Proc. Indian natn. Sci. Acad., **B 59**, No. 6, pp. 617-622 (1993)

Short-Term Nitrate Inhibition of Nitrogenase Activity in the Cultured Cyanobiont *Nostoc* ANTH

SURENDRA SINGH*

Department of Biochemistry, North-Eastern Hill University, Shillong 793 014

(Received on 10 July 1992; after revision 7 May 1993; Accepted on 5 May 1993)

The Short-term effect of nitrate on the nitrogenase activity was studied in the cultured cyanobiont *Nostoc* ANTH. High levels of nitrate uptake and nitrogenase were observed, when the cyanobiont was grown on elemental nitrogen (N_2). Nitrate inhibited the nitrogenase activity only after a lag of 1 hr. The inhibition of ammonium assimilation by L-methionine-DL-sulphoximine (MSX), an inhibitor of glutamine synthetase, alleviates the inhibitory effect of nitrate on nitrogenase activity without impairing the ability of the cells to take up nitrate, indicating that nitrate effect on nitrogenase activity is indirect and the assimilation of ammonium resulting from nitrate reduction is required for the repression of nitrogenase activity to occur. MSX-treated cells showed simultaneous nitrate uptake and nitrogenase activities. These cells also generated ammonium from both nitrate and N_2 simultaneously.

Key Words: Cyanobiont, Nitrate uptake, Nitrogenase, *Nostoc* ANTH

Introduction

The assimilatory nitrate reduction and nitrogen-fixation represent the processes by which nitrate and N_2 are converted to ammonium. In heterocystous cyanobacteria nitrogenase is localized in heterocysts (Rai et al. 1989) and nitrate reductase in vegetative cells (Kumar et al. 1985) only. Ammonium is required for the repression of both the enzymes to occur (Bagchi et al. 1985, Singh et al. 1985). Nitrogen-fixation is an energy-expensive process and therefore, the synthesis and activity of nitrogenase are subject to regulation by different nitrogen sources (Gallon & Chaplin 1987). *Nostoc* ANTH has ability to fix N_2 in association with the liverwort *Anthoceros punctatus*.

Present Address: Department of Microbiology, Barakatullah University, Bhopal 462 026 (M.P.)

Regulation of nitrogen-fixation in relation to ammonium metabolism has been reported (Reich et al. 1986). However, the information on the short-term nitrate inhibition of nitrogenase in cyanobacteria is scarce, conflicting and poorly understood. In several organisms, repression of nitrogenase synthesis by nitrate occurs by a mechanism which does not require the reduction of nitrate to ammonium (Gallon & Chaplin 1987). In *Anabaena* sp. CA the process does not involve the participation of ammonium or its derivatives, but is directly linked to nitrate reduction (Bottomely et al. 1979, Tabita et al. 1981). The role of ammonium, however, is reported in nitrate-mediated inhibition of nitrogenase in free-living cyanobacterium *Anabaena* sp. strain ATCC 33047 (Ramos & Guerrero 1983) but nothing is known about the role of

nitrate in the regulation of nitrogenase activity in the symbiotic cyanobacteria.

The paper aims to determine the role of nitrate in the regulation of nitrogenase activity in the cultured cyanobiont *Nostoc* ANTH and provides evidence here showing that nitrate inhibition of nitrogenase activity is mediated by products of ammonium assimilation via glutamine synthetase rather than nitrate itself. For this purpose L-methionine-DL-sulphoximine (MSX) treated cells of *Nostoc* ANTH have been used in which ammonium assimilation is blocked. Such cells simultaneously take up nitrate and reduce N_2.

Materials and Methods

Nostoc ANTH isolated from the gametophyte of *Anthoceros punctatus* was axenically grown in BG-$11_0(N_2)$ medium (Rippka et al. 1979) at 25°C and a photon fluence rate of 50 μmol $m^{-2}s^{-1}$. Exponentially growing cultures (7 days) were used in all experiments, since this was the age at which the nitrogenase activity was found to be maximum (Singh 1989). It was desirable to obtain cells having on detctable intracellular nitrate but exhibiting nitrate reductase activity. For this purpose, the cells were washed and resuspended in 2 μM KNO_3. After 12 hr of incubation, the cells were collected by centrifugation, washed with sterile medium and resuspended in nitrate-free growth medium. According to table 1, L-methionine-DL-sulphoximine (MSX, 50 μM) was added to the cultures 2 hr prior to the addition of nitrate and incubated under normal growth conditions so as to allow the entry of the chemical into the cells. The medium was buffered to *p*H 7.5 with 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES)/NaOH. Hundred-fifty ml sample (in triplicate) of the above desired cell suspension was incubated at 25°C in 250 ml sealed conical flasks. Two of the samples were used for nitrogenase and nitrate uptake assays and third one was used for the assay of ammonium liberation.

Nitrogenase activity: It was measured using acetylene reduction technique (Stewart et al. 1967). Five ml cell suspensions placed in 15 ml serum vials with 10% (v/v) acetylene were incubated at 25°C and at a photon fluence rate of 50 μmol $m^{-2}s^{-1}$ with continuous shaking. Gas samples (1 ml) was collected after 60 min and analyzed for estimating C_2H_4 with a Gas Chromatograph (Model Tractor 540) fitted with a flame ionization detector and Porapak T column. Nitrogen was used as the carrier gas. The chlorophyll content was estimated by the method of Mackinney (1941).

Nitrate uptake: It was assayed by measuring the depletion of nitrate from the external medium. Exponentially growing cells were harvested by centrifugation at 5000g for 15 min, washed with sterile N_2-medium and resuspended in the same medium to a final density of 200 μg protein ml^{-1}. Assay was carried out in open flasks under aerobic conditions with continuous shaking at 25°C and at a photon fluence rate of 50 μmol $m^{-2}s^{-1}$. The experiments were started by the addition of KNO_3 (1 mM, final concentration) to the cell suspension at zero time. From the aliquots withdrawn at regular intervals (30 min), the cells were removed by rapid centrifugation and cell-free supernatants were analysed for the residual nitrate following the method of Avissar (1985). The linear portions of the curves (uptake during 0-30 min) were used to calculate the uptake rate.

Ammonium: Ammonium in the external medium was estimated by the method of Solorzano (1969) and was expressed in terms of nmol ammonium liberated μg^{-1} Chl *a* hr^{-1}.

Results and Discussion

The data in table 1 show the changes in nitrogenase activity and nitrate uptake levels in *Nostoc* ANTH cells grown in N_2-medium

Table 1 *Nitrate induced changes in nitrogenase activity and nitrate uptake levels in the cultured cyanobiont Nostoc ANTH*

Incubation period (hr)	Nitrate uptake (nmol μg^{-1} Chl *a* h^{-1})		Nitrogenase activity (nmol C_2H_4 μg^{-1} Chl *a*hr^{-1})	
	NO_3^-	NO_3^- + MSX	NO_3^-	NO_3^- + MSX
0	0.0	0.0	11.30	13.94
1	210.6	240.7	10.72	12.18
2	425.8	543.5	7.34	11.90
3	490.3	635.5	6.20	11.96
4	493.7	639.4	5.58	12.04
5	491.2	645.1	3.94	11.98

Nostoc ANTH cells grown under N_2-fixing conditions were suspended in N_2-medium supplemented with 1 mM KNO_3 at zero time and nitrate uptake as well as nitrogenase activity were determined at the times indicated. MSX (50 μM) was added to the cultures 2 hr prior to the addition of nitrate and incubated under normal growth conditions so as to allow the entry of the chemical into the cells. The values in each column are the mean of three independent determinations.

and then transferred to nitrate-medium. *Nostoc* ANTH cells grown with N_2 as sole source (zero time) exhibited the maximum nitrogenase activity. The addition of nitrate to this culture resulted in the uptake of nitrate, accompanied by a repression in nitrogenase activity. The repression in nitrogenase activity level started only after a lag period of 1 hr (table 1). This time lag was consistent with the necessity for nitrate to be first reduced to nitrite and then to ammonium which may be the actual repressor in this case. In three to four hours after the addition of nitrate, the cells exhibited an appreciable nitrate uptake (490 nmol μg^{-1} chl *a* hr^{-1}) whereas the nitrogenase activity level dropped to about 50% of the initial value. These results also support the finding of the earlier report that short-term uptake of nitrate is free from its subsequent reduction (Rai & Singh 1982) and it may therefore be concluded that nitrate induced nitrogenase inhibition is not exerted by nitrate itself but it requires its reduction.

To further determine whether nitrate promoted nitrogenase repression could either be exerted by nitrate itself or by a product of ammonium metabolism, experiments were carried out in which glutamine synthetase, the first enzyme of ammonium assimilation had been inactivated by MSX. MSX induces a drastic irreversible inhibition of glutamine synthetase, thus, preventing the assimilation of ammonium by this route (Stewart & Rowell 1975). The addition of MSX to the cultures prevented the nitrate inhibition of nitrogenase activity without impairing the ability of the cells to take up nitrate (table 1).

Table 2 *The relative contribution of N_2 and nitrate in the combined generation of ammonium by normal untreated and MSX-treated cells of* Nostac ANTH

Addition or Incubation conditions	Ammonium in the medium (nmol μg^{-1} Chl *a* h^{-1})
N_2	0.0
N_2 + MSX	9.8
KNO_3	0.0
KNO_3 + MSX	219.6

Nostoc ANTH cells grown in N_2-medium were suspended in N_2 and nitrate media. MSX (50 μM) was added to the cultures 2 hr prior the addition of nitrate (5 mM) and was present during the experiments. Ammonium in the outer medium was measured as described in Materials and Methods. The data are the mean of three determinations

Thus, nitrate may inhibit nitrogenase activity in *Nostoc* ANTH only when glutamine synthetase is operative. Similar results were also reported in glutamine auxotrophic mutants of *Anabaena cycadeae* where the loss of glutamine synthetase derepressed the nitrate uptake (Singh et al. 1985). Since, the same treatment also abolished the ammonium inhibition of nitrogenase activity in *Nostoc* ANTH (Singh 1991), the present result reinforces my proposal that the repression of nitrogenase activity exerted by nitrate is neither by itself nor as a result of its reduction to ammonium, but the assimilation of the latter compound is required.

Further, the cells treated with MSX, not only showed the recovery of nitrogenase activity from nitrate inhibition but exhibited an induced level of nitrogenase activity (table 1). Thus, the regulatory system modulating nitrogenase activity seems to involve the products of ammonium assimilation via glutamine synthetase.

Furthermore, in MSX-treated *Nostoc* ANTH, about 90% of the ammonium resulting from the reduction of either N_2 or nitrate is released to the external medium (Singh 1992 a,b). Ammonium production can thus be used for determining the extent at which nitrate reduction and nitrogen-fixation occur. The data in table 2 show the relative contribution of N_2 and nitrate in the ammonium production by MSX-treated cells of *Nostoc* ANTH having high levels of both nitrogenase and nitrate uiptake activities (table 1). No detectable ammonium was liberated by normal untreated cells of *Nostoc* ANTH either in the presence of N_2 or nitrate. However, the MSX-treated cells quantitatively released in the external medium ammonium resulting from the reduction of N_2 and nitrate individualy or jointly (table 2). In the presence of N_2 and in the absence of nitrate these cells liberated ammonium at a rate of 9.8 nmol μg^{-1} Chl *a* hr^{-1} which increased upto 219.1 nmol μg^{-1} Chl *a* hr^{-1} in response to nitrate addition. These results ascertain the relative contribution of nitrate and N_2 in ammonium production when present together and also suggest that the interaction between nitrate reduction and N_2-fixation takes place at the level of ammonium assimilation and not at the level of nitrate uptake and N_2 reduction.

Acknowledgement

The author thanks, OSTP, CSIR, New Delhi for financial assistance and the Head, Department of Biochemistry, North-Eastern Hill University, for necessary laboratory facilities.

References

Avissar Y J 1985 Induction of nitrate assimilation in the cyanobacterium *Anabaena variabilis*; *Physiol. plant.* **63** 105-108

Bagchi S N, Rai A N and Singh H N 1985 Regulation of nitrate reductase in cyanobacteria. Repression-derepression control of nitrate reductase apoprotein in the cyanobacterium *Nostoc muscorum*; *Biochim. Biophys. Acta.* **838** 370-373

Bottomely P J, Grillo J F, Van Baalen C and Tabita F R 1979 Synthesis of nitrogenase and heterocysts by *Anabaena* sp. CA in the presence of high levels of ammonia; *J. Bacteriol.* **140** 938-943

Gallon J R and Chaplin A E 1987 *An Introduction to Nitrogen Fixations;* (London: Cassel Educational Ltd.).

Kumar A P, Rai A N and Singh H N 1985 Nitrate reductase activity in the isolated heterocyst of the cyanobacterium *Nostoc muscorum; FEBS Lett,* **179** 125-128

Mackinney G 1941 Absorption of light by chlorophyll solutions; *J. Biol. Chem.* **140** 315-322

Rai A K and Singh S 1982 Regulation of nitrate uptake in *Nostoc muscorum* by glutamine synthetase; *FEMS Microbiol. Lett.* **14** 303-306

Rai A N, Borthakur M, Singh S and Bergman B 1989 *Anthoceros-Nostoc* symbiosis: Immunoelectron-microscopic localization of nitrogenase, glutamine synthetase, phycoerythrin and ribulose-1, 5-bi-phosphate carboxylase/oxygenase in the cyanobiont and cultured (free-living) isolate *Nostock* 7801; *J. Gen. Microbiol.* **135** 385-395

Reich S, Almon H and Boger F 1986 Short-term effect of ammonia on nitrogenase activity of *Anabaena variabilis* (ATCC 29413); *FEMS Microbiol. Lett.*, **34** 53-56

Rippka R, Deruelles J, Waterbury J B, Herdman M and Stanier R Y 1979 Generic assignment, strain histories and properties of pure cultures of cyanobacteria; *J. Gen. Microbiol.* **111** 1-61

Singh H N, Rai A N and Bagchi S N 1985 Evidence for a common genetic regulation of glutamine synthetase and nitrate uptake and reductase in the cyanobacterium *Anabaena cycadeae; Mol. Gen. Genet.* **198** 367-368

Singh S 1989 Acetylene reduction activity of some blue-green algae; *Curr. Sci.* **58** 816-817

—— 1991 Involvement of ammonium assimilation in the ammonium inhibition of nitrogenase activity in the cyanobacterium *Nostoc* Anth; *Indian J. Exp. Biol.* **29** 496-497

—— 1992a Regulation of nitrite uptake in the cyanobiont *Nostoc* ANTH; *Indian J. Exp. Biol.* **30** 288-291.

—— 1992b Photobiological production of ammonia by an L-methionine-DL-sultfoximine-resistant mutant of cyanobiont *Nostoc* ANTH; *Indian J. Exp. Biol.* **30** 284-287

Solorzano L 1969 Determination of ammonia in natural waters by the phenol hypochlorite method; *Limnol. Oceanogr.* **14** 799-801

Stewart W D P, Fitzgerald G P and Burris R H 1967 *In situ* studies on N_2-fixation using the acetylene reduction technique; *Proc. Natl. Acad. Sci.*, USA **58** 2071-2078

—— and Rowell P 1975 Effect of L-methionine-DL-sulfoximine on the assimilation of newly fixed NH_3, acetylene reduction and heterocyst production in *Anabaena cylindrica; Biochem. Biophys. Res. Commun.* **65** 846-856

Tabita F R, Ramsden D K and Van Baalen C 1981 Nitrate control of heterocyst and nitrogenase synthesis in *Anabeena* sp. CA; in *Current Perspective in Nitrogen Fixation* pp 452 ed. A H Gibson and W E Newton (Amsterdam: Elsevier/North Holland)