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Physiological Studies of Oxygen Protection Mechanisms in the Heterocysts of *Anabaena cylindrica*

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The mechanism of O_2 protection of nitrogenase in the heterocysts of *Anabaena cylindrica* was studied in vivo. Resistance to O_2 inhibition of nitrogenase activity correlated with the O_2 tension of the medium in which heterocyst formation was induced. O_2 resistance also correlated with the apparent K_m for acetylene, indicating that O_2 tension may influence the development of a gas diffusion barrier in the heterocysts. The role of respiratory activity in protecting nitrogenase from O_2 that diffuses into the heterocyst was studied using inhibitors of carbon metabolism. Reductant limitation induced by 3-(3,4-dichlorophenyl)-1,1-dimethylurea increased the O_2 sensitivity of in vivo acetylene reduction. Azide, at concentrations (30 mM) sufficient to completely inhibit dark nitrogenase activity (a process dependent on oxidative phosphorylation for its ATP supply), severely inhibited short-term light-dependent acetylene reduction in the presence of O_2 but not in its absence. After 3 h of aerobic incubation in the presence of 20 mM azide, 75% of cross-reactive component I (Fe-Mo protein) in nitrogenase was lost; less than 35% was lost under microaerophilic conditions. Sodium malonate and monofluoroacetate, inhibitors of Krebs cycle activity, had only small inhibitory effects on nitrogenase activity in the light and on cross-reactive material. The results suggest that oxygen protection is dependent on both an O_2 diffusion barrier and active respiration by the heterocyst.

The photosynthetic production of O_2 presents a unique problem to the functioning of nitrogenase in the cyanobacteria (blue-green algae). In common with other bacterial nitrogenases, cyanobacterial nitrogenase is irreversibly inactivated by O_2 (20, 38). Despite this, a wide variety of species is capable of growth on atmospheric N_2 under aerobic conditions, even in waters supersaturated with O_2 (21, 23). Members of the family *Nostocaceae* have evolved a unique system to allow the simultaneous operation of these basically incompatible processes: the heterocyst.

There may be several complementary mechanisms by which the heterocyst is able to maintain the reducing environment required for activity of the O_2-labile nitrogenase. The loss of O_2-evolving photosystem II components is well documented (2, 13, 41, 43). It has been suggested that four unique glycolipids in the laminated layer of the heterocyst envelope provide a passive barrier to the diffusion of O_2 (25). The isolation of mutants deficient in these envelope glycolipids which have little or no nitrogenase activity when assayed aerobically (19) is consistent with the idea of a diffusion barrier. O_2-sensitive mutants with normal glycolipid composition were also isolated (19); they presumably have lesions in genes coding for other O_2 protection mechanisms. Comparison of dark O_2 uptake rates as a function of O_2 tension showed diffusion-limited kinetics (28) associated with the presence of heterocysts in *Anabaenopsis arnoldii* (K. S. Rhoades, Ph.D. thesis, University of Michigan, Ann Arbor, 1981) and *Anabaena hos-aquae* (22). Similarly, a diffusion barrier by the heterocyst envelope was suggested by the finding that the apparent K_m of nitrogenase for acetylene is 10-fold higher in vivo than in vitro in *Anabaena cylindrica* but only slightly higher in the nonheterocystous cyanobacterium *Plectonema boryanum* (18).

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It is unlikely, however, that O_2 is completely excluded from the heterocyst. Isolated heterocysts are capable of oxidative phosphorylation (40). Dark nitrogenase activity is O_2 dependent (16, 44), presumably because activity is dependent on ATP production by oxidative respiration. Moreover, enhanced levels of superoxide dismutase, an enzyme normally restricted to aerobic organisms (31), have been observed in the heterocysts of *Anabaena cylindrica* (11, 30). Finally, if the heterocyst cell wall provided a truly impermeable gas diffusion barrier, then N_2, in addition to O_2, would presumably be excluded from the site of N_2 fixation.

There are several possible means by which the heterocyst could remove any O_2 that entered the cell. Enhanced rates of O_2 uptake in isolated heterocysts (17, 35) and enriched levels of dehydrogenases in differentiated cultures (46) suggest the operation of a respiratory protection mechanism, perhaps analogous to that proposed for *Azotobacter chroococcum* (12, 48). The O_2 sensitivity of nitrogenase activity in linear-phase cultures, but not in exponential-phase cultures (44), also supports this view, although O_2 sensitivity may also be due to damaged heterocysts under nonexponential growth conditions. Hemoproteins, localized in the "honeycomb" region and the periphery of heterocysts of *Anabaena cylindrica* by diaminobenzidine, may act to reduce O_2 diffusing through the heterocyst envelope and from the junction with the photosynthetic vegetative cells (33). An uptake hydrogenase which is localized in the heterocysts (36) and was shown to couple with O_2 reduction (15, 35) may provide another means of scavenging O_2 from the site of nitrogen fixation. The kinetics of O_2 inactivation and recovery of nitrogenase activity in *Anabaena* spp. suggested the operation of a mechanism preventing irreversible denaturation of nitrogenase (37), perhaps similar to the conformational protection of nitrogenase in *Azotobacter* spp. (39).

The present study is concerned with the nature of these O_2 protection mechanisms, their relative role in protecting the nitrogenase enzyme, and the effect of environmental parameters on their development.

MATERIALS AND METHODS

Anabaena cylindrica 629 was grown axenically as previously described (32) in 2-liter batch cultures with a modified (5×10^{-3} M $NaHCO_3$) medium (1) sparged with air-CO_2 (99.7%:0.3%). Heterocyst-free cultures were obtained by successive transfer in the medium supplemented with 5 mM NH_4Cl. Heterocyst differentiation was initiated by centrifuging the culture and suspending the filaments in N-free medium. The cell suspension was divided into 250-ml cylindrical bottles and incubated under the desired gas phase at a light intensity of 2×10^4 ergs cm^{-2} s^{-1}. Log-phase cultures were obtained by dividing a low-density 2-liter culture into several 250-ml cylindrical bottles and incubating at 28 to 29°C (under lowered light intensities) with the desired gas phase for at least 12 h before initiating experimentation. The light intensity was doubled to 2×10^4 ergs cm^{-2} s^{-1} when the cultures reached a density of 40 to 50 Klett units. The doubling time under these conditions ranged between 13 and 15 h. Reductant-limited cultures were obtained by addition of 2×10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to log-phase cultures sparged with N_2-CO_2. Growth was measured with a Klett-Summerson colorimeter (red filter no. 66). The conversion factor between dry weight of *A. cylindrica* and Klett units was 3.2 μg/ml for 1 Klett unit.

The O_2 tension of the culture medium was measured with a galvanic dissolved-O_2 probe with D. O. Analyzer model DO-40 (New Brunswick Scientific Co., New Brunswick, N.J.). The probe was inserted into the neck of the 250-ml magnetically stirred culture bottle immediately after removing the gas sparger, and dissolved O_2 was read within 30 s. Heterocyst and proheterocyst frequencies were estimated with a phase-contrast microscope at ×1,000. Acetylene reduction assays were performed in triplicate (standard deviation, ± 5%) in argon-filled 5.5-ml Fernback flasks which contained all of the gases indicated in the text (32). Except for the K_m measurements, 15% acetylene was used. Assay mixtures were incubated at 30°C with shaking and saturating light (2×10^4 ergs cm^{-2} s^{-1}) from below for 30 min. The assay remained linear for over 1 h. Photosynthetic carbon fixation was measured by injecting 50 μl (10 μCi) of $NaH^{14}CO_3$ into argon-filled 5.5-ml Fernbach flasks containing 2 ml of cells. The samples were incubated as described above for acetylene reduction. After 30 min of incubation, 1 ml of cells was removed, filtered on a 2.5-cm membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and washed with acidified distilled water (pH 4.0) to remove soluble bicarbonate. The filters were dissolved in 10 ml of Filter-Solv (Beckman Instruments, Inc., Fullerton, Calif.) for liquid scintillation counting.

Component I (Fe-Mo protein) of nitrogenase was quantified by rocket immunoelectrophoresis with goat immunoglobin G monospecific to component I (31a). Samples (20 to 40 ml) of algae were harvested (10 min, 10,000 × *g*), suspended in 0.5 to 1.0 ml of 50 mM Tris–1 mM $CaCl_2$ buffer (pH 8.6), and frozen immediately. α-*N*-tosyl-L-phenylalanine chloromethyl ketone (1 mM) was added to each sample to prevent proteolytic degradation of O_2-inactivated component I during preparation. Just before immunoelectrophoresis, the samples were rapidly thawed and sonicated for 2 min (Ultrasonic model W200) in an ice bath, and then six to seven dilutions were prepared in the Tris buffer. Of each dilution, 7.5 μl was applied to 3-mm wells punched in 0.75% (vol/vol) antiserum–1% agarose (J. T. Baker Chemical Co., Phillipsburg, N.J.) molded plates (200 by 100 by 1.5 mm). Electrophoresis was carried out for 20 h at 10.5 mA cm^{-2}. The plates were washed and stained according to the procedure of Weeke (45). Protein content of the crude extracts was determined by the procedure of Lowry et al. (29). Linear regression analysis was used to determine the slope of the curve relating precipitin peak height to amount of cellular protein applied. Component I in the experimental samples was estimated by comparison with a standard curve prepared with highly purified component I.

RESULTS

O_2 sensitivity of in vivo nitrogenase activity. Undifferentiated cultures of *A. cylindrica* were induced to form heterocysts under several O_2 tensions. Under all culture conditions, most proheterocysts appeared under the light microscope to have matured into heterocysts within 24 h after the removal of ammonia. Large differences, however, were found in the response of in vivo nitrogenase activity to O_2 tension of the assay gas phase (Fig. 1). Nitrogenase activity in aerobically induced cultures was very resistant to O_2 in the assay gas phase. Indeed, the culture induced under very high O_2 levels (280% of air saturation) actually required exogenous O_2 for maximal activity. Nitrogenase activity was much more sensitive to O_2 in cultures sparged with anaerobic gases during the induction period. The most O_2-sensitive culture was induced under an atmosphere of argon-CO_2, in which O_2 tensions were the lowest due to the severely reduced photosynthetic O_2 production under nitrogen-limiting conditions. These results are similar to those of Mackey and Smith (30). A marked decline in phycocyanin pigments is apparent under all culture conditions within 12 h of N removal. Associated with this loss is a dramatic decline in

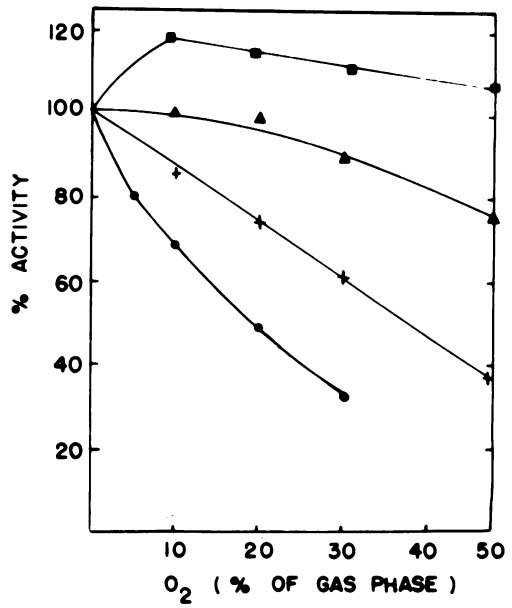

FIG. 1. O_2 Sensitivity of in vivo nitrogenase activity in *A. cylindrica* 24 h after heterocyst induction under various gas phases. During the induction period, cultures were incubated under atmospheres of N_2-O_2 (50:50; 672 μM dissolved O_2) (■), air (278 μM O_2) (▲), N_2 (55 μM O_2) (+), and argon (15.3 μM O_2) (●). CO_2 was included in the gas phase in each case at ca. 0.5%. Aliquots (2 ml) were assayed for acetylene reduction in the light for 30 min. The specific activities of the four cultures measured under argon were 11.5, 12.9, 12.5, and 24.3 nmol of C_2H_4 min^{-1} mg (dry weight)$^{-1}$, respectively.

TABLE 1. Apparent K_m for acetylene reduction by *A. cylindrica* cultures 24 h after induction under various O_2 tensions[a]

O_2 tension of induction medium (% saturation)	K_m (atmospheres of acetylene [$\times 10^{-3}$])
278	29.6
120	22
23	12.9
6.4	8.4
In vitro	1.85[b]

[a] Experimental conditions were as described in the legend to Fig. 1. Assays were performed in triplicate by sparging cells with argon to remove dissolved O_2 and injecting 2-ml aliquots into micro-Fernbach flasks containing the appropriate concentrations of acetylene. The flasks were vented to atmospheric pressure and uncubated for 30 min at a light intensity of 6.0×10^4 ergs cm^{-2} s^{-1} on a thermostated (30°C) reciprocal shaker. The K_ms were determined by a linear regression analysis of all data points, using a standard double-reciprocal plot. The correlation coefficient in each case was greater than 0.97. Values represent the average of at least two experiments.

[b] From Hallenbeck et al. (18).

photosynthetic capacity (9, 34). Under an atmosphere of air or N_2, but not argon, phycobiliproteins and photosynthesis are restored in the vegetative cells as heterocysts mature and nitrogen fixation is initiated. Within 24 h of initiating induction, photosynthetic $^{14}CO_2$ incorporation in the argon cultures was 10 to 15% of the values observed in the cultures induced with N_2 in the sparging gases (data not shown).

This observed correlation between resistance to O_2 inhibition of nitrogenase activity and increasing O_2 tension of the induction medium does not appear to reflect reductant limitations to the heterocyst, which would impair respiratory protection. In contrast to results obtained with reductant-limited cultures of this species (3), H_2 included in the assay medium had no stimulatory effect on nitrogenase activity at any time during the induction period under each O_2 tension used. Furthermore, acetylene reduction rates in the dark were relatively high, and DCMU was not inhibitory (data not shown).

Development of the heterocyst gas diffusion barrier. To determine whether the influence of ambient O_2 tension on O_2 sensitivity of nitrogenase involved a differential effect on development of the postulated gas diffusion barrier, in vivo K_ms for acetylene were measured as an indication of heterocyst permeability to gas diffusion. The in vivo K_m for acetylene was determined by standard double-reciprocal plots in cultures induced for 24 h under various O_2 tensions, and this K_m was compared with the in vitro K_m (Table 1). In the culture induced under the lowest O_2 tension (6.4% of air saturation), the apparent K_m for acetylene was about four times that for nitrogenase in vitro. The apparent K_m was 16-fold greater in cultures induced under the highest O_2 tensions (280% of air saturation) than the K_m in vitro. In contrast, no significant difference in the in vitro K_m was seen between cell extracts prepared from aerobically and from microaerophilically (argon-CO_2) induced filaments.

Respiratory protection mechanisms of heterocysts. Reductant limitation was induced by treating a log-phase, microaerophilic (N_2-CO_2) culture with DCMU for 4 h. Within this period, nitrogenase activity declined 25%. Activity was restored to the original level, however, by inclusion of 15% H_2 in the assay mixture, indicating that reductant limited enzyme activity. Nitrogenase activity in the DCMU-treated culture was considerably more sensitive to O_2 than was activity in the control culture (Fig. 2). In log-phase cultures, DCMU has little effect on O_2 sensitivity in short-term (15- to 30-min) assays.

The role of the cytochrome system in scavenging O_2 from the site of N_2 fixation was studied in vivo by use of the cytochrome inhibitor sodium azide. Under anaerobic conditions, azide had no inhibitory effect on nitrogenase activity until concentrations above 20 mM were reached (Fig. 3). Dark acetylene reduction was 90% inhibited by 20 mM azide. Dark nitrogenase activity is apparently dependent on oxidative phosphorylation for ATP, since O_2 is required (16, 43). Atmospheric levels of O_2 which had only a small inhibitory effect on nitrogenase activity under these conditions (Fig. 1) inhibited nitrogenase activity by almost 70% when 20 mM sodium azide was included in the assay. As seen from the anaerobic control, azide at this concentration does not affect nitrogenase directly, nor are azide-sensitive reactions involved in substrate supply. Thus, it appears that cytochrome activity is an important factor in O_2 protection of nitrogenase. The inhibitory effects of high azide concentrations under anaerobic conditions may be attributed to inhibition of reductant or ATP generation in heterocysts or to competitive inhibition of nitrogenase (47).

To identify the metabolic pathways responsible for providing substrate for nitrogenase activity and O_2 protection, we studied selective inhibitors of carbon catabolism for their effect on short-term acetylene reduction and photosynthetic ^{14}C incorporation in log-phase cultures (Table 2). Monofluoroacetate, an inhibitor of aconitate hydratase, had no effect on nitrogenase activity in the light. Dark nitrogenase activity, however, was inhibited substantially. These results are similar to those obtained earlier with the same cyanobacterium (26). However, in contrast to that study (26), we consistently observed a slight but reproducible loss of photosynthetic capacity with monofluoroacetate. Sodium malonate, a competitive inhibitor of the Krebs cycle enzyme succinate dehydrogenase, was ineffective at concentrations

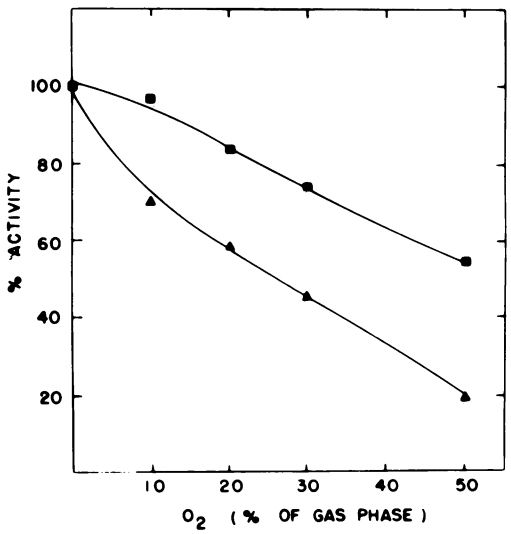

FIG. 2. Effect of O_2 tension on nitrogenase activity in log-phase (■) and reductant-limited (DCMU-treated) (▲) cultures of *A. cylindrica* incubated under N_2-CO_2. Nitrogenase activity (under argon) was 10.6 nMol of C_2H_4 min^{-1} mg (dry weight)$^{-1}$ in the log-phase culture and 8.1 nmol of C_2H_4 min^{-1} mg (dry weight)$^{-1}$ in the culture treated with DCMU for 4 h. Inclusion of 15% H_2 stimulated activity to 10.8 nmol of C_2H_4 min^{-1} mg (dry weight)$^{-1}$ in the DCMU-treated culture but had no effect on the log-phase culture.

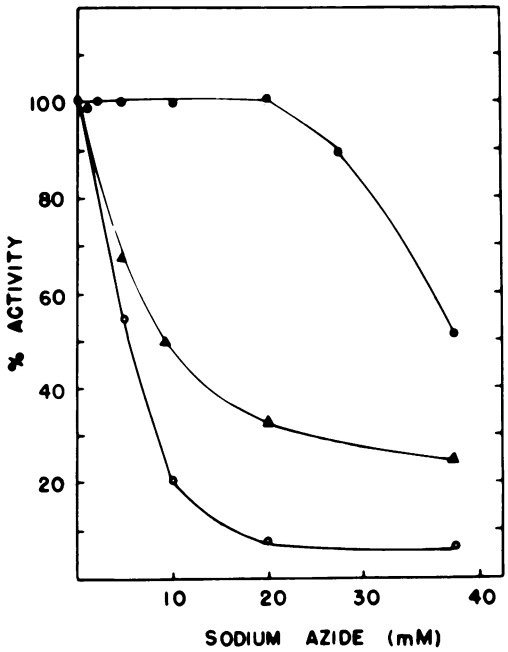

FIG. 3. Effect of sodium azide on nitrogenase activity in N_2-grown log-phase *A. cylindrica*. Cultures were assayed in the light under argon (●) and under argon plus 20% O_2 (▲), and in the dark under argon plus 20% O_2 (○); 100% activity was 11.2, 10.6, and 4.95 nmol of C_2H_4 min^{-1} mg (dry weight)$^{-1}$, respectively.

of up to 10 mM in inhibiting nitrogenase activity under any of the assay conditions. However, a significant loss of photosynthetic capacity was observed.

To differentiate between the effects of these inhibitors on nitrogenase activity and the amount of enzyme actually present, immunoelectrophoretic determination of component I of nitrogenase was compared with in vivo acetylene reduction. After 3 h of aerobic incubation in the presence of azide, nitrogenase activity was nearly abolished (Table 3). Loss of enzyme activity is partially due to an azide-effected loss of reductant pools, since inclusion of H_2 in the assay system stimulated residual acetylene reduction. However, more than 75% of the immunoprecipitable component I was lost in the 3-h period. This apparently reflects a proteolytic degradation of this protein. Loss of both enzyme activity and component I was much smaller with azide treatment under microaerophilic incubation, suggesting that O_2 was responsible for the loss of enzyme under these conditions. Monofluoroacetate treatment inhibited acetylene reduction only slightly under aerobic and microaerophilic conditions, and this inhibitory effect appeared to be at the level of reductant supply, since H_2 stimulated enzyme activity. A small loss of component I was observed under aerobic but not under microaerophilic conditions.

TABLE 2. Effects of Krebs cycle inhibitors on nitrogenase activity and photosynthetic capacity ($^{14}CO_2$ uptake) in log-phase, aerobic cultures of *A. cylindrica*[a]

Assay conditions	Inhibitor[b]	Acetylene reduction (% of control)	^{14}C incorporation (% of control)
Light			
Argon	None	100	
	MFA	97	
	Mal	101	
Argon + 20% O_2	None	100	100
	MFA	102	85.4
	Mal	102	69.6
Dark			
Argon + 20% O_2	None	100	
	MFA	55	
	Mal	106	

[a] All assays were for 30 min at 30°C. The specific activities for acetylene reduction of the control cultures were 9.79, 8.45, and 4.6 nmol of ethylene min^{-1} mg (dry weight)$^{-1}$ under argon and argon plus 20% O_2 in the light and under argon plus 20% O_2 in the dark, respectively. The photosynthetic capacity of the control culture was 116.0 cpm min^{-1} mg (dry weight)$^{-1}$.

[b] MFA, Monofluoroacetate (5 mM); Mal, sodium malonate (2.5 mM).

TABLE 3. Effect of azide and monofluoroacetate on nitrogenase activity and component I levels after 3 h of treatment of log-phase cultures of *A. cylindrica*

Gas phase	Inhibitor[a]	Acetylene reduction (% of time-zero level)[b]		Component I (% of time-zero level)[c]
		Argon	Argon + H_2	
Air-CO_2	None	100	100	97
	NaN_3	0.3	6.4	23
	MFA	69.8	86.5	88
N_2-CO_2	None	100	105	103
	NaN_3	42	50	67
	MFA	81.1	97	106

[a] MFA, Monofluoroacetate (2.5 mM). NaN_3 was at 20 mM.

[b] Air-grown control cultures had reduced 10.5 nmol of C_2H_4 min^{-1} mg (dry weight)$^{-1}$; N_2-CO_2-grown cultures had reduced 11.6 nmol of C_2H_4 min^{-1} mg (dry weight)$^{-1}$.

[c] Control cultures contained ca. 10 ng of component I per μg of total cellular protein.

DISCUSSION

The results presented here provide evidence that at least two mechanisms, a gas diffusion barrier and respiratory protection, are involved in O_2 protection of nitrogenase. The O_2 sensitivity of nitrogenase activity in cultures induced under low partial O_2 pressure (pO_2) apparently reflects inadequate development of an O_2 protection mechanism that is distinct from the protection afforded nitrogenase by the availability of carbohydrate pools. The correlation between O_2 sensitivity and the apparent K_m for acetylene suggests that ambient O_2 tension may influence the development of the passive barrier of the heterocyst envelope. If the affinity of nitrogenase for acetylene is the same under all of the induction conditions used, then the variation in apparent K_m in vivo could be interpreted as an indication of the intracellular level of acetylene and thus as an indirect measure of the resistance of the heterocyst envelope to gas diffusion. This approach has been used to determine the permeability of actinorhizal nodules (46a). In vitro, the K_m for N_2 and acetylene in cell-free nitrogenase preparations apparently is not always constant. In *Rhizobium* extracts the value is dependent on a variety of factors, including pH and ATP and dithionite levels (6). Furthermore, the K_m for N_2 in intact nodule systems (4) and bacteroid suspensions (5) was shown

to depend on the pO_2: both K_m and V_{max} increased concomitantly in response to the O_2 tension of the assay system. Bergersen and Turner suggested (6) that varying the pO_2 in intact systems could alter ATP concentrations and thus influence these values.

Thus, it is possible that the variation in apparent K_m for acetylene observed here may reflect a difference in substrate availability rather than an effect on the gas diffusion barrier. However, since under all induction conditions the carbohydrate pools appear to be quite high, reductant supply is unlikely to be a significant factor. It is known that in organisms such as *Azotobacter chroococcum* the cytochrome content increases in response to increasing pO_2 of the culture medium (14). An analogous situation may exist in the heterocyst. However, several lines of evidence indicate that in the light, cyclic photophosphorylation is the major source of ATP production in the heterocysts (8, 10, 26). Little is known about the influence of O_2 on this process. However, if the large variation in K_m was due to substrate supply, then comparable differences in V_{max} would also be expected (6). Enzyme activity was similar in all three cultures induced in the presence of N_2 (Fig. 1). The argon-induced culture is not directly comparable to the N_2-induced cultures because under conditions of nitrogen starvation, the actual enzyme concentration is much greater. In conclusion, we interpret the varying K_ms to support the conclusions that a gas diffusion barrier in heterocysts is present and that its biosynthesis is regulated by ambient O_2 tensions.

Reduced carbon compounds produced in the vegetative cells are transported into the heterocysts to provide substrates for nitrogenase activity. With prolonged DCMU treatment the heterocysts become depleted of reductant, and the O_2 sensitivity of nitrogenase activity increases substantially. This observation corroborates the finding of O_2-sensitive nitrogenase activity in aging (light-limited) *A. cylindrica* cultures (44) and suggests that photosynthate plays an important role not only in substrate supply but also in O_2 protection of nitrogenase.

The strong inhibitory effect of sodium azide on in vivo nitrogenase activity and the loss of component I in the presence of O_2 indicate that cytochrome activity is an important factor in O_2 protection of nitrogenase, both in the light and in the dark. Although azide and the other inhibitors used in this study also affect vegetative cell functions, it is clear that azide-sensitive processes, whether in the heterocyst or in vegetative cells, are not important in substrate supply, since no inhibition of nitrogenase activity was observed in the light under anaerobic conditions at azide concentrations which abolish dark aerobic nitrogenase activity. This observation supports the earlier suggestion (8) that cyclic photophosphorylation can supply all of the ATP for maximum nitrogenase activity. Krebs cycle activity is apparently not an important source of reductant for either enzyme activity or O_2 protection in the light. However, in the dark, monofluoroacetate, but not malonate, inhibited nitrogenase activity substantially. Thus, reductant (and ATP) for dark nitrogenase activity may be provided by isocitrate or its metabolites. Isocitrate was shown to support acetylene reduction in isolated heterocysts (27). This may explain the occurrence of greatly enhanced levels of the Krebs cycle enzyme isocitrate dehydrogenase in the heterocysts (7).

The finding that the diffusion barrier of the heterocysts is more or less developed depending on ambient oxygen tensions was already suggested from earlier studies which showed that the laminated layer of the heterocyst cell wall was poorly developed in cultures grown under anaerobic or microaerophilic conditions (24, 38). Such an adaptive biosynthetic regulation implies that this protection mechanism carries a significant metabolic penalty and that its presence was not consistently required in the environment in which these algae evolved. Studies correlating pO_2 to nitrogenase activity by natural blooms must take into account not only existing pO_2 but also the history of the cultures. A similar argument may apply to the respiratory protection mechanism, although no direct evidence for such adaptation is presented here. Of course, the availability of respiratory substrates, which is dependent on prior rates of CO_2 fixation, will determine the effectiveness of this respiratory protection mechanism. The presence of two major, independent, and variably active oxygen protection mechanisms results in a flexible and dynamic response to the environmental oxygen tensions, a key factor in nitrogen fixation by cyanobacteria in nature.

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