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Protection of Nitrogenase Levels in Dark-incubated Cultures of *Anabaena* sp. strain CA by Various Carbon Sources, and Restoration of Nitrogenase Activity by Oxygen*

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Photoautotrophically grown, nitrogen-fixing cultures of *Anabaena* sp. strain CA lost nitrogenase activity completely after 4 h of incubation in the dark. The original level of nitrogenase activity was restored within 3 h of re-illumination and was apparently dependent on *de novo* protein synthesis. Several organic carbon sources protected nitrogenase activity. The heterocysts isolated from photoautotrophically grown cultures incubated in the dark (35 min) showed negligible nitrogenase activity. When these heterocysts were exposed to oxygen, glucose or fructose during isolation, normal activity was observed only with O₂. Oxygen also enhanced the rate of initial H₂ evolution from isolated heterocysts.

For most nitrogen-fixing cyanobacteria it is the heterocysts that, by virtue of their unique physiology and biochemistry, allow the reduction of dinitrogen to NH₄⁺ (Stewart, 1980). Nitrogen fixation requires both ATP and a source of reducing power. Although heterocysts have the ability to generate ATP to photosystem I mediated cyclic photophosphorylation, the reductant for N₂ fixation is dependent upon a supply of carbon compounds from the photosynthesizing vegetative cells. Moreover, the apparently wasteful nitrogenase-mediated H₂ evolution accounts for about 30% of the total energy flow through the nitrogenase enzyme system. Clearly there is a need for a better understanding of this aspect of the N₂ and H₂ metabolism (Eisbrenner & Evans, 1983).

The effects of light intensity and quality on nitrogenase activity (Fay, 1970, 1976; Lex & Stewart, 1973; Mullineaux, Chaplin & Gallon, 1980; Yoch & Gotto, 1982), and on nitrogen-mediated photoproduction of H₂ (Kumar & Kumar, 1988) have been studied. Ramos, Madueno & Guerrero (1985) have studied the regulation of nitrogenase activity

by various physiological factors including light and dark incubation. However, we know of no previous report on the regulation of nitrogenase level by various carbon sources, nor of any information on an O₂ requirement for enhanced nitrogenase or nitrogenase mediated H₂ production by isolated heterocysts. In this paper we describe the possible role of O₂ in enhancing nitrogenase activity, nitrogenase-catalysed H₂ production and protection of nitrogenase activity by various carbon sources in a heterocystous cyanobacterium.

MATERIALS AND METHODS

The organism used for these studies was *Anabaena* sp. strain CA (ATCC 33047), a filamentous, heterocystous, marine cyanobacterium. Cultures were grown in Pyrex culture tubes (22 × 175 mm) containing 20 ml of ASP-2 medium (Van Baalen, 1962) with no combined nitrogen and 5 g NaCl l⁻¹. The cultures were grown in 1% CO₂-in-air at 39 ± 0.1°C. The culture bath was illuminated with four F36T12/D/HO fluorescent lamps (Westinghouse, Bloomfield, NJ) on either side of the bath 12 cm from the centre of the growth tube at an average intensity of 300 μE m⁻² s⁻¹. The growth rate was determined turbidimetrically with a model 502 Lumetron colourimeter equipped with broad-band pass filter set with peak transmission centred at 660 nm. Dry weights

*Dedicated to late Prof Chase Van Baalen by his student (1984-1986).

were determined by harvesting whole filaments or isolated heterocysts on a 0.4 μM polycarbonate filter and drying to a constant weight in a vacuum oven at 45°C over P_2O_5 .

The procedure for heterocyst isolation was essentially that of Kumar, Tabita & Van Baalen (1982) with some modifications. A suspension of 20 to 30 ml of cells at a density of 0.10 to 0.12 mg (dry weight) ml^{-1} was washed two times in ASP-2 medium with the total concentration of KCl raised to 0.03 M and NaCl to 0.37 M (assay medium). Exogenous thiols were not required and therefore were not added during isolation for any of the experiments. The cells were resuspended in 5 ml of the assay medium containing 1 mg ml^{-1} lysozyme and sparged for 5 min with 1% CO_2 +99% Ar. Cells were then transferred to a 15 ml stoppered serum tube containing the same gas mixture, using a gastight syringe, and the tube was placed in a 39°C bath shaking at 60 rpm. The bath was illuminated from the bottom by six F48T12/CW/HO fluorescent lamps (Sylvania, Danvers, Mass.) at an average intensity of 300 $\mu\text{E m}^{-2} \text{s}^{-1}$. After 35 min, the suspension was sonicated for 18 s with two interruptions of 1 s each on a model W-10 sonicator (Heat Systems Ultrasonics, Inc., Plainview, NY) set at full power. The sonicated suspension was transferred to a 7 ml vacutainer tube and centrifuged at 2000 rpm for 5 min. The supernatant was removed, and the pellet resuspended on a 4 ml pre-gassed assay medium and centrifuged at 1500 rpm for 5 min. The final pellet was resuspended in either 2 ml volume for acetylene reduction assays or 3 ml for use on the hydrogen electrode.

For acetylene reducing activity, a suspension of 2 ml of either whole filaments or isolated heterocysts was placed in 7 ml stoppered serum tubes containing 10% C_2H_2 and placed in the shaker bath. The bath was illuminated from the bottom by six F48T12/CW/HO fluorescent lamps (Sylvania) at an average intensity of 300 $\mu\text{E m}^{-2} \text{s}^{-1}$. The suspension was shaken at 60 rpm. The ethylene formed was measured by injecting 0.2 ml of the gas phase into Antek model 464-IPC gas chromatograph equipped with a 182 cm chromosorb 104 column (Johns Manville, Celite Division, Denver, Colo.) at 50°C with helium as the carrier gas.

Amperometric measurement of hydrogen was performed using a 5331 electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) incorporated into a 1.8 ml water-jacketed electrode chamber maintained at 39°C. The contents of the electrode chamber were continuously mixed with a small magnetic stirring bar. The electrode signals were detected and amplified using model 150B microvolt-ammeter (Keithley Instruments, Inc., Cleveland, Ohio) and recorded. Actinic light was provided by a projector with Day-DAK

500 W lamp, screened by no. 34-01-2 hot mirror (Baird, Atomic, Inc., Bedford, Mass.). The lamp was operated at 100 V, and the intensity incident on the electrode chamber was 1200 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Biochemicals were from Sigma and were of reagent grade quality or better.

RESULTS

The cellular nitrogenase activity levels found in *Anabaena* sp. strain CA grown diazotrophically in 1% CO_2 -in-air were 1.4 ± 0.2 $\mu\text{moles C}_2\text{H}_4$ formed $\text{mg dry wt}^{-1} \text{h}^{-1}$ (80 ± 10 $\mu\text{moles C}_2\text{H}_4$ formed $\text{mg Chl } a^{-1} \text{h}^{-1}$). About 10% of the cells in the filaments differentiated as heterocysts. When light-grown cultures were transferred to darkness, the level of nitrogenase activity decreased with time (Fig. 1) to negligible activity 4 h after transfer from light to darkness. Cultures kept in light maintained high nitrogenase activity. When the dark-incubated cultures were re-illuminated, the activity was restored after about 3 h. Addition of 50 $\mu\text{g ml}^{-1}$ each of both chloramphenicol (inhibitor of translation) and rifampicin (inhibitor of transcription) prevented restoration of activity on re-illumination, suggesting involvement of *de novo* protein synthesis in the reactivation process (Fig. 1). Glycerol (5 mM), glucose (10 mM), erythrose (5 mM) and fructose (50 mM) all showed the same trend of protection of nitrogenase levels in dark-incubated cultures (Fig. 1), but the degree of protection appeared greatest with erythrose and least with glycerol.

Heterocysts isolated after 35 min dark incubation showed almost negligible activity (Fig. 2). However, when different concentrations of O_2 (0.2, 1.0, 5.0%) were injected preceding the 35 min dark incubation for the isolation of heterocysts, the nitrogenase activity increased, in the case of 5% O_2 almost reaching the control value. This increase in activity was independent of KCN. Fructose (50 mM) did not restore nitrogenase activity in the dark-incubated isolated heterocysts (Fig. 2).

A typical hydrogen electrode tracing from suspensions of heterocysts isolated under light was biphasic, with initial higher rates of

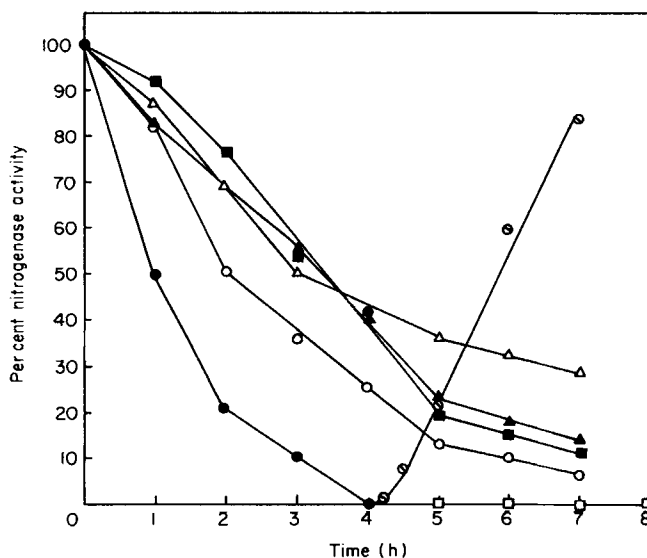


FIG. 1. Effect of darkness and re-illumination on nitrogenase activity of photoautotrophically grown *Anabaena* sp. strain CA. Cultures were transferred to darkness and nitrogenase activity was determined at the times indicated. At $t = 0$ the suspension was divided into several aliquots which were supplemented with various carbon sources, or were illuminated in the absence and presence of chloramphenicol and rifampicin. Dark incubation alone (●); dark incubation with 5 mM glycerol (○); 50 mM fructose (■); 10 mM glucose (▲) or 5 mM erythrose (△); re-illumination in the absence (○) or presence (□) of chloramphenicol and rifampicin ($50 \mu\text{g ml}^{-1}$).

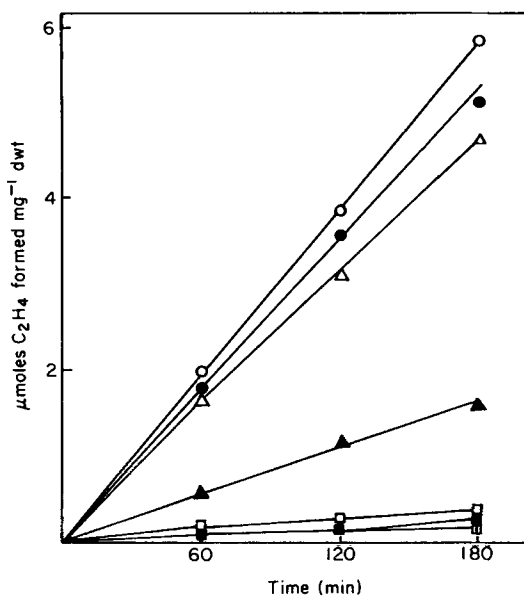


FIG. 2. Effects of O_2 , glucose and fructose addition on the nitrogenase activity of isolated heterocysts of *Anabaena* sp. strain CA under 90% H_2 + 10% C_2H_2 . During heterocyst isolation, the culture was darkened with aluminium foil for 35 min. All additions to cultures were made preceding the dark-phase. Control with no dark incubation (○); dark incubation alone (□); dark incubation with 10 mM glucose or 50 mM fructose (■); dark incubation with 0.2, 1.0, 5.0% O_2 v/v (□, ▲, ● respectively); dark incubation with 5% O_2 + 100 μM KCN (△).

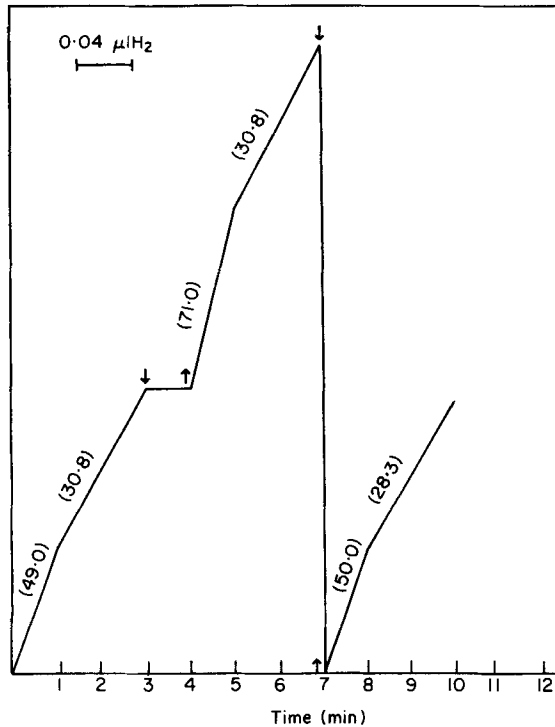


FIG. 3. Original hydrogen electrode trace (reduced in scale) of H_2 production by isolated heterocysts from strain CA. Cells were grown under photoautotrophic conditions as described in the text. Heterocysts were transferred directly to the electrode chamber, where they were gassed for 2 min with 100% Ar. Other conditions were as described in the text. The light ($1200 \mu E m^{-2} s^{-1}$) was on at time 0; \uparrow indicates light on and \downarrow indicates light off. Measurements were made after an interval of 2 min dark. At the second arrow (\uparrow) $2 \mu l$ pure O_2 was injected in the electrode chamber. At the third arrow (\downarrow) 100% Ar was bubbled for 2 min and then activity was measured. Values in parentheses are calculated rates of hydrogen evolution ($\mu l H_2 mg \text{ dry wt}^{-1} h^{-1}$).

H_2 production (Fig. 3). This burst typically lasted only 1 or 2 min. The lower, steady state rate was constant for at least 20 min on the hydrogen electrode. When $2 \mu l$ of O_2 -saturated water was injected into a 1.8 ml electrode chamber, the H_2 burst increased from 49.0 to 71.0 $\mu l H_2$ produced ($mg \text{ dry wt}^{-1} h^{-1}$) and lasted only about 1 min. This increase was re-checked again by bubbling 100% Ar into the chamber for 2 min followed by reinjection of O_2 saturated water, when the same kinetics of H_2 production were obtained.

DISCUSSION

The strain of *Anabaena* used in this work is photoautotrophic and does not fix nitrogen in the dark. Fay (1976) concluded that CO_2 impedes the utilization of glucose

for dark nitrogen fixation, but the ability of carbon sources to protect nitrogenase activity in a photoautotrophically grown culture has not been studied previously. In our study various carbon sources were added just before the onset of dark incubation so that the organism was not pre-fed during the light phase. Supplementation with various carbon sources during light phase affects the initial nitrogenase activity (Fay, 1976; Mulleneaux, Chaplin & Gallon, 1980; Misra, Jha & Kumar, 1985), and consequently increases the duration of nitrogenase activity in dark. It appears probable that these carbon sources prevent the deactivation of some protein(s) when the organism is incubated in the dark.

Oxygen-incubation of argon-sparged cultures restored the nitrogenase activity of isolated heterocysts to the normal level.

However, this restoration process was independent of KCN inhibition, suggesting oxidation of substrates by a so called "KCN-insensitive oxidase", KCN-insensitive respiration, or to ferredoxin-NADP⁺ oxidoreductase activity in the dark (Sturzl, Scherer & Böger, 1984). Addition of O₂ also leads to a substantial increase in energy change, but with very low rates of acetylene reduction (Ernst, Bohme & Böger, 1983); this rules out any role of energization in the restoration process.

The biphasic kinetics of H₂ evolution and significant enhancement by O₂ in isolated heterocysts constitute novel observations. The burst pattern was not seen with whole filaments on the H₂ electrode. A burst of H₂ can be seen when the two subunits of the nitrogenase complex are brought together under conditions of low electron flux, or when Fe-protein is limiting (Chatt, 1980), or there is ATP limitation (Yates & Walker, 1980). In our work low electron flow does not seem to be the explanation. H₂ production could be increased four-fold by simply shifting the gas phase from N₂ to Ar (Smith *et al.*, 1985). Ernst, Bohme & Böger (1983) also reported a rapid depletion and subsequent accumulation of ATP pools in heterocysts upon transition from dark to light.

The significance of the observed reaction between O₂ and nitrogenase is not presently understood. Nor is it clear if this reaction is related to other known reactions of the nitrogenase system involving H₂. Exposure of nitrogenase components to O₂, when no H₂ is present, has been reported to inactivate the enzyme irreversibly (Grillo *et al.*, 1979; Smith, Van Baalen & Tabita, 1987; Tabita *et al.*, 1988). However, fully active reduced MoFe has been obtained even after exposure to 1–5% level of O₂ under an atmosphere of argon+10% H₂ (Wang & Watt, 1984). These results suggest the possibility of a regulatory role of O₂ in cyanobacterial nitrogen metabolism.

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