Control of Nitrogenase Recovery from Oxygen Inactivation by Ammonia in the Cyanobacterium Anabaena sp. Strain CA (ATCC 33047)

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The control of nitrogenase recovery from inactivation by oxygen was studied in Anabaena sp. strain CA (ATCC 33047). Nitrogenase activity (acyteline reduction) in cultures grown in 1% CO2 in air was inhibited by exposure to 1% CO2-99% O2 and allowed to recover in the presence of high oxygen tensions. Cultures exposed to hyperbaric levels of oxygen in the presence of 10 mM NH4NO3 were incapable of regaining nitrogenase activity, whereas control cultures returned to 65 to 80% of their original activity within about 3 h after exposure to high oxygen tension. In contrast to the regulation of heterocyst differentiation and nitrogenase synthesis, recovery from oxygen inactivation in this organism was shown to be under the control of NH4+ rather than NO3-.

Among nitrogen-fixing bacteria, the ability to perform oxygenic photosynthesis during diazotrophic growth is an attribute of only a few genera of cyanobacteria. While this affords the luxury of using photomediated reactions for the generation of high levels of ATP and reducing equivalents needed to power the nitrogenase machinery, it also requires a highly specialized system for coping with the deleterious effects of oxygen on the labile enzymes of the nitrogenase complex. The actual mechanism for the protection of nitrogenase remains unclear (3, 6, 8, 9, 18). In several heterocystous Anabaena spp., high levels of nitrogenase activity may be realized in the presence of up to 100% O2 after the initial loss of about 90% of the initial whole-filament activity (6, 9). In Anabaena strains CA and 1F, recovery from O2 inactivation took between 2.5 and 3.0 h, during which the cells were continuously exposed to 100% O2 (9). Recovery was shown to be dependent on protein synthesis (chloramphenicol sensitive) but not de novo synthesis of nitrogenase, and neither phosphorylation nor [14C]leucine incorporation into intact filaments was affected by hyperbaric oxygen tensions. Furthermore, cells which were allowed to recover from oxygen inactivation were less sensitive to subsequent rounds of exposure to high O2 levels. Experiments with cell extracts indicate that the loss of oxygen sensitivity following recovery correlates with an increased stability of the proteins to air in vitro (9), suggesting a system similar to that in Azotobacter spp. for conformational stabilization of the complex (14, 22). More recently it was found that both subunits of the iron protein (nitrogenase reductase) of the nitrogenase complex are specifically modified or altered in the presence of hyperbaric oxygen in two Anabaena species (13, 15, 19), which for Anabaena sp. strain CA is a reversible process (15).

In addition to conformational protection, several hypotheses for oxygen protection have been proposed, such as mechanical restriction of oxygen from the site of nitrogen fixation (in the form of a largely impervious, specialized cell membrane around the heterocysts [21]) and physiological adaptations in response to oxygen (5, 7). In any case, it is important to determine if the regulation of the synthesis of the recovery or protection system might be coordinated with the expression of the nitrogenase enzymes. In this study, we have examined the development of the recovery system under conditions in which the synthesis of both nitrogenase and heterocysts is easily manipulated (16).

The organism used for these studies was Anabaena sp. strain CA (ATCC 33047), a filamentous, heterocyst-forming marine cyanobacterium (17). Cultures were routinely grown in Pyrex culture tubes (22 by 175 mm) in 20 ml of medium ASP-2 (20) with an NaCl content of 5 g/liter. When required, NH4NO3 was added to a final concentration of 10 mM, NH4Cl was added to 10 mM, and NaNO3 was added to 12.5 mM. The cultures were routinely grown in an atmosphere of 1% CO2 in air at 39°C. Culture tubes were sealed with a rubber stopper through which a cotton-plugged Pasteur pipette (15 cm) and ventilation tube had been inserted, and air was bubbled through the cultures at a rate of 20 to 25 ml/min. The growth bath was illuminated at an average intensity of 200 microeinsteins/m2 per s with four F36T12/D/HO fluorescent lamps (Westinghouse) positioned on either side 12 cm from the center of the growth tubes. Oxygen inactivation and recovery of nitrogenase activity were performed as previously described (9). A nitrogen-fixing culture at a density of 0.10 to 0.12 mg (dry weight) of cells per ml was switched from an atmosphere of 1% CO2 in air to an atmosphere of 1% CO2-99% O2 with continuous sparging. Samples for acetylene reduction (1.0 ml) were taken directly from the growth tube at appropriate intervals and assayed for ethylene production. Suspensions of whole filaments (1.0 ml) were placed in stoppered 7.0 ml tubes containing 10% C2H2 in air and incubated with shaking in a 39°C light bath similar to the one used for growth. Ethylene production was measured by injection of 0.2 ml of the gas phase into an Antek model 464-IPC gas chromatograph equipped with a 182-cm-long Chromosorb 104 column (Johns-Manville Prod-
Inactivation per se of the nitrogenase enzymes. Contrary to the situation in the purple, nonsulfur bacteria (10), a rapid inactivation of nitrogenase does not occur upon the addition of fixed nitrogen to heterocystous cyanobacteria at physiological pH levels (1, 4, 11, 12, 16, 23). Under highly alkaline conditions, however, a rapid switching off of nitrogenase activity was observed when ammonia was added to intact filaments of *Anabaena variabilis* (13).

In contrast to the control of nitrogen fixation in most other diazotrophs, the regulation of nitrogenase derepression (and heterocyst differentiation) in *Anabaena* sp. strain CA is specifically mediated by the metabolism of nitrate rather than ammonia (2, 12). Cultures grown in the presence of nitrate differentiate no heterocysts and possess no acetylene-reducing capacity. However, when grown in the presence of up to 20 mM NH$_4$Cl, filaments express about 5 to 6% heterocysts and 30% of the nitrogenase activity compared with cultures grown with N$_2$ as the sole nitrogen source (2). To determine if ammonia or nitrate (or both) affected the recovery from oxygen inactivation, either NH$_4$Cl or NaNO$_3$ was added concomitantly with oxygen. The results clearly indicate that the metabolism of ammonia, and not nitrate, inhibits the recovery of nitrogenase activity after the culture has been subjected to hyperbaric oxygen (Table 1). Thus, the recovery or protective system appears to be regulated independently of the synthesis of nitrogenase and the development of heterocysts. Furthermore, these results strengthen the arguments that de novo nitrogenase synthesis is not necessary for recovery from oxygen inactivation (4, 9).

The recovery of nitrogenase activity in response to oxygen inactivation in the presence of NH$_4$Cl or NaNO$_3$ is shown in Table 1. Recovery of nitrogenase activity was followed by measuring acetylene reduction at time zero and at 0.5 and 3 h after the addition of NH$_4$Cl or NaNO$_3$. The results show that recovery of nitrogenase activity is dependent on the addition of ammonia or nitrate, respectively. The recovery of nitrogenase activity was more rapid in the presence of ammonia than in the presence of nitrate.

![Graph showing recovery of nitrogenase activity](image)

**FIG. 1.** Recovery of nitrogenase activity from oxygen inactivation in the absence or presence of NH$_4$Cl. At time zero, cultures (20 ml) of *Anabaena* sp. strain CA at a density of 0.10 to 0.12 mg (dry weight) of cells per ml were switched (with one exception) from an atmosphere of 1% CO$_2$ in air to one of 1% CO$_2$--99% O$_2$, with continuous bubbling. At appropriate time points, 1.0-ml samples were taken, injected into stopped 7.0-ml tubes containing 10% C$_2$H$_2$--90% air, and placed in a shaking bath in the light at 39°C. Each time point represents the rate of ethylene production as measured over 1 h at 15-min intervals. The zero time point represents nitrogenase activity prior to treatment with oxygen. Symbols: ○, cultures sparged with 1% CO$_2$--99% O$_2$; ●, cultures with 10 mM NH$_4$NO$_3$ added at time zero and remaining in 1% CO$_2$ in air; □, cultures sparged with 1% CO$_2$--99% O$_2$ with 10 mM NH$_4$NO$_3$ added at time zero.

**TABLE 1.** Recovery of nitrogenase activity in response to oxygen inactivation in the presence of NH$_4$Cl or NaNO$_3$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrogenase activity (μmol of C$_2$H$_2$ formed/mg [dry wt] of cells per h) at*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>None</td>
<td>1.94</td>
</tr>
<tr>
<td>NH$_4$NO$_3$, 1 mM</td>
<td>1.96</td>
</tr>
<tr>
<td>NH$_4$Cl, 10 mM</td>
<td>1.93</td>
</tr>
<tr>
<td>NaNO$_3$, 12.5 mM</td>
<td>1.93</td>
</tr>
</tbody>
</table>

* Acetylene reduction assays were performed as described in the legend to Fig. 2. Each time point represents the first-order rate of ethylene production measured over 1 h at 15-min intervals. The zero time point represents nitrogenase activity prior to treatment with oxygen. Cells were initially grown in an atmosphere of 1% CO$_2$ in air and switched to an atmosphere of 1% CO$_2$--99% O$_2$ for the indicated lengths of time.

The results of Gottos et al. (4) on the relationship between oxygen sensitivity and L-methionine-DL-sulfoximine resistance and those of Ramos et al. (12) on L-methionine-DL-sulfoximine inhibition of ammonium repression of the recovery of nitrogenase activity in dark-inactivated cells may now be addressed. Gottos et al. (4) found that the nitrogenase systems of mutants selected for resistance to L-methionine-DL-sulfoximine (a potent inhibitor of ammonium assimilation) or DL-7-azatryptophan showed an increased sensitivity to oxygen. One of their conclusions was that at least one component of the system was not related to nitrogenase directly. If ammonia (or one of its metabolites) does repress some protective mechanism for nitrogenase against oxygen, than a mutant which is resistant to the inhibition of ammonium uptake or incorporation and is therefore exposed to the inhibiting species should show higher-than-normal levels of oxygen sensitivity. That is, the protective mechanism would not be expressed in such mutants because its synthesis would be repressed by ammonia.
These conclusions are supported also by Ramos et al. (12), who demonstrated that the development of nitrogenase activity in Anabaena sp. strain CA incubated in the dark under fully aerobic conditions (conditions which they recognized might be related to oxygen-mediated inactivation), express only a limited level of acetylene-reducing activity (30%) upon the addition of ammonia but recover normally in the presence of L-methionine-DL-sulfoximine and ammonia.

In conclusion, we have demonstrated a direct effect of ammonia metabolism on at least one component of the nitrogenase system of Anabaena sp. strain CA. Ammonia-metabolizing cells, while fully capable of synthesizing active nitrogenase (2), are not able to recover from oxygen-mediated nitrogenase inactivation. Perhaps ammonia metabolism leads to the production of a repressor of the expression of a specific recovery or protective system(s). The nature of this component or system, how it interacts with the nitrogenase complex, and whether this putative component actually allows for nitrogenase activity in the presence of oxygen are questions awaiting further study.

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LITERATURE CITED