Heterocyst envelope thickness, heterocyst frequency and nitrogenase activity in *Anabaena flos-aquae*: influence of exogenous oxygen tension

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The heterocyst envelope of the N₂-fixing cyanobacterium *Anabaena flos-aquae* thickened as exogenous O₂ partial pressure (pO₂) was increased from 5 to 40 kPa. The majority of the thickening occurred in the glycolipid layer area of the envelope. Such thickening appears to be an O₂-induced mechanism for providing a greater O₂ diffusion barrier against O₂ inhibition of nitrogenase. Nitrogenase activity decreased at pO₂ levels above ambient (20 kPa), indicating that a thicker envelope is not completely effective as a barrier to O₂ diffusion. However, when cultures grown at 10 kPa and 40 kPa pO₂ were transferred to ambient pO₂, the 40 kPa pO₂ cells showed higher nitrogenase activity 24 h after transfer compared to those grown at 10 kPa or ambient pO₂, indicating O₂-protection of nitrogenase by thicker heterocyst envelopes. Heterocyst frequency was lowest at 20 kPa O₂.

Introduction

In oxygenic phototrophic cyanobacteria, exogenous oxygen tension is the net product of consumption through respiration, release through oxygenic photosynthesis and physical processes such as diffusion. The nitrogenase enzyme complex responsible for the conversion of molecular nitrogen to ammonium in diazotrophs is inactivated by oxygen (Fay & Cox, 1967; Robson & Postgate, 1980). Various physiological, morphological and ecological strategies exist among different diazotrophs to overcome this dilemma (e.g. Carlton & Paerl, 1989; Carpenter & Price, 1976; Kangatharalingam *et al.*, 1991). An important structural feature providing protection against oxygen inactivation of nitrogenase in certain cyanobacteria is the multi-component envelope of the heterocyst (Wolk, 1982). Despite the presence of heterocysts, filamentous cyanobacteria such as *Anabaena* and *Aphanizomenon* exhibit some degree of nitrogenase inhibition when exogenous oxygen levels are elevated above ambient. This indicates that the oxygen diffusion barrier afforded by the heterocyst envelope is not completely effective against increases in external oxygen tension (Murry *et al.*, 1984; Murry & Wolk, 1989).

Structures analogous to heterocysts, called vesicles in

the actinorhizal diazotroph *Frankia*, have thin walls in cultures grown at low partial pressure of O₂ (pO₂) while having thicker walls at high pO₂ (Parsons *et al.*, 1987). This phenomenon has not been reported among cyanobacterial heterocysts, except for the observation that in *Anabaena* spp. the heterocyst envelope is poorly developed under low-pO₂ and anaerobic conditions (Kulasooriya *et al.*, 1972; Rippka & Stanier, 1978). Only short-term effects of pO₂ or O₂-supersaturation on nitrogenase activity in cyanobacteria have been reported (Murry *et al.*, 1984; Paerl, 1978).

Physiological (environmental) factors such as light intensity, combined nitrogen availability and assimilable organic carbon sources affect the frequency of heterocyst formation (Fogg, 1949). Heterocyst frequency of *Anabaena cylindrica* under highly elevated CO₂ levels with no O₂ was shown to be higher than that in air (Kulasooriya *et al.*, 1972). However, we are unaware of reports on the effect of varied pO₂ alone on heterocyst frequency. Because heterocysts are the primary sites of nitrogenase synthesis (Elhai & Wolk, 1990), their frequency is often positively related to corresponding nitrogenase activity (Smith *et al.*, 1987).

We have examined the influence of external pO₂ on heterocyst envelope thickness, heterocyst frequency and nitrogenase activity in *Anabaena flos-aquae*. This study provides information on the inter-relationships of these factors and the physiological implications of exogenous pO₂.

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Abbreviations: Chl *a*, chlorophyll *a*; TEM, transmission electron microscopy.

Methods

Cultures and general experimental conditions. *Anabaena flos-aquae*, isolated from Hebgen Lake, Montana, was grown and maintained in batch cultures at $25 \pm 1^\circ\text{C}$ in ASM medium (Allen, 1968) without an inorganic nitrogen source (ASM⁻). An irradiance of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ was supplied by 'cool white' fluorescent lamps under a 12 h light/dark cycle.

Nine replicate sealed serum bottles (unit capacity 117.5 ml) were used per pO_2 treatment, each containing 20 ml ASM⁻ medium and 0.5 ml 5-d-old *A. flos-aquae* inoculum in exponential growth. Temperature and irradiance were the same as for culture maintenance. The bottles were incubated with agitation at 60 r.p.m. on a gyrotary shaker. Gas mixtures were replenished every 5 d during the 12 d course of the experiment (see below).

Chlorophyll assay. Chlorophyll *a* (Chl *a*), used to normalize nitrogenase activity, was determined on samples vacuum-filtered onto Whatman GF/C filters. Warm (70°C) 95% (v/v) ethanol was added to the sample filter, which was then vortexed for 2 min and allowed to cool to room temperature for > 12 h (Sartory & Grobbelaar, 1984). Either a spectrophotometer (Varian DMS 80) or a fluorimeter (Turner model 112; Sequoia-Turner Corporation) was employed to quantify the amount of Chl *a* in the supernatant of centrifuged extracts (Winternans & De Mots, 1965). Chl *a* concentration was calculated using the absorption coefficient reported by Winternans & De Mots (1965); fluorimetric determinations were calibrated using pure *Anacystis* Chl *a* (Sigma) in 95% ethanol.

Gas mixtures. Gas mixtures were made using a two-way gas flowmeter/proportioner (Matheson 7300 series) in which O_2 and N_2 were combined to produce mixtures containing 5, 10, 20, and 40 kPa O_2 . The gas mixtures were introduced by gas displacement of the headspace in the 117.5 ml sealed serum bottles containing 20 ml culture medium. CO_2 was then introduced into the culture bottles using a syringe fitted with a 23-gauge hypodermic needle to a constant level of 0.04 kPa. Filled bottles were then agitated gently for about 1 min to equilibrate the gas and liquid phases.

Heterocyst envelope thickness. On day 12 of the experiment, a 1 ml sample from each of two replicate serum bottles per pO_2 treatment was viewed under dark-field microscopy using a Nikon Labophot photomicroscope. Photomicrographs of the samples on Kodak Panatomic-x film were enlarged to achieve approximately 2000-fold magnification of *A. flos-aquae* heterocysts. Heterocyst envelope thickness in the photomicrographs of samples grown at various pO_2 values was measured using a calibrated electronic digitizer connected to a microcomputer. Fifteen heterocysts per pO_2 treatment were randomly selected for wall thickness measurements.

Because heterocyst envelope thickness of low- pO_2 samples reached the limit of resolution of light microscopy (approx. $0.3 \mu\text{m}$), we repeated the entire experiment utilizing transmission electron microscopy (TEM) to verify responses observed with light microscopy. In this second experiment, samples collected on day 12 of incubation from each pO_2 treatment were fixed with 2% (v/v) glutaraldehyde, transferred to BEEM capsule chambers (Day, 1974), postfixed for 1 h in aqueous 1% (w/w) OsO_4 and dehydrated using an ethanol series. This method of fixation does not stain the laminated glycolipid layer of the heterocyst (Fay & Lang, 1971). Hence, we refer to the region occupied by the laminated glycolipid layer as the glycolipid layer area. Our fixation procedure may also cause shrinkage in the various layers of the heterocyst envelope. Although we were unable to evaluate the degree of shrinkage, it should be the same for all samples processed, so that evaluation of relative changes in layer or layer area thickness should be valid. The dehydrated filaments were then embedded (with care given to orientation of filaments) and thin-sectioned (Brawner & Cutler,

1987). A Zeiss model EM 10C/CR electron microscope was used to photograph and measure directly the thickness of the glycolipid layer area of the heterocyst envelope (using a digitizing attachment on the microscope) of 30 randomly selected heterocysts from each pO_2 treatment. Measurements were made at magnifications ranging from 8000 to 10000. Three or four measurements were made on the lateral portions of each heterocyst envelope, avoiding polar regions where wall thickness varied considerably. The entire envelope was measured on the dark-field photomicrographs; only the glycolipid layer area of the envelope was repeatedly measured on the TEM preparations.

Nitrogenase activity. Nitrogenase activity of two replicate samples from each experimental pO_2 level was assayed, without altering pO_2 , 6 and 12 d (late exponential and stationary phase, respectively) after the beginning of the experiment using the acetylene reduction assay (Stewart *et al.*, 1967). Acetylene was added at 10% of the bottle volume; incubation was for 3 h under the same experimental conditions. Ethylene produced was determined by flame-ionization gas chromatography (Carle AGC series 100) based on ethylene standards. Separate experiments in our laboratory have shown that nitrogenase activity in *A. flos-aquae*, at the rates observed, is essentially linear for at least 6 h. The rate of ethylene production for each sample was normalized to Chl *a*. Nitrogenase activity was also determined initially and at 24 and 48 h intervals following transfer to 20 kPa pO_2 of samples grown for 12 d at 10, 20 and 40 kPa pO_2 .

Heterocyst frequency. On days 6 and 12 of the experiment, heterocyst frequency (mean number of heterocysts expressed as a percentage of total number of heterocysts plus vegetative cells counted per trichome) of four subsamples from each of two replicate incubation bottles per pO_2 treatment was determined by light microscopy. All heterocysts and vegetative cells from individual trichomes were included in the counting procedure. This protocol resulted in approximately 300 vegetative cell counts per subsample per pO_2 treatment. Vegetative cell lengths (longitudinal) were obtained from enlarged photomicrographs on 20 random cells (from different trichomes) from each replicate vial of each pO_2 treatment.

Growth rate. Growth rates of *A. flos-aquae* at various pO_2 values were determined by changes in Chl *a* in a 2 ml sample removed just before starting the experiment and on days 6 and 12 of incubation. The change in Chl *a* concentration was divided by the integral average Chl *a* level (assuming exponential growth) between each time interval to yield Chl *a* specific growth.

Fig. 1. Heterocyst envelope thickness of *A. flos-aquae* on day 12 of incubation at various pO_2 levels. Measurements were obtained from digitized (dark-field) photomicrographs ($n=15$) (●) and digitized on-line TEM fields ($n=30$) (■). Error bars, denoting standard error, were all smaller than the symbols. TEM measurements represent the thickness of the glycolipid layer area only. See text for details.

Fig. 2. Photomicrographs from light microscopy (a-c) and TEM (d-f) of *A. flos-aquae* grown at various pO_2 levels showing typical heterocyst envelope thickness at 10 kPa, 20 kPa and 40 kPa. Arrows on the light photomicrographs show the heterocyst envelope (the white line surrounding each heterocyst). H, homogeneous polysaccharide layer; L, laminated glycolipid layer area. Terminology for heterocyst microstructure after Wolk (1982). Bars, 7.5 μm (a-c) and 0.6 μm (d-f).

Results

Heterocyst envelope thickness of *A. flos-aquae*, measured by light microscopy and TEM (glycolipid layer area only), following 12 d of incubation at various pO_2 is shown in Fig. 1; samples grown at 5 kPa were not included in TEM analysis. Selected photo- and electron micrographs of whole and thin-sectioned *A. flos-aquae* heterocysts in cultures grown at 10, 20 and 40 kPa pO_2 are shown in Fig. 2. Light microscopy and TEM both revealed that the thickest heterocyst envelopes consistently occurred at 40 kPa pO_2 . Envelope thickness decreased as pO_2 was lowered to 10 kPa. TEM revealed that the glycolipid layer area of the envelope responded to changes in pO_2 more than the polysaccharide layer. The difference in thickness of the polysaccharide layer between cells grown at 10 kPa and 40 kPa pO_2 was about 2.5-fold, whereas a 16-fold difference in thickness occurred in the glycolipid layer area. No significant difference in heterocyst envelope thickness among pO_2 levels was observed on day 6 of incubation (data not shown). The internal structure of the heterocysts within and between each treatment showed considerable variation. Currently we have no data which could explain this variability. The electron micrographs shown reflect typical envelope thickness for each treatment and the typical range of internal structure observed within and among treatments.

Nitrogenase activity on both day 6 and 12 of the incubation was highest at 20 kPa pO_2 (Fig. 3). When cultures grown at 10 kPa, 20 kPa and 40 kPa pO_2 were transferred to 20 kPa pO_2 , the 40 kPa pO_2 culture had the highest nitrogenase activity following 24 h of incubation (Table 1).

Heterocyst frequency of *A. flos-aquae* on day 6 of the incubation was highest at 5 kPa and lowest at 20 kPa pO_2 . On day 12, 40 kPa pO_2 produced the highest heterocyst frequency, although it was not statistically different from that at 5 kPa; the lowest heterocyst frequency again occurred at 20 kPa pO_2 (Fig. 4). A high proportion of elongated vegetative cells showing no indications of septation occurred at 5 and 40 kPa pO_2 . Measurements of 20 random cells on day 12 revealed that mean vegetative cell lengths ($\mu\text{m} \pm$ standard error) were 7.80 ± 0.36 at 5 kPa, 6.25 ± 0.29 at 10 kPa, 5.81 ± 0.23 at 20 kPa, and 7.19 ± 0.25 at 40 kPa pO_2 . Elongated cells were rarely found at 20 kPa. Chlorophyll *a* specific growth (d^{-1} ; mean $\pm$ standard error) of *A. flos-aquae* at 5, 10, 20 and 40 kPa O_2 was 0.068 ± 0.000 , 0.347 ± 0.000 , 0.380 ± 0.003 and 0.218 ± 0.014 during the first 6 d of incubation, and 0.024 ± 0.024 , 0.025 ± 0.005 , 0.016 ± 0.006 and 0.014 ± 0.014 between days 6 and 12 of incubation, respectively. These growth data showed that

Fig. 3. Nitrogenase activity of *A. flos-aquae* grown at various pO_2 levels, on days 6 (○) and 12 (●) of incubation. Error bars denote standard error. When not present, error bars were smaller than the symbols. $n = 2$.

Fig. 4. Heterocyst frequency of *A. flos-aquae* grown at various pO_2 levels, on days 6 (○) and 12 (●) of incubation. Error bars denote standard error. When error bars are not shown they were smaller than the symbols. $n = 8$.

Table 1. Nitrogenase activity of *A. flos-aquae* transferred to 20 kPa pO_2 from 10, 20 or 40 kPa pO_2 after 12 d growth

Initial growth condition (pO_2 , kPa)	Nitrogenase activity*		
	Before transfer	After transfer	
		24 h	48 h
10	4.28 ± 0.48	3.20 ± 0.60	2.45 ± 0.45
20	24.60 ± 2.10	19.40 ± 0.80	20.90 ± 0.30
40	4.41 ± 0.38	20.90 ± 0.40	22.85 ± 0.75

* Mean ($\pm$ standard error; $n = 2$) nitrogenase activity, $\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} (\text{mg Chl } a)^{-1}$, before transfer, and at 24 and 48 h after transfer to 20 kPa pO_2 .

the highest rates generally occurred between 10 and 20 kPa O_2 . The high errors associated with the specific growth rates at 5 and 40 kPa O_2 between days 6 and 12 of incubation make these particular rates suspect.

Discussion

Heterocyst envelope layers provide a passive barrier to O₂ diffusion (Murry *et al.*, 1984; Murry & Wolk, 1989; Stanier & Cohen-Bazire, 1977). Our results indicate that both the homogeneous polysaccharide layer and the laminated glycolipid layer area of the heterocyst envelope in the cyanobacterium *A. flos-aquae* increased in thickness in response to prolonged exposure (12 d) to high exogenous pO₂, an observation similar to that noted in *Frankia* vesicle wall morphogenesis (Parsons *et al.*, 1987). Conditions of prolonged O₂ supersaturation have been measured in the surface waters during late spring in Hebgen Lake, the site from which *A. flos-aquae* was isolated (J. C. Priscu, unpublished data). Hence, the ability to regulate envelope thickness may enhance the fitness of this organism, and possibly other heterocystous cyanobacteria, in nature.

The culture grown at 40 kPa pO₂ showed a considerable increase in nitrogenase activity following transfer to 20 kPa pO₂, while the two cultures grown at 10 and 20 kPa pO₂ showed no increase following transfer. These results imply an inducible nature of heterocyst envelope thickness in response to pO₂ which can protect nitrogenase by mediating inward O₂ diffusion. Our observation is in agreement with previous reports of poor development of heterocyst walls in *Anabaena* species under anaerobic or microaerobic conditions (Kulasooriya *et al.*, 1972; Rippka & Stanier, 1978). Heterocyst envelope components, such as the laminated glycolipid layer and the homogeneous polysaccharide layer, have been shown to be important as gas diffusion barriers (Murry & Wolk, 1989). According to Walsby (1985) the maximum rate of oxygen diffusion into a heterocyst is nearly half that of nitrogen when the inside concentration of these gases is zero. This is because the molar concentration of oxygen dissolved in water equilibrated with air (258 µM) is nearly half the equilibrium concentration of nitrogen (506 µM). These diffusion rates will change rapidly when the environmental oxygen/nitrogen ratio is changed. The permeability of the heterocyst envelope to oxygen/nitrogen depends on the number of layers of glycolipid in the heterocyst envelope (Walsby, 1985). Producing a thick heterocyst envelope (either the glycolipid layer or the polysaccharide layer) could be a metabolically 'expensive' process so that organisms presumably form thicker envelopes only when necessary.

That optimum nitrogenase activity of *A. flos-aquae* occurred at 20 kPa pO₂ under prolonged growth indicates that nitrogenase activity was regulated by other factors as well as external pO₂. Prolonged growth at 40 kPa pO₂ apparently induced physiological stress leading to reduced nitrogenase activity. Optimum nitro-

genase activity should have occurred at 5 or 10 kPa pO₂ instead of 20 kPa if regulated directly by exogenous pO₂. Our data imply that 20 kPa pO₂ provides a general physiological optimum. The observation that the culture transferred to 20 kPa from 40 kPa pO₂ showed higher nitrogenase activity, compared to those transferred from 10 and 20 kPa pO₂, indicates that thicker heterocyst envelopes offer better protection of nitrogenase from O₂-inactivation. This observation also implies a degree of recovery from physiological stress at 40 kPa pO₂. Kulasooriya *et al.* (1972) showed that short-term anaerobic growth of *A. cylindrica* with highly elevated CO₂ levels increased nitrogenase activity, followed by a rapid decline. Azide, an inhibitor of cytochrome activity, strongly suppressed nitrogenase activity in *A. cylindrica* under aerobic conditions, indicating a reliance of nitrogenase activity on active respiration (Murry *et al.*, 1984). This contention assumes that azide does not inhibit nitrogenase directly. Presumably, the negative effect of energy limitation in *A. flos-aquae* at low pO₂ (5 and 10 kPa) counters the positive influence of reduced O₂ inhibition of nitrogenase at 5 and 10 kPa pO₂. High pO₂, apart from its direct inhibitory effect on nitrogenase, could also inhibit the fixation of inorganic C by photosynthesis through photooxidation and photorespiration, which are energy-consuming processes (Van Liere & Walsby, 1982).

Our data indicate that prolonged exposure to elevated oxygen tension or microaerobiosis is detrimental to *A. flos-aquae* because the fundamentally important processes of CO₂ and N₂ fixation can be affected. We conclude that *A. flos-aquae* achieves optimal growth at ambient atmospheric pO₂ (near 20 kPa) despite its ability to produce a thicker heterocyst envelope at elevated pO₂. The tradeoff between morphogenic and physiological adaptations to varying pO₂ conditions in all likelihood involves a complicated interplay of genetic flexibility, bioenergetics (including the metabolic 'costs' of altering and maintaining cell morphology in response to environmental stress) and biochemical limitations (to O₂ stress, N starvation, and availability of energy and reductant). The ability to regulate heterocyst envelope thickness may have selective advantages for *A. flos-aquae* in N-deficient, O₂-rich environments such as those found in the near surface waters of Hebgen Lake (the site of isolation). The biosynthetic processes responsible for altering heterocyst envelope thickness may be of physiological and evolutionary significance when considering long-term increases in oxygenation of the biosphere during the inception and development of oxygenic photosynthesis.

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