Nitrogen Fixation in Cultures of the Cyanobacterium *Gloeocapsa* (*Gloeotheca*) sp. 1430/3 Incubated in the Dark

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Gloeocapsa sp. 1430/3 fixed N_2 in the dark, but at a lower rate than in the light. Following the transfer of exponentially growing cultures to the dark, the rate of N_2 fixation increased for between 2 and 5 h and then decreased, becoming negligible after 12 h. No substantial increase in activity occurred for about 10 h following re-illumination. It is suggested that the decrease in nitrogenase activity which occurred about 5 h after transfer to the dark was not caused by exhaustion of carbon reserves but by a cessation of nitrogenase synthesis coupled with an irreversible inactivation of the enzyme, probably by O_2 . Subsequent recovery of activity apparently depended upon resynthesis of nitrogenase.

INTRODUCTION

After 4 h incubation in the dark, cultures of the unicellular cyanobacterium *Gloeocapsa* sp. LB795 fixed N_2 at approximately 40% of the rate in the light (Gallon *et al.*, 1973). In this respect, *Gloeocapsa* differs from the heterocystous cyanobacteria *Anabaena cylindrica* and *Anabaenopsis circularis* in which nitrogenase activity declined exponentially almost to zero during incubation for 4 h in the dark (Fay, 1976; Bottomley & Stewart, 1977).

In both *Chlorogloea fritschii* (Fay, 1965) and *Anabaenopsis circularis* (Watanabe & Yamamoto, 1967; Bottomley & Stewart, 1977), N_2 fixation in the dark could be maintained by the addition of a suitable exogenous carbon source, suggesting that in these species the limitation of carbon supply probably determines both the rate and duration of nitrogenase activity. During the first minute after transition from light to dark, the rate of N_2 fixation by cultures of *Gloeocapsa* sp. 1430/3 dropped to 40% of that in the light and this lower rate was maintained for up to 5 min (Mullineaux *et al.*, 1980) and probably for much longer (Gallon *et al.*, 1973). However, Mullineaux *et al.* (1980) concluded that limitations in neither the supply of ATP nor the availability of storage glucan could explain this lower rate of N_2 fixation.

The object of the work reported here was to study the ability of *Gloeocapsa* sp. 1430/3 to fix N_2 in the dark and also to investigate some of the factors which might influence this process.

METHODS

Growth of cultures. *Gloeocapsa* (*Gloeotheca*) sp. CCAP 1430/3 (Culture Centre of Algae and Protozoa, Cambridge) was grown in 15 l of sterile medium free of combined nitrogen (Gallon *et al.*, 1978) and harvested during the period of exponential growth, 6 to 8 d after inoculation.

Acetylene reduction (N_2 fixation). This was measured as described by Tözüm *et al.* (1977) except that cultures were assayed immediately after transfer to 150 ml Erlenmeyer flasks and acetylene was added to a concentration of 10% (v/v). Where indicated, the incubation medium contained chloramphenicol (0.1 mg ml^{-1}) or NH_4Cl (2 mM).

Acetylene reduction under anaerobic conditions was measured after bubbling the cultures with N_2 for 2 to 5 min prior to sealing the incubation vessels. That anaerobic conditions were maintained during the incubation period was demonstrated by including in these vessels a 1.5 ml centrifuge tube containing 1 ml of decolorized methylene blue (50 mg l^{-1}) in 2 mM-NaOH.

Estimation of glucan and protein. Cultures were incubated in the light in an atmosphere containing $^{14}\text{CO}_2$ for 14 h as described by Mullineaux *et al.* (1980). The light source was then removed for 10 h after which the cultures were re-illuminated for a further 4 h. Methanol extracts were prepared and examined for glucan or protein as described by Mullineaux *et al.* (1980). Protein synthesis was also measured in methanol extracts from a culture of *Gloeocapsa* (50 ml) which had been concentrated 10-fold and incubated with $1.85 \text{ MBq [U-}^{14}\text{C]protein hydrolysate [1.85 GBq (mmol C)}^{-1}\text{]}$ under the above conditions.

O_2 consumption/evolution. This was measured polarographically at 25°C (Mullineaux *et al.*, 1980).

Measurement of the cell density of cultures. This was performed as described by Tözüm & Gallon (1979).

RESULTS AND DISCUSSION

Gloeocapsa sp. 1430/3 reduced acetylene after 4 h in the dark, though at a lower rate than in the light (Fig. 1). This confirms the results of Gallon *et al.* (1973) with the non-axenic strain, *Gloeocapsa* sp. LB795. In cultures harvested during the exponential phase of growth (6 to 8 d after inoculation), the rate of acetylene reduction steadily increased during incubation for up to 7 h in the light (Fig. 1). The rate in cultures transferred to darkness also increased over a period which varied between 2 and 5 h, but it subsequently steadily decreased (Fig. 1). Occasionally, cultures were harvested during a phase in which acetylene reduction activity in the light was decreasing; the rate of acetylene reduction also decreased when such cultures were transferred to the dark. In all cases, the rate of acetylene reduction during the first 2 to 5 h of darkness was about 40% of the rate in the light. Addition of either chloramphenicol, which inhibits protein synthesis in prokaryotes, or NH_4^+ , an inhibitor of nitrogenase synthesis, prevented any increase in nitrogenase activity in both light and dark, suggesting that the increases were probably the result of a net synthesis of nitrogenase.

There were therefore three distinct phases in the response of N_2 fixation in cultures of *Gloeocapsa* sp. 1430/3 to darkness. During the first minute the rate of N_2 fixation fell sharply below the rate in an illuminated control culture (Mullineaux *et al.*, 1980), probably as a result of limitation of ATP and/or reductant. For up to 5 h after the start of the dark period, the rate of N_2 fixation by cultures harvested in the exponential phase of growth increased, although the rate was always lower than in the light. Subsequently the rate of N_2 fixation decreased.

In the light $^{14}\text{CO}_2$ was incorporated into storage glucan (Fig. 2*a*), but following transfer to the dark there was a net loss of radioactivity from the previously labelled storage glucan. Upon re-illumination of the culture, the incorporation of $^{14}\text{CO}_2$ recommenced immediately, at a rate slightly greater than that during the previous light period. These data suggest that storage glucan is synthesized in the light and broken down in the dark in order to provide the energy requirements of the organism. However, this pattern of synthesis and breakdown did not correlate with the pattern of N_2 fixation (Fig. 2*b*). Whilst the breakdown of storage glucan began immediately illumination was discontinued, nitrogenase activity did not start to decline until 4 h later. At the end of the dark period, the rate of acetylene reduction had fallen almost to zero even though the glucan pool was not completely exhausted. Furthermore, when cultures were re-illuminated, storage glucan synthesis started immediately but there was only a small increase in the rate of N_2 fixation. Thus it appears that the decrease in the rate of N_2 fixation which occurred after 5 h in the dark was not simply a result of a lack of storage glucan as a source of energy and reducing power.

O_2 evolution ceased immediately the cultures were transferred to the dark, but it recommenced as soon as they were re-illuminated (Fig. 2*c*). Thus the inability of *Gloeocapsa* to fix N_2 in the light after a period in the dark cannot be caused by the deprivation of ATP, and/or reducing power, derived directly from photosynthesis. After 12 h in the dark, there was no sustained nitrogenase activity for 10 h after re-illumination even though photosynthesis and glucan synthesis were proceeding normally.

Fig. 1. Effect of transfer from light to dark (at zero time) on acetylene reduction by *Gloeocapsa* sp. 1430/3 (●). The control culture (○) was maintained in the light. The data shown here are from a single experiment but are typical. In a number of such experiments, the absolute rate of acetylene reduction varied considerably. However, the rate of acetylene reduction was always lower in the dark than in the light.

Fig. 2. Comparison of (a) ^{14}C in storage glucan, (b) acetylene reduction, (c) O_2 exchange, and (d) ^{14}C in protein in a culture of *Gloeocapsa* sp. 1430/3 subjected to a light-dark-light regime. The black areas indicate the periods of darkness. In (c), a positive value indicates O_2 evolution and a negative value indicates O_2 uptake. The data shown are from a single experiment but are typical.

Measurements of the incorporation of $^{14}\text{CO}_2$ into protein during a light-dark-light regime (Fig. 2*d*) indicated that the rate of protein synthesis was lowered in the dark, to about 15 % of that in the light. These results were confirmed by measurements of the incorporation of radioactivity from $[\text{U-}^{14}\text{C}]$ protein hydrolysate into protein. The rate of incorporation fell by 85 % from $33 \text{ Bq h}^{-1} (10^6 \text{ cells})^{-1}$ in the light to $5.5 \text{ Bq h}^{-1} (10^6 \text{ cells})^{-1}$ in the dark. Protein synthesis increased to its pre-dark rate immediately after the cells were re-illuminated (Fig. 2*d*), but nitrogenase activity continued to decline (Fig. 2*b*). Therefore, the decrease in nitrogenase activity which occurred after a period in the dark was probably not the result of a general inhibition of protein synthesis.

In cyanobacteria, it seems unlikely that the activity of nitrogenase is modulated by intermediary metabolites (Stewart *et al.*, 1975; Murry & Benemann, 1979). N_2 fixation would therefore continue at a rate determined largely by the supply of ATP and/or reductant or by the amount of nitrogenase in the cyanobacterial cell. Control of the latter might be determined by the relative rates of nitrogenase synthesis and inactivation, particularly by O_2 (Bone, 1972).

In cultures transferred to the dark under anaerobic conditions, nitrogenase activity initially increased more rapidly than in cultures under dark aerobic conditions. However, after 5 h, the rate under anaerobic conditions became constant whereas the rate under aerobic conditions began to decrease (results not shown). The initial increase in the rate of acetylene reduction in the dark under both aerobic and anaerobic conditions could be prevented by the addition of chloramphenicol or NH_4^+ and it therefore appeared to result from synthesis of nitrogenase. Synthesis apparently ceased after 5 h in the dark, leading, under aerobic conditions, to a net loss of nitrogenase activity as a consequence of inactivation of the enzyme by O_2 . However, under dark anaerobic conditions, O_2 inactivation did not occur.

Nitrogenase activity recovered 10 h after re-illumination of a culture which had been incubated aerobically in the dark for 12 h. The recovery was prevented by the addition of chloramphenicol or NH_4^+ either at the beginning or end of the dark period. The delay of 10 h in the recovery of nitrogenase activity in the light after a 12 h dark period may therefore result from delayed synthesis of new nitrogenase enzyme.

Treatment of *Gloeocapsa* with 1 mM-EDTA for 20 min under aerobic conditions irreversibly inactivated any existing nitrogenase. Subsequent treatment of the culture with 5 mM- CaCl_2 relieved the inhibition by EDTA and nitrogenase activity slowly recovered, probably as a result of *de novo* enzyme synthesis (Hamadi & Gallon, 1979). When cultures of *Gloeocapsa* were incubated in the dark for various periods (between 0.5 and 6.5 h) and nitrogenase was inactivated with EDTA as described above, the initial rate of synthesis of nitrogenase in both light and dark decreased with increasing length of dark pre-incubation (Fig. 3). In the dark, nitrogenase activity increased for between 1 and 3 h after addition of CaCl_2 and then decreased, while in the light, activity increased for at least 5 h.

These results could be explained by postulating either the accumulation in the dark of a compound which inhibits nitrogenase synthesis or activity, or the disappearance in the dark of a compound which is necessary for nitrogenase synthesis or activity. An effect on the activity of nitrogenase seems less likely, because there was no inhibition of nitrogenase activity in cultures incubated anaerobically in the dark. Since our results suggest that the initial increase in nitrogenase activity in the dark may be the result of nitrogenase synthesis, the cessation of the increase after 2 to 5 h in the dark may be caused by an interruption of this synthesis.

One inhibitor of nitrogenase synthesis that might accumulate in the dark is ammonia. Because N_2 fixation initially continued in the dark but protein synthesis, and hence the utilization of ammonia, was markedly decreased, it is possible that ammonia might have accumulated to a level at which it would have repressed nitrogenase synthesis. However, this appears unlikely because 3 mM-L-methionine-DL-sulphoximine, which is known to reverse the repression of nitrogenase synthesis by ammonia in the light (Gallon *et al.*, 1978), did not reverse the inhibition of nitrogenase synthesis which occurred after 5 h in the dark. An

Fig. 3. Effect of dark incubation on nitrogenase synthesis by cultures of *Gloeocapsa* sp. 1430/3. Cultures harvested during the exponential phase of growth were incubated for (a) 0.5 h, (b) 2.5 h, (c) 4.5 h and (d) 6.5 h in the dark, and then treated with 1 mM-EDTA followed 20 min later by 5 mM- CaCl_2 . The cultures were then incubated in the dark (●) or in the light (O) and the rate of acetylene reduction was measured hourly. The data shown are from a single experiment but are typical.

example of a compound necessary for nitrogenase synthesis which might disappear from cultures of *Gloeocapsa* incubated in the dark is mRNA, which has been shown to be unstable in the cyanobacterium *Anabaena variabilis* (Carr, 1973).

The above examples are purely speculative and there is, so far, no direct evidence that the disappearance of nitrogenase activity in the dark is caused by an effect on nitrogenase synthesis. However, in general terms, an inhibitor of nitrogenase synthesis might either be produced only in the dark or produced both in the light and in the dark but broken down more rapidly in the light. Either of these alternatives would allow the accumulation of the inhibitor in the dark. Subsequent transfer of cultures to the light would require the breakdown of this inhibitor before nitrogenase synthesis could begin. Since the intracellular level of inhibitor would depend upon the length of dark incubation, an inverse relationship between the rate of nitrogenase synthesis on transfer to the light and the length of pre-incubation in the dark might be expected. Similar arguments could be applied to the disappearance of a component essential for the synthesis of nitrogenase.

Whatever the mechanism, the response of acetylene reduction in *Gloeocapsa* to dark incubation appeared to differ markedly from that of heterocystous cyanobacteria such as *Anabaena cylindrica* and *Anabaenopsis circularis*. In these latter organisms, N_2 fixation declined exponentially to zero within 4 h of transfer to darkness but resumed immediately

upon re-illumination, although in *Anabaenopsis circularis* it could be maintained for long periods in the dark provided that an exogenous carbon source was supplied (Fay, 1965, 1976; Bottomley & Stewart, 1977). This suggests that in these species, unlike *Gloeocapsa*, the rate of N_2 fixation in the dark is limited mainly by the supply of energy and reducing power.

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