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Oxygen Inactivation and Recovery of Nitrogenase Activity in Cyanobacteria

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Exposure of nitrogen-fixing cultures of *Anabaena* spp. to 100% oxygen resulted in the rapid decline of nitrogenase activity. When oxygen-treated cells were transferred to 100% argon, nitrogenase activity was quickly restored in a process that required protein synthesis. Anaerobiosis was not essential for the recovery process; in fact, cells of *Anabaena* sp. strains CA and 1F will recover nitrogenase activity after prolonged incubation in 100% oxygen. Oxygen treatment acted directly on the intracellular nitrogenase and did not affect other metabolic processes. Examination of crude extracts of oxygen-treated *Anabaena* sp. strain CA indicated that both components of nitrogenase are inactivated. However, several lines of evidence suggest that oxygen treatment does not result in irreversible denaturation of nitrogenase, but rather results in a reversible inactivation which may serve as a protection mechanism. Nitrogenase present in crude extracts from cells of *Anabaena* sp. strain 1F which had been incubated for a prolonged period in 100% oxygen was less sensitive to oxygen in vitro than was nitrogenase of a crude extract of untreated cells.

Nitrogenase, the enzyme that catalyzes the reduction of dinitrogen to ammonia, is an extremely oxygen-labile enzyme (6), and yet many nitrogen-fixing organisms are able to thrive in aerobic environments. The mechanism of protection of nitrogenase from oxygen denaturation has been established in some detail in *Azotobacter vinelandii* and *Azotobacter chroococcum* (16). These organisms utilize two basic modes of protection. The first, respiratory protection, refers to the maintenance of low levels of intracellular oxygen by adjustment of components of the electron transport chain to allow high rates of respiration. The second mode, conformational protection, refers to the formation of an inactive but stable complex consisting of nitrogenase component I (also known as dinitrogenase and MoFe protein), component II (also known as dinitrogenase reductase and Fe protein), and a third protein, Fe-S protein II (5, 9). When the oxygen concentration is lowered, this complex dissociates spontaneously and nitrogenase activity is restored.

The problem of aerobic nitrogen fixation is especially intriguing in some species of cyanobacteria (blue-green algae) which must cope not only with atmospheric oxygen, but also with photosynthetically produced oxygen. In an algal bloom, surface water can become supersaturated with oxygen, and yet nitrogen fixation can continue (14). Certain filamentous cyanobacteria produce specialized cells, heterocysts, which are the sites of aerobic nitrogen fixation for all cells of the filament (11). These cells are capable of providing a low oxygen environment for nitrogenase because they do not contain photosystem II and thus produce no oxygen photosynthetically. In addition, it has been suggested that their thick walls serve as a barrier to oxygen diffusion.

However, if a passive barrier does play a protective role, it does not appear to be acting alone. Bothe et al. (4) demonstrated that oxygen is less inhibitory to nitrogenase activity in *Anabaena cylindrica* when assays are performed in the presence of hydrogen than when assays are done in the absence of hydrogen. They concluded that hydrogenase, by transferring electrons from hydrogen to the respiratory chain and thus reducing oxygen to form water and ATP, might be important in maintaining a microaerobic environment inside the heterocyst.

Another approach makes use of oxygen-sensitive mutants. Heterocystous cyanobacteria are amenable to this work because they can grow and fix nitrogen microaerobically as well as aerobically (8, 12). Gotto et al. (8) isolated several oxygen-sensitive mutant strains of *Anabaena* sp. strain CA. Nitrogenase activity in one

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of these ceases in an atmosphere of air, but this effect is reversed when microaerobiosis is restored. These observations suggest that protection of nitrogenase requires more than a simple barrier to oxygen, because the effect of oxygen would not be rapidly reversible in a strain with a defective barrier.

Lambert and Smith (13) showed that nitrogenase activity could be completely eliminated in *Anabaena cylindrica* after 30 min of treatment with 100% oxygen, but that activity was partially restored after subsequent anaerobic incubation. We considered the possibility that this inactivation and recovery might be a protection mechanism similar to the conformational protection of nitrogenase in *Azotobacter* spp. This study represents an attempt to probe the mechanism of protection of nitrogenase against oxygen denaturation by pushing cyanobacteria beyond the limit of their tolerance.

(A preliminary account of this work has appeared [P. T. Pienkos, S. Bodmer, and F. R. Tabita, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K89, p. 151].)

MATERIALS AND METHODS

Organisms and growth conditions. *Anabaena* sp. strain 1F (7), *Anabaena* sp. strain CA (ATCC 33047 [18]), and the oxygen-sensitive mutant strain GM9, which was derived from strain CA (8), have been described previously. All were cultured in ASP-2 medium (20) at 39°C with constant illumination and bubbling with air enriched with 1% CO_2 (18). When strain GM9 was grown under nitrogen-fixing conditions, cultures were bubbled with 98% N_2–2% CO_2 to maintain microaerobic conditions (8). *Anabaena variabilis* Kutz (ATCC 29413), supplied by C. P. Wolk, was grown under the same conditions of illumination and aeration as strains CA and 1F, but at 35°C in CG-10 medium (21) with *N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid buffer (1.0 g/liter) used to replace glycylglycine. *Azotobacter vinelandii* (ATCC 12837), supplied by O. Wyss, was grown at 30°C with vigorous shaking in modified Burk medium (19) lacking Na_2MoO_4 and containing 5 μM Na_2WO_4. Tungstate was used to replaced molybdate to produce cells which had synthesized active component II and inactive component I (15). The cells were derepressed for nitrogenase synthesis by growth on limiting NH_4Cl (15).

Protein determinations. Whole-cell protein analyses for strains CA and 1F were calculated from a linear curve relating cell protein and culture turbidity. An absorbance value of 1.0 at 660 nm in a Spectronic 20 spectrophotometer, using tubes with a 2.5-cm light path, is equivalent to 220 μg of protein per ml for *Anabaena* sp. strain CA and 174 μg of protein per ml for *Anabaena* sp. strain 1F. Cells of *Anabaena variabilis* were washed once with distilled water and digested in 0.1 N NaOH at 100°C for 20 min. The digested cells and other proteinaceous samples were then analyzed by the modified Lowry technique described by Bensadoun and Weinstein (2).

Nitrogenase assays. Nitrogenase in whole cells was assayed by the acetylene reduction technique (17). Assays of strains CA and 1F were performed at 39°C; *Anabaena variabilis* was assayed at 35°C.

In vitro nitrogenase assays were performed in 23.5-ml vials fitted with sleeve stoppers. All reagents and enzyme preparations were made up in HP buffer [30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 30 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)], 1.0 mM $MgCl_2$, pH 7.4]; all reagents were stored and all assays were done in atmospheres of ultrapure argon. Samples of nitrogenase were mixed with 0.1 ml of 10 mM EDTA, 0.1 ml of 100 mM sodium dithionite, and 0.5 ml of an ATP-generating system (12 mg of ATP per ml, 14 mg of creatine phosphate per ml, 0.5 mg of creatine phosphokinase per ml [140 U/mg of protein], 20 mM $MgCl_2$ in HP buffer). The volume was adjusted to 2.0 ml with HP buffer. The vials were equilibrated to 39°C, and assays were initiated by the addition of 2.5 ml of C_2H_2. Gas samples were removed either periodically to ensure linear reduction of C_2H_2 or after the assay was stopped by the injection of 0.1 ml of 30% trichloroacetic acid. A unit of nitrogenase is defined as the amount necessary to form 1 nmol of C_2H_4 in 1 min.

Photophosphorylation. Immediately after the oxygen inactivation or the control treatment with argon, whole spheroplasts were prepared according to a method published by Binder et al. (3). Cyclic photophosphorylation with phenazine methosulfate and ascorbate was assayed at 39°C for 10 min in a Warburg apparatus with the same illumination as used for the whole-cell nitrogenase assays (17); [^{32}P]ATP formed was measured in the manner described by Avron (1).

Oxygen evolution. Oxygen evolution was measured with 1.7-ml samples of cultures of *Anabaena* sp. strain CA at 39°C with a Clark-type oxygen electrode. Illumination was provided by a 300-W slide projector with 10 cm of water as heat filter.

Incorporation of [^{14}C]leucine. The assimilation of radioactive leucine was used as an indicator of protein synthesis. To 10-ml cultures of *Anabaena* sp. strain CA, 2 μCi of L-[*U*-^{14}C]leucine (specific activity, 343 mCi/mmol) was added, and 0.5-ml samples were removed at intervals and filtered through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.). The filters were extensively washed with growth medium and counted in a Beckman LS-100C scintillation counter.

Preparation and handling of crude extracts. Cells of *Azotobacter vinelandii* and *Anabaena* sp. strain 1F were harvested at room temperature by centrifugation. The pellets were washed once with 5 to 10 volumes of HP buffer containing 2 mM sodium dithionite. From this point, all manipulations were carried out anaerobically under either ultrahigh-purity argon or nitrogen. The washed cells were suspended in 3 to 5 volumes of HP buffer containing 2 mM sodium dithionite and broken with a French pressure cell at 20,000 lb/in^2.

Anabaena sp. strain CA produces a thick slime layer which makes centrifugation and anaerobic manipulations difficult. The slime could be removed from the cells by suspending the pellet collected from 1 to 3 liters of culture in 100 ml of HP buffer containing 4 mM sodium dithionite. This suspension was then homogenized anaerobically with a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, N.Y.) at low

power. Centrifugation of this preparation resulted in a small tight cell pellet which could then be washed and broken in the manner described for *Anabaena* sp. strain 1F.

All crude extracts were stable for months when stored frozen under an atmosphere of argon or nitrogen.

Partial purification of *Anabaena* sp. strain 1F component I. All manipulations were performed anaerobically, using reagents which had been flushed with argon. All buffers contained 2 mM sodium dithionite. The crude extract was first clarified by treatment with 0.16% (wt/vol) protamine sulfate (10) and centrifuged at 12,000 × *g* for 30 min. The supernatant was loaded onto a DEAE-cellulose column which had been equilibrated with HP buffer. After the column was washed with 1 bed volume of HP buffer, partially purified component I was eluted with HP containing 0.15 M NaCl.

Gases and reagents. Gases were obtained from Big 3 (Houston, Tex.) or Wilson Oxygen (Austin, Tex.). All biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). The source of [^{14}C]leucine and ^{32}P was New England Nuclear (Boston, Mass.). DEAE-cellulose was obtained from Whatman (Clifton, N.J.). All glassware was acid washed before using, and all reagents were made up in glass-distilled water.

RESULTS

Loss of nitrogenase activity with oxygen treatment. Treatment of *Anabaena cylindrica* with 100% O_2 for 30 min results in the inactivation of nitrogenase (13). This activity can be partially restored in a reaction requiring protein synthesis if oxygen-treated cells are incubated anaerobically. We wished to determine whether the same reaction would occur with the marine cyanobacterium *Anabaena* sp. strain CA. For this, samples of nitrogen-fixing cells were injected into 23.5-ml rubber-stoppered serum vials containing atmospheres of 100% O_2 or 100% Ar as a control. The vials were incubated at 39°C in a Warburg apparatus with illumination and shaking for 30 min, and then 2.0-ml samples of the cultures were removed and injected into C_2H_2 reduction assay vials containing an atmosphere of 89% Ar–10% C_2H_2–1% CO_2. Duplicate samples of oxygen-treated and argon-treated (control) cells were injected into assay vials containing 40 μg of chloramphenicol (CAM) to inhibit protein synthesis. These vials were incubated at 39°C with illumination and shaking, and gas samples were removed every 15 min for analysis of C_2H_4 formation. Argon-treated *Anabaena* sp. strain CA control cells reduced C_2H_2 at a linear rate corresponding to a specific activity of 40.4 U/mg of protein; when assayed in the presence of CAM the cells also reduced C_2H_2 at a linear rate, although at a slightly lower rate (Fig. 1A). Nitrogenase activity in the oxygen-treated cells dropped nearly to zero as evidenced by the low level of C_2H_4 formed after 15 min, but the initial low rate of C_2H_2 reduction increased rapidly with time when the cells were assayed in the absence of CAM, and by 60 min the rate was approximately 55% that of the control. On the other hand, the oxygen-treated cells, assayed in the presence of CAM, did not show an increase in activity during the assay, but rather gave a low linear rate of C_2H_2 reduction corresponding to a specific activity of 2.6 U/mg of protein.

Similar effects were also observed after oxygen treatment of *Anabaena* sp. strain 1F and *Anabaena variabilis* (Fig. 1B and C). It is interesting that strain 1F was not inactivated to the same degree as was strain CA or *Anabaena variabilis*. The latter showed almost 100% inactivation, but was much slower to recover than either strain CA or strain 1F.

Oxygen treatment has a specific effect on nitrogenase activity. The dissolved oxygen found in cultures treated for 30 min with 100% O_2 was 706 nmol/ml, an increase of 3.4-fold over the level observed in normally growing cultures. Such a high oxygen concentration could have a general deleterious effect on *Anabaena* spp. rather than a specific effect on nitrogenase. We therefore investigated the effect of oxygen treatment on various key physiological activities of *Anabaena* sp. strain CA (Table 1). Although the oxygen treatment resulted in 90% inactivation of nitrogenase activity, it did not affect the rate of photophosphorylation of spheroplasts, photosynthetic oxygen evolution, or incorporation of [^{14}C]leucine into intact filaments. Acetylene reduction requires a steady flow of electrons and ATP to nitrogenase (6), and oxygen treatment could interrupt these processes and produce the observed results while having no effect on the nitrogenase enzyme itself. To test this, we analyzed cell-free preparations from *Anabaena* sp. strain CA, since in vitro C_2H_2 reduction assays bypass the physiological sources of reductant and ATP. The crude extract from the untreated control cells had a specific activity of 3.7 U of nitrogenase per mg of protein. The in vitro nitrogenase specific activity was much lower than the in vivo activity, probably due to the unusual handling necessary to produce a crude extract of *Anabaena* sp. strain CA (see Materials and Methods). An additional factor may be that the artificial reductant is less effective than the natural source (6). The crude extract from the oxygen-treated cells showed no nitrogenase activity. To determine whether one or both nitrogenase components were affected by the oxygen treatment, samples of components I and II, obtained from *Anabaena* sp. strain 1F and *Azotobacter vinelandii*, respectively, were added to the extract prepared from oxygen-treated *Anabaena* sp. strain CA. Components I and II from these sources have been tested with the

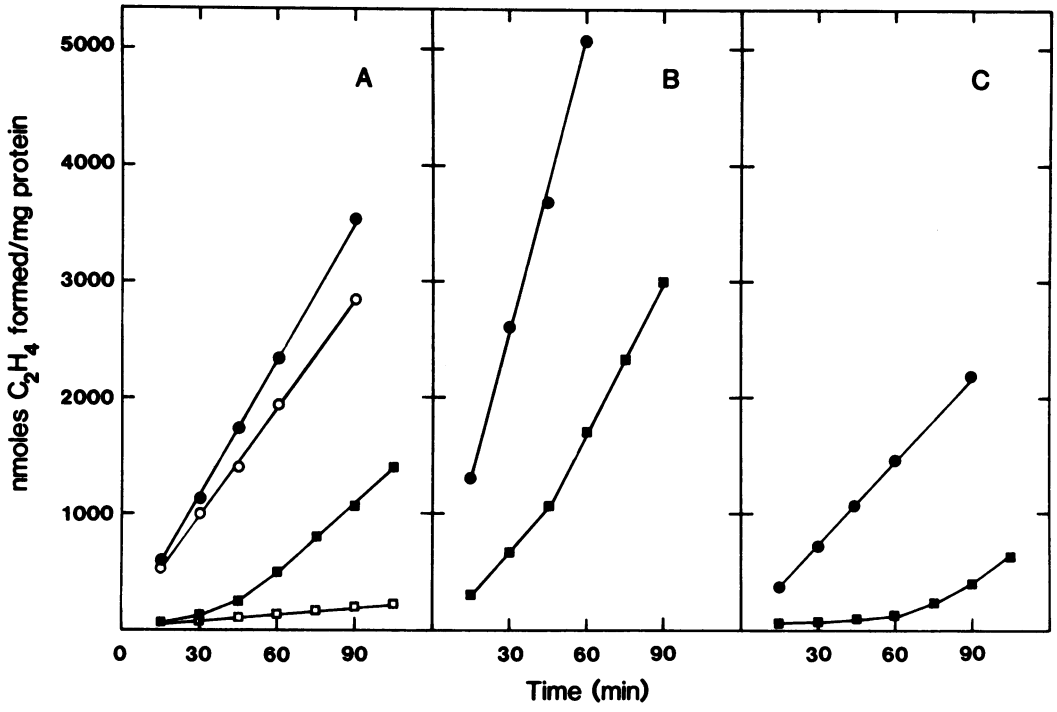

FIG. 1. Oxygen inactivation and recovery of nitrogenase activity in *Anabaena* spp. Cells of *Anabaena* sp. strains CA (A) and 1F (B) and *Anabaena variabilis* (C) were grown under nitrogen-fixing conditions to midlog phase. Samaples (5 ml) of cultures were injected into two 23.5-ml rubber-stoppered vials containing 100% O_2 or 100% Ar. After 30 min of incubation at ambient temperature (39°C for strains CA and 1F; 35°C for *Anabaena variabilis*) with shaking and illumination in a Warburg apparatus, 2-ml samples were removed and injected into 23.5-ml assay vials containing 89% Ar–10% C_2H_2–1% CO_2 in the presence and absence of 40 μg of CAM. These vials were also incubated at ambient temperature with illumination and shaking in the Warburg apparatus, and gas samples were removed every 15 min for analysis of C_2H_2 formation. The zero time point refers to the time the culture samples were injected into the C_2H_2 reduction vials. Symbols: Argon-treated cells minus CAM, ●; argon-treated cells plus CAM, ○; oxygen-treated cells minus CAM, ■; oxygen-treated cells plus CAM, □.

appropriate component from *Anabaena* sp. strain CA and found to form active nitrogenase (data not shown). Neither of these restored nitrogenase activity. These data suggest that oxygen treatment of *Anabaena* sp. strain CA inactivated both components of nitrogenase.

Conditions of recovery of nitrogenase activity. After a nitrogen-fixing culture of *Anabaena* sp. strain CA was treated with 100% O_2 for 30 min, samples of culture were removed and injected into C_2H_2 reduction vials containing 0, 20, and 40% O_2. The recovery of activity was identical in 0 and 20% O_2, but appeared to be inhibited by 40% O_2 (Fig. 2).

We devised a method to separate the recovery process from the C_2H_2 reduction step. In this procedure a nitrogen-fixing culture of *Anabaena* sp. strain CA was injected into a vial containing 100% O_2 or 100% Ar as control and incubated for 30 min with illumination and shaking. The

TABLE 1. Effect of treatment with 100% oxygen on different physiological activities of *Anabaena* sp. strain CA

Treatment of cells	Nitrogenase activity (nmol of C_2H_4 formed/min per mg of protein)	Photophosphorylation of spheroplasts (μmol of ATP/mg of chlorophyll per h)[a]	Oxygen evolution (μmol/mg of chlorophyll per h)	L-[*U*-^{14}C]leucine incorporation (cpm, after 60 min)
100% Ar for 30 min	46.3	348	130	83,960
100% O_2 for 30 min	4.2	383	130	84,320

[a] Phosphorylation with phenazine methosulfate-ascorbate.

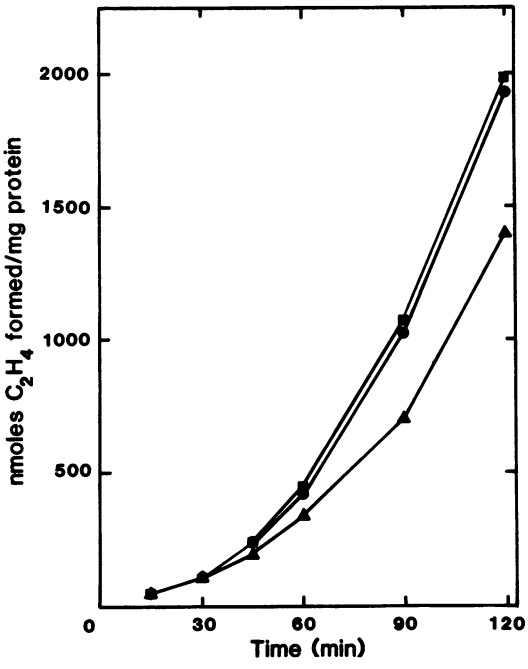

FIG. 2. Recovery of nitrogenase activity at various oxygen concentrations. A 7-ml sample of a nitrogen-fixing culture of *Anabaena* sp. strain CA was injected into a 23.5-ml vial containing 100% O_2. After 30 min of incubation at 39°C with shaking and illumination, 2-ml samples were removed and injected into 23.5-ml vials containing: 89% Ar–10% C_2H_2–1% CO_2, ●; 20% O_2–69% Ar–10% C_2H_2–1% CO_2, ■; or 40% O_2–49% Ar–10% C_2H_2–1% CO_2, ▲.

cultures were then transferred to new vials containing 100% Ar, and samples of culture were removed every 30 min for C_2H_2 reduction assays in the presence of 20 μg of CAM per ml, which would halt the recovery process at that point (Fig. 3). The specific activity of the argon-treated cells continued to rise throughout the experiment because these cells were starved for nitrogen and thus began to hyperproduce nitrogenase (unpublished data). The nitrogenase activity of the oxygen-treated cells also rose, but at a higher rate after a 1-h lag, until the cells reached the original activity of the control cells (47 U/mg of protein). At this time, the rate of increase began to fall off.

Recovery is not simply de novo nitrogenase synthesis. One explanation for the results previously obtained was that oxygen treatment led to the denaturation of both components of nitrogenase, and recovery resulted from their de novo synthesis. However, the observation that recovery of activity occurred at a faster rate than nitrogenase synthesis (Fig. 3) suggested that this might not be the only reason for recovery.

One method to determine whether oxygen inactivation was a result of nitrogenase denaturation was to determine the oxygen sensitivity of *Anabaena* sp. strain CA after one round of inactivation and recovery. If recovery were merely the result of de novo synthesis of nitrogenase, one would expect recovered cells to be just as sensitive to a second round of oxygen treatment as cells not exposed to oxygen. When a culture of strain CA was oxygen treated, allowed to recover in argon, and then oxygen treated a second time, the pattern of recovery was different than that of cells which were inactivated only once (Fig. 4). The nitrogenase activity of twice-treated cells was only reduced to approximately 14 U of nitrogenase per mg of protein, whereas the cells treated once had a specific activity of about 1.5 U/mg of protein. Clearly, the nitrogenase of recovered cells was not as sensitive to oxygen inactivation as was that of control (once-treated) cells.

The question of de novo synthesis playing a role in the recovery process was addressed by repressing nitrogenase synthesis with $NaNO_3$ (17). This repression requires approximately one generation time (about 5 h), a time during which nitrate reductase is being synthesized (D. Rams-

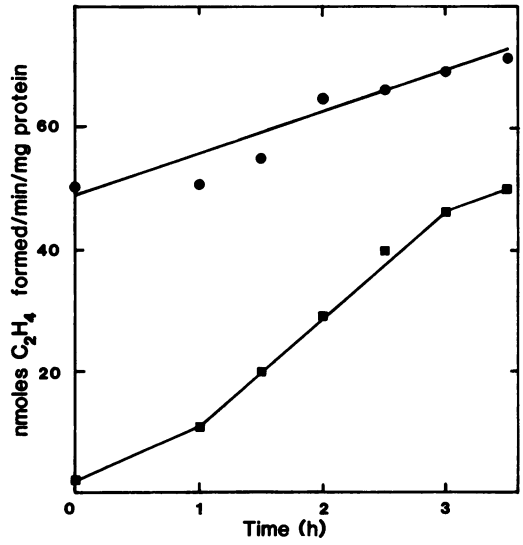

FIG. 3. Separation of the recovery of nitrogenase activity from the C_2H_2 reduction assay. Samples (20 ml) of nitrogen-fixing cultures of *Anabaena* sp. strain CA were injected into 60-ml vials containing 100% O_2 or 100% Ar. After 30 min of incubation at 39°C with illumination and shaking, the two cultures were removed and injected into new 60-ml vials containing 100% Ar. Every 30 min, 2-ml samples were removed and injected into assay vials containing 40 μg of CAM. Rates of C_2H_2 formation were calculated at each time point. Symbols: Argon-treated cells, ●; oxygen-treated cells, ■.

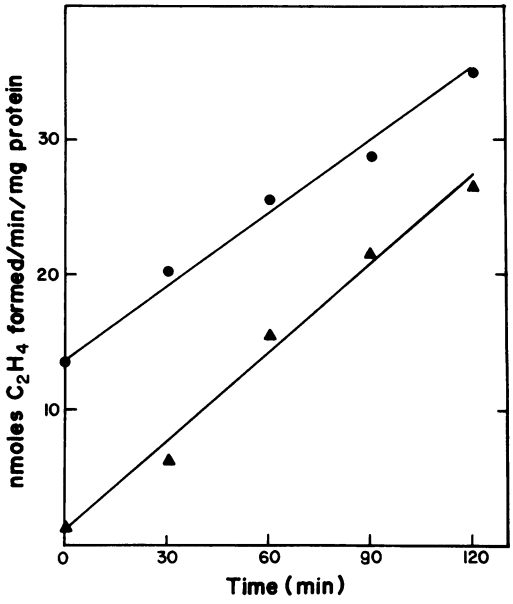

FIG. 4. Recovery of nitrogenase activity after two exposures to oxygen. Samples (20 ml) of nitrogen-fixing cultures of *Anabaena* sp. strain CA were injected into 60-ml vials containing either 100% O_2 or 100% Ar. After 30 min of incubation at 39°C with illumination and shaking, the two cultures were removed and injected into new 60-ml vials containing 100% Ar and incubated under the same conditions for an additional 2 h. Then both cultures were injected into vials containing 100% O_2 and incubated under the same conditions for 30 min. Finally, the cultures were injected into vials containing 100% Ar, and 2-ml samples were removed at 30-min intervals and injected into C_2H_2 reduction assay vials containing 40 μg of CAM. Rates of C_2H_4 formation were determined at each time point. Symbols: Cells treated with oxygen twice, ●; cells treated with oxygen once, ▲.

den, personal communication). In Fig. 5 we see the results of oxygen treatment at 0, 3, and 6 h after the addition of nitrate. Between 0 or 3 h the units of nitrogenase per milliliter of culture increased and the specific activity remained approximately equal, indicating that the cells were still synthesizing nitrogenase. These cells also demonstrated the expected reaction to oxygen treatment. By 6 h, the number of units per milliliter of culture had stopped increasing and the specific activity had dropped, indicating that nitrate-mediated repression had begun. Even though de novo nitrogenase synthesis was blocked by the presence of nitrate, oxygen-

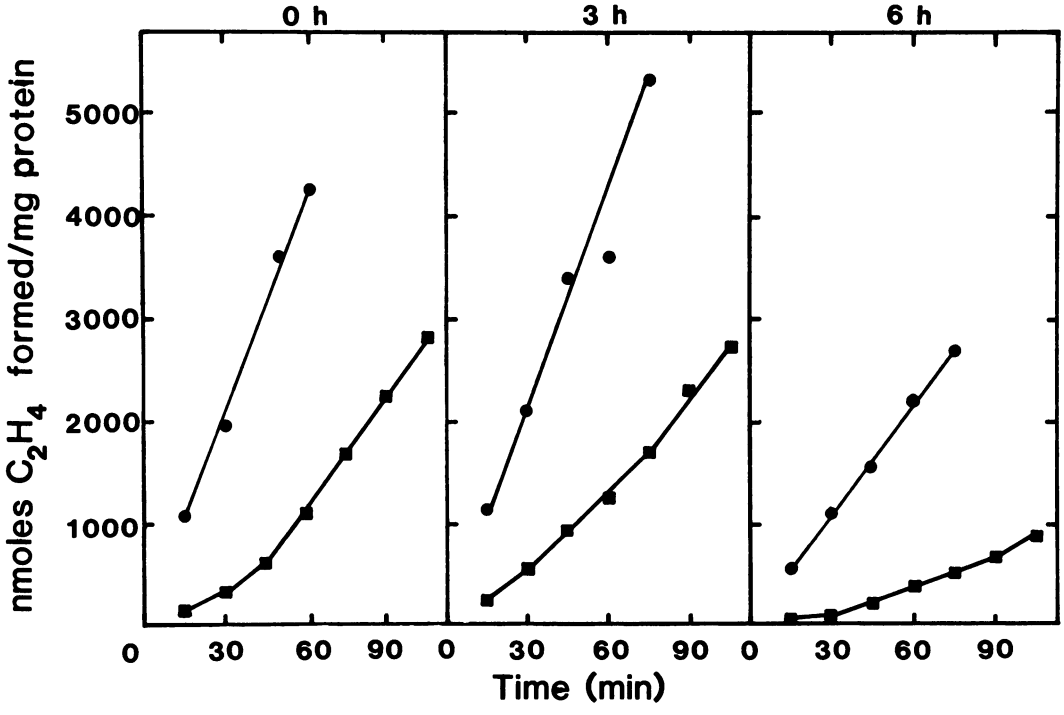

FIG. 5. Recovery of nitrogenase activity in cells repressed for nitrogenase synthesis. A culture of *Anabaena* sp. strain CA grown up under nitrogen-fixing conditions was made 10 mM with KNO_3. Samples were removed after O, 3, and 6 h of nitrate treatment and treated with 100% Ar and 100% O_2 exactly as described in the legend to Fig. 1. Symbols: Argon-treated cells, ●; oxygen-treated cells, ■.

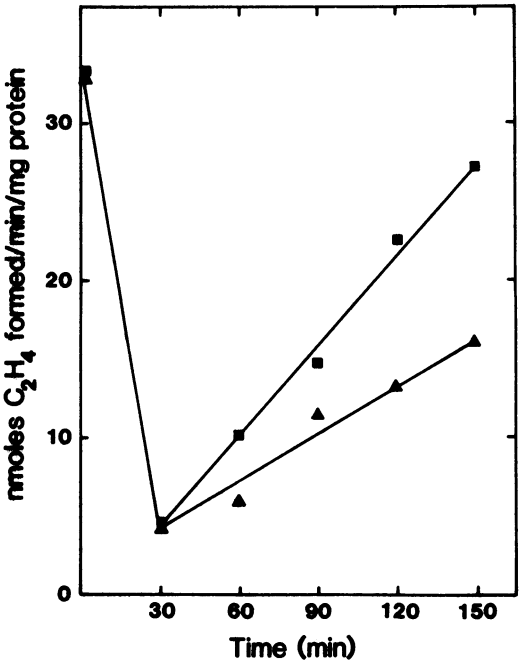

FIG. 6. Recovery of nitrogenase activity in 100% O_2. Samples (20 ml) of nitrogen-fixing cultures of *Anabaena* sp. strain CA were injected into two 60-ml vials containing 100% O_2. After 30 min of incubation at 39°C with illumination and shaking, the cultures were removed and injected into vials containing either 100% O_2 or 100% Ar, and the incubation was continued under the same conditions. At 30-min intervals, 2-ml samples were removed and injected into C_2H_2 reduction assay vials containing 40 μg of CAM. The rate of C_2H_4 formation was determined at each time point. The zero time point is the nitrogenase specific activity before oxygen treatment, and the 30-min time point is the specific activity before the cultures were transferred from 100% O_2 to 100% Ar or 100% O_2. Symbols: Argon-recovered cells, ■; oxygen recovered cells, ▲.

treated cells were still capable of recovery. In a similar experiment done with ammonia-treated *Anabaena* sp. strain 1F, identical results were obtained (data not shown).

A third indication that the recovery from oxygen inactivation was not simply a matter of de novo synthesis of nitrogenase came from the observation that prolonged oxygen treatment of *Anabaena* sp. strain CA did not result in increased inactivation or poorer recovery. In fact, cells incubated with 100% O_2 for longer than 30 min began to recover nitrogenase activity (Fig. 6). Although the rate of recovery was slower in 100% O_2 than in 100% Ar, the nitrogenase specific activity steadily increased from a basal level of 5 U/mg of protein during the 150 min of oxygen treatment.

Oxygen stability of nitrogenase in crude extracts. This physiological adaptation to prolonged exposure to 100% O_2 led us to investigate the phenomenon in a cell-free system. We wished to know whether nitrogenase obtained from crude extracts of *Anabaena* sp. strain 1F which had been bubbled with 100% O_2 for 3.5 h (i.e., inactivated and allowed to recover) was less oxygen sensitive than that obtained from crude extracts of untreated cells. Strain 1F was chosen for this experiment because it is easier to harvest and manipulate anaerobically (unpublished data). We found that nitrogenase in crude extracts obtained from untreated cells of *Anabaena* sp. strain 1F was extremely oxygen labile (Fig. 7), much more so than nitrogenase crude extracts obtained from *Azotobacter vinelandii* (unpublished data). The control crude extract was diluted in HP buffer containing 2 mM sodium dithionite and 10 mg of bovine serum albu-

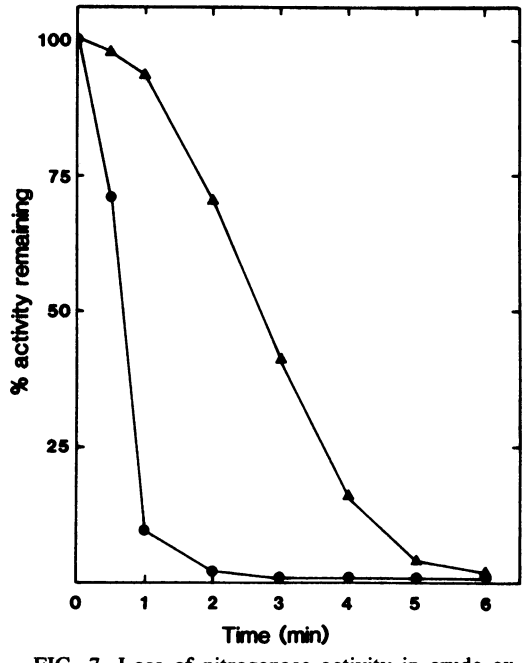

FIG. 7. Loss of nitrogenase activity in crude extracts exposed to air. Crude extracts were prepared from nitrogen-fixing cells of *Anabaena* sp. strain 1F which were either bubbled with 100% O_2 for 4 h (▲) or left untreated (●). The number of nitrogenase units per milliliter of each extract was determined, and the extract of the untreated cells was diluted with a solution of 10 mg of bovine serum albumin per ml of HP buffer plus 2 mM sodium dithionite so that the final specific activity was approximately equal for both extracts. Samples (5 ml) of both extracts were injected into open 60-ml vials which were then incubated at 39°C with shaking on the Warburg apparatus. Samples were removed periodically and injected into assay vials containing all the requirements for in vitro C_2H_2 reduction assays.

min per ml such that the initial nitrogenase activity would be the same in crude extracts from control and oxygen-treated cells. It was found that nitrogenase from the control cells lost 85% of its activity after 1 min and fell to zero by 3 min. The nitrogenase from oxygen-treated cells was more stable, showing more than 40% of the activity remaining after 3 min.

Oxygen-sensitive mutant of *Anabaena* sp. strain CA. The oxygen-sensitive strain GM9 and the wild-type strain CA were grown under nitrogen-fixing conditions microaerobically by continuously bubbling the cultures with 98% N_2–2% CO_2 (8). Samples of the two strains were incubated for 30 min with 100% Ar, air, and 100% O_2 and then injected into acetylene reduction assay vials containing 89% Ar–10% C_2H_2–1% CO_2.

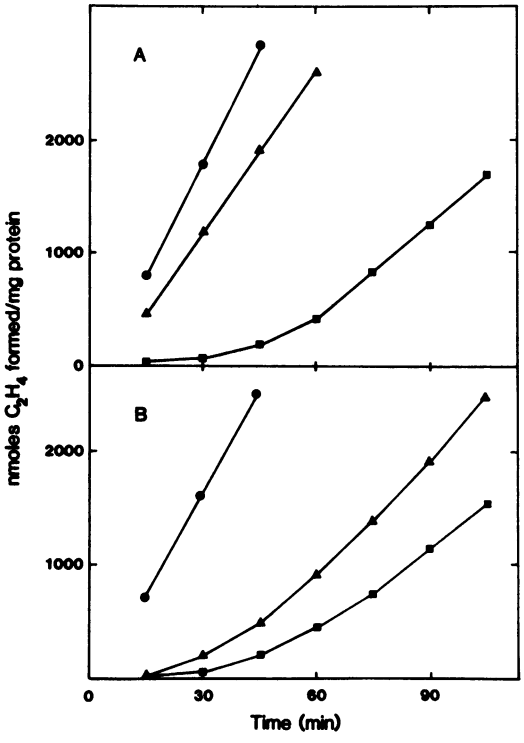

FIG. 8. Oxygen sensitivity of mutant strain GM9. Cultures of *Anabaena* sp. strain CA (A) and the mutant strain derived from it, GM9 (B):, were grown aerobically in the presence of 10 mM KNO_3. These cultures were used to inoculate nitrogen-fixing cultures which were grown microaerobically by bubbling with 98% N_2–2% CO_2. Samples were removed from these nitrogen-fixing cultures and injected into 23.5-ml vials containing 100% Ar, 100% O_2, and air. After 30 min of incubation at 39°C with illumination and shaking, 2-ml samples of culture were removed from each vial and injected into C_2H_2 reduction assay vials. Gas samples were removed and tested for C_2H_4 formation at 15-min intervals. Symbols: Argon-treated cells, ●; oxygen-treated cells, ■; air-treated cells, ▲.

Oxygen treatment of strain CA produced the expected result of nitrogenase inactivation; this activity dropped somewhat after air treatment, but the cells reduced C_2H_2 at a linear rate (Fig. 8A). Air treatment of strain GM9, on the other hand, resulted in almost 95% inactivation of nitrogenase activity followed by rapid recovery (Fig. 8B), which is comparable to the recovery of aerobically grown strain CA after exposure to oxygen (Fig. 1A). When strain GM9 was treated with 100% O_2, the level of inactivation was about the same as that seen in air-treated cells, but the rate of recovery was slower than after air treatment.

DISCUSSION

When a nitrogen-fixing culture of *Anabaena* sp. strain CA is exposed to 100% O_2 for 30 min, the disolved oxygen content rises from 208 to 706 nmol of O_2 per ml of culture. This treatment causes a loss of 90 to 95% of the nitrogenase activity, but if the oxygen concentration is lowered, the activity is rapidly restored in a reaction that requires protein synthesis. Although photosynthetic oxygen evolution during algal blooms can raise the dissolved oxygen to 50% over normal air saturation (14), treatment with 100% O_2 is probably not physiologically significant. Even so, the loss of nitrogenase activity is not a result of general damage to cellular functions; photophosphorylation, photosynthetic oxygen evolution, and [^{14}C]leucine incorporation are unaffected by oxygen treatment. In addition, protein synthesis continues even in the presence of 100% O_2 (unpublished data). The loss of activity is not due simply to an interruption of reductant or ATP to nitrogenase, since studies with cell-free extracts showed that both components of nitrogenase are inactivated by oxygen treatment of whole cells.

It was essential to prove that inactivation and subsequent recovery of nitrogenase activity are not simply matters of irreversible denaturation and de novo synthesis of nitrogenase itself. The observation that cells which are oxygen treated and allowed to recover are no longer as sensitive to subsequent exposures to oxygen argues strongly against irreversible denaturation. A second line of evidence comes from the ability of *Anabaena* sp. strain CA to recover nitrogenase activity during prolonged exposure to oxygen. If nitrogenase were irreversibly denatured by oxygen treatment, one would not expect an increase in activity as the oxygen treatment continued.

The strongest argument against recovery being simply a matter of de novo nitrogenase synthesis comes mainly from the ability of strains CA and 1F to recover even when nitrogenase synthesis is repressed by nitrate or ammonia. This is not to say that de novo synthesis

does not play a role in the recovery process. We have observed that cells labeled with [^{14}C]leucine after oxygen treatment synthesize nitrogenase (unpublished data). However, we feel that our results indicate that the recovery process is more complicated than simple de novo synthesis of nitrogenase. We submit that the phenomenon of oxygen inactivation and recovery of nitrogenase activity is part of a mechanism of protection of nitrogenase against irreversible denaturation. This reaction may be similar to the mechanism of conformational protection observed in *Azotobacter* spp. in which components I and II form a complex with the Fe-S protein II that is inactive but relatively oxygen stable (16). The oxygen-sensitive mutant strain GM9 may form the inactive complex at lower oxygen tensions so that nitrogen fixation can only occur microaerobically. The obvious difference between the two systems is that, whereas the *Azotobacter* complex dissociates spontaneously in lowered oxygen tensions to yield active nitrogenase, in *Anabaena* spp. the restoration of activity requires the synthesis of a new protein or proteins. The recovery protein(s) may be responsible for the in vitro increase in oxygen stability of nitrogenase obtained from cells treated with 100% O_2 for 3.5 h. If so, it should be possible to purify this protein(s), just as the Fe-S protein II was purified from *Azotobacter vinelandii* (9).

We will continue to probe into the molecular mechanism of inactivation and recovery, using wild-type and oxygen-sensitive mutant strains of *Anabaena* sp., and attempt to identify new proteins synthesized in response to oxygen treatment.

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LITERATURE CITED

1. **Avron, M.** 1960. Photophosphorylation by Swiss chard chloroplasts. Biochim. Biophys. Acta **40**:257–272.
2. **Bensadoun, A., and D. Weinstein.** 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. **70**:241–250.
3. **Binder, A., E. Tel-Or, and M. Avron.** 1976. Photosynthetic activities of membrane preparations of the blue-green alga *Phormidium luridum*. Eur. J. Biochem. **67**:187–196.
4. **Bothe, H., J. Tennigkeit, and G. Eisbrenner.** 1977. The utilization of molecular hydrogen by the blue-green alga *Anabaena cylindrica*. Arch. Microbiol. **114**:43–49.
5. **Dervartanian, D. V., Y. I. Shethna, and H. Beinert.** 1969. Purification and properties of two iron-sulfur proteins from *Azotobacter vinelandii*. Biochim. Biophys. Acta **194**:548–563.
6. **Eady, R. R., and J. R. Postgate.** 1974. Nitrogenase. Nature (London) **249**:805–810.
7. **Gotto, J. W., F. R. Tabita, and C. Van Baalen.** 1979. Isolation and characterization of rapidly growing marine, nitrogen-fixing strains of blue-green algae. Arch. Microbiol. **121**:155–159.
8. **Gotto, J. W., F. R. Tabita, and C. Van Baalen.** 1979. Mutants of *Anabaena* strain CA altered in their ability to grow under nitrogen-fixing conditions. J. Bacteriol. **140**:327–332.
9. **Haaker, H., and C. Veeger.** 1977. Involvement of the cytoplasmic membrane in nitrogen fixation by *Azotobacter vinelandii*. Eur. J. Biochem. **77**:1–10.
10. **Hallenbeck, P. C., P. J. Kostel, and J. R. Benemann.** 1979. Purification and properties of nitrogenase from the cyanobacterium *Anabaena cylindrica*. Eur. J. Biochem. **98**:275–284.
11. **Haselkorn, R.** 1978. Heterocysts. Annu. Rev. Plant Physiol. **29**:319–344.
12. **Haury, J. F., and C. P. Wolk.** 1978. Classes of *Anabaena variabilis* mutants with oxygen-sensitive nitrogenase activity. J. Bacteriol. **136**:688–692.
13. **Lambert, G. R., and G. D. Smith.** 1980. Hydrogen metabolism by filamentous cyanobacteria. Arch. Biochem. Biophys. **205**:36–50.
14. **Pearl, H. W., and P. E. Kellar.** 1979. Nitrogen-fixing *Anabaena*: physiological adaptations instrumental in maintaining surface blooms. Science **204**:620–622.
15. **Pienkos, P. T., S. Klevickis, and W. J. Brill.** 1981. In vitro activation of inactive nitrogenase component I with molybdate. J. Bacteriol. **145**:248–256.
16. **Robson, R. L., and J. R. Postgate.** 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. **34**:183–207.
17. **Stacey, G., P. J. Bottomley, C. Van Baalen, and F. R. Tabita.** 1979. Control of heterocyst and nitrogenase synthesis in the cyanobacteria. J. Bacteriol. **137**:321–336.
18. **Stacey, G., C. Van Baalen, and F. R. Tabita.** 1977. Isolation and characterization of a marine *Anabaena* sp. capable of rapid growth on molecular nitrogen. Arch. Microbiol. **114**:197–201.
19. **Strandberg, G., and P. W. Wilson.** 1968. Formation of N_2 fixing system in *Azotobacter*. Can. J. Microbiol. **14**:25–31.
20. **Van Baalen, C.** 1962. Studies on marine blue-green algae. Bot. Mar. **4**:129–139.
21. **Van Baalen, C.** 1967. Further observations on growth of single cells of coccoid blue-green algae. J. Phycol. **3**:154–157.