glutaraldehyde for the periods noted. Incubation was terminated by centrifugation. The cells were washed several times in 50 mM Tris, 0.5 mM $CaCl_2$, pH 8.6, concentrated and sonicated for 5 min at 50% pulse. Rocket immunoelectrophoresis was carried out on 0.75% (v/v) antiserum concentration

Table 2. Distribution of ferritin molecules on heterocysts and on formvar support film versus vegetative cells of aerobically and anaerobically induced *A. cylindrica*

	N[b]	Ferritin molecules per unit area[a]			
		Formvar	Vegetative cell	Formvar/vegetative cell	Heterocyst/vegetative cell
Aerobic cells	15	1778	1686	1.05	104.5
Anaerobic cells	15	1415	1444	0.98	128.6

[a] Number of ferritin molecules in 2.5 cm^2 area, 3 to 5 blocks were counted/micrograph
[b] Number of micrographs

heterocyst envelope (Kulasooriya et al. 1972; Rippka and Stanier 1978), which is postulated to act as a barrier to O_2 diffusion (Lambein and Wolk 1973). O_2 may similarly influence development of respiratory protection mechanisms. Thus, O_2 sensitivity of nitrogenase activity in anaerobically induced cultures may reflect poorly developed O_2 protection mechanisms normally operative in the heterocyst rather than a vegetative cell pool of nitrogenase.

In conclusion, these results indicate that even under environmental conditions that enable nitrogenase function, heterocyst differentiation is a prerequisite for nif gene expression in *A. cylindrica*. The restriction of nitrogenase to the heterocysts is likely to have adaptive value. In aquatic environments, periods of anaerobiosis frequently develop especially at night when photosynthetic O_2 production ceases. The derepression of nitrogenase in vegetative cells during anaerobic conditions would result in its subsequent inactivation as O_2 was reintroduced with the resumption of photosynthesis. Thus, a futile cycle of nitrogenase synthesis and destruction would occur. The restriction of nitrogenase to the heterocysts would avoid this problem and increase the competitive advantage of heterocystous over non-heterocystous cyanobacteria.

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