JOURNAL OF BACTERIOLOGY, Jan. 1981, p. 175-180
0021-9193/81/010175-06$02.00/0

Vol. 145, No. 1

Regulation of Nitrate Reductase Levels in the Cyanobacteria *Anacystis nidulans*, *Anabaena* sp. Strain 7119, and *Nostoc* sp. Strain 6719

ANTONIA HERRERO, ENRIQUE FLORES, AND MIGUEL G. GUERRERO*

Departamento de Bioquímica, Facultad de Biología y Consejo Superior de Investigaciones Cientificas, Universidad de Sevilla, Seville, Spain

The effect of the nitrogen source on the cellular activity of ferredoxin-nitrate reductase in different cyanobacteria was examined. In the unicellular species *Anacystis nidulans*, nitrate reductase was repressed in the presence of ammonium but de novo enzyme synthesis took place in media containing either nitrate or no nitrogen source, indicating that nitrate was not required as an obligate inducer. Nitrate reductase in *A. nidulans* was freed from ammonium repression by L-methionine-D,L-sulfoximine, an irreversible inhibitor of glutamine synthetase. Ammonium-promoted repression appears therefore to be indirect; ammonium has to be metabolized through glutamine synthetase to be effective in the repression of nitrate reductase. Unlike the situation in *A. nidulans*, nitrate appeared to play an active role in nitrate reductase synthesis in the filamentous nitrogen-fixing strains *Anabaena* sp. strain 7119 and *Nostoc* sp. strain 6719, with ammonium acting as an antagonist with regard to nitrate.

Ferredoxin-nitrate reductase from cyanobacteria catalyzes the reduction of nitrate to nitrite, the first step in the assimilatory reduction of nitrate (10, 11, 26). Ammonium, the end product of the nitrate-reducing pathway, behaves as a very effective antagonist of nitrate assimilation in procaryotic and eucaryotic algae, and different inhibitory effects of this compound on nitrate uptake, nitrate reductase activity, and nitrate reductase synthesis have been described (10, 24, 26).

Regulation of nitrate reductase synthesis in eucaryotic algae has been examined to some detail, and in most cases it has been proposed that ammonium acts as a repressor (8, 13, 25). Although the role of nitrate as an obligate inducer has been claimed, general agreement has been reached recently that this is not the case, since synthesis of NAD(P)H-nitrate reductase has been shown to take place either in nitrogen-free medium or in the presence of amino acids, both in green (8, 20, 23, 25) and in red algae (17).

Available information regarding control of nitrate reductase synthesis in procaryotic algae (cyanobacteria) is scarce and confusing. Whereas induction by nitrate has been reported to occur in both the unicellular strain *Agmenellum quadruplicatum* (21) and the filamentous nitrogen fixer *Anabaena cylindrica* (7, 14), repression by ammonium has also been claimed to take place in the unicellular strains *Anacystis nidulans* (11) and *Agmenellum quadruplicatum* (21).

In the experiments described below, we have attempted to determine the role of nitrate and ammonium in the regulation of nitrate reductase synthesis in representative strains of cyanobacteria, a unicellular non-nitrogen fixer and two filamentous nitrogen fixers. Significant differences have been found between both types of cyanobacteria with regard to the effect of nitrate on the synthesis of this enzyme. The glutamine synthetase inhibitor L-methionine-D,L-sulfoximine (MSX) has been used to show that ammonium metabolism is required for the repression of nitrate reductase formation by ammonium.

(A preliminary report of this investigation was presented at the 8th Congress of the Spanish Biochemical Society, Murcia, Spain, 1979, abstr. no. 36.)

MATERIALS AND METHODS

Organisms and culture condition. *A. nidulans* L 1402-1 was obtained from the Göttingen University Algal Culture Collection; *Nostoc* sp. strain PCC 6719 was from the Pasteur Institute Culture Collection; and *Anabaena* sp. strain 7119, previously classified as *Nostoc muscorum* 7119, was a gift of D. I. Arnon (Berkeley, Calif.). All of these cyanobacterial strains were grown photoautotrophically under continuous fluorescent illumination (25 W m^{-2}), at 39°C (*Anacystis*) or 30°C (*Nostoc* and *Anabaena*), in a stream of 5% CO_2 in air on a synthetic medium containing (millimoles per liter): KNO_3, 20; $MgSO_4$, 0.5; $CaCl_2$, 0.1; NaCl, 2; Na_2MoO_4, 0.004; K_2HPO_4, 12; EDTA, 0.5; $FeSO_4$, 0.5; $NaHCO_3$, 10. A supplement of 1 ml of a modified A_5

trace metal solution per liter was added (1), containing (millimoles per liter): H_3BO_3, 46; $MnCl_2$, 9; $ZnSO_4$, 0.77; and $CuSO_4$, 0.32. When $(NH_4)_2SO_4$ replaced KNO_3 as the nitrogen source, its final concentration was 10 mM for *A. nidulans* and 2 mM for both *Nostoc* sp. and *Anabaena* sp.

For the transfer of cells to a medium containing a nitrogen source other than that used for growth, cells were harvested by filtration on a Millipore HA 0.45-μm pore size filter, washed on the filter with a large volume of the new medium, and finally suspended to a density of 20 to 100 μg of cell protein per ml of culture.

Determination of nitrate reductase activity. For the estimation of cellular nitrate reductase activity levels, an in situ assay was used. To a 1-ml portion of cell suspension 20 μl of toluene was added. After continuous and vigorous shaking for 2 min, a portion of this preparation was added to a reaction mixture for nitrate reductase activity determination (11). The solution contained in a final volume of 1 ml: $NaHCO_3$-Na_2CO_3 buffer, pH 10.5, 100 μmol; KNO_3, 20 μmol; methyl viologen, 4 μmol; and 10 μmol of $Na_2S_2O_4$ in 0.1 ml of 0.3 M $NaHCO_3$. The reaction mixture was incubated for 5 min at 30°C, and nitrite was determined. Corrections were applied for the amount of nitrite present at zero time. Activity units correspond to μmol of nitrite produced per min.

Analytical methods. Nitrite was estimated by the method of Snell and Snell (18). Ammonium in the medium was determined, after removal of the cells, with glutamate dehydrogenase (3). Cellular protein was estimated by the Lowry procedure as modified by Bailey (2) after pretreating the cells with cold 10% trichloroacetic acid; bovine serum albumin was used as a standard.

Chemicals. ADP, MSX, rifampin, chloramphenicol, and L-glutamic dehydrogenase (type II; from bovine liver) were purchased from Sigma Chemical Co., St. Louis, Mo. NADPH was from Boehringer Mannheim Corp., New York, N.Y., and kanamycin was from Laboratorio Rether, Barcelona, Spain. Other chemicals were products of E. Merck AG, Darmstadt, Germany.

RESULTS

The cellular activity levels of ferredoxin-nitrate reductase found in *A. nidulans* cells grown on media containing nitrate as the nitrogen source were about 200 mU/mg of protein, whereas cells grown on ammonium exhibited only basal activity levels in the range of 5 to 10 mU/mg of protein. Figure 1 shows the development of nitrate reductase activity after transfer of ammonium-grown cells to a nitrate medium. An increase in nitrate reductase specific activity took place in the presence of nitrate, with the maximal level being reached in about 5 h. The specific activity of nitrate reductase remained stationary thereafter at a level corresponding to that typically found in nitrate-grown cells. The constancy of the specific activity level after 5 h shows that the increase in total nitrate reductase activity parallels that in protein content of the growing culture. Figure 1 also shows that there was a substantial increase in the cell growth rate between 3 and 4 h after transfer to nitrate medium once a significant level of nitrate reductase activity was attained. This might be taken as an indication that lower enzyme levels are not able to sustain a maximal growth rate on nitrate as the nitrogen source.

To assess whether this development of nitrate reductase activity was due to the presence of nitrate or merely to ammonium removal, the time course of changes in nitrate reductase activity upon transferring cells from ammonium to media containing either no nitrogen source, nitrate, ammonium, or both forms of inorganic nitrogen, was examined. As shown in Fig. 2,

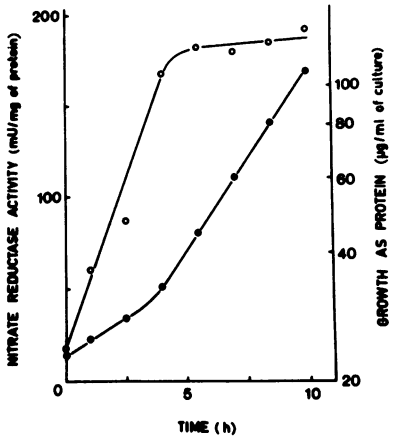

FIG. 1. *Development of nitrate reductase activity in A. nidulans upon transfer from ammonium to nitrate medium. Cells grown on ammonium were transferred to a medium containing 20 mM KNO_3.* (○) *Nitrate reductase specific activity;* (●) *protein.*

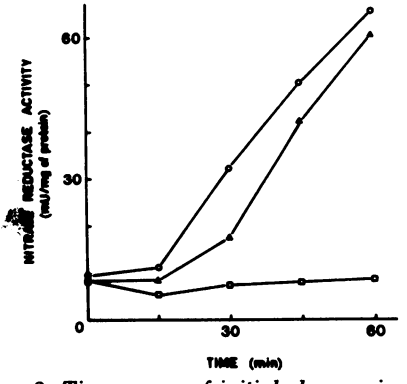

FIG. 2. *Time course of initial changes in nitrate reductase activity in A. nidulans upon transfer from ammonium to different media. Cells grown on ammonium were transferred to media containing either 20 mM KNO_3* (○)*, no nitrogen source* (△)*, or 10 mM $(NH_4)_2SO_4$ or NH_4NO_3* (□).

nitrate reductase activity also developed in a medium from which nitrate was absent, the kinetics of the activity increase being quite similar to that observed in the presence of nitrate. A lag period of about 15 min was always observed in both cases. On the other hand, the low initial nitrate reductase level remained unchanged in the presence of ammonium, even if nitrate was simultaneously present. These results indicate that the presence of nitrate is not an obligatory requirement for the development of nitrate reductase and also that the presence of ammonium overrides any positive effect of nitrate. It thus appears that the presence of ammonium is the factor which governs the establishment of low nitrate reductase activity levels.

Chloramphenicol and rifampin are specific inhibitors of protein synthesis which act at the stages of translation and transcription, respectively, and which have been reported to be effective in cyanobacteria (9). Results shown in Table 1 show that both inhibitors were equally effective in preventing the increase in nitrate reductase activity, which otherwise took place in their absence in both nitrate and nitrogen-free media. Analogous results (not shown) were obtained with kanamycin, another inhibitor of the translation step in procaryotes. Therefore, the existence of only basal activity levels in the presence of ammonium seems to point to repression of nitrate reductase synthesis. Kinetic studies of the short-term development of nitrate reductase in the presence of rifampin (data not shown) did not provide any evidence that the ammonium effect was located at the post-transcriptional level. It therefore seems likely that ammonium affects the enzyme production at the level of transcription, though the possibility still remains that the half-life of the specific mRNA was too short to be detected by the employed procedure.

TABLE 1. *Effect of chloramphenicol and rifampin on the development of nitrate reductase activity in A. nidulans after ammonium removal*[a]

Additions	Nitrate reductase sp act[b] (mU/mg of protein)	
	KNO_3	minus N
None	58	40
Chloramphenicol (50 μg/ml)	5	1
Rifampin (50 μg/ml)	2	13

[a] *A. nidulans* cells grown on ammonium medium were transferred to media containing 20 mM nitrate or no nitrogen source as indicated and incubated for 1 h under growth conditions with the indicated supplements.

[b] Initial specific activity was 9 mU/mg of protein.

From the above considerations it follows that transfer of the cells from nitrate to ammonium-containing medium should result in a cessation or a decreased rate of nitrate reductase synthesis. Results shown in Fig. 3 indicate this to be the case, since the cellular nitrate reductase activity level decreased from 300 to 60 mU/mg of protein in about 12 h. Most of the decay in specific activity could be accounted for by the increase in total protein of the cell culture during this time interval, though the total nitrate reductase activity also decreased at a low rate. It is not clear whether there is a specific nitrate reductase-inactivating system present or whether the slow decline in total enzyme activity is due to nonspecific protein degradation.

The repressive effect on nitrate reductase synthesis exercised by ammonium could either be caused by ammonium itself or be due to a product of ammonium metabolism or a related compound. To decide between these alternatives, we have made use of MSX, an irreversible inhibitor of glutamine synthetase (16), the first enzyme of the major pathway for ammonium assimilation in the strain of *Anacystis* used throughout this work (J. L. Ramos, E. Flores, and M. G. Guerrero, Abstr. II Fed. Europ. Soc. Plant Physiol., 1980). As shown in Fig. 4, addition of 1 μM MSX (final concentration) to cells growing on ammonium medium resulted in the development of nitrate reductase activity after a lag period of about 1 h. The initial appearance of nitrate reductase was concomitant with inhibition of both ammonium utilization (Fig. 4) and cell growth (data not shown), the latter effects being expected results of the inactivation of glutamine synthetase. The lag period between the addition of MSX and the manifestation of the effects

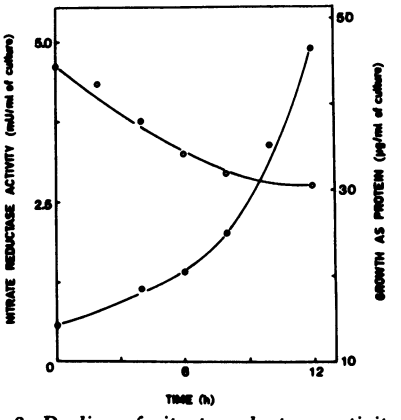

FIG. 3. *Decline of nitrate reductase activity in A. nidulans after transfer from nitrate to ammonium medium. Cells grown on nitrate were transferred to a medium containing 10 mM $(NH_4)_2SO_4$.* (○) *Nitrate reductase activity;* (●) *protein.*

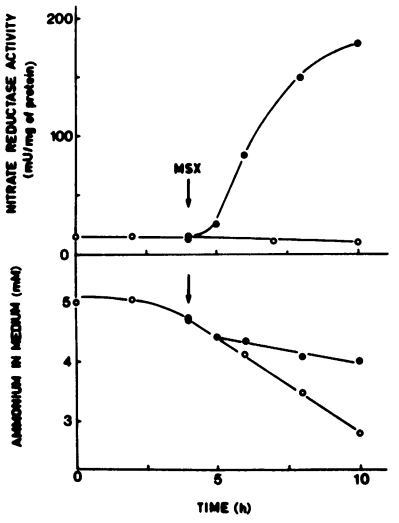

FIG. 4. *Effect of MSX on ammonium repression of nitrate reductase in A. nidulans. Cells grown on ammonium were incubated in a medium containing 2.5 mM $(NH_4)_2SO_4$ at an initial density of 31 μg of cell protein per ml. At 4 h, the cell suspension was divided into two halves, and MSX was added to one of them to reach a final concentration of 1 μM.* (○) *Control;* (●) *MSX present.*

described above probably represents in its main part the time required for the effective inactivation of glutamine synthetase. The increase in specific activity of nitrate reductase caused by the action of MSX in the presence of ammonium was particularly striking because this increase took place under conditions of diminished total net protein synthesis. Under these conditions the synthesis of nitrate reductase must be largely supported by degradation of other proteins, which is also the case for enzyme synthesis in a nitrogen-free medium.

Table 2 shows the nitrate reductase activity levels of *A. nidulans* cells incubated in media containing different nitrogen sources both in the absence and in the presence of MSX. In the presence of MSX, high activity levels were always found, especially when a utilizable source of nitrogen was present, either nitrate or ammonium. It is worth noting that, in the presence of MSX, the activity level attained was always markedly higher when both nitrate and ammonium were present, rather than with each present separately. In the absence of MSX, the repressive effect of ammonium was manifest even if nitrate was simultaneously present.

Taken together, the results in Fig. 4 and Table 2 clearly indicate that ammonium must be metabolized through glutamine synthetase before it can exert a negative effect on nitrate reductase synthesis.

The effect of the nature of the nitrogen source on the nitrate reductase activity level has also been investigated in the filamentous cyanobacteria *Anabaena* sp. strain 7119 and *Nostoc* sp. strain 6719 (both nitrogen fixers). In this type of cyanobacteria, ammonium assimilation is also effectively inhibited by MSX (12). Table 3 shows the results obtained with *Nostoc* sp. strain 6719 both in the absence and presence of MSX; similar results (not shown) were obtained with *Anabaena* sp. strain 7119. As in the case of *A. nidulans*, high levels of nitrate reductase were found in the nitrate medium, whereas low levels were present in ammonium-containing media. In contrast to *A. niudulans*, nitrate reductase levels of *Nostoc* or *Anabaena* cells in a medium without a source of combined nitrogen were as low as those of cells in ammonium-containing media. Also, in contrast to *A. nidulans*, MSX allowed the synthesis of nitrate reductase in the presence of ammonium, but only if nitrate was simultaneously present. These results indicate a positive role of nitrate in the regulation of nitrate reductase synthesis in these filamentous cyanobacteria. Kinetic experiments (data not shown) indicated that for the filamentous strains used, the

TABLE 2. *Effect of the nitrogen source and MSX on cellular nitrate reductase activity in A. nidulans*[a]

Source of nitrogen	Nitrate reductase sp act (mU/mg of protein)	
	Without MSX[b]	With MSX[b]
KNO_3 (20 mM)	272	224
None	150	53
$(NH_4)_2SO_4$ (10 mM)	8	187
KNO_3 (10 mM) and $(NH_4)_2SO_4$ (5 mM)	8	497

[a] *A. nidulans* cells grown on ammonium medium were transferred to media containing the indicated source of nitrogen and incubated for 6 h under growth conditions.

[b] Where indicated, 1 μM MSX was present in the corresponding medium.

TABLE 3. *Effect of the nitrogen source and MSX on cellular nitrate reductase activity in Nostoc sp. strain 6719*[a]

Source of combined nitrogen	Nitrate reductase sp act (mU/mg of protein)	
	Without MSX[b]	With MSX[b]
KNO_3 (20 mM)	72	69
None	19	16
$(NH_4)_2SO_4$ (2 mM)	19	18
KNO_3 (20 mM) and $(NH_4)_2SO_4$ (2 mM)	18	76

[a] *Nostoc* cells grown on ammonium medium were transferred to media containing the indicated source of combined nitrogen and incubated for 7 h under growth conditions.

[b] Where indicated, 10 μM MSX was present in the corresponding medium.

time required to reach the characteristic nitrate reductase levels of nitrate-grown cells after a transfer from ammonium to nitrate medium is about 5 to 6 h.

DISCUSSION

The studies on the metabolic control of ferredoxin-nitrate reductase in *A. nidulans* reported in this investigation support two main conclusions: (i) nitrate reductase appears to be a derepressible rather than a nitrate-inducible enzyme; and (ii) although ammonium behaves as a nutritional repressor of nitrate reductase, this effect is not direct but seems to be a result of its metabolism. With regard to the lack of inducibility of the enzyme by its substrate, the behavior of *A. nidulans* nitrate reductase resembles that of some eucaryotic algae (8, 17, 20, 23, 25) and some aerobic bacteria (27), in which ammonium appears to act as a repressor. Nitrogen metabolite repression of nitrate reductase at the transcriptional level is also well documented for the case of fungi (5, 19).

Our results from experiments with MSX and *A. nidulans* strongly suggest that ammonium per se is not the true repressor, but that it has to be metabolized through glutamine synthetase before a repressive effect on nitrate reductase is manifested. From experiments with wild-type and mutant strains of the fungus *Neurospora crassa,* a decisive role of glutamine synthetase in the "ammonium repression" effect on nitrate reductase synthesis has also been reported recently (4, 15). It has been proposed that either glutamine or some particular derivative of glutamine is the actual repressor (15) or that glutamine synthetase itself is the putative regulator in nitrogen metabolite repression (4). Although our results do not allow a decision about the nature of the true repressor, they clearly show that inactivation of glutamine synthetase resulted in the disappearance of the repressive effect of ammonium.

Nitrogenase, another enzyme system involved in the assimilation of inorganic nitrogen, appears to be subjected to repression by ammonium in a similar indirect way, since inactivation of glutamine synthetase also alleviates the inhibitory effect of ammonium on nitrogenase synthesis (22).

In the nitrogen-fixing cyanobacterial strains *Anabaena* sp. strain 7119 and *Nostoc* sp. strain 6719, nitrate exhibits a positive effect on the regulation of nitrate reductase. These observations agree with the previous proposal that the enzyme of *Anabaena cylindrica* is induced by its substrate (7, 14). Also in the nitrogen-fixing aerobic bacterium *Azotobacter chroococcum,* an inducer role has been proposed for nitrate in the regulation of nitrate reductase synthesis (6). Although an active role of ammonium as a negative regulator of nitrate reductase synthesis in *Anabaena* and *Nostoc* species cannot be ruled out absolutely, present results (Table 3) could more simply be explained by the inhibitory effect of ammonium on the uptake of nitrate, the positive regulator. This inhibition of nitrate uptake is effectively prevented by MSX (E. Flores, M. G. Guerrero, and M. Losada, Arch. Microbiol., in press).

It is interesting that nitrate plays a positive role in regulating nitrate reductase in those cells that fix N_2, the diazotrophs, whereas in *A. nidulans* and *Acinetobacter calcoaceticus,* procaryotes which cannot fix N_2, the ammonium repression seems more important in controlling enzyme levels. Further comparative studies with different species are necessary to show whether one can generalize these differences in regulatory patterns.

ACKNOWLEDGMENTS

This work was supported by grants from Philips Research Laboratories (The Netherlands) and Centro de Estudios de la Energía (Spain).

We are deeply grateful to Manuel Losada for his generous encouragement and help and to Birgit Vennesland and Pedro Candau for a critical reading of the manuscript. We wish to thank Pepa Pérez de León and Antonia Friend for skillful secretarial assistance.

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