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Modification of Dinitrogenase Reductase in the Cyanobacterium *Anabaena variabilis* Due to C Starvation and Ammonia

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In the heterocystous cyanobacterium *Anabaena variabilis*, a change in nitrogenase activity and concomitant modification of dinitrogenase reductase (the Fe protein of nitrogenase) was induced either by NH_4Cl at pH 10 (S. Reich and P. Böger, FEMS Microbiol. Lett. 58:81–86, 1989) or by cessation of C supply resulting from darkness, CO_2 limitation, or inhibition of photosystem II activity. Modification induced by both C limitation and NH_4Cl was efficiently prevented by anaerobic conditions. Under air, endogenously stored glycogen and added fructose protected against modification triggered by C limitation but not by NH_4Cl. With stored glycogen present, dark modification took place after inhibition of respiration by KCN. Reactivation of inactivated nitrogenase and concomitant demodification of dinitrogenase reductase occurred after restoration of diazotrophic growth conditions. In previously C-limited cultures, reactivation was also observed in the dark after addition of fructose (heterotrophic growth) and under anaerobiosis upon reillumination in the presence of a photosynthesis inhibitor. The results indicate that modification of dinitrogenase reductase develops as a result of decreased carbohydrate-supported reductant supply of the heterocysts caused by C limitation or by increased diversion of carbohydrates towards ammonia assimilation. Apparently, a product of N assimilation such as glutamine is not necessary for modification. The increase of oxygen concentration in the heterocysts is a plausible consequence of all treatments causing Fe protein modification.

In the cyanobacterium *Anabaena variabilis*, aerobic nitrogen fixation by the enzyme complex nitrogenase (EC 1.18.6.1) is located in the heterocysts (12, 13). Heterocysts are devoid of an oxygenic photosystem II and ribulose-1,5-bisphosphate carboxylase, which are operative in vegetative cells. Thus, N_2 fixation is spatially separated from water photolysis and coupled ATP formation. The necessary ATP is generated in the heterocysts by photosystem I-mediated photophosphorylation and by oxidative phosphorylation. Reductants are produced by dissimilation of fixed carbon imported from vegetative cells into heterocysts (for a review, see reference 41).

It is accepted that cyanobacterial nitrogenase activity is controlled at the level of gene expression (13) and by the ability of cells to modulate the supply of reductants and ATP (7, 8). Under air, nitrogenase activity in vivo is known to be inhibited by dark treatment (9, 33) and by photosynthesis inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which is considered to be due to reductant limitation of heterocysts and concurrent oxygen inactivation of the heterocystous nitrogenase enzyme complex (24, 25). The repressing effect of excess ammonia and other nitrogenous compounds was attributed to enzyme turnover and regulation of enzyme biosynthesis (see, for example, references 4, 13, and 33). Some authors interpreted these effects in terms of inhibition of nitrogenase activity, possibly exerted by glutamine (24, 26, 29, 45) or carbamoyl phosphate (19) as intermediates of N assimilation.

Recently, it was noticed that addition of ammonia or an oxygen shock abolished nitrogenase activity in two *Anabaena* strains and that the dinitrogenase reductase (the Fe protein of nitrogenase) was modified (36, 39). Fe protein modification as another means of activity control was first observed in *Rhodospirillum rubrum* (22, 23, 32). Here the molecular basis for the inactivation is the reversible ADP ribosylation at a specific arginine residue (Arg-101) of one of the Fe protein polypeptides (30). Inactivation and activation of nitrogenase are catalyzed by specific enzymes (21, 38). This reversible in vivo loss of nitrogenase activity was called switch-off (46) and interpreted as an ammonia-dependent feedback inhibition (42). Factors leading to the switch-off include ammonia, glutamine, darkness, and phenazine methosulfate (see, for example, references 15 and 27). The competence to switch off nitrogenase is determined by the metabolic state (C and N supply) of the cell (1, 44, 45). No uniform concept has yet emerged with regard to the metabolic signals regulating the modifying enzymes in members of the family *Rhodospirillaceae* (16, 20).

Little is known about the molecular basis of enzyme modification observed in *Anabaena* spp. In this study, experimental conditions known to influence in vivo nitrogenase activity in heterocystous cyanobacteria were correlated with concomitant enzyme modification. To evaluate whether the regulation of existing nitrogenase is caused by ATP, reductant supply, or enzyme modification, the assay conditions were adjusted independently of the cultivation conditions. To provide for ATP, nitrogenase activity was assayed in the light. This allows for photophosphorylation in heterocysts, which is more efficient than oxidative phosphorylation (8). Excess reductant was supplied by molecular hydrogen (7). Endogenous reductant supply was varied by fructose addition, which causes a build-up of glycogen. The glycogen content of the filaments was determined by a coupled enzymatic assay (9). Enzyme modification was visualized by Western blotting (immunoblotting), applying a specific antibody raised against the purified Fe protein of *Anabaena* nitrogenase (36).

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MATERIALS AND METHODS

Growth conditions. *A. variabilis* (ATCC 23914) was cultivated in mineral medium (2) without combined nitrogen at 30°C in a thermostat-regulated water bath (Kniese-Edwards, Marburg, Federal Republic of Germany) illuminated with continuous light (130 μE m^{-1} s^{-2}). The growth rate (μ = 0.08 h^{-1}) was similar to that reported previously (6). The inoculum, equivalent to 0.4 μg of chlorophyll *a* (Chl) ml^{-1}, was from cultures grown for 2 days only. DCMU and L-methionine-D,L-sulfoximine (MSX) were added directly to the culture medium. Aluminum tubes fitting the growth vessels were used to examine the dark inactivation of nitrogenase. Care was taken not to change the CO_2 supply (1.5% [vol/vol] in air) or other conditions during the experiment. Gas flow was controlled by connecting the gas outlets of each tube with gas meters (Kuehne, Dortmund, Federal Republic of Germany).

Nitrogenase activity. Acetylene reduction was measured in short-time assays (15 min) at 30°C with white light at 650 μE m^{-2} s^{-1} as described previously (6). Either air, hydrogen, or argon was used as the gas phase in the assays supplemented with acetylene (13% [vol/vol]). Ethylene was determined by gas chromatography, using an apparatus from Carlo Erba (Hofheim, Federal Republic of Germany) fitted with a flame ionization detector and a Porapak R column. Traces of oxygen were present in the gas phases other than air (0.5 ± 0.2%; analyses performed with a Hewlett-Packard gas chromatograph [Hewlett-Packard Co., Avondale, Pa.]; see reference 43 for details).

Ammonium switch-off of nitrogenase in cyanobacteria is achieved only at pH 10, leading to ammonia inundation of metabolism (17, 36). Glass vessels (37 ml) with a 5-ml cell suspension (equivalent to 20 μg of Chl ml^{-1}) were placed in a water bath under assay conditions. Ammonia switch-off was initiated by addition of 1.2 mM NH_4Cl (final concentration).

Enzyme assays. For glycogen determination, 1 ml of culture suspension (equivalent to 3 to 30 μg of Chl) was concentrated by centrifugation (15 min, 11,000 × *g*), boiled with KOH, and enzymatically digested as described previously (9). The resulting glucose was determined spectrophotometrically. The final reaction mixture contained, in 1 ml, 50 mM triethanolamine (pH 7.6), 8 mM $MgSO_4$, 5 mM EDTA, 2 mM ATP, 0.5 mM NADP, 0.28 U of hexokinase, 0.14 U of glucose-6-phosphate dehydrogenase, and an appropriate amount of hydrolyzed cell extract. Fructose in the medium was determined with a similar enzyme assay that included hexose phosphate isomerase (0.7 U). Nitrogenase activity and enzyme tests were run in triplicate.

Immunological methods. For immunospecific Western blotting, 3 to 6 ml of cells was harvested by filtration (HATF [0.45-μm pore size] or RA [1.2-μm pore size]; Millipore Corp., Bedford, Mass.) and quickly frozen in liquid nitrogen. Before separation on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (18% [wt/vol] acrylamide, 3 M urea; thickness of gels, 1 mm; gel electrophoresis apparatus, GE 2/4 LS [Pharmacia, Uppsala Sweden]), nitrogenase was extracted by heating the cells in 0.4 ml of SDS buffer (18). The equivalent of 1 μg of Chl was used per slot of the polyacrylamide gel. A polyclonal antibody prepared against the SDS-denatured Fe protein of nitrogenase (dinitrogenase reductase) from *A. variabilis* (36) was used for immunological identification after transfer to polyvinylidene difluoride-paper (Millipore). Staining was performed by using a peroxidase-conjugated anti-rabbit antiserum (Dianova, Hamburg, Federal Republic of Germany). It should be emphasized that separation and immunological visualization of modified and unmodified dinitrogenase reductase could be achieved only within a narrow protein concentration range.

RESULTS

Inactivation and modification of dinitrogenase reductase upon transfer to darkness. Batch cultures grown for 24, 48, and 72 h under continuous light were darkened, and the decreases in nitrogenase activity (Fig. 1A) and glycogen content (Fig. 1B) were monitored over a 1- to 7-h period. In addition, cell extracts were separated on SDS-polyacrylamide gels and analyzed for Fe protein alteration by immunospecific Western blotting. At start of the dark treatment, only one form of Fe protein was present (Fig. 1C). Progressively, a second form with a slower electrophoretic mobility became apparent. In a glycogen-rich culture (after a 1-day cultivation), the second form became detectable when the glycogen content of the filaments dropped below 4 mg of glycogen per mg of Chl. About half of the subunits of the Fe protein present were modified when the glycogen pool contained less than 2 mg of glycogen per mg of Chl. At this time, nitrogenase activity had decreased to about 10% of the initial rate. When growth proceeded from the exponential phase to light limitation, the glycogen content of cells decreased (cf. reference 9). After darkening of a culture with lowered glycogen content (Fig. 1B, day 2), transformation of the Fe protein into the inactive form was accelerated (Fig. 1C, day 2) and proceeded up to complete modification of all Fe protein present. Under strong light limitation and very low glycogen (after a 3-day cultivation), traces of the modified form were already present in the light (t = 0). The activity was lost quickly after darkening, and the Fe protein was almost completely modified within less than 1 h. The results indicate that modification of half of the Fe protein is associated with loss of most enzyme activity and that the completion of modification adds little to further inactivation.

Protection of nitrogenase by addition of fructose. *A. variabilis* is able to efficiently use fructose for heterotrophic growth (14). Fructose (1 mM) was added 5 to 10 min before darkening to obtain additional evidence for the protective effect of photosynthates on nitrogenase alteration. The addition of fructose delayed the activity decrease and enzyme modification in darkened, aerobic cultures (Fig. 2). Fructose was consumed within 3 h of dark incubation, a fraction of it being used for build-up of glycogen (Fig. 3). After consumption of fructose, the glycogen content decreased. Under heterotrophic (dark) conditions, the altered form of nitrogenase reductase appeared at a lower glycogen content (2 mg/mg of Chl) than in autotrophic cultures (Fig. 1).

Influence of oxygen and respiration. When oxygen was excluded during dark treatment of a culture grown for 2 days (low glycogen content), no modification was observed in samples removed under anaerobiosis and frozen immediately after concentration on a filter (the exposure to air could be limited to 1 min; Fig. 3B). Samples for nitrogenase assays were removed under identical conditions. Nitrogenase activity decreased slowly over a 7-h incubation period when the assays were performed in a hydrogen atmosphere but without DCMU to inhibit photosynthetic oxygen evolution (microaerobic conditions; Fig. 3A, upper trace). When assayed in presence of air, the decrease in activity was greater (Fig. 3A, middle trace) but was still less than the activity switch-off observed in the culture bubbled with air during dark treatment (Fig. 3A, lower traces). The results indicate that

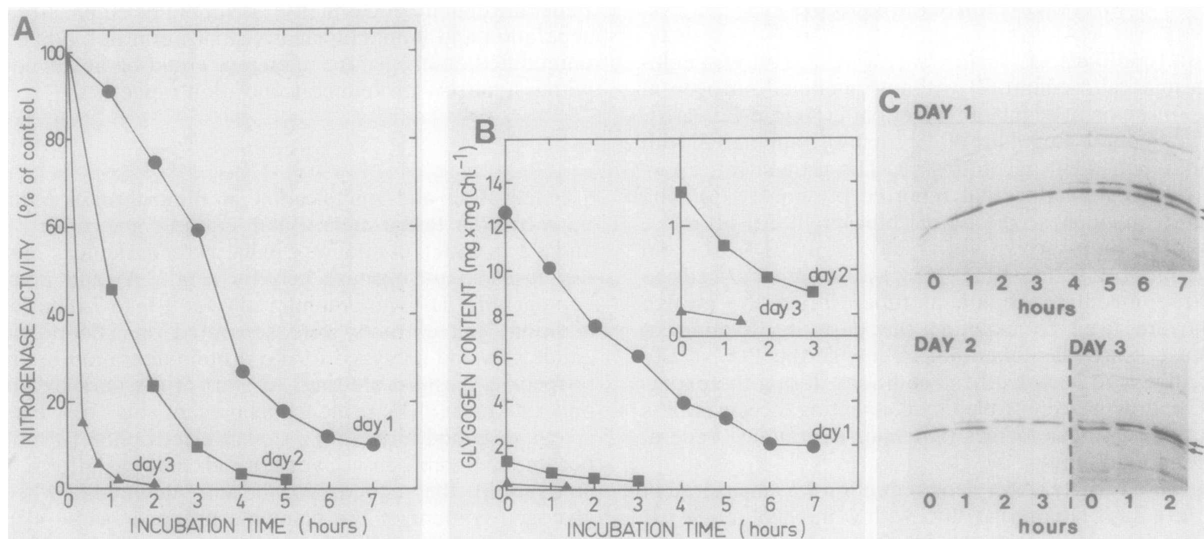

FIG. 1. Influence of darkness on nitrogenase activity, glycogen content, and dinitrogenase reductase modification. (A) Effect on nitrogenase activity. Cultures grown for 24 h (day 1), 48 h (day 2) and 72 h (day 3) were darkened. Samples were removed, and nitrogenase was assayed by acetylene reduction in the light under air plus C_2H_2 over a 15-min period. Nitrogenase activity (micromoles of C_2H_4 produced per milligram of Chl per hour) and Chl concentration (micrograms per milliter), respectively, of the cultures were as follows: day 1, 57.1 ± 2.1 and 3.9; day 2, 37.5 ± 3.3 and 14.6; and day 3, 15.4 ± 1.4 and 32.8. (B) Dissimilation of glycogen in the darkened cultures described above. Glycogen was determined after KOH extraction and enzymatic digestion, using a coupled enzymatic assay for glucose. The insert shows the decay of glycogen in light-limited cultures (days 2 and 3). (C) Western blots of samples removed from the darkened cultures described for panel A. The samples were concentrated on filters and extracted with boiling SDS buffer. A sample equivalent to 1 μg of Chl was used per slot of the SDS-polyacrylamide gel. An antibody prepared against SDS-denatured dinitrogenase reductase of *A. variabilis* was used for immunological identification. The bands indicated by arrows represent the active Fe protein monomer (lower band) and the inactive (modified) protein monomer (upper band). Traces of dimers and degradation products of dinitrogenase reductase are seen on several blots above and below the monomers. The bending of the bands results from increased conductivity at the edges of the gels, which could not be avoided with the instrument used. The blots have been cut to show the Fe protein subunits only.

the unmodified Fe protein exhibits an increasing sensitivity to oxygen evolved during or present in the assay. It is noteworthy that activity of the modified enzyme (after dark incubation in the presence of air) did not differ significantly when measured under hydrogen or air (Fig. 3A, both lower traces).

To further obtain evidence for a role of oxygen in the dark modification of dinitrogenase reductase, we used a glycogen-rich culture (day 1 of cultivation) and inhibited respiration by KCN. A 50 μM concentration of KCN was sufficient to block 90% of oxidase activity of isolated heterocysts (data not shown). KCN greatly accelerated the decay of nitrogenase activity and enzyme modification (Fig. 4). With KCN present, half of the Fe protein was modified within 1 h, whereas the control reached this state after a 4-h dark treatment. Addition of KCN to an illuminated culture was less effective in causing modification (data not shown), indicating that a detoxification process is operative in the light. Conceivably, carbohydrates stored as glycogen prevent dark modification when respiration is operative.

Inactivation by DCMU and CO_2 limitation in the light. Modification and inactivation were also achieved in illuminated cultures under air when photosynthesis was inhibited by DCMU (4 μM) or by a decrease of CO_2 from 1.5% (vol/vol) to the air level (Fig. 5). The decrease in nitrogenase activity with DCMU present exceeded that of a darkened culture at a similar physiological stage (Fig. 1A, day 2) and resembled the fast decay of a culture that had reached strong light limitation after a 3-day growth period (Fig. 1A, day 3). Reduction of the CO_2 supply was less efficient in causing inactivation and alteration of dinitrogenase reductase (Fig. 5; cf. Fig. 1A, day 2). The inefficiency of CO_2 limitation may be due to accumulated inorganic carbon, which allows for a limited continuation of photosynthesis (3).

Reversibility of Fe protein alteration and reactivation of nitrogenase. Complete modification of the Fe protein was obtained by aerobic dark treatment (2.5 to 3.75 h) of cultures grown for 2 days (Fig. 1C, day 2). Reactivation of dark-inactivated, modified nitrogenase occurred after illumination or addition of fructose. In the presence of oxygen, a short lag phase was observed during light and dark activation that was not seen under anaerobic reactivation in the light (Fig. 6A). Activation by light resulted in a higher nitrogenase activity than did activation with 1 mM fructose in the dark. Furthermore, in the dark, nitrogenase activity declined after consumption of fructose (Fig. 3A). Inhibition of photosynthesis by DCMU prevented reactivation in air, but reactivation occurred without a lag phase when the culture was kept anaerobic by gassing with hydrogen (Fig. 6A) or argon (not shown) in the presence of DCMU. The immunoblots (Fig. 6B) indicate that demodification was completed within 1 h (anaerobic reactivation) or 2 h (aerobic reactivation), whereas activity measured as acetylene reduction continued to increase over 3 h until the original activity was reached. This result indicates that de novo synthesis of nitrogenase may account for part of the recovered activity.

Inhibition of ammonium switch-off by omission of oxygen. Rapid entry of ammonia at alkaline pH (ammonia inundation) causes switch-off of in vivo nitrogenase activity and a nearly complete loss of extractable nitrogenase activity (34). Immunological analysis showed modification of half of the Fe protein present (36). In contrast to modification induced by C starvation, the ammonia switch-off was operative in glycogen-rich cultures (day 1 of cultivation) and in the

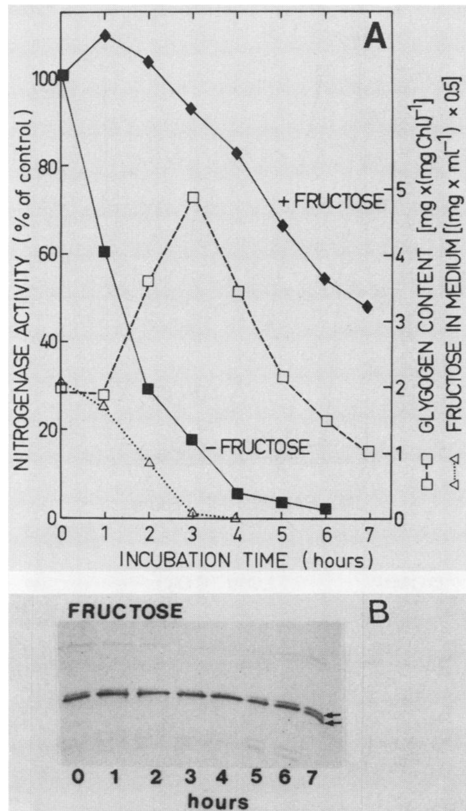

FIG. 2. Influence of fructose on nitrogenase activity and modification of dinitrogenase reductase in darkened cultures. (A) Enzyme assays. A culture grown for 48 h and equivalent to 17.4 μg of Chl per ml in an autotrophic medium was supplied with 1 mM fructose 5 min before darkening. A control (14.1 μg of Chl per ml) was darkened without fructose. Nitrogenase activity was assayed in the light under air-C_2H_2. Activities (100%) at the start of the dark incubation were 33.6 ± 1.8 and 36.6 ± 3.1 μmol of C_2H_4 per mg of Chl per h in the cultures with and without fructose, respectively. In the culture supplemented with fructose, the carbohydrate of the medium and glycogen of the filaments were determined by coupled enzymatic assays. (B) Western blots of cells removed every hour from the darkened tubes with (+) fructose.

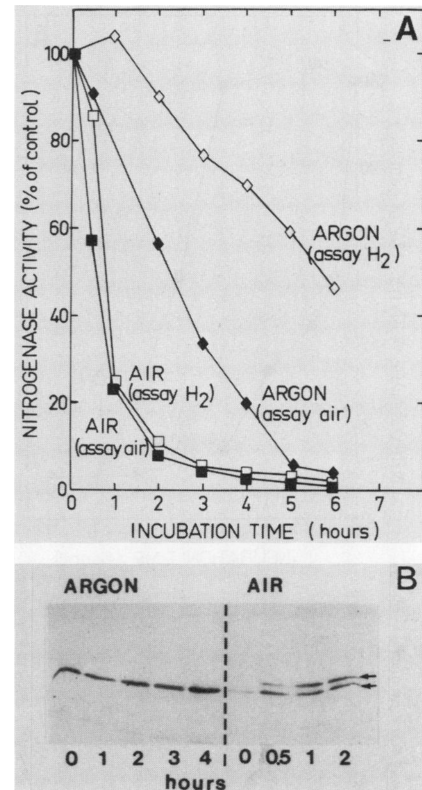

FIG. 3. Comparison of nitrogenase activity upon dark anaerobic (argon) and dark aerobic (air) treatment. (A) Enzyme assays. Cultures grown for 48 h (argon and air, respectively, equivalent to 16.0 and 16.9 μg of Chl per ml) were darkened and treated either with air-CO_2 (98.5/1.5% [vol/vol]) or with argon. Nitrogenase assays were performed in the light with gas phases of either air-C_2H_2 (assay air) or H_2-C_2H_2 (assay H_2). Initial activities (micromoles of C_2H_4 per milligram of Chl per hour) were 45.6 ± 2.1 (assay H_2) and 36.8 ± 3.1 (assay air) in the argon-treated cultures 45.6 ± 2.1 (assay H_2) and 36.8 ± 3.1 (assay air) in the argon-treated culture and 35.3 ± 3.4 (assay H_2) and 31.6 ± 2.4 (assay air) in the air-treated culture. (B) Western blots of filaments removed at 60-min intervals from darkened cultures treated with either argon or air-CO_2.

presence of fructose (Table 1; Fig. 7). The ammonia switch-off disappeared under anaerobic conditions (Table 1); no modification was observed during a 30-min incubation (Fig. 7). The anaerobic atmosphere was critical, since modification was observed neither in an argon atmosphere nor in the presence of hydrogen. The partial inhibition of nitrogenase activity seen in Table 1 is attributed to uncoupling of photophosphorylation (34).

Effect of carbamoyl phosphate. Carbamoyl phosphate is an intermediate in arginine and cyanophycin biosynthesis that is derived from glutamine. It was reported to inhibit nitrogenase activity and suggested to be a regulatory intermediate in heterocystous cyanobacteria. Since carbamoyl phosphate is labile and degradation products like the CNO radical also inhibit nitrogenase (19), a carbamoyl phosphate concentration that inhibits nitrogenase within 30 min was considered to be specific in *Anabaena cylindrica* (19). A similar inhibition of nitrogenase was observed with our *Anabaena* strain (Table 2). However, no modification of nitrogenase reductase was found (Fig. 7), indicating that a different mechanism is involved in the inhibition by carbamoyl phosphate.

DISCUSSION

Under autotrophic growth conditions, carbohydrates are required in heterocysts for the maintenance of nitrogenase activity, for respiratory oxygen protection, and to produce acceptors for ammonia. Thus, C starvation caused by a decrease of light intensity, CO_2 concentration, or darkening may affect nitrogenase activity either by reductant and ATP limitation, oxygen inactivation, or accumulation of the products of N assimilation. As shown by our experiments, nitrogenase activity decreases and remains inactive under C starvation even when assayed over a short period (15 min) in the light and under hydrogen (Fig. 3), conditions that efficiently support reductant and ATP supplies of nitrogenase in isolated heterocysts (7, 8). Accordingly, the decrease in activity indicates that the amount of active enzyme has decreased. In this report, we document for the first time that the inactivation observed in the presence of oxygen after darkening (Fig. 1) and under C starvation in the light (Fig. 5) is accompanied by a reversible modification of *Anabaena* dinitrogenase reductase. The onset of the modifying process is delayed by endogenously stored glycogen (Fig. 1). In this

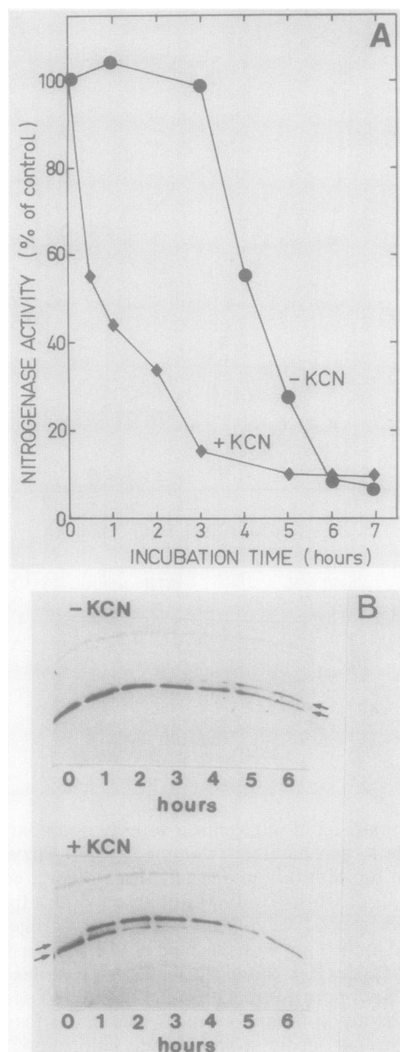

FIG. 4. Influence of KCN on nitrogenase activity and dinitrogenase reductase modification. (A) Enzyme assays. Glycogen-rich cultures grown for 25 h in the light were darkened, and one culture was supplemented with 50 μM KCN. Samples were assayed for nitrogenase activity in the light under air-C_2H_2. Initial activities (micromoles of C_2H_4 per milligram of Chl per hour) were 52.4 ± 4.2 (with KCN) and 62.4 ± 3.5 (without KCN), and chlorophyll concentrations (micrograms per milliliter) were 3.26 (with KCN) and 3.19 (without KCN). (B) Western blots of samples removed each hour from the cultures described above.

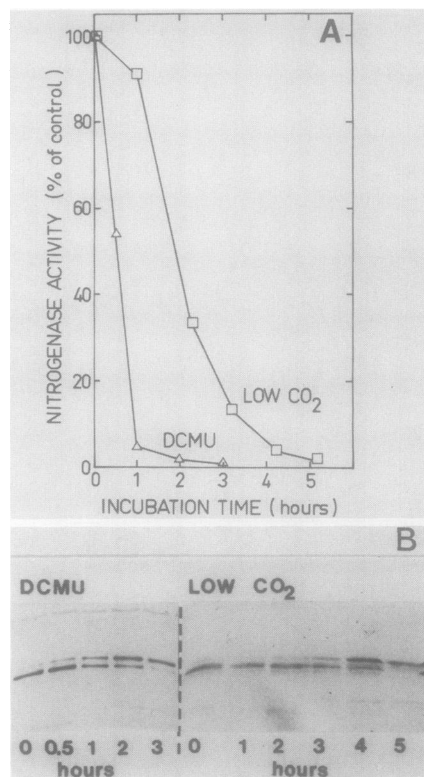

FIG. 5. Influence of DCMU and CO_2 limitation on nitrogenase activity and modification of dinitrogenase reductase. (A) Enzyme assays. A culture grown for 50 h (18.9 μg of Chl ml^{-1}) under air-CO_2 was incubated under air without additional CO_2 (low CO_2). Initial light-stimulated nitrogenase activity (100%) was 32.8 ± 1.6 μmol of C_2H_2 mg of Chl^{-1} h^{-1}. Another culture (20.2 μg of Chl ml^{-1}) was supplemented with 4 μM DCMU. Activity without the inhibitor (100%) was 33.4 ± 3.1 μmol of C_2H_2 mg of Chl^{-1} h^{-1}. Nitrogenase assays were performed every hour with an air-C_2H_2 gas phase. (B) Western blots of samples removed each hour from the cultures described above.

case, the modification process starts after the glycogen content of the filaments has dropped below 4 mg mg of Chl^{-1}. With a lower initial glycogen content, modification starts immediately after inhibition of photosynthesis. The results are in accordance with a previously reported threshold value of 3.6 mg of glycogen mg of Chl^{-1}, above which nitrogenase resists oxygen inactivation during dark incubation (9).

With exogenously supplied fructose, nitrogenase modification was prevented in darkened cultures carrying a low initial glycogen content (Fig. 2). This finding further supports the conclusion that carbohydrates are effective in regulating modification, whereas darkening itself is not a signal.

No modification of dinitrogenase reductase takes place under dark anaerobic conditions (Fig. 3B). This precludes a regulatory function of a (low) ATP/ADP ratio in Fe protein modification, as the energy charge of heterocysts remains at its minimum under dark anaerobic conditions (7, 8). Similarly, adenylate pools were not found to be instrumental in the regulation of *Rhodospirillum* enzyme modification (20, 28).

Although no modification was observed during anaerobic dark pretreatment of low-glycogen cultures (Fig. 3B, argon), enzyme activity decreased (Fig. 3A, two top curves). In these assays, greater inhibition of nitrogenase was observed under air than under hydrogen, indicating that the unmodified nitrogenase becomes increasingly oxygen sensitive during dark pretreatment under argon. On the other hand, no significant difference was observed in nitrogenase assays under air and hydrogen when dark pretreatment took place in air (Fig. 3A, two bottom curves) after modification of half of the Fe protein (Fig. 3B, air). This finding supports the suggestion by Smith et al. (39) that the modified nitrogenase, having low (or negligable) enzyme activity, is less affected by oxygen. The sensitivity to oxygen of unmodified nitrogenase in the activity assay explains the apparent lack of a strict correlation between subunit composition and loss of whole-cell activity (for example, Fig. 1, day 1). It should be mentioned that a strict correlation was demonstrated in

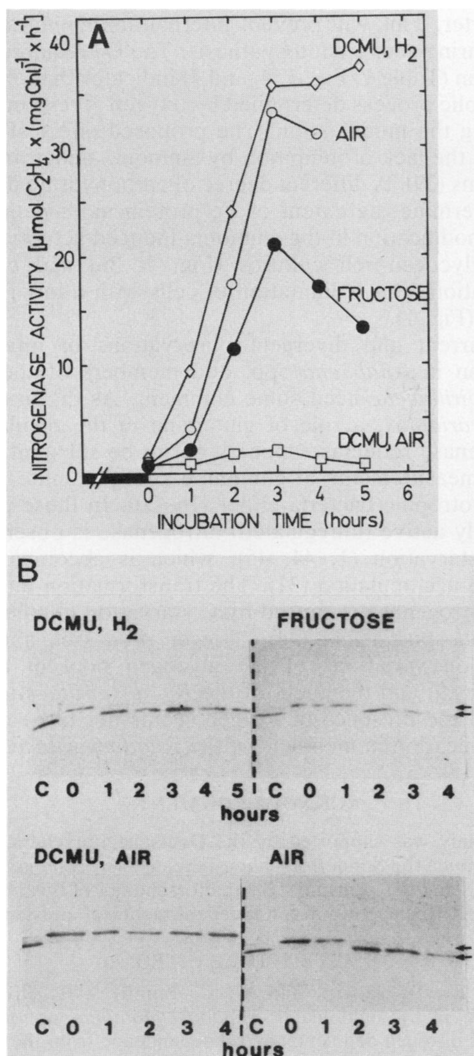

FIG. 6. Reactivation of nitrogenase after dark inactivation. (A) Enzyme assays. Cultures grown for 42 to 48 h (2 days) were inactivated by dark aerobic treatment for 2.5 to 3.75 h. At the start (t = 0), reactivation was initiated either by addition of fructose (dark) or by illumination. When indicated, 2.5 µM DCMU was added before illumination. During reactivation, cultures were treated with either air-CO_2 or H_2. All assays were performed in the light. Chl concentrations (micrograms per milliliter) were 14.0 (H_2 plus DCMU), 16.0 (air), 17.5 (air plus DCMU), and 19.0 (fructose). (B) Western blots of samples removed each hour during activation from the culture described above. Lane C, Control, dinitrogenase reductase monomers before dark treatment.

glycogen-rich cells, using ammonia as a trigger of modification (36).

KCN applied at 50 µM was low enough to avoid interference with enzymes other than cytochrome oxidase, which is present in high amounts in heterocysts (11). Fe protein modification and concomitant loss of nitrogenase activity were promoted by KCN in a 1-day culture despite the presence of a high glycogen level (Fig. 4). Obviously, carbohydrates prevent Fe protein modification essentially through supply of reductants for respiratory oxygen consumption.

In cyanobacteria, NH_4Cl added to a culture at pH 7 causes a slow decrease of nitrogenase activity that was previously

TABLE 1. Nitrogenase switch-off after addition of ammonia[a]

Addition	Nitrogenase activity[b]		% Inhibition of control (no. of replicates)
	Control	NH_4Cl (1.2 mM)	
Air	71.2 ± 13.7	3.6 ± 2.5	95 (6)
Air, 1 mM fructose	63.0 ± 9.3	7.8 ± 3.8	88 (3)
Argon, 4 µM DCMU	122.3 ± 19.8	56.4 ± 9.7	54 (6)
Hydrogen, 4 µM DCMU	140.2 ± 23.2	84.1 ± 12.5	40 (6)

[a] Cultures grown for 1 day (glycogen rich) were harvested and suspended (at 20 µg of Chl ml^{-1}) in 25 mM (3-cyclohexylamino)-1-propanesulfonic acid buffer (pH 10) just before activity measurement. Fructose was added to the culture 2 h before ammonia treatment. Nitrogenase modification was analyzed by immunospecific Western blotting of samples removed 20 to 30 min after the addition of ammonia (Fig. 7).
[b] Calculated after 15 to 30 min of incubation in the light and expressed as micromoles of ethylene produced per milligram of Chl per hour.

ascribed to enzyme turnover (10, 13). The inhibitory effect of ammonia on nitrogenase synthesis and activity is absent under anaerobic conditions (29) and, at neutral pH, in N-starved cells (25, 33), indicating that carbohydrates counteract the repressive effect of ammonium or derivatives (10). At pH 10, the ammonia switch-off resulting from ammonia inundation is operative in the presence of a high glycogen level and oxygen (Table 1; Fig. 7). The participation of oxygen points to a similar regulatory mechanism underlying the switch-off induced by either ammonia or C starvation. Under C starvation, modification was induced after cessation of a reductant supply in the presence of oxygen. Ammonia inundation strongly diverts carbohydrates for ammonia assimilation, causing a decreased reductant supply of respiration and leading to modification.

This conclusion requires revision of the previous suggestion assigning glutamine a role in the regulation of enzyme modification (34–36). This assumption was based on the effect of MSX in eliminating modification after ammonia addition at pH 10 (34), since this inhibitor prevents formation of glutamine (40). Furthermore, methylamine not being converted to glutamine by glutamine synthetase does not induce modification (35). Our present conclusion is based on the following reasoning.

(i) MSX treatment as reported in reference 34 causes a strong accumulation of glycogen (after a 12-h treatment with 20 µM MSX, a 1-day culture contained three times more glycogen than did the untreated control [A. Ernst et al., manuscript in preparation]). Decreased ammonia assimilation (due to MSX) and an increased level of carbohydrates will ensure a reductant supply for respiration and therefore prevent modification.

TABLE 2. Nitrogenase switch-off after addition of carbamoyl phosphate[a]

Cultivation time (h)	Nitrogenase activity[b]		% Inhibition of control
	Control	Carbamoyl phosphate (10 mM)	
30	59.9 ± 5.0	19.9 ± 4.5	67
55	46.1 ± 3.0	8.5 ± 3.5	82

[a] Cultures (three replicates) grown for the times indicated were suspended in 20 mM morpholinoethanesulfonic acid buffer (pH 6.0; 8 µg of Chl ml^{-1}). Nitrogenase modification was analyzed by immunospecific Western blotting of samples removed 20 to 30 min after the addition of ammonia (Fig. 7).
[b] See Table 1, footnote b.

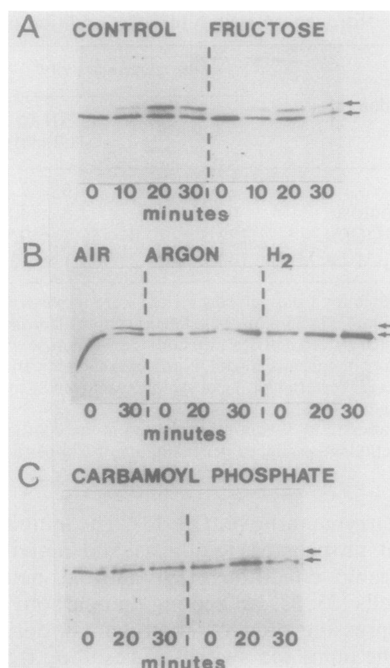

FIG. 7. Status of the Fe protein after ammonia treatment in the presence of fructose, under aerobic or anaerobic conditions, and after addition of carbamoyl phosphate. Shown are Western blots of samples removed at the indicated intervals. Cells were treated as described for Table 1.

(ii) Methylamine, not being metabolized, will affect neither the carbohydrate pool nor respiratory activity and does not cause modification.

(iii) When hydrogen is present during the ammonia switch-off experiment, additional reductant and ATP can be supplied by the uptake hydrogenase present in heterocysts. This allows for increased ammonia assimilation via glutamine synthetase and glutamate synthase. A higher glutamine/glutamate ratio is expected to result when 2-oxoketoglutarate, the ammonia acceptor in the glutamate synthetase reaction, becomes limiting (10). However, no modification is observed after ammonia addition under hydrogen or argon (Fig. 7).

(iv) An accumulation of glutamine upon darkening seems unlikely because the glutamine synthetase activity of cyanobacteria decreases in the dark (37).

(v) Reactivation of nitrogenase in cultures depleted of carbohydrates occurs without photosynthetic C assimilation or feeding of fructose (Fig. 6), which would be necessary if an inhibitory surplus of glutamine had to be removed.

(vi) Carbamoyl phosphate inhibits in vivo nitrogenase activity without Fe protein modification (Table 2; Fig. 7).

Protection against modification by the C supply allows one to interpret the different responses of nitrogenase upon ammonia addition at pH 7 and 10 and explains the divergent results reported for ammonia inhibition of nitrogenase at high light intensity in references 35 and 45. At pH 10, CO_2 assimilation is limited by inorganic carbon (3); the ammonia inundation at this pH promotes depletion of the carbohydrate pool in the light as a result of the reductant requirement of ammonia assimilation. At pH 7 (and in the presence of 4% CO_2 [45]), a greater CO_2 supply improves photosynthesis and ammonia assimilation; furthermore, at this pH the ammonia uptake is restricted by the capacity of an ammonia transporter. This will prevent internal ammonium accumulation during active photosynthesis. The O_2 requirement for regulation (Table 1; Fig. 3, 4, and 7) indicates that oxygen or a metabolic process determined by oxygen is responsible for triggering the modification. The proposed effect of oxygen explains the lack of inhibition by ammonia under anaerobic conditions (29). A different degree of carbohydrate depletion may determine the extent of Fe protein modification, i.e., partial modification in the ammonia-induced activity switch-off in glycogen-rich cultures (Fig. 7; 36) and complete modification after C limitation of cells with a low glycogen content (Fig. 1).

Concurrent and divergent observations of nitrogenase regulation in *Anabaena* spp. and members of the family *Rhodospirillaceae* need some comment. As discussed here for *A. variabilis*, a role of glutamine in the regulation of dinitrogenase reductase appears not to be substantiated by careful measurements of glutamine and glutamate pools in the phototrophic bacteria either (16, 20). In these procaryotes, fully active (unregulated) nitrogenase is observed under N starvation (1, 44, 45), which is accompanied by glycogen accumulation (31). The transformation into a regulated nitrogenase is caused by C starvation in members of the *Rhodospirillaceae* (44). Trigger processes apparently depend on variations of the glycogen pool in both *A. variabilis* (9) and members of the *Rhodospirillaceae* (5). In contrast, the presence of oxygen seems not to be required for modification in members of the *Rhodospirillaceae*.

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