Calcium Ions, Oxygen and Acetylene Reduction (Nitrogen Fixation) in the Unicellular Cyanobacterium *Gloeocapsa* sp. 1430/3

By A. FALAH HAMADI AND JOHN R. GALLON*

Department of Biochemistry, University College of Swansea, Singleton Park,
Swansea SA2 8PP, U.K.

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Cultures of *Gloeocapsa* sp. 1430/3 rapidly and irreversibly lost their ability to reduce acetylene when incubated with 1 mM-EDTA, either in the light or aerobically in the dark. However, EDTA did not inhibit acetylene reduction by cultures of *Gloeocapsa* incubated anaerobically in the dark. It is suggested that EDTA depletes the cyanobacterial cells of Ca^{2+} and thereby destroys a Ca^{2+} -dependent process by which nitrogenase is protected from inactivation by oxygen.

INTRODUCTION

Acetylene reduction (N_2 fixation) by cultures of several species of cyanobacteria is inhibited by the addition of chelating agents such as EDTA (Gallon, 1978). In most strains simultaneous addition of MgCl_2 , CaCl_2 , SrCl_2 or BaCl_2 counteracts the effect of EDTA, but in cultures of the unicellular cyanobacterium *Gloeocapsa* sp. (*Gloeotheca* sp.), only CaCl_2 prevents the inhibition: MgCl_2 , SrCl_2 and BaCl_2 themselves inhibit acetylene reduction (Gallon, 1978). Because EDTA is a polar molecule which does not readily enter cells (Albert, 1973), it probably exerts its effect on acetylene reduction indirectly. Addition of EDTA to a culture would decrease the concentration of free divalent cations in the medium and could thereby cause their efflux from the cyanobacterial cells. The consequent lowering of the intracellular concentrations of these ions might then inhibit acetylene reduction. Acetylene reduction in *Gloeocapsa* appears specifically to require Ca^{2+} (Gallon, 1978).

This paper describes investigations into the nature of the Ca^{2+} requirement for acetylene reduction in *Gloeocapsa* sp. 1430/3.

METHODS

Growth of cultures. *Gloeocapsa* sp. 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).

Preparation of extracts. For assay of nitrogenase and reductant-dependent ATPase, extracts of *Gloeocapsa* were prepared at room temperature (20 °C) by a modification of the method of Gallon *et al.* (1972). Between 5 and 7 d after inoculation, 5 l of culture was removed and allowed to settle under gravity for 2 h. As much as possible of the culture medium was carefully removed and the cells were resuspended in an equal volume of 20 mM-N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)/NaOH buffer pH 7.5, transferred to a measuring cylinder and allowed to settle once more. The supernatant was discarded, and the settled cells (usually less than 10 ml) were transferred to a darkened test tube and bubbled with N_2 for 15 min. Sodium dithionite was added to a final concentration of 1 mM, and the cells were broken in a French press at 138 MPa and collected, under N_2 , in a darkened vessel. A cell-free extract was obtained by centrifugation at 10 000 g for 20 min in a sealed tube flushed with N_2 . Nitrogenase activity could then be detected in the supernatant, in contrast to the preparations of Gallon *et al.* (1972). Alternatively, settled cells, prepared as above, were bubbled with N_2 for

15 min and then broken under N_2 by four passages through a Yeda press at 17.2 MPa. Nitrogenase prepared in this way could be sedimented by anaerobic centrifugation at 10000 g for 20 min.

For the assay of Ca^{2+} -dependent ATPase, extracts of *Gloeocapsa* were prepared at 4 °C as described by Tözüm & Gallon (1979).

Enzyme assays. N_2 fixation by intact cells was measured by the acetylene reduction technique of Tözüm *et al.* (1977), or using cultures concentrated 20- to 50-fold and 5 min incubation with acetylene (Tözüm & Gallon, 1979). HEPES/NaOH buffer pH 7.5 (5 mM) was added to all incubations. Anaerobic conditions were produced by flushing cultures with N_2 or Ar for 2 to 5 min prior to sealing the incubation vessels. To demonstrate that anaerobic conditions persisted throughout the incubation, a 1.5 ml centrifuge tube containing 1 ml methylene blue (50 mg l^{-1} in 2 mM-NaOH) decolorized by heating with D-glucose (20 g l^{-1}) (Meynell & Meynell, 1970) was included in these vessels. Occasionally, a tube containing 1 ml pyrogallol (50 g l^{-1} in 10 M-KOH) was also included, to absorb any residual traces of oxygen. However, the effects of anaerobiosis on acetylene reduction were identical in the presence or absence of alkaline pyrogallol.

Nitrogenase activity in extracts was measured at 25 °C as described by Gallon *et al.* (1972), except that the reactants were enclosed in 25 ml sealed serum bottles and the reaction was started by injection, through the seal, of 0.5 ml of extract. Control incubations, containing either boiled extract or unbroken cells, were included in all assays. Unbroken cells were usually, but not always, inactive under the conditions of assay; all results were corrected, where necessary, for the activity of any unbroken cells in the enzyme preparation.

Reductant-dependent ATPase was assayed under the same conditions as nitrogenase except that the reaction mixture contained (in a total volume of 2 ml) Tris/HCl buffer pH 7.4 (50 μ mol), $MgCl_2$ (10 μ mol), Tris-ATP (10 μ mol) and sodium dithionite (10 μ mol). Ca^{2+} -dependent ATPase activity was measured at 25 °C as described by Rorive & Kleinzeller (1974), with 0.5 ml of extract. In both ATPase assays inorganic phosphate produced by hydrolysis of ATP was measured by the method of LeBel *et al.* (1978). Boiled enzyme controls and controls lacking sodium dithionite or Ca^{2+} were included in all assays.

Other measurements. Photosynthetic O_2 evolution was measured polarographically at 25 °C under illumination at 2500 lx, in a Clark-type oxygen electrode (Rank Bros, Bottisham, Cambs). Intracellular ATP was measured as described by Mullineaux *et al.* (1980) after incubation of cultures with or without 1 mM-EDTA. Protein was measured by the method of Bailey (1962). Cell counts were performed in a Hawksley haemocytometer.

Special chemicals. N' -(3,4-Dichlorophenyl)- N,N -dimethylurea (DCMU) was obtained from DuPont (U.K.), London. 6-Chloro- N -ethyl- N' -(1-methylethyl)-1,3,5-triazine-2,4-diamine (atrazine) was kindly supplied by Ciba-Geigy. Ionophore A23187 was a generous gift from Dr R. L. Hamill, Lilly Research Laboratories, Indianapolis, U.S.A.

RESULTS AND DISCUSSION

EDTA and acetylene reduction

On addition of 1 mM-EDTA to illuminated, aerobically grown cultures, nitrogenase activity disappeared with a half-life of about 2.5 min and had fallen almost to zero within 20 min (Fig. 1). Following 20 min exposure to EDTA, no nitrogenase activity was detected within 4 h of resuspension in fresh medium. On the other hand, addition of 5 mM- $CaCl_2$ within 15 min of adding EDTA almost completely prevented any inhibition of acetylene reduction during the following 60 min (Fig. 2). Thus, EDTA appeared to cause a rapid, reversible, loss of nitrogenase activity, followed by irreversible inactivation of the enzyme after more than 15 min exposure.

When 20 min or more elapsed between the addition of EDTA and that of $CaCl_2$, little or no nitrogenase activity could be detected in aerobic, illuminated cultures during the following 30 to 60 min. Subsequently, however, the ability to reduce acetylene gradually reappeared (Fig. 3). After an initial lag period (normally about 30 min) following the addition of $CaCl_2$, this reappearance of nitrogenase activity was linear, and by 7 h the activity was about 80% of that in a control culture to which no EDTA had been added (Fig. 3). Thereafter, nitrogenase activity continued to increase, though at a reduced rate; by 12 h the activity of experimental cultures frequently exceeded that of the controls. No reappearance of nitrogenase activity was observed when $MgCl_2$ was added instead of $CaCl_2$.

The reappearance of nitrogenase activity was completely abolished by addition of chloramphenicol (0.1 mg ml^{-1}) or 2 mM- NH_4Cl . Chloramphenicol at this concentration markedly inhibits incorporation, by *Gloeocapsa*, of radioactivity from $Na_2^{14}CO_3$ into protein (A. F.

Fig. 1

Fig. 2

Fig. 1. Effect of 1 mM-EDTA (added at zero time) on acetylene reduction by cultures of *Gloeocapsa* sp. 1430/3. After incubation for the period indicated, the rate of acetylene reduction was measured over the following 5 min. Activity is expressed relative to the activity of a control to which no EDTA was added. Each point represents the mean of at least five determinations.

Fig. 2. Effect of CaCl_2 on acetylene reduction by cultures of *Gloeocapsa* sp. 1430/3 incubated with EDTA. CaCl_2 (5 mM) was added to cultures preincubated for the period indicated in the presence of 1 mM-EDTA (added at zero time). The rate of acetylene reduction was then measured over the following 60 min. Activity is expressed relative to a control lacking EDTA. Each point represents the mean of at least five determinations.

Fig. 3. Reappearance of nitrogenase activity in cultures of *Gloeocapsa* sp. 1430/3 treated with 1 mM-EDTA, followed 20 min later by 5 mM- CaCl_2 . After further incubation for the period indicated, either in the light (○) or in the dark (●), the rate of acetylene reduction was measured over the following 60 min. Activity is expressed relative to the activity of similar cultures to which no EDTA had been added. Each point represents the mean of at least six determinations.

Hamadi, unpublished observation), whilst NH_4^+ represses nitrogenase biosynthesis, directly or indirectly, in N_2 -fixing organisms, including cyanobacteria (Stewart, 1977). Thus, addition of EDTA to illuminated, aerobic cultures leads, after 20 min, to an irreversible inactivation of existing nitrogenase, and the ability to reduce acetylene reappears following addition of CaCl_2 , probably as a result of the synthesis of new enzyme.

The ability to reduce acetylene also reappeared when aerobic, illuminated cultures treated with EDTA and then, 20 min later, with CaCl_2 were incubated in the dark. However, in contrast to the response of cultures incubated in the light, there was no lag period and, although the activity of nitrogenase was lower in the dark than in the light, the initial rate of reappearance of this activity was greater in the dark (Fig. 3). One possible explanation for this is that, in the dark, there is no photosynthetic O_2 evolution to inactivate newly synthesized nitrogenase (see Tözüm & Gallon, 1979) or, perhaps, to inhibit nitrogenase synthesis, as occurs in other N_2 -fixing organisms (Eady *et al.*, 1978; Robson, 1979*a*). In the dark, acetylene reduction reached a peak after about 4 h and then slowly declined (Fig. 3), suggesting that *Gloeocapsa* synthesizes nitrogenase in the dark for only limited periods.

Exposure to 1 mM-EDTA for 60 min completely inhibited acetylene reduction by illuminated cultures incubated under an atmosphere of N_2 , Ar, air or Ar/ O_2 (4:1, v/v). In the dark, however, EDTA stimulated, rather than inhibited, acetylene reduction after incubation for 60 min under either N_2 or Ar, though under air or Ar/ O_2 the chelating agent was as inhibitory as in the light (Hamadi & Gallon, 1979). Similarly, 5 mM- MgCl_2 , which inhibited acetylene reduction by illuminated, aerobic cultures (Gallon, 1978), did not inhibit acetylene reduction by cultures incubated for 60 min anaerobically in the dark (Hamadi & Gallon, 1979). The Ca^{2+} requirement for N_2 fixation by *Gloeocapsa* is apparently evident only in the presence of O_2 . In the light, anaerobic conditions cannot be established because of photosynthetic O_2 evolution.

During longer dark, anaerobic incubation with 1 mM-EDTA, the ability to reduce acetylene was lost with a half-life of about 2 h. In contrast to the response of aerobic, illuminated cultures, either 5 mM- CaCl_2 or 5 mM- MgCl_2 , added simultaneously with EDTA, markedly decreased the rate of this disappearance (Fig. 4). EDTA appears to inhibit several metabolic processes in *Gloeocapsa* (Hamadi & Gallon, 1979) but its rapid effect on acetylene reduction in the presence of O_2 is unique in that it is prevented only by Ca^{2+} and not by Mg^{2+} .

When cultures were incubated in the presence of 1 mM-EDTA for 60 min in the dark, complete inhibition of acetylene reduction was obtained only when the partial pressure of O_2 was 0.02 atm or more (Fig. 5), while complete inhibition in illuminated cultures was achieved in the absence of added O_2 . It appeared, therefore, that photosynthesis generated a higher intracellular concentration of O_2 in illuminated cells than existed in cultures incubated in the dark with an O_2 partial pressure below 0.02 atm. However, even in the presence of 10 μM -DCMU or 10 μM -atrazine, which completely abolished photosynthetic O_2 evolution, 1 mM-EDTA caused an 80% inhibition of acetylene reduction by illuminated cultures incubated under Ar or N_2 .

Three possible explanations for the different effect of EDTA under anaerobic conditions in the light and dark were examined. Firstly, EDTA may inhibit acetylene reduction only when the synthesis of ATP is coupled to electron transport. Secondly, addition of EDTA to cultures of *Gloeocapsa* might disrupt a Ca^{2+} -dependent mechanism which normally protects nitrogenase from inhibition by O_2 . Although no O_2 evolution could be measured in illuminated cultures of *Gloeocapsa* exposed to DCMU or atrazine, there may still be sufficient production of O_2 in the presence of these inhibitors to allow EDTA partially to inhibit nitrogenase in illuminated cultures incubated anaerobically. Thirdly, it is possible that EDTA fails to inhibit acetylene reduction by cultures incubated anaerobically in the dark because under these conditions there is little or no efflux of Ca^{2+} from the cyanobacterial cells.

EDTA and ATP synthesis

Addition of 1 mM-EDTA to illuminated cultures caused an immediate fall in the intracellular concentration of ATP from 2.20 to 1.75 pmol (10^6 cells) $^{-1}$. However, within 1 min, this concentration had returned to that found in control cultures. Similarly, when 1 mM-

Fig. 4

Fig. 5

Fig. 4. Effect of 1 mM-EDTA (added at zero time) on acetylene reduction by cultures of *Gloeocapsa* sp. 1430/3 incubated anaerobically in the dark (●). Other additions, made simultaneously with EDTA, were 5 mM-CaCl₂ (■) or 5 mM-MgCl₂ (▲). After incubation for the period indicated, the rate of acetylene reduction was measured over the following 60 min. Activity is expressed relative to the activity of similar cultures to which no additions had been made. Each point represents the mean of at least three determinations.

Fig. 5. Effect of O₂ on acetylene reduction by cultures of *Gloeocapsa* sp. 1430/3 incubated for 60 min in the dark with (■) or without (●) 1 mM-EDTA. The data shown are from a single experiment but are typical.

EDTA was added to cultures incubated aerobically in the dark, only a transient decrease in the intracellular concentration of ATP was observed. EDTA therefore evidently disrupts neither photosynthetic phosphorylation nor oxidative phosphorylation to any great extent.

In the presence of 10 μ M-(3-chlorophenyl)hydrazonopropanedinitrile (CCCP) or 10 μ M-2,4-dinitrophenol, which are known to uncouple respiratory ATP synthesis (Heytler, 1979), cultures incubated for 60 min aerobically in the dark reduced acetylene at rates similar to those of cultures incubated anaerobically without additions. EDTA inhibited acetylene reduction in aerobic cultures incubated with CCCP or 2,4-dinitrophenol, but not in anaerobic cultures. Assuming that CCCP and 2,4-dinitrophenol had been effective, ATP for N₂ fixation could be supplied only by mechanisms which generate ATP under anaerobic conditions, particularly substrate level phosphorylation. This suggests that the effect of EDTA is not related to the mechanism of ATP synthesis within the cyanobacterial cells.

The addition of 5 mM-CaCl₂ stimulated the ATPase activity of extracts of *Gloeocapsa*. This stimulation was most marked in extracts from cultures pretreated for 20 min with 1 mM-EDTA, in which Ca²⁺-dependent ATPase activity reached 0.25 to 0.5 nkat (mg protein)⁻¹, i.e. 15 to 30% of the total ATPase activity. Presumably this ATPase would be inhibited in cells treated with EDTA but there is no evidence to link its activity with either electron transport or N₂ fixation. Consequently, it remains unlikely that EDTA inhibits acetylene reduction as a result of any effect on ATP synthesis linked to electron transport.

EDTA and O₂

Gloeocapsa nitrogenase is inactivated by O₂ in vivo and in vitro (Gallon *et al.*, 1972, 1974). When broken cell preparations of *Gloeocapsa* prepared with the French press were preincubated for 10 min in a gas phase containing 0.01 atm O₂, nitrogenase activity (assayed anaerobically) was markedly inhibited. The presence of 5 mM-CaCl₂ during the pre-

Table 1. *Effects of EDTA, CaCl₂ and O₂ on Gloeocapsa nitrogenase activity*

Enzyme activity was measured, under anaerobic conditions, in broken cells and a cell-free preparation, as shown. Additions were made directly to the assay mixture except that, where indicated, broken cell preparations were exposed to 0.01 atm O₂ for 10 min, in the presence or absence of CaCl₂, prior to their assay for nitrogenase.

Preparation	Addition or treatment	Acetylene reduction [pkat (mg protein) ⁻¹]
Broken cells (French press)	None	3.78
	5 mM-CaCl ₂	5.02
	1 mM-EDTA	3.97
	0.01 atm O ₂	0.64
	0.01 atm O ₂ + 5 mM-CaCl ₂	1.02
Cell-free extract (French press)	None	10.58
	5 mM-CaCl ₂	7.43
Broken cells (Yeda press)	None	1.70
	5 mM-CaCl ₂	1.47

incubation did not prevent this inhibition (Table 1). On the other hand, when added to broken cell preparations that had not been exposed to O₂, 5 mM-CaCl₂ stimulated nitrogenase activity (Table 1), and also increased reductant-dependent ATP hydrolysis from 0.1 to 0.121 nkat (mg protein)⁻¹. The stimulatory effect of CaCl₂ on acetylene reduction has not been investigated, but it may be significant that 5 mM-CaCl₂ inhibited, rather than stimulated, the nitrogenase activity of cell-free extracts from the French press, and broken cell preparations from the Yeda press (Table 1).

The fact that preincubation in the presence of 0.01 atm O₂ inhibited nitrogenase activity *in vitro* even in the presence of 5 mM-CaCl₂ suggests that Ca²⁺ does not interact directly with *Gloeocapsa* nitrogenase to form an O₂-stable complex. However, such a complex might be formed by interaction between Ca²⁺, nitrogenase and a second protein, analogous to that found in *Azotobacter vinelandii* by Haaker & Veeger (1977). In *Azotobacter chroococcum*, Mg²⁺ is involved in the formation of such a complex (Robson, 1979*b*); it is possible that in *Gloeocapsa* Ca²⁺ is required.

In certain organisms, O₂ inhibits both nitrogenase synthesis and activity (Eady *et al.*, 1978; Robson, 1979*a*) and, in aerobically incubated cultures of *Gloeocapsa*, inhibition of nitrogenase synthesis (by, for example, chloramphenicol or NH₄⁺) leads to a loss of nitrogenase activity with a half-life of about 3.3 h (see data of Tözüm *et al.*, 1977). However, in the presence of EDTA, nitrogenase activity in *Gloeocapsa* cultures decayed with a half-life of about 2.5 min (Fig. 1), so it is unlikely that EDTA inhibits acetylene reduction simply by inhibiting nitrogenase synthesis or by exacerbating any inhibition of nitrogenase synthesis by O₂.

Addition of 10 µM-methyl viologen to 10 d batch cultures of *Gloeocapsa* increased the inhibition of acetylene reduction by O₂ (Tözüm & Gallon, 1979). We found that this effect could be prevented by simultaneous addition of 5 mM-CaCl₂, suggesting that Ca²⁺ might protect nitrogenase from the damaging effects of H₂O₂ produced by reaction of O₂ with reduced methyl viologen. In support of this hypothesis, 5 mM-CaCl₂ also reversed the inhibition of acetylene reduction caused by 0.1 mM-H₂O₂. Although 5 mM-NaCl or, to a lesser extent, 5 mM-KCl had a similar effect to CaCl₂ on the inhibition of acetylene reduction caused by either methyl viologen or H₂O₂, neither NaCl nor KCl prevented 1 mM-EDTA from inhibiting acetylene reduction. Furthermore, addition of 5 mM-sodium ascorbate or catalase (EC 1.11.1.6; 2.5 µkat ml⁻¹), which reverse the effect of methyl viologen (Tözüm & Gallon, 1979), did not reverse the inhibition of acetylene reduction by EDTA. H₂O₂ cannot, therefore, be implicated in the O₂-dependent inhibition of acetylene

Table 2. *Effect of CaCl₂ on the inhibition by organic solvents and sodium lauryl sulphate of acetylene reduction by Gloeocapsa sp. 1430/3*

Cells were collected from batch cultures after 10 d growth. They were then incubated aerobically in the light for 60 min with the compounds indicated and the rate of acetylene reduction was measured over the following 60 min. The rates of acetylene reduction are typical; the percentages are the means of at least seven observations.

Addition	Acetylene reduction [pmol min ⁻¹ (ml cells) ⁻¹]	Percentage of uninhibited activity
None	27.3	100
13 mM-Butan-1-ol	18.3	67
13 mM-Butan-1-ol + 5 mM-CaCl ₂	36.6	134
54 mM-Toluene	18.0	66
54 mM-Toluene + 5 mM-CaCl ₂	30.0	110
1.7 mM-Sodium lauryl sulphate	2.2	8
1.7 mM-Sodium lauryl sulphate + 5 mM-CaCl ₂	28.7	105

reduction which follows the addition of EDTA, and there is no evidence that EDTA inhibits the mechanisms by which H₂O₂ is destroyed in this organism.

Addition of 1 mM-EDTA to cultures of *Gloeocapsa* stimulated the incorporation of ¹⁴CO₂ into membrane lipids, particularly monogalactosyldiacylglycerol. The extent of stimulation varied from 35 to 118% but was always observed. Furthermore, 5 mM-CaCl₂ prevented the inhibition of *Gloeocapsa* nitrogenase activity that was produced by the organic solvents butan-1-ol and toluene and by the surface active agent sodium lauryl sulphate (Table 2). Although the significance of these observations is not clear, it is possible that certain membranes protect nitrogenase from inactivation by O₂, either by acting as a diffusion barrier or by providing conformational protection for the enzyme.

EDTA and intracellular Ca²⁺

There is no direct evidence that EDTA causes an efflux of Ca²⁺ from cells of *Gloeocapsa*. However, such a mechanism is consistent with the idea that EDTA does not enter living cells (Albert, 1973) and is supported by the observation that Ca²⁺ caused a greater stimulation of ATPase activity in extracts of cultures pre-treated with EDTA than in controls.

Ionophore A23187, which carries divalent cations across membranes (Reed & Lardy, 1972), has been used by Gallon (1978) to render sensitive to inhibition by EDTA acetylene reduction by certain, usually resistant, cyanobacteria. However, 1 mM-EDTA did not inhibit acetylene reduction by cultures of *Gloeocapsa* incubated anaerobically in the dark for 60 min with 8 µM-A23187. Furthermore, when illuminated cultures of *Gloeocapsa* were incubated with 1 mM-EDTA for 20 min and then transferred to dark, anaerobic conditions, the ability to reduce acetylene reappeared as new nitrogenase was synthesized. Under dark anaerobic conditions, therefore, factors other than a barrier to Ca²⁺ efflux must protect nitrogenase from inactivation by EDTA because, if EDTA acts as assumed, intracellular Ca²⁺ would already have been depleted during incubation in the light.

In conclusion, therefore, the most likely explanation for the rapid inhibition of acetylene reduction in *Gloeocapsa* caused by 1 mM-EDTA is that a Ca²⁺-dependent mechanism, which normally protects nitrogenase from O₂, is inactivated.

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