Studies on the Effects of Oxygen on Acetylene Reduction (Nitrogen Fixation) in *Gloeotheca* sp. ATCC 27152

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Cultures of *Gloeotheca* sp. ATCC 27152 did not reduce acetylene when exposed to O₂ concentrations greater than 0.7 atm. However, following exposure to 1 atm O₂ for up to 12 h or to 0.8 atm O₂ for up to 14 d, the ability to reduce acetylene recovered rapidly when cultures were returned to air. Complete recovery required active protein synthesis, probably for *de novo* synthesis of nitrogenase. Respiratory O₂ consumption by cultures of *Gloeotheca* was stimulated under elevated concentrations of O₂. This respiration may contribute to the protection of nitrogenase from inactivation by O₂ at concentrations up to 0.4 atm. The role of Ca²⁺ in nitrogen fixation may be related to respiratory protection.

INTRODUCTION

Among the various organisms that fix N₂, the properties of nitrogenase (EC 1.18.2.1) are remarkably constant. For example, nitrogenase is extremely sensitive to inactivation by O₂ and it is now well-documented that N₂-fixing organisms have evolved a variety of mechanisms in order to protect this enzyme from damage by O₂ (Gallon, 1981). Cyanobacteria are unique among N₂-fixing organisms in that they evolve O₂ as a result of their photosynthesis. Accordingly, N₂-fixing cyanobacteria are able to protect nitrogenase from both photoevolved and atmospheric O₂. Precisely how this protection is achieved is not understood, but it appears to involve a combination of different mechanisms (Gallon, 1981; Gotto *et al.*, 1979).

Gloeotheca (formerly *Gloeocapsa*) is a unicellular cyanobacterium capable of aerobic N₂ fixation. It does not possess the differentiated heterocysts that characterize most aerotolerant N₂-fixing cyanobacteria (Haselkorn, 1978). In *Gloeotheca*, N₂ fixation and photosynthetic O₂ evolution coexist within a single, undifferentiated cell type. Although cultures of *Gloeotheca*, grown under an alternating cycle of light and darkness, effect an almost complete temporal separation of photosynthesis and N₂ fixation (Mullineaux *et al.*, 1981), cultures grown under constant illumination evolve O₂ and fix N₂ simultaneously. Like heterocystous cyanobacteria, *Gloeotheca* appears to use a variety of strategies in order to protect nitrogenase from O₂ damage. Among these are a superoxide-metabolizing system (Tözüm & Gallon, 1979) and an unidentified Ca²⁺-dependent mechanism (Hamadi & Gallon, 1981). However, these may well be supplemented by other systems.

In this paper, we describe a more detailed study of additional mechanisms by which the nitrogenase of *Gloeotheca* sp. ATCC 27152 might be protected from inactivation by O₂. In addition, we attempt to identify the role of Ca²⁺ in O₂ protection.

METHODS

Growth of cultures. *Gloeotheca* sp. ATCC 27152 (American Type Culture Collection, Rockville, Md., USA) was grown aerobically in 15 litres of sterile medium, free of combined nitrogen (Tözüm & Gallon, 1979). *Plectonema boryanum* UTEX594 (University of Texas Culture Collection, Austin, Texas, USA) was grown similarly, either

Abbreviation: Disulfiram, tetraethylthioperoxydicarbonic diamide.

aerobically in medium containing 2 mM- NaNO_3 , or in nitrogen-free medium sparged with N_2 containing 0.03% (v/v) CO_2 .

Acetylene reduction (N_2 fixation). During incubation of cultures of *Gloeotheca* under various gas phases, acetylene reduction was measured as described by Tözüm *et al.* (1977). The effect of transient exposure to O_2 was measured as follows. Exponentially growing cultures of *Gloeotheca* (10^5 – 10^6 cells ml^{-1}), harvested 6 to 9 d after inoculation, were concentrated five-fold by settling under gravity and 50 ml samples were incubated aerobically, with stirring, at 25 °C, in sealed 150 ml Erlenmeyer flasks. Illumination at 2.5 klx ($17 \mu\text{E m}^{-2} \text{s}^{-1}$) was provided by Osram white fluorescent lights. Acetylene was added to 0.01 atm and 0.5 ml samples of the gas phase were removed after various incubation periods for determination of ethylene by gas chromatography. After 20 min incubation, the experimental culture was bubbled with either O_2 or O_2/N_2 (1:1, v/v) for 2 min, followed by 1 min with air. Control cultures were bubbled with air for 3 min. The flasks were then resealed, acetylene added to 0.01 atm and further 0.5 ml samples of the gas phase removed at suitable intervals during the following 2 to 3 h.

Longer-term exposure to O_2 was achieved by bubbling cultures of *Gloeotheca* (2–15 litres) with O_2 or with O_2/N_2 (4:1, v/v) containing 0.03% (v/v) CO_2 . Samples (50 ml) were then removed and incubated aerobically for a suitable period after which acetylene reduction was measured as described by Tözüm *et al.* (1977).

Respiratory O_2 consumption. O_2 consumption by 3 ml samples of exponentially growing cultures of *Gloeotheca* was measured polarographically at 25 °C in the dark, in a Clark-type O_2 electrode (Rank Bros., Bottisham, Cambs., UK). The cultures used were either unconcentrated or concentrated threefold by settling under gravity. The electrode was calibrated with air-saturated water and the concentration of dissolved O_2 was altered by bubbling with O_2 or N_2 and also by illumination of cultures at 6 klx ($66 \mu\text{E m}^{-2} \text{s}^{-1}$) under a tungsten lamp, before measurement of O_2 consumption. Control incubations, with boiled cells, were included throughout and all data are corrected for O_2 exchange in these controls.

O_2 consumption by broken cell preparations of *Gloeotheca*, prepared in 50-mM-HEPES/NaOH (pH 7.5) as described by Tözüm & Gallon (1979), was also measured polarographically. The reaction mixture (3 ml) contained 32 mM-HEPES/NaOH (pH 7.5), 1 mM- MgCl_2 and 0.5 ml of a broken cell preparation of *Gloeotheca*. NADH or NADPH (5 μmol) was added to start the reaction followed, in some cases, 2 to 5 min later by addition of ADP (5 μmol) and KH_2PO_4 (5 μmol).

Intracellular Ca^{2+} . To minimize contamination by Ca^{2+} , all reagents were of the highest purity available (BDH) and were dissolved in fresh double-distilled water. All solutions were stored in polypropylene containers and polypropylene vessels were used for all experimental manipulations.

Exponentially growing cultures of *Gloeotheca* (100 ml) were harvested 6 to 8 d after inoculation and were incubated in 5 mM-HEPES/NaOH (pH 7.5) for 30 min with or without 1 mM-EDTA. The cells were collected by centrifugation at 10000 g for 10 min. They were then suspended in double-distilled water, washed twice with 0.2 mM- MgCl_2 to saturate the chelating capacity of any EDTA present in solution (Azzi & Chance, 1969), and then twice with double-distilled water and finally resuspended in 2 ml double-distilled water. Cells were broken in a French press at 138 MPa and centrifuged at 32000 g for 20 min. KCl was added to the supernatant to a final concentration of 1.35 M and, after standing for 15 min, the precipitate was removed by centrifugation at 35000 g for 20 min. The Ca^{2+} concentration of a sample (0.1 ml) of the supernatant was measured using the bioluminescent method of Blinks *et al.* (1978). Each assay contained, in a final volume of 1 ml, 5 mM-PIPES/NaOH (pH 6.5) and 0.15 M-KCl. The reaction was started by addition of 20 μg aequorin (Sigma) dissolved in 2 mM-KCl and passed through a column of Sephadex G-25 immediately before use. Total luminescence during a 10 s integration time was measured at 30 °C using a Biolumat LB 9500 photon counter (Laboratory Implex, Twickenham, Middlesex, UK). Calibration curves were obtained by taking through the extraction procedure standards containing 0.1 pmol to 1 μmol CaCl_2 , with and without 1 mM-EDTA.

Other measurements. H_2 evolution was measured as described by Bothe *et al.* (1977) using exponentially growing cultures of *Gloeotheca*, concentrated 20-fold by settling under gravity and incubated in sealed 25 ml bottles for 6 to 18 h.

Lipoxygenase (EC 1.13.11.12) was assayed polarographically as described by Wallace & Wheeler (1979). The assay mixture (3 ml) contained 50 mM-sodium phosphate buffer, pH 6.8 and 12 mM-sodium linoleate. The reaction was started by addition of 0.5 ml of a broken cell preparation of *Gloeotheca*.

The purity of cyanobacterial cultures was routinely monitored by streaking cultures onto nutrient agar or onto 1% (w/v) agar containing growth medium supplemented with 10 mM-glucose and 0.02% (w/v) Casamino acids (Rippka *et al.*, 1979).

Cell numbers were measured in a haemocytometer (Tözüm & Gallon, 1979).

RESULTS AND DISCUSSION

O₂ and acetylene reduction

Illuminated cultures of *Gloeotheca* sp. ATCC 27152, growing exponentially under aerobic conditions, exhibited maximum rates of acetylene reduction (N_2 fixation) under 0.1 atm O_2

Fig. 1

Fig. 2

Fig. 1. Effect of O_2 on acetylene reduction by *Gloeotheca*. Exponentially growing cultures (50 ml) were incubated for 60 min under N_2 containing O_2 to the concentration indicated. Acetylene reduction was then measured during the following 60 min. Incubation was either in the dark (●) or under illumination at 2.5 klx with no additions (○), with 5 mM- $CaCl_2$ (□) or with 5 mM-sodium ascorbate (△). Each value of the rate of acetylene reduction is the mean of at least eight separate observations and is expressed as a percentage of the rate in illuminated cultures with no additions, incubated under 0.2 atm O_2 .

Fig. 2. Effect of transient exposure to 1 atm O_2 on acetylene reduction by *Gloeotheca*. Cultures (50 ml, concentrated fivefold) were incubated with 0.01 atm acetylene and samples removed for measurement of acetylene reduction as indicated. At the time marked by the arrow, cultures were bubbled for 3 min with air (○, ▽), or for 2 min with 1 atm O_2 followed by 1 min with air (●, ▼). At the same time, 0.1 mg chloramphenicol ml^{-1} was added to two of the cultures (▽, ▼). Because of variations in the absolute rate of acetylene reduction, the data shown are from a single experiment. However, the effects are typical of five independent experiments.

(Fig. 1). As the concentration of O_2 was increased, the rate of acetylene reduction decreased, reaching zero above 0.7 atm O_2 . Batch cultures of *Gloeotheca* in the stationary phase of growth (10 to 14 d after inoculation) were rather more sensitive to elevated concentrations of O_2 . For example, whilst exponentially growing cultures consistently reduced acetylene 1 h after transfer to 0.6 atm O_2 (Fig. 1), acetylene reduction by stationary phase cultures was completely inhibited under these conditions (data not shown).

The rate of acetylene reduction by illuminated cultures also decreased as the concentration of O_2 in the gas phase fell below 0.1 atm (Fig. 1). It appears, therefore, that photosynthesizing cultures of *Gloeotheca* need atmospheric O_2 in order to sustain maximum rates of acetylene reduction. Possibly, some respiratory activity is needed to supply reduced ferredoxin, ATP or carbon skeletons for N_2 fixation. In this context, it may be significant that there is no unequivocal evidence that photosynthesis directly provides reductant for N_2 fixation in *Gloeotheca* (Gallon, 1980).

Maximum rates of acetylene reduction also occurred under 0.1 atm O_2 when exponentially growing cultures of *Gloeotheca* were incubated in the dark. It appears, therefore, that atmospheric O_2 is needed to sustain maximum rates of acetylene reduction in the dark, as in the light. However, because cultures of *Gloeotheca* were able to fix N_2 anaerobically in the dark (Fig. 1), O_2 is not an essential requirement for this process.

Acetylene reduction by cultures of *Gloeotheca* incubated under 0.2 to 0.6 atm O_2 was stimulated by addition of 5 mM-sodium ascorbate (Fig. 1). Sodium ascorbate also, to some extent, prevented methyl viologen from inhibiting acetylene reduction, and it may destroy toxic radicals produced by reduction of O_2 (Tözüm & Gallon, 1979). It is possible, therefore, that elevated concentrations of O_2 may, in part, inhibit nitrogenase as a result of the production of toxic oxygen radicals.

Fig. 3

Fig. 4

Fig. 3. Effect of 12 h exposure to 1 atm O_2 on acetylene reduction by *Gloeotheca*. Cultures (2 litres) were bubbled with O_2 for 12 h. Samples (50 ml) were then removed and, after aerobic incubation for the period indicated, were assayed for acetylene reduction. To these cultures was added 5 mM-HEPES/NaOH, pH 7.5 (●), along with 0.1 mg chloramphenicol ml^{-1} (▼), or 2 mM- NH_4Cl (■). Although the data shown are from a single experiment they are typical of 12 experiments involving 1 to 12 h exposure to O_2 .

Fig. 4. Effect of 14 d exposure to 0.8 atm O_2 on acetylene reduction by *Gloeotheca*. Cultures (15 litres) were bubbled with O_2/N_2 (4:1, v/v) containing 0.03% (v/v) CO_2 for 14 d, after which 50 ml samples were removed. To these was added HEPES/NaOH (5 mM), pH 7.5 (●), along with 2 mM- NH_4Cl (■). Acetylene reduction was then measured after aerobic incubation for the period indicated. The data shown are from a single experiment but are typical of six experiments involving 1 to 14 d exposure to 0.8 atm O_2 .

Illuminated cultures of *Gloeotheca*, exposed to an inhibitory concentration (1 atm) of O_2 for 2 min, resumed acetylene reduction after a lag period of about 30 min following return to air. However, within 2 h the rate of acetylene reduction in treated cultures was comparable to that in control cultures which had not been exposed to O_2 (Fig. 2). Exposure for 2 min to 0.5 atm O_2 had a similar effect although, in this case, acetylene reduction did not cease completely and recovered to its rate in control cultures within 1 h (results not shown). Recovery of acetylene reduction, following 2 min exposure to either 1 atm O_2 (Fig. 2) or 0.5 atm O_2 , was completely prevented by addition of 0.1 mg chloramphenicol ml^{-1} , implying that recovery was the result of *de novo* protein synthesis.

The length of the lag period that preceded reappearance of nitrogenase activity may be related to the length of exposure to 1 atm O_2 . For example, whilst cultures of *Gloeotheca*, exposed to 1 atm O_2 for between 2 min and 12 h recovered the ability to reduce acetylene within 30 to 60 min (Fig. 2, Fig. 3), cultures exposed to 1 atm O_2 for 24 h recommenced acetylene reduction only after a lag period of about 7 h. Following 36 h exposure to 1 atm O_2 , the lag period was 13 h and following 100 h exposure, a lag of 23 h preceded reappearance of acetylene reduction (data not shown). On the other hand, when cultures were exposed to 0.8 atm O_2 for between 24 h and 14 d, the ability to reduce acetylene consistently returned within 1 h of transfer to air (Fig. 4). The pattern of recovery of nitrogenase activity following exposure of *Gloeotheca* to elevated concentrations of O_2 therefore appears to vary with respect to the concentration of O_2 used, as well as with respect to the length of exposure to O_2 .

Nitrogenase synthesis appears to continue for up to 4 h in cultures of *Gloeotheca* incubated under 1 atm O_2 (Mullineaux *et al.*, 1983). This is in contrast to the repressive effect of O_2 in some other N_2 -fixing organisms (Eady, 1981). The relatively short lag period before nitrogenase activity reappears following up to 12 h exposure to 1 atm O_2 or up to 14 d exposure to 0.8 atm O_2 suggests that synthesis of the enzyme might continue for periods longer than 4 h under elevated

concentrations of O_2 . In contrast to the effect of O_2 , 12 h exposure of immobilized cultures of *Gloeotheca* to 0.2 mM- NH_4Cl , which represses nitrogenase synthesis, resulted in no reappearance of nitrogenase activity for 7 to 12 h after removal of NH_4Cl (Gallon *et al.*, 1984). However, nitrogenase synthesis may not continue indefinitely under 1 atm O_2 because a lag period of several hours preceded reappearance of nitrogenase activity following 24 to 100 h exposure to O_2 . Possibly, the carbon and/or nitrogen reserves of the cell became depleted during prolonged incubation under 1 atm O_2 , thereby inhibiting nitrogenase synthesis.

Although *Gloeotheca* may continue to synthesize nitrogenase during exposure to elevated concentrations of O_2 , acetylene reduction was not observed at any time during 100 h exposure to 1 atm O_2 or during 14 d exposure to O_2/N_2 (4:1, v/v) containing 0.03% (v/v) CO_2 . In the absence of N_2 -fixation, these cultures would become nitrogen-deficient during exposure to either 1 atm or 0.8 atm O_2 . In view of this, it is more likely that depletion of cellular carbon, rather than cellular nitrogen, interrupted nitrogenase synthesis during 24 to 100 h exposure to 1 atm O_2 .

Pienkos *et al.* (1983) have reported that cultures of *Anabaena* sp. CA, a heterocystous cyanobacterium, recover the ability to reduce acetylene within 30 to 60 min whilst still exposed to 1 atm O_2 . In this organism, as in *Gloeotheca*, nitrogenase synthesis apparently continues under elevated concentrations of O_2 , although the two organisms differ in their ability to maintain an active nitrogenase under these conditions. However, in both *Gloeotheca* (Fig. 2, Fig. 3) and *Anabaena* CA (Pienkos *et al.*, 1983) brief exposure to O_2 , followed by return to air, results in only a short lag period before acetylene reduction reappears. In contrast, in *Anabaena flos-aquae*, recovery of nitrogenase activity was not complete until 8 to 10 h after exposure for 10 min to 1 atm O_2 (Bone, 1971). In some species of cyanobacteria, therefore, transient exposure to O_2 may repress nitrogenase synthesis.

Addition of either 0.1 mg chloramphenicol ml^{-1} or 2 mM- NH_4Cl completely abolished recovery of nitrogenase activity following transfer to air of cultures of *Gloeotheca*, previously exposed to 1 atm O_2 for 12 h (Fig. 3). The involvement of protein synthesis in this recovery may therefore be for *de novo* synthesis of nitrogenase itself. However, although addition of NH_4Cl to cultures exposed to 0.8 atm O_2 for 14 d markedly decreased the recovery of acetylene reduction, it did not prevent it completely (Fig. 4). In this latter case, as in *Anabaena* CA (Pienkos *et al.*, 1983), it is possible that only part of the nitrogenase activity that reappears is the result of *de novo* synthesis of the enzyme. Some of the inactivation of nitrogenase by 0.8 atm O_2 may therefore be freely reversible.

The N_2 -fixing aerobe *Azotobacter* exhibits a reversible conformational protection of nitrogenase during transient exposure to elevated concentrations of O_2 (Robson & Postgate, 1980). A similar mechanism may exist in *Gloeotheca* but there is little evidence for this (Hamadi & Gallon, 1981). On the other hand, in cultures of *Gloeotheca* exposed to 1 atm O_2 for 12 h, and in cultures in which O_2 inactivation of nitrogenase had been caused by addition of 1 mM-EDTA (Hamadi & Gallon, 1981; Gallon, 1980), reappearance of nitrogenase activity may be entirely a consequence of synthesis of new enzyme. In these cases, inactivation of nitrogenase by O_2 may have been irreversible.

It appears, therefore, that in *Gloeotheca* synthesis of nitrogenase continues for several hours under 1 atm O_2 and for several days under 0.8 atm O_2 . Nevertheless, the enzyme is rapidly and, largely, irreversibly inactivated by exposure to O_2 at these concentrations. Although continued synthesis of nitrogenase during O_2 stress appears wasteful it may be advantageous in allowing rapid recovery of nitrogenase activity following return to less extreme conditions.

Plectonema boryanum is a non-heterocystous, filamentous cyanobacterium capable of N_2 fixation under microaerobic, but not aerobic, conditions. However, Wang *et al.* (1982) have reported that, when incubated with extracts of *Gloeotheca*, cultures of *P. boryanum* can fix N_2 aerobically. Extracts of *Gloeotheca* contain ascorbate and other reducing agents (Tözüm & Gallon, 1979) and, in view of its stimulation of acetylene reduction in *Gloeotheca* (Fig. 1), it is possible that ascorbate is responsible for the effect on *P. boryanum* of extracts of *Gloeotheca*. In support of this, addition of 5 mM-sodium ascorbate to cultures of *P. boryanum* UTEX 594 resulted in low, but consistent, rates of acetylene reduction during aerobic incubation. However,

Fig. 5. Effect of O_2 concentration on O_2 uptake by cultures of *Gloeotheca*. Both the rate of O_2 uptake and the prevailing concentration of O_2 were measured polarographically in 3 ml samples of *Gloeotheca* either with no additions (○) or in the presence of 1 mM-KCN (Δ). Each point represents an individual measurement, but the lines are drawn with reference to additional points which, for reasons of clarity, are not shown here. A concentration of O_2 in the gas phase of 0.8 atm was equivalent to a dissolved O_2 concentration of $1.03 \mu\text{mol ml}^{-1}$.

addition of sodium ascorbate did not allow aerobic growth of *P. boryanum* in nitrogen-free medium.

Respiration in *Gloeotheca*

The rate of O_2 consumption by cultures of *Gloeotheca*, incubated in the dark, increased as the dissolved O_2 concentration increased (Fig. 5). In this respect, *Gloeotheca* resembles *Azotobacter*, where respiratory O_2 consumption increased by a factor of five when the concentration of O_2 was increased from 0.05 to 0.55 atm (Dalton, 1980). In *Gloeotheca* the equivalent increase was 2.7-fold (Fig. 5). In *Azotobacter* respiration may function as an O_2 -scavenging mechanism which thereby protects nitrogenase from O_2 (Yates & Eady, 1980). Natural populations of *Gloeotheca* probably fix most of their N_2 in the dark (Mullineaux *et al.*, 1981), and under these conditions respiration may have a major role in protecting nitrogenase from inactivation by O_2 .

The greatest stimulation of respiratory O_2 consumption in response to increasing concentrations of O_2 took place between 0 and 0.4 atm O_2 (Fig. 5). Between 0.4 and 0.8 atm O_2 , little further stimulation occurred and it is unlikely that respiratory O_2 consumption would be very effective in protecting *Gloeotheca* nitrogenase above 0.4 atm O_2 . Consistent with this, exposure of *Gloeotheca* to more than 0.7 atm O_2 led rapidly to complete cessation of acetylene reduction (Figs 1–4). The stimulatory effect of O_2 on respiratory O_2 consumption (Fig. 5) was measured within 15 min following exposure of cultures to the new concentration of O_2 . It is not known for how long these rates of respiration would be maintained, but it is unlikely to be for more than 1 h. In support of this, preliminary data suggest that cultures of *Gloeotheca* incubated in the dark for 1 h under 1 atm O_2 exhibit rates of CO_2 evolution and O_2 consumption markedly lower than those in control cultures. It cannot therefore be assumed that respiratory O_2 consumption could protect nitrogenase during prolonged exposure of cultures of *Gloeotheca* to O_2 .

In *Azotobacter*, the respiratory chain is branched (Haddock & Jones, 1977). The different branches have different sensitivities to various inhibitors, including CN^- (Jones & Redfearn, 1967). As a result of this branched electron transport chain, at high concentrations of O_2 respiration in *Azotobacter* is poorly coupled to ATP synthesis and apparently functions primarily to consume O_2 .

Addition of 0.1 mM- or 1 mM-KCN (Fig. 5) to cultures of *Gloeotheca* partially inhibited respiratory O_2 consumption. However, there was always residual O_2 uptake of about 35% of the

Table 1. *Effect of various additions on O₂ uptake by cultures of Gloeotheca*

O₂ uptake was measured polarographically in air using cultures (3 ml) concentrated threefold under gravity. All incubations also contained 5 mM-HEPES/NaOH, pH 7.5. The rates of oxygen consumption are typical.

Addition	O ₂ consumption [nmol min ⁻¹ (10 ⁶ cells) ⁻¹]
None	1.00
0.1 mM-KCN	0.37
1 mM-KCN	0.32
1 mM-Salicylhydroxamate	1.00
15 µM-Disulfiram	1.00
1 mM-EDTA	0.60
5 mM-CaCl ₂	1.04
5 mM-MgCl ₂	0.81
1 mM-EDTA + 5 mM-CaCl ₂	0.91
1 mM-EDTA + 5 mM-MgCl ₂	0.63
1 mM-EDTA + 1 mM-KCN	0.12

uninhibited rate (Table 1). In higher plants, CN⁻-insensitive respiration has been equated with a branched electron transport chain (Lance, 1981), although recently this has been questioned on the grounds that lipoygenase (EC 1.13.11.12) activity could explain CN⁻-resistant O₂ uptake (Kelly, 1982). However, unlike CN⁻-resistant respiration in the mitochondria of higher plants, O₂ uptake in *Gloeotheca* was not inhibited by either salicylhydroxamate, an inhibitor of lipoygenase (Parish & Leopold, 1978), or disulfiram (Table 1). Furthermore, no lipoygenase activity has been detected in extracts of *Gloeotheca*.

The rate of CN⁻-resistant respiration in cultures of *Gloeotheca* increased with increasing O₂ concentration (Fig. 5). Between 0.1 atm and 0.2 atm O₂, the increase in the rate of CN⁻-resistant respiration could entirely account for the increase in respiration observed in the absence of CN⁻. However, above 0.2 atm O₂, although CN⁻-resistant respiration continued to increase, the increase was insufficient to explain the increase in O₂ consumption by uninhibited cultures of *Gloeotheca*. Presumably, CN⁻-sensitive respiration was also stimulated over this range of O₂ concentrations.

It is therefore possible that *Gloeotheca*, like *Azotobacter*, possesses a branched electron transport chain and that electron flow down a CN⁻-resistant branch is mainly, though not exclusively, stimulated under elevated concentrations of O₂. By analogy with *Azotobacter*, this CN⁻-resistant branch may be poorly coupled to ATP synthesis and may function mainly to consume O₂. Cultures of *Gloeotheca* may, therefore, exhibit respiratory protection of nitrogenase.

In some species of cyanobacteria, H₂ donates electrons via an 'uptake' hydrogenase to an electron transport chain with O₂ as terminal acceptor (Bothe *et al.*, 1980). The resulting O₂ consumption may contribute to the protection of nitrogenase from damage by O₂, particularly under conditions of carbon deficiency. Cultures of *Gloeotheca* evolved little, if any, H₂ either in the light or, aerobically or anaerobically, in the dark. However, in the presence of 12.4 mM-C₂H₂ and 0.124 mM-CO (Bothe *et al.*, 1977), H₂ evolution could be measured at a rate of 24.6 pmol h⁻¹ (10⁶ cells)⁻¹ in the light and of 4.8 pmol h⁻¹ (10⁶ cells)⁻¹ in the dark. This suggests that *Gloeotheca* possesses an 'uptake' hydrogenase and may, therefore, recycle H₂ evolved by the action of nitrogenase, possibly in an O₂-consuming system. However, there is no direct evidence that H₂ has a role in protecting *Gloeotheca* nitrogenase from inactivation by O₂ (Tözüm & Gallon, 1979).

Ca²⁺ and acetylene reduction

Addition of EDTA to cultures of *Gloeotheca* inhibits acetylene reduction by disruption of a Ca²⁺-dependent process by which nitrogenase is protected from O₂ (Hamadi & Gallon, 1981). EDTA acts by depleting the cyanobacterial cells of Ca²⁺. The intracellular concentration of

Ca^{2+} in cultures of *Gloeotheca* was 62 pmol (10^6 cells) $^{-1}$ under normal growth conditions (equivalent, approximately, to 0.6 mM), but fell to 12 pmol (10^6 cells) $^{-1}$ following addition of 1 mM-EDTA.

Addition of 5 mM- CaCl_2 to N_2 -fixing cultures decreased the inhibitory effect on acetylene reduction of 0.2 to 0.5 atm O_2 , but was without effect at O_2 concentrations outside these limits (Fig. 1).

EDTA (1 mM) inhibited respiratory O_2 uptake (Table 1). This inhibition was prevented by simultaneous addition of 5 mM- CaCl_2 but not of 5 mM- MgCl_2 . In this respect, the effect of EDTA on respiration resembles its effect on acetylene reduction (Gallon, 1978). However, EDTA did not permanently affect the intracellular concentration of ATP (Hamadi & Gallon, 1981). It is possible, therefore, that EDTA inhibits a relatively uncoupled respiratory O_2 uptake. In support of this, the effects of EDTA and CN^- appear to be additive (Table 1).

In broken cell preparations of *Gloeotheca*, addition of 5 mM- CaCl_2 stimulated by 50% both NADH- and NADPH-dependent O_2 uptake. Unstimulated rates were 10.9 and 14.8 nmol min^{-1} (mg protein) $^{-1}$ respectively. Stimulation was observed whether or not ADP and inorganic phosphate were also present. Furthermore, although oligomycin, an inhibitor of oxidative phosphorylation, at a concentration of 10 $\mu\text{g ml}^{-1}$, decreased the rate of NADH- and NADPH-dependent O_2 consumption to about 45% of their uninhibited rate, this residual O_2 uptake was still stimulated by addition of 5 mM- CaCl_2 . The effect of CaCl_2 on respiratory O_2 consumption does not, therefore, depend upon simultaneous ATP synthesis and may be the result of stimulation of uncoupled electron flow.

CaCl_2 (5 mM) did not greatly affect the recovery of nitrogenase activity following treatment of cultures of *Gloeotheca* with 0.8 atm O_2 for 2 to 7 d. It is unlikely, therefore, that Ca^{2+} ions are specifically involved in this recovery.

In conclusion, therefore, the role of Ca^{2+} in N_2 fixation by *Gloeotheca* may be linked to respiratory protection of nitrogenase from O_2 . The mechanism by which Ca^{2+} ions affect respiration is not clear but may be related to membrane integrity. In support of this, Ca^{2+} prevented surface-active compounds from inhibiting acetylene reduction by *Gloeotheca* (Hamadi & Gallon, 1981).

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