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Short-Term Nitrate (Nitrite) Inhibition of Nitrogen Fixation in *Azotobacter chroococcum*

FRANCISCO J. CEJUDO AND ANTONIO PANEQUE*

Departamento de Bioquímica, Facultad de Biología y C.S.I.C., 41080 Sevilla, Spain

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Nitrate-grown *Azotobacter chroococcum* ATCC 4412 cells lack the ability to fix N_2. Nitrogenase activity developed after the cells were suspended in a combined nitrogen-free medium and was paralleled by a concomitant decrease in nitrate assimilation capacity. In such treated cells exhibiting transitory nitrate assimilation and N_2-fixation capacity, nitrate or nitrite caused a short-term inhibitory effect on nitrogenase activity which ceased once the anion was exhausted from the medium. The glutamate analog L-methionine-DL-sulfoximine, an inhibitor of glutamine synthetase, prevented inhibition of nitrogenase activity by nitrate or nitrite without affecting the uptake of these antions, which were reduced and stoichiometrically released into the external medium as ammonium. Inhibition of nitrogenase by nitrate (nitrite) did not take place in *A. chroococcum* MCD1, which is unable to assimilate either. We conclude that the short-term inhibitory effect of nitrate (nitrite) on nitrogenase activity is due to some organic product(s) formed during the assimilation of the ammonium resulting from nitrate (nitrite) reduction.

Repression of nitrogenase synthesis by different sources of nitrogen, such as ammonium, nitrate, or urea, has been known for many years (10, 21, 31, 32). Furthermore, an immediate inhibition of nitrogenase activity in whole organisms, but not in extracts, in response to exogenous ammonium ions has been described to occur in photosynthetic bacteria (1, 2, 4, 33) and in some azotobacters (7, 9, 13, 17). Inhibition and reactivation of nitrogenase takes place shortly after the addition or depletion of ammonium, respectively, and it is known as the switch-off/switch-on effect.

To explain the short-term effect of ammonium on nitrogen fixation in *Azotobacter vinelandii*, Laane et al. (17) have suggested that ammonium uptake switches off the flow of reducing equivalents to nitrogenase by lowering the electrical component of the proton motive force. Aside from this substrate-dependent inactivation, we have reported that in *Azotobacter chroococcum* some organic product derived from ammonium assimilation may be the actual inhibitor of nitrogenase (7).

So far, no effects of either nitrate or nitrite on nitrogenase activity have been reported comparable to the short-term ammonium inhibition discussed above (21).

In this communication we present results showing for the first time that nitrate and nitrite also exert a rapid and reversible inhibitory effect on nitrogenase. The results indicate that this inhibition is mediated by metabolic intermediates generated from the ammonium formed in the enzymatic reduction of those anions.

MATERIALS AND METHODS

Chemicals. ADP, MOPS (morpholinopropanesulfonic acid), L-methionine-DL-sulfoximine (MSX), and L-glutamic dehydrogenase (type II, from bovine liver) were purchased from Sigma Chemical Co., St. Louis, Mo. NADPH was from Boehringer, Mannheim, Federal Republic of Germany. All other chemicals were of analytical grade and were acquired from Merck (Darmstadt, Federal Republic of Germany).

Organisms and culture conditions. *A. chroococcum* ATCC 4412 (from the Valencia University Collection, Valencia, Spain) and *A. chroococcum* MCD1 (a gift of R. Robson, Brighton, U.K.) were grown heterotrophically on nitrogen-free Burk medium supplemented with sucrose (1%) as the sole energy and carbon source. When nitrate-grown cells were used, 10 mM KNO_3 was added to the medium. Growth conditions were as previously described (7).

Mid-logarithmic-growth cells (A_{560} of ca. 0.5) were used for each experiment. The cells were harvested at room temperature by centrifugation at 1,000 × *g* for 10 min, washed with nitrogen-free medium, and resuspended to a cell density of about 70 μg of cell protein per ml of the same medium.

Analytical procedures. For nitrogenase activity and nitrate or nitrite uptake assays, three 20-ml samples of the above-described cell suspension were preincubated at 30°C with shaking (100 strokes min^{-1}) in 50-ml sealed conical flasks. Two of the samples were used for nitrogenase assay and the third one was used for nitrate or nitrite uptake assay. At zero time the atmosphere in each flask was made 10% in acetylene. Nitrogenase activity was determined by analyzing the appearance of ethylene in the flask atmosphere. Nitrate and nitrite consumption and ammonium released were determined by estimating the concentration of the corresponding ion in the medium. This estimation was done with samples of the cell suspension after the cells had been rapidly removed by filtration in a Millipore system with glass microfiber Whatman paper.

Nitrate reductase activity was determined in situ as follows. A 5-ml sample of cell suspension was centrifuged at 9,000 × *g* for 5 min at 4°C, washed once with 50 mM MOPS-KOH buffer (pH 7.5), and suspended in 0.5 ml of the same buffer supplemented with 0.25 mM EDTA. A portion of this suspension was added to the reaction mixture to determine nitrate reductase activity with dithionite reduced-methyl viologen as the electron donor (12) and 20 μg of alkyltrimethylammonium bromide mixture added to permeabilize the cells. Activity units correspond to micromoles of nitrite produced per minute.

Nitrite reductase activity was assayed in situ using the same cell suspension as used for nitrate reductase activity determination. A sample of the cell suspension was added to

* Corresponding author.

TABLE 1. Nitrate-induced changes in nitrogenase activity and nitrate uptake activity levels in diazotrophically grown *A. chroococcum* ATCC 4412 cells[a]

Incubation time (h)	Nitrate uptake (nmol mg^{-1} of protein min^{-1})	Nitrogenase activity (%)
0	0	100
1	0	95
2	37.5	53
3	66.2	55

[a] *A. chroococcum* cells grown under nitrogen-fixing conditions, washed, and resuspended in N-free medium, were supplied with 5 mM KNO_3 (final concentration) at zero time, and nitrate uptake rate and nitrogenase activity were determined at the indicated times. Nitrogenase activity of 100% corresponds to 220 nmol of C_2H_4 mg^{-1} of protein min^{-1}.

the reaction mixture for nitrite reductase activity as described by Ramirez et al. (22). The reaction mixture also contained 20 μg of alkyltrimethylammonium bromide per ml. Activity is expressed as micromoles of nitrite removed per minute.

Ethylene was measured with a Pye-Unican 204 gas chromatograph equipped with a column filled with Porapak Q and a flame ionization detector.

Nitrate was determined spectrophotometrically at 210 nm in acid solution (6). Nitrite was estimated by the method of Snell and Snell (27). Ammonium determination was carried out with the glutamate dehydrogenase assay (8). Whole-cell protein was estimated by the procedure of Lowry et al. as modified by Markwell et al. (18), using bovine serum albumin as standard.

RESULTS

Preparation of *A. chroococcum* cells with N_2-fixing activity and nitrate (nitrite) assimilation ability. Table 1 shows changes in nitrogenase activity levels and nitrate assimilation rates in *A. chroococcum* cells that had been grown in combined nitrogen-free medium and then transferred to nitrate-containing medium. Cells grown under N_2-fixing conditions (zero time) were not able to assimilate nitrate. On incubation in the presence of nitrate, development of the assimilatory nitrate uptake took place, accompanied by a decrease in nitrogenase activity. After 2 to 3 h, cells exhibited an appreciable nitrate assimilation rate (40 to 66 nmol of NO_3^- mg^{-1} of protein min^{-1}) and nitrogenase activity was about 50% of the original value.

Alternatively, nitrate-grown cells diminished their nitrate uptake ability simultaneously with derepression of nitrogenase development when transferred to combined nitrogen-free growth medium. Figure 1 shows the loss of nitrate uptake ability and development of nitrogenase activity; after 30 min of incubation, cells displayed both nitrate uptake activity and nitrogenase activity in assayable amounts.

Short-term nitrate or nitrite inhibition of nitrogen fixation in *A. chroococcum*. As we proposed that, in *A. chroococcum*, the short-term ammonium inhibition of nitrogenase activity is due to products derived from ammonium assimilation (7), the next group of experiments were designed to determine whether nitrate or nitrite inhibited nitrogenase activity in *A. chroococcum* cells exhibiting the ability to assimilate nitrate and fix nitrogen simultaneously. Addition of low amounts (0.3 mM final concentration) of KNO_3 to such cells promoted a short-term inhibition of nitrogenase activity (Fig. 2). The inhibition was reversible, since it ceased once the added nitrate was used up by the cells. Although not shown, nitrate

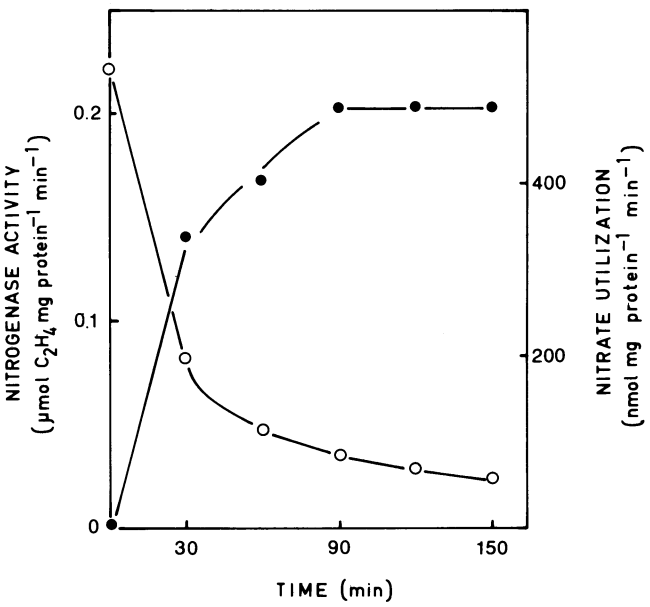

FIG. 1. Time course of nitrogenase development and loss of nitrate uptake activity in *A. chroococcum*. Nitrate-grown cells, washed and suspended in N-free medium, were incubated at 30°C under growth conditions described in the text. At the indicated times, nitrogenase activity (●) and nitrate uptake activity (○) were determined.

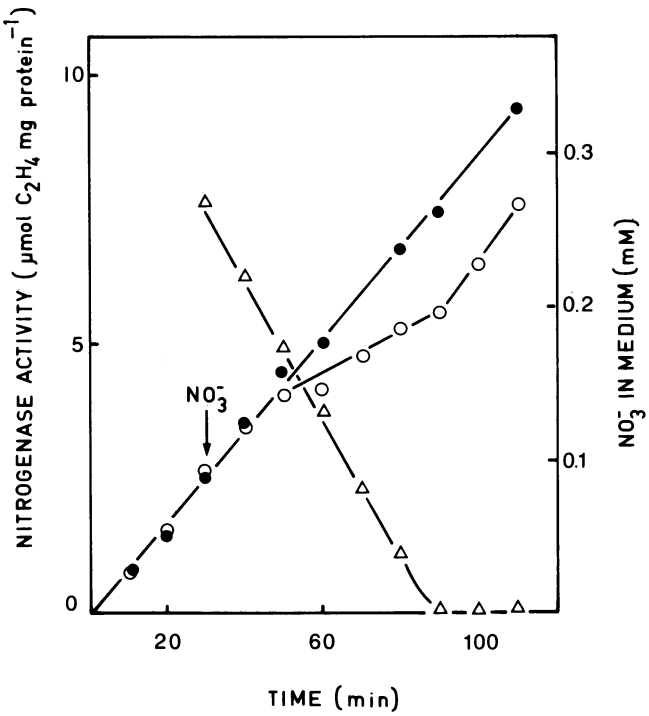

FIG. 2. Inhibition of nitrogenase activity by NO_3^- and its reversal after NO_3^- depletion. Nitrate-grown cells, washed and suspended in N-free medium, were incubated at 30°C under growth conditions. After 1 h, cells were assayed for nitrogenase activity in the absence (●) and in the presence (○) of 0.22 mM KNO_3, which was added when indicated. Nitrate disappearance from the medium was also followed (△).

inhibition of nitrogenase activity was independent of protein synthesis since no difference was observed in the presence of rifampin (100 μg ml^{-1}), an inhibitor of translation in procaryotic organisms.

The assimilation of nitrate requires its reduction to nitrite and ammonium so a similar effect of nitrite on nitrogenase activity in the described cells was expected. It could be seen that nitrite inhibition of nitrogenase activity is rather similar to that produced by nitrate (data not shown).

Similar experiments were carried out using cells that had been grown under nitrogen-fixing conditions and hence were incapable of taking up nitrate or nitrite (24). In these cells, none of the ions, at the concentration used (up to 0.3 mM), had any effect on nitrogenase activity in vivo (not shown).

These results strongly suggested that products from ammonium assimilation were responsible for either nitrate or nitrite inhibition of nitrogenase activity. To test whether the nitrate (nitrite)-promoted nitrogenase switch-off was due to the metabolism of the internally generated ammonium, experiments were carried out in *A. chroococcum* cells in which glutamine synthetase, the first enzyme involved in ammonium assimilation, had been inactivated by 15 min of prior incubation with 5 mM MSX. Nitrate had no effect on nitrogenase activity in MSX-treated cells (Fig. 3). However, nitrate uptake and reduction was not prevented, as nitrate disappeared from the medium and ammonium accumulated in it stoichimetrically (Fig. 3B). Similar results were found when nitrite was used instead of nitrate.

To further corroborate that the short-term nitrate inhibition of nitrogenase activity is due to metabolic intermediates generated from ammonium assimilation, *A. chroococcum* MCD-1 (26) was used. This strain is unable to use nitrate or nitrite as the sole nitrogen source under an Ar-O_2 atmosphere. When grown under air in the presence of nitrate, strain MCD-1 cells exhibited undetectable nitrate (nitrite) uptake and only negligible levels of nitrate reductase activity and nitrite reductase activity (10% of those exhibited by nitrate-grown strain ATCC 4412), whereas nitrogenase activity was similar to that found in *A. chroococcum* ATCC 4412 (0.166 μmol of C_2H_4 mg^{-1} of protein min^{-1}). As expected, the addition of nitrate (0.3 mM, final concentration) to *A. chroococcum* MCD-1 cells grown under an air atmosphere in the presence of nitrate had no effect on nitrogenase activity. However, as was the case for *A. chroococcum* ATCC 4412 (7), addition of low amounts of NH_4Cl (0.1 mM, final concentration) to strain MCD-1 which was actively reducing acetylene resulted in a rapid inhibition of this activity (not shown). This inhibition was reversible and was prevented by MSX.

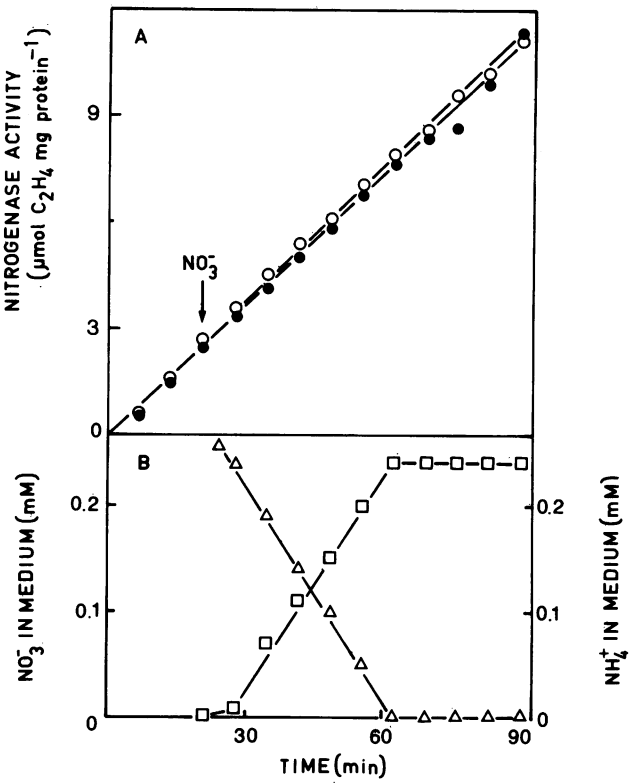

FIG. 3. Prevention of NO_3^- inhibition of nitrogenase activity by MSX. Nitrate-grown cells, washed and suspended in N-free medium, were incubated at 30°C under growth conditions. After 1 h, cells were supplied with 5 mM MSX and kept for 15 min with continous shaking (100 strokes min^{-1}). Cell suspension samples were then incubated in sealed conical flasks under an air atmosphere made 10% in acetylene, and (A) nitrogenase activity in the absence (●) and in the presence (○) of 0.26 mM KNO_3 and (B) nitrate disappearance from the medium (△) and ammonium appearance in the medium (□) were followed. Nitrate reductase activity at zero time was 50 mU mg^{-1} of protein.

DISCUSSION

The nitrogen-fixing, aerobic bacterium *A. chroococcum* ATCC 4412 is able also to use nitrate, nitrite, or ammonium as a nitrogen source. When cells were grown in the presence of some of these nitrogen compounds, no nitrogenase activity was detectable, and it is accepted that repression of nitrogenase is mediated by products generated in the assimilation of those ions (21).

Results presented in this work have shown that it is possible to get *A. chroococcum* cells with both nitrogenase activity and nitrate (or nitrite) assimilatory uptake activity. In these cells nitrate and nitrite induced a rapid inhibition of nitrogenase activity, the inhibition ceasing once nitrate or nitrite had been exhausted from the medium, as occurred in the ammonium-induced nitrogenase switch-off (7). Since the same results were reached in experiments carried out in the presence of rifampin, an inhibitor of translation, the restoration of activity involved the reuse of preexisting nitrogenase, not the synthesis of new enzyme.

Several lines of evidence have indicated that nitrogenase switch-off is strictly dependent on nitrate (nitrite) assimilation via ammonium ions. First, nitrate or nitrite at the concentration used had no effect on nitrogenase activity in cells lacking an assimilatory nitrate uptake system. In connection with these results, it is worth mentioning that nitrite also inhibited nitrogen fixation in whole *Rhizobium japonicum* bacteroids (16, 25) and cultured cowpea *Rhizobium* sp. (20), as well as in the photosynthetic bacterium *Rhodopseudomonas capsulata* (14). However, the reversibility of this effect and the mechanism of inhibition were not studied. In vitro inhibition of *A. vinelandii* nitrogenase by nitrate (28) and by nitrite (5) has been also described, but these effects clearly differ from that studied in the present work, as does the nitrite inhibition of nitrogenase in soybean bacteroids (30).

That ammonium, the product of nitrate reduction, is a required intermediate in the generation of the nitrogenase activity presumptive inhibitor(s) could be inferred from studies with the glutamate analog MSX. This glutamine synthetase inhibitor has been used to show nitrogenase

synthesis in the presence of excess ammonium (11, 29) or nitrate (23). MSX prevented nitrogenase activity inhibition by nitrate without impairing the ability of the cells to take up nitrate (nitrite) and reduce it to ammonium (Fig. 3A). Since the cation could not be incorporated into carbon skeletons, it was released into the medium (Fig. 3B). These facts clearly indicate that MSX-treated *A. chroococcum* cells escape nitrogenase activity regulation by nitrate because the production of ammonium derivatives is interrupted. Since the same treatment also abolished the ammonium-induced nitrogenase activity switch-off (7), the present data reinforce our proposal that nitrogenase activity regulation by ammonium is mediated by products from its assimilation rather than by ammonium itself.

Results obtained with *A. chroococcum* MCD1 confirm that nitrogenase inhibition by nitrate (nitrite) involves the assimilation of the anion. Since nitrogenase activity in strain MCD-1 cells grown in air was not affected by the presence of nitrate, the results presented in this work corroborate that nitrate repression of nitrogenase takes place via ammonium (19, 21) and agree with data showing that nitrate reductase-negative mutants of diazotrophs do not show nitrate repression of nitrogenase (15, 20, 28).

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