Adaptation of the cyanobacterium *Anabaena cylindrica* to high oxygen tensions

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The cyanobacterium *Anabaena cylindrica* can adapt to protect its nitrogenase activity against greater than atmospheric (up to 100%) oxygen tension. The adaptation is light-dependent and involves induction of superoxide dismutase and catalase. After adaptation, the cells are resistant to photooxidation as measured by phycocyanin release and microscopic examination.

Cyanobacterium *Nitrogenase* *Oxygen* *Catalase* *Superoxide dismutase* *Photooxidation*

1. INTRODUCTION

Cyanobacteria (blue–green algae) are frequently observed to dominate the algal population of the surface waters of nutrient-rich lakes and ponds, and this has been attributed to their ability to fix atmospheric nitrogen [1]. However, in such habitats they are subject to potentially damaging photooxidative conditions due to high irradiation and oxygen levels. Oxygen tensions in a cyanobacterial bloom may considerably exceed those in air when photosynthetic activity is high [2], and hence cells must have protective mechanisms that can cope with enhanced oxygen tensions if they are to avoid photooxidative death. The oxygen sensitivity of nitrogenase poses an additional problem, since although it is protected at aerobic oxygen tensions by the heterocysts of heterocystous cyanobacteria [3], the protective mechanisms can be overcome at enhanced tensions [4,5].

Although the precise mechanism by which oxygen inhibits nitrogenase is not known, general photooxidative damage and death can now be attributed, at least in part, to the formation of the reduced oxygen species superoxide, hydrogen peroxide and hydroxyl radicals at a rate which overloads the protective machinery of cells [6]. Hence photooxidative protection mechanisms must include means for removing these species. Cyanobacteria vary considerably in their sensitivity to photooxidation [7] and hence in the efficiency of their protective mechanisms. Here, we report that a strain of *Anabaena cylindrica* shows considerable adaptive capacity to oxygen tensions higher than atmospheric, in terms of its nitrogenase stability, and that it is associated with significantly enhanced superoxide dismutase and catalase levels.

2. MATERIALS AND METHODS

2.1. *Algae and their growth*

Anabaena cylindrica (ATCC 29414) was obtained from the American Type Culture Collection and grown as in [8], but with the growth medium at 1/8 strength with respect to all components except phosphate. Cells were harvested by centrifugation (6000 × *g*, 10 min) at ~100 Klett units as measured by a Klett-Summerson colourimeter, where 100 Klett units are equivalent to 3.3 mg dry wt/10 ml [9].

For experiments in which cells were grown or sparged with gas mixtures other than air/0.3% CO_2, the mixtures were made by passing the

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respective gases through flowmeters (Fischer and Porter) at defined rates. For all growth and preincubation conditions, a light intensity of 7000 lux (fluorescent daylight tubes) was used.

2.2. *Nitrogenase assays*

Nitrogenase activity was measured by the reduction of acetylene to ethylene, which was determined gas chromatographically as in [5]. Cyanobacteria were at ~300 Klett units for these assays. Gas mixtures were created by evacuating the assay tubes via a manifold system connected to a vacuum pump, a manometer and to cylinders of the various gases. The mixtures were then made by restoring the gas pressure to atmospheric by adding each gas to its particular partial pressure. Rates of acetylene reduction are expressed as initial velocities measured from 4–6 samples taken over a period of 2–6 h.

2.3. *Dry weight determinations*

All results are expressed in terms of dry weight of cyanobacteria, determined by centrifuging duplicate samples (10 ml), washing once with water and then drying overnight at 85°C.

2.4. *Catalase and superoxide dismutase assays*

Catalase was assayed in a Clark-type oxygen electrode (Rank Bros., Bottisham). Cells or cell extracts (in 50 mM phosphate buffer, pH 7.0) were made 30 μM with respect to dichlorophenyldimethyl urea (DCMU) to inhibit photosynthetic O_2 formation, and added to the electrode chamber. A stream of argon (Ar) gas was bubbled through the solution until at least 99% of the dissolved oxygen had been displaced, and after equilibration for a few minutes hydrogen peroxide (100 μl), in the same buffer and also pregassed with Ar, was added to give 33.5 mM final conc. The chamber was then rapidly stoppered to minimize diffusion of air. The initial velocity was measured and corrected for non-enzymic H_2O_2 decomposition. Initial experiments showed that rates for whole cells and cell extracts were the same.

Superoxide dismutase was measured on cell extracts produced by passage through a French pressure cell at 20000 lb/in^2 and then cleared of cell debris by centrifugation (7000 × g, 4°C, 10 min). The cells were initially suspended in 0.05 M phosphate buffer, containing 0.1 mM Na_2-EDTA (pH 7.8). Activity was measured as inhibition of ferricytochrome c reduction by superoxide radicals generated by xanthine/xanthine oxidase [10]. One unit of activity is defined as the amount required to inhibit the rate of reduction of cytochrome c by 50% [10]. Protein was determined by the Lowry method [11].

2.5. *Chemicals and gases*

Xanthine oxidase (cow milk) was obtained from Boehringer. All gases were of highest purity available from Commonwealth Industrial Gases (Canberra ACT).

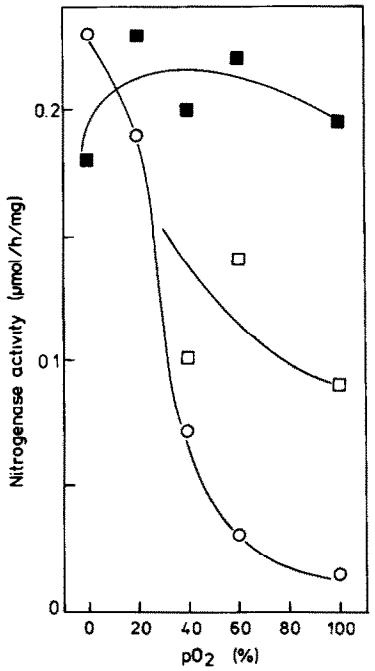

Fig.1. The effect of different partial pressures of oxygen on nitrogenase activity both with (squares) and without (circles) a 16 h preincubation at the indicated oxygen level. In one set of experiments (○) cells were grown in the usual way (in air/0.3% CO_2) and were then assayed in argon supplemented with oxygen. In the second set of experiments cells were grown as normal, then preincubated for 16 h whilst sparging with N_2 supplemented with 0.3% CO_2 and O_2 as indicated; nitrogenase was then assayed under argon supplemented with the same oxygen tension as used in the preincubation (□) or in pure argon (■). All results are expressed on the basis of cyanobacterial dry weight and rates were calculated from initial velocities either at time zero or after a short lag when this occurred.

3. RESULTS

3.1. *Adaptation of nitrogenase to elevated oxygen tensions*

When cells are grown normally and then assayed for nitrogenase activity at different oxygen tensions a progressive decrease in activity is observed as the oxygen tension is increased (fig.1). Interestingly, when cells were grown and then sparged for 16 h with 100% N_2 the nitrogenase activity was significantly reduced by even aerobic levels of O_2 (20%), when compared with cells treated similarly but assayed in the absence of O_2 (fig.2). This suggests that after the 16 h period in the absence of O_2 the cells are less well equipped to protect their nitrogenase from oxygen.

This result prompted a series of experiments in which, following normal growth, the cells were subjected to 16 h periods of exposure to oxygen tensions from 20–100%, and then assayed for nitrogenase activity at the same O_2 tension. It is seen (fig.1) that the nitrogenase had substantially recovered from its initial inactivation, indicating that the cells had adapted to the elevated O_2 tensions. When such cells were assayed in an anaerobic environment (Ar) their activity was seen to be virtually no different from control values (fig.1).

The effect of light on the adaptation process was determined by preincubating cells for 16 h in a gas phase of N_2/40% O_2/0.3% CO_2 in the dark and then assaying in the presence and absence of 40% O_2 in argon. The results (fig.3) showed that the elevated O_2 tension had substantially damaged the capacity of the nitrogenase to recover in the presence of O_2 whereas a steady recovery was observed in the initially anaerobic environment. This suggests that light is required for the development of protective mechanisms for the nitrogenase.

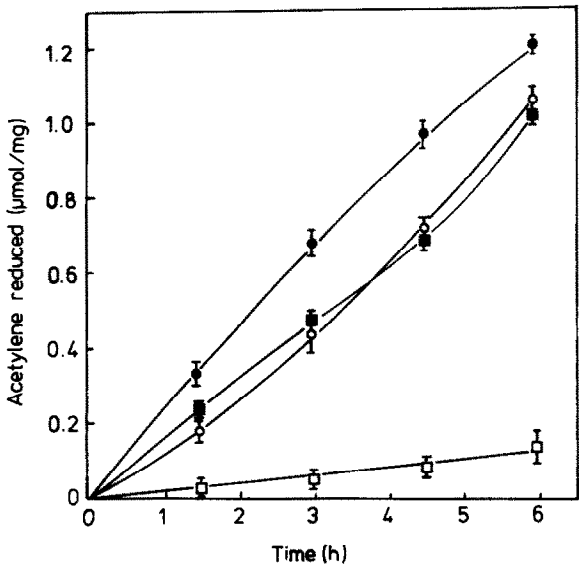

Fig.2. The effect of oxygen on nitrogenase activity following a 16 h anaerobic preincubation in N_2. Cells were grown as usual, preincubated in N_2 and then assayed in either pure argon (■) or argon supplemented with 20% O_2 (□). These results are compared with controls in which the cells were grown in air/0.3% CO_2 and then directly assayed in either argon (●) or Ar/20% O_2 (○). Results are expressed on a dry weight basis.

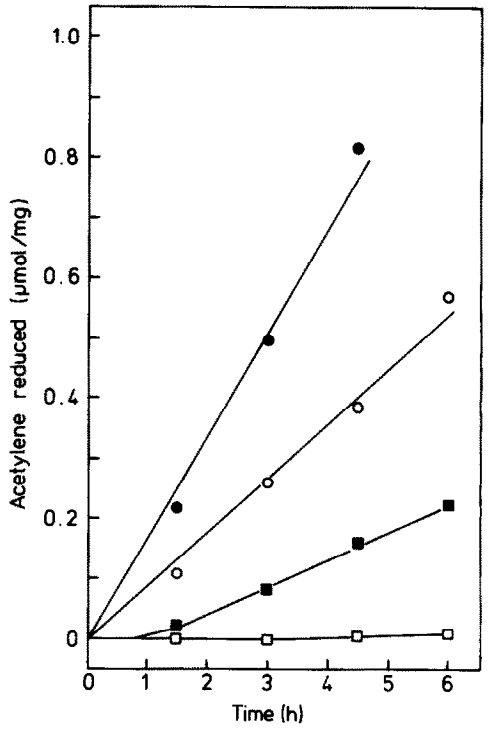

Fig.3. The effect of a dark preincubation on the subsequent sensitivity of the nitrogenase to 40% O_2. Cells were preincubated for 16 h in N_2/40% O_2/0.3% CO_2 in the dark and then assayed under pure argon (■) or argon/40% O_2 (□). These are compared to cells grown continuously in N_2/40% O_2/0.3% CO_2 in the light and then assayed in pure argon (●) or Ar/40% O_2 (○). Results are expressed on a dry weight basis.

3.2. *Induction of superoxide dismutase and catalase*

Cells were subjected to 16 h preincubations in the elevated oxygen tensions used for the nitrogenase assays (in the light) and then assayed for superoxide dismutase and catalase. The activities of the enzymes showed that catalase was induced to a level proportional to the oxygen tension in the preincubation whereas superoxide was elevated at all tensions except 100% oxygen (fig.4). Identical experiments were performed with preincubations being done in the dark. The results (not shown) revealed that no stimulation of either enzyme occurred in the dark.

To test the time course of the increase in catalase activity and also to check that it represented protein synthesis, we assayed the enzyme regularly from cells incubated under N_2/40% O_2/0.3% CO_2 in the presence and absence of chloramphenicol. The induction was abolished by chloramphenicol, but in its absence the enzyme induction occurred within 2 h.

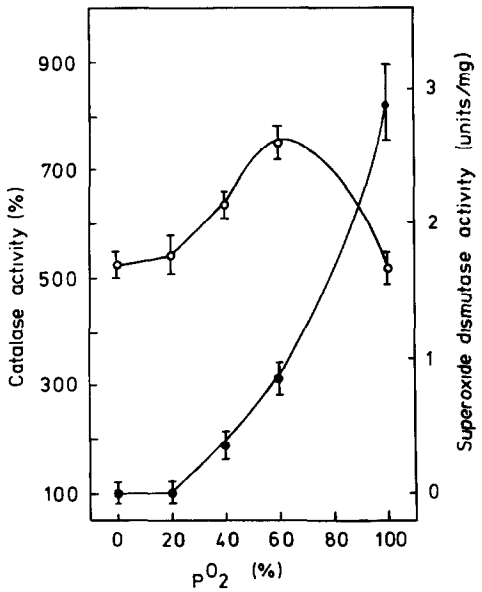

Fig.4. Superoxide dismutase and catalase activities after preincubation of cells for 16 h in N_2 supplemented with 0.3% CO_2 and the indicated O_2 levels. Superoxide dismutase activity (○) is expressed in terms of mg protein in the extract and catalase (●) in terms of the control level (cells grown in air/0.3% CO_2). This varied from one batch of cells to another and hence results are expressed in terms of the rate measured on a given day. This was 100–150 μmol $O_2 . min^{-1}$ mg dry wt $cells^{-1}$.

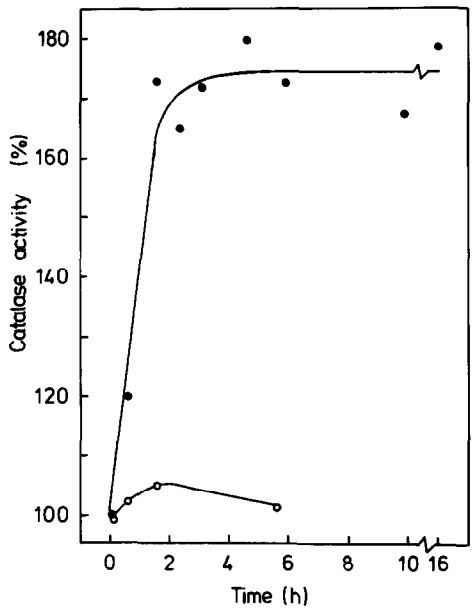

Fig.5. The time course of induction of catalase activity. Cells were grown in air/0.3% CO_2 and then incubated under a gas phase of N_2/40% O_2/0.3% CO_2 either with (○) or without (●) chloramphenicol (20 μg/ml) added to the solution. Catalase was assayed at the indicated times. The base level of catalase (100%) was shown to vary from one batch of cells to another but was always 100–150 μmol $O_2\ min^{-1} . mg$ dry wt $cells^{-1}$.

4. DISCUSSION

A major conclusion of this work is that although it has been known for some time that the heterocysts of filamentous cyanobacteria have properties which protect nitrogenase from oxygen inhibition [3] *A. cylindrica* has additional protective mechanisms which are induced by exposure to elevated oxygen tensions. The nitrogenase can regain activity even after exposure to 100% oxygen (fig.1).

Although the precise nature of the protective mechanism remains to be elucidated it seems that its induction is oxygen-dependent (fig.2), light-dependent (fig.3) and involves induction of superoxide dismutase and catalase (fig.4,5). Therefore, it may be postulated that the Mehler reactions of photosynthesis are enhanced by the elevated oxygen tension [6], and possibly also is photorespiration; although there has been controversy concerning the significance of photo-

respiratory reactions in cyanobacteria [13,14] they are more likely to occur at elevated oxygen tensions [15]. Both pathways produce H_2O_2 and would be consistent with elevated catalase levels. The enhanced superoxide dismutase activity is indicative of enhanced O_2 consumption by the Mehler pathway but unfortunately the transitory nature of the superoxide radical makes measurement of the maximum velocity of this enzyme, and hence of the steady state level of each step in the pathway, difficult. Interestingly, superoxide dismutase has also been shown to be an inducible enzyme in *Anacystis nidulans* [16]. In that organism the enzyme was elevated by increased oxygen partial pressures but was greatly depressed at 100% O_2, ultimately resulting in cell death; the enzyme was undetectable in the absence of O_2. In *A. cylindrica*, by contrast, the enzyme seems to exist at its aerobic level both in the absence of O_2 and at 100% O_2 (fig.4). In *A. nidulans* H_2O_2 accumulation itself is observed under certain conditions but this is not the case with *A. cylindrica* [17], suggesting that the species vary considerably in their ability to remove this substance.

From this work it is not possible to conclude that there is a causal relationship between the production of activated oxygen species and nitrogenase inhibition. It may be that nitrogenase is not inactivated directly by these species so much as by general photooxidative damage which could affect the energetics, for example, of nitrogenase function. In experiments (not reported) designed to assess resistance of the organism to elevated oxygen tension, we found that cells adapted to 100% oxygen under the conditions reported herein were subsequently much less susceptible than a control culture grown in air/0.3% CO_2 to photooxidative damage; photooxidation was induced by bubbling the cells with 100% O_2 at high light intensity (50000 lux) and measured as phycocyanin released from the cells or by microscopic detection of filament degradation and cell disruption.

In conclusion, it is clear that cyanobacteria vary considerably in their capacity to protect themselves from photooxidative damage and some strains have considerable adaptive capacity to meet changing degrees of photooxidative stress. Measurements of the type reported herein may prove useful in developing monitoring criteria for photooxidative susceptibility and in understanding the dynamics of mixed algal populations.

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