*Proc. Indian natn. Sci. Acad.*, **B60**, No. 3, pp. 243-254 (1994)

# Cyanobacterial Nitrate Reduction: Process and Regulation

SUVENDRA NATH BAGCHI

*Department of Biological Sciences, R.D. University, Jabalpur* 482 001 (*M.P.*)

(Received on 3 August 1992; after 2nd revision 28 December 1993; Accepted on 23 February 1994)

Higher plants and micro-organisms reduce nitrate to ammonia by activities of nitrate and nitrite reductases. In cyanobacteria, nitrate is actively transported by a 48 kDa protein, a product of *ntr* gene. Nitrate reductase, product of at least three *nar* genes, is composed of a molybdenum-cofactor and an apo-enzyme. Nitrite reductase resembles enzymes of higher plant chloroplasts and is coded by a *nir* gene. Several artificial electron donors and ferrodoxin serve as reductants for reductases. Photosynthetic regulation of nitrate reduction is at the level of nitrite reductase activation and of supply of ATP, reductants and carbon-skeleton. Competition for reductants occurs upon light unsaturation. Ammonia, via assimilatory product(s), represses the genes for 48 kDa protein and reductases in a co-ordinate manner, and involves *ntc* gene product responsible for positive nitrogen control. Enzyme expression may or may not require nitrate, the putative inducer. Nitrate also protects the pre-formed enzyme from inactivation by $H_2O_2$ and oxygen radicals and proteolysis. Future emphasis is on molecular genetic analysis so as to maximize the utility of the process in scavenging nitrate pollution and in production of biofertilizer/ ammonia.

**Key Words: Cyanobacteria, Nitrate reduction, Ammonia, Nitrate pollution**

## Introduction

Majority of the plants utilize nitrogen via a two step reduction of nitrate to ammonia, catalysed by assmilatory nitrate reductases (NR's) and nitrite reductases (NiR's). Above $10^4$ megatons of nitrates are assimilated annually by these organisms. Highly soluble nitrate can leach from the soil and accumulate in ground water. Bacterial reduction of nitrates produces nitrous oxides and other toxic substances which can be injurious to human health and pollute the air. Nitrate concentration beyond 50 ppm in drinking water has been implicated in causing methanoglobinemia in infants. Immobilized NR's along with some other enzymes are presently being used in reducing toxic levels of nitrate in polluted waters. Use of biological systems to scavenge nitrates from the ecosystems will depend on efficient nitrate uptake by the prospective organisms. It is therefore essential to understand the entire mechanism of nitrate reduction to maximise the benefits.

In higher plants, eukaryotic algae, yeasts and filamentous fungi, assimilatory NR is a soluble, multicenter redox enzyme that catalyses the two-electron reduction to nitrite using pyridine nucleotide as the electron donor. There are three closely related forms of NR: NADH NR(EC 1.6.6.1), NAD(P)H NR(EC 1.6.6.2) and NADPH NR(EC 1.6.6.3), NADH NR is the most common form in higher plants, algae and yeasts, although some of them also contain NAD(P)H NR. NADPH NR occurs only in fungi. Because monoclonal antibodies could be raised against plant NR's, it was possible to examine structure and function of every component of this enzyme. Broadly, NR is a

homodimer with each subunit of ~ 100 kDa polypeptide and three cofactors; FAD, iron-heme and molybdenum-pterin, in a 1:1:1 ratio.

Bacterial NR's are different from those of eukaryotic forms. Both assimilatory and dissimilatory NR's in several prokaryotic organisms receive electrons from ferredoxin. There are three subunits of *Escherichia coli* dissimilatory NR, but great majority of assimilatory NR's are single polypeptide with a molecular weight of 80-90 kDa.

NiR catalyses six-electron reduction of nitrite to ammonia and is generally considered to be a soluble, multicenter redox enzyme. Higher plant and algal NiR's (EC 1.7.7.1) use reduced ferredoxin as the electron donor and are found in chloroplasts, but is encoded by a nuclear gene with its polypeptide synthesized in cytoplasm. Ferredoxin NiR is a monomeric protein with molecular weight of 63 kDa, minus the transit peptide responsible for targetting the enzyme to the organelle. Fungal and bacterial NiR (EC 1.6.6.4) are homodimers with a peptide of 881-140 kDa and use NAD(P)H as electron donor. Both forms of NiR contain siroheme- Fe and terranuclear FeS centers, while the fungal and bacterial forms also contain FAD and perhaps an FeS center not associated with siroheme.

Nitrate assimilation in cyanobacteria has been exhaustively reviewed by Guerrero and Lara (1987), Guerrero et al. (1981) and Flores et al. (1983a).

## Nitrate Uptake

Nitrate is transported by an active transport system, before being available for reduction. In vacuolated algae and higher plants, variety of ions including nitrate are accumulated (Syrett 1988), which indicates that uptake and reduction of nitrate are independent processes. However, in non-vacuolated organisms including cyanobacteria, it is not possible to demonstrate this distinction. This is because of the experimental design, in which disappearence of nitrate from surrounding medium represents the uptake process.

To overcome this problem, utilization of nitrate was determined independently by developing NR-mutants or pre-incubating the algal cells in presence of tungsten to inactivate the enzyme. Using *Nostoc muscorum*, Bagchi and Singh (1984) found that short term nitrate intake was non-interrupted in the mutants. Further, influx of nitrate in tungsten-treated *Synechococcus* R2 (Shearer et al. 1991) was uninhibited, though about 80% of the accumulated nitrate was released, due to an efflux mechanism. Different properties of cyanobacterial nitrate uptake have been considered in a review by Guerrero and Lara (1987). In addition, a 48kDa nitrate transport protein, a product of *nrt* A gene has been characterized in cytoplasmic membrane (Madueno et al. 1988b, Omata et al. 1989).

## Nitrate Reduction

### *Structure, Location and Catalytic Activities of Nitrate and Nitrite Reductases*

NR from the unicellular cyanobacterium *Anacystis nidulans* has been purified to homogeneity and characterized (Candau 1979) as single protein with a molecular weight of 75,000 Da having only one polypeptide chain and exhibiting a $K_m$ value of 0.7 mM for nitrate. NR was also purified from a filamentous cyanobacterium *Plectonema boryanum* and characterized (Ida & Mikami 1983, Mikami & Ida 1984, 1986) as a single polypeptide with a molecular weight 85,000 Da containing 0.95 atoms of molybdenum and four atoms each of iron and acid labile sulfur per molecule. The $K_m$ for nitrate was nearly 0.75 mM. NR from all sources have a common molybdenum-cofactor which can restore the activity in the apoprotein fractions of cofactor-free

molybdoenzymes such as xanthine oxidase, sulfite oxidase as well as NR (Hewitt 1975, Ketchum & Swarin 1973, Lee et al. 1974, Singh et al. 1978a, b). This property of molybdenum cofactor was tested in a cell-free system containing the cofactor and a source of cofactor-free NR apoprotein of *Neurospora crassa nit*-1 mutant (Muller & Grafe 1978, Fernandez & Cardenas 1981, Miller & Amy 1988) or *Nostoc muscorum* tungsten-resistant mutant (Bagchi et al. 1985b). The cofactor activity could be defined by its capacity to reconstitute mutant NR in a complementation assay (Muller & Grafe 1978, Lee et al. 1974). In cyanobacteria, molybdenum-cofactor was characterized only in *Nostoc muscorum* (Bagchi et al. 1987b) where, it was found in the soluble fraction of cell-free preparations and was distributed between two pools; protein-bound (molecular weight, 30,000 Da and a sedimentation coefficient at 25°C, 2.5) and protein-free. Molybdenum cofactor from several sources has been chemically analysed and shown to contain pterin moiety (Rajagopalan et al. 1981).

A significant feature of all cyanobacterial NR is its close association with photosynthetically active thylakoid membranes and dependence upon ferredoxin as the sole natural electron donor (Manzano et al. 1976, Ortega et al. 1976, Hattori & Myers 1967). Iron starvation caused replacement of ferredoxin by flavodoxin as an electron donor (Flores et al. 1983a). Ferredoxin reduced by illuminated thylakoid membranes (Manzano et al. 1976, Ortega et al. 1976, 1977), or by illuminated 5-diazariboflavin in the presence of suitable electron donor like EDTA (Candau et al. 1980), or by low concentrations of sodium dithionite (Flores et al. 1983a) can donate electrons to NR. High concentrations of dithionite had no effect (Manzano et al. 1976, Hattori & Myers 1967), presumably due to the formation of a stable and inactive complex between reduced ferredoxin and NR. Ferredoxin can be effectively replaced by other electron donors such as reduced FAD and FMN (Hattori 1970) and dithionite reduced methylviologen (Manzano et al. 1976, Ortega et al. 1977). The later is a routine reactant in the cell-free assay of cyanobacterial NR. Nitrate reduction associated with anoxygenic photosynthesis has been achieved with chlorophyll-containing particles in presence of ferredoxin and suitable electron donor like DCPIP, ascorbate or $H_2$ (Flores et al. 1983a). Broadly, the properties of cyanobacterial NR's resemble those of the bacterial NR's, particularly the clostridial enzyme (Mikami & Ida 1984).

NiR from *A. nidulans* (Manzano et al. 1976, Guerrero et al. 1974) and *Anabaena* 7119 (Mendez et al. 1981, Mendez & Vega 1981) has been partially purified and characterized. The enzyme from both the cyanobacteria exhibited a $K_m$ value for nitrite in the range of 70 to 100 μM and possessed a single polypeptide chain with a molecular weight of about 52,000 Da, containing iron, presumably as siroheam. Cyanobacterial NiR also receives electrons from ferredoxin, which is a typical plant-like character (Manzano et al. 1976, Hattori & Myers 1967, Mendez et al. 1981, Ortega et al. 1976). Under iron starvation, flavodoxin takes the responsibility of ferredoxin (Bothe 1977). Ferredoxin also serves as an artificial electron donor for NiR reduced by illuminated photosynthetic preparations (Manzano et al. 1976, Mendez et al. 1981, Ortega et al. 1976) or by illuminated 5-azariboflavin in presence of a suitable electron donor (Candau et al. 1980). Dithionite-reduced ferredoxin can also donate electrons to NiR (Manzano et al. 1976, Vega et al. 1980, Mendez et al. 1981). And in the cell-free system, dithionite reduced methylviologen serves as the reductant source. Cyanobacterial enzyme, therefore, resembles the enzyme from higher plants. Recently, Lague et al. (1993)

sequenced the NiR gene (*nir*) from *Synechococcus* sp. and found 1536 nucleotides to be similar to NiR from higher plants.

*Genetic and Molecular Properties of Nitrate Reductase Genes*

The approach has been to score NR-mutants and mutant altered in regulation of this enzyme. Complementation in these mutants using the genomic library of the wild type and selection of $NR^+$ phenotype provided an idea on the nature of NR genes.

Spontaneous mutants of *N. muscorum* scored on chlorate failed to assimilate nitrate due to defect in nitrate uptake or NR or both (Bagchi & Singh 1984). In some mutants, loss of NR was accompanied with a loss of nitrogenase activity (Singh et al. 1977). In the non $N_2$ fixer *Phormodium uncinatum*, chlorate-resistant nitrate reduction persisted in a resistant mutant (Bagchi et al. 1992).

$NR^-$ mutants of *A. nidulans* were obtained by chemical (MNTG) and transposone (Tn 901) mutagenesis and selected for poor growth on nitrate (Kuhlemeier et al. 1984a). Establishment of the gene cloning system here enabled the complementation test. A cosmid gene bank of the strain R2 (PCC 7942) was constructed in shuttle cosmid pPUC 29 and used to transform $NR^-$ mutants. The *nar*$^+$ characteristic was chosen among the transformants. Using this technique, at least three genes termed *nar* A, *nar* B and *nar* C for NR were identified and cloned (Kuhlemeier et al. 1984a, b). Of these *nar* B appears to be the structural gene for NR enzyme and is clustured with the genes of NiR (*nir*) and the 48 kDa nitrate transport protein (*nrt* A). These genes are co-ordinately regulated and are co-transcribed (Laque et al. 1992). Two more genes responsible for nitrate assimilation have been identified by transformation work on additional and varied classes of defective mutants (Madueno et al. 1988a). Unfortunately, none of these genes correspond to the bacterial *nar* genes responsible for apoenzyme synthesis, regulatory genes or genes for molybdenum-cofactor synthesis (Marzulf 1981).

*Relationship between Nitrate Reductase and other Molybdo-enzymes with Respect to Molybdenum Processing*

Various molyboenzymes, excluding nitrogenase, which contains an iron-molybdenum-cofactor (Ugalde et al. 1985), share a common molybdenum-cofactor (Hewitt 1975, Ketchum & Swarin 1973, Lee et al. 1974). It is nevertheless, certain that molybdenum-cofactor and iron-molybdenum-cofactor share a common route of synthesis after molybdenum-intake (Ugalde et al. 1985). Therefore it would not be surprising if nitrogenase and NR share a precursor of molybdenum-cofactor. Singh et al. (1978a) first hypothesized this in *N. muscorum* and subsequently confirmed by Bagchi and Singh (1984) and Bagchi et al. (1985b). *N. muscorum* grown in the absence of combined nitrogen showed nitrogenase activity confined to the heterocysts and the non-operative NR was localized in the vegetative cells (Bagchi & Singh 1984). Molybdenum-cofactor activity of $N_2$ cultures was much lower than the nitrate cultures (Bagchi et al. 1985b), lacking active nitrogenase. This observation suggests a competition for molybdenum-components between NR and nitrogenase. Substantiating the above view, Kumar et al. (1985) detected molybdenum-cofactor activity in the isolated heterocysts showing nitrogenase acttivity but lacking NR activity. Conditions that favoured expression of NR in *N. muscorum* led to an excessive synthesis of the apoprotein moiety whereas the molybdenum-cofactor synthesis became limiting, which regulated the level of net cellular NR protein (Bagchi et al. 1985b). These ob-

servations suggest that molybdenum control of NR is at the level of cofactor synthesis.

*Post-synthesis Modifications of Nitrate Reductase Protein*

Preformed cyanbacterial NR in *A. nidulans* is prone to changes inside the cells (Herrero et al. 1984). These factors strongly influence the enzyme activity by modifying and/or degrading the protein. Apparently, this enzyme is decayed in a biphasic manner. In the first phase, the oxidative modification of the active centres causes massive reversible inactivation. This is followed by a more rapid and irreversible proteolytic degradation of the enzyme. Actively photosynthesizing cells can generate superoxide anion ($O_2^-$) and $H_2O_2$ as a consequence of excess reductant load at PS I centre, which in turn would reduce molecular oxygen to these toxic radicals. Such radicals and $H_2O_2$ can directly interact with NR protein, leading to inactivation. Infact, inactivation of *N. muscorum* NR was achieved following $H_2O_2$-treatment (Bagchi et al. 1987b). Purified NR from *Plectonema boryanum* lost its activity following xanthine/xanthine oxidase treatment, causing production of superoxide (Mikami & Ida 1986). Presently a great variety of enzymes, including cyanobacterial nitrogenase (Bagchi et al. 1991), have been found to respond to the oxygen radicals in similar pattern. Proteolytic degradation of modified NR could be partially protected by external nitrogenous compounds such as nitrate (Herrero et al. 1984). It has been reported that superoxide ion is involved in $NADH_2$ mediated inactivation of green algal and higher plant enzymes, including NR (Mikami & Ida 1986).

*Regulation after Infection with a Virus*

Although several cyanobacteria are known to serve as hosts responsible for host specific cyanophage multiplication, limited host-virus systems were studied for the likely changes in the host nitrate assimilatory pathway. Both with *N. muscorum*/N-1 (Bagchi et al. 1987a) and *P. uncinatum*/LPP-1 (Bisen et al. 1986, Bagchi & Kaloya 1987) as test systems, it was found that infection caused a massive increase in the nitrate utilization capacity, in general, and enhancement of molybdenum-cofactor activity and thereby NR activity, in specific. Further, the enzyme from infected host managed to escape the $H_2O_2$ caused oxidative inactivation, normally observed with the uninfected counterparts (Bagchi et al. 1987, Bagchi & Kaloya 1987). These adjustments were necessary to meet the high demand of nitrogenous compounds required for virus multiplication.

*Regulation by Photosynthesis*

Photoautotrophically growing cyanobacteria derive the assimilatory power from photosynthesis. The first evidence for a close and stoichiometric relation between nitrate metabolism and photosynthesis was obtained using thylakoid preparations of *A. nidulans* (Candau et al. 1976). These preparations contained both the processes intact but lacked $CO_2$-fixation ability. One molecule of nitrate reduced resulted in an evolution of two molecules of $O_2$. Studies on illuminated subcellular particles of *Anabaena* 7119 (Ortega et al. 1976) revealed a positive correlation between photosynthetic $O_2$ evolution and nitrate reduction. These workers developed an assay system in which ferredoxin received electrons from $NADPH_2$ instead of $H_2O$ via a coupled enzyme $NADP^+$-ferredoxin reductase (FNR). Reduced ferredoxin eventually donated electrons to NR (Manzano et al. 1976).

Using intact cells of *A. nidulans*, Flores et al. (1983b) demonstrated a close correlation between rates of nitrate utilization and of photosplitting of water. Care was taken to minimize reductant wastage by not allowing ammonia to further metabolize, by adding

MSX, the inhibitor of glutamine synthetase (GS). A photosynthesis (PS II) inhibitor DCMU abruptly ceased $O_2$ evolution accompanied with concomitant block of nitrate entry, suggesting a tight coupling of the two processes. Apparently, cyanobacterial nitrate reduction is more close to PS II reaction than the conventional $CO_2$ fixation. Nitrate can be considered as Hill reagent. Some indirect evidences, such as nitrate/nitrite-induced quenching of chlorophyll fluorescence of *Anabaena* 7119 and *Nostoc* 6719 (Serrano et al. 1981, 1982) further confirmed the photosynthesis dependent activity of cyanobacterial nitrate reduction. It is now well established that the energy requirement for nitrate intake (Flores et al. 1983b) and reductants needed for its reduction (Candau et al. 1976) are met by photophosphorylation and electron transport from water.

In green plants and algae it has been suggested that light effects on nitrate reduction may involve participation of phytochrome and/or blue-light absorbing pigments (Hewitt et al. 1976). Thus the question arose, whether light directly interacts with the cyanobacterial enzymes. This possibility was first checked in *A. nidulans* (Flores et al. 1983b) in which darkness caused inactivation of nitrate utilization but had hardly any influence on the cellular NR activity. Eventually, Tischner and Schmidt (1984) eleborately studied such effects using another unicellular form *Synechococcus leopoliensis*. This work revealed that NiR and not NR is stimulated by light, via ferredoxin-ferrodoxin thiredoxin oxidoreductase-thioredoxin complex in the cells. In a cell-free system, NiR activity could be reductively activated several folds by including dithiothreitol (DTT) whose action was to reduce thioredoxin of the extract. Several enzymes including GS and those of carbon metabolism are stimulated by light (Tischner & Schmidt 1984). This brings out a possibility that photosynthetic regulation of nirate utilization, at least in part, involves enzyme activation.

Most of the cellular experiments were conducted in presence of MSX to avoid errors caused by $NH_4^+$ metabolism. The treated cells did not continue nitrate incorporation for longer period as synthesis of organic nitrogen from a combination of ammonia (resulting from nitrate reduction) and photosynthate (resulting from $CO_2$-fixation) was not possible. Similar situation could arise if $CO_2$-fixation was inhibited. Therefore, for continuous nitrate assimilation a continuous supply of carbon skeleton has to be ensured (Romero et al. 1985b). A positive effect of $CO_2$ supply on nitrate utilization was shown in *A. nidulans* (Lara et al. 1984). Since $CO_2$-fixation directly depends on assimilatory power generated during photosynthesis, one can assume that photosynthesis indirectly regulates nitrate utilization. One very basic metabolic arrangement by any organism is an adjustment between carbon and nitrogen utilization. Carbon dioxide enriched photosynthetic cells would utilize nitrate at an optimum rate. Once $CO_2$ is excluded or limited the net nitrate entry is immediately checked, in order to maintain the C:N balance. In fact, it has been obseved in green algae (Azuara & Aparicio 1984) and cyanobacterium *Syechochoccus* (Kramer & Schmidt 1989) that under $CO_2$ limitation, the photoreduction of nitrate followed an excretion of the resulting nitrite. This would be a mechanism to balance the C:N ratio and to minimize the wastage of reductants as nitrite reduction to $NH_4^+$ requires 6 electrons.

Since assimilation of nitrate consumes photosynthetically generated assimilatory power. It may compete with the process of carbon fixation. This interaction is still a matter of controversy in photosynthetic

organisms and was tested in *A. nidulans* (Romero & Lara 1987), by exposing the cells to graded light intensity. Under light limiting conditions, $CO_2$ fixation was depressed by the addition of nitrate whereas at photon fluxes saturating condition for $CO_2$ fixation, the addition of nitrate had no negative effect. At low light intensity, a strong competition between nitrate utilization and $CO_2$-fixation existed which reduced with increasing light intensity. Therefore, if other factors are not involved, an optimal nitrate utilization would be operative under saturating carbon status and light intensity.

In all, photosynthesis of a cyanobacterial cell operates and regulates nitrate utilization at four distinct levels namely : (a) ATP for nitrate transport (Flores et al. 1983b), (b) reductants for reduction to ammonia (Candau et al. 1976), (c) supply of photosynthates for ammonium assimilation (Flores et al. 1983c) and (d) enzyme modification (Tischner & Schmidt 1984).

The regulation of nitrate assimilation in some bloom-forming cyanobacteria is, however, exceptional. Planktonic *Oscillatoria redecki* continued nitrate assimilation even in darkness (Foy & Smith 1980). In *P. uncinatum* sustained nitrate uptake and reduction to nitrite was observed even in photosynthetically impaired cells (Bagchi et al. 1989). Reserve glycogen synthesized during photosynthesis seems to support the process by dissimilation via oxidative pentose pathway. For the first time chemo- and photoheterotrophic mode of nitrate assimilation was also proposed in this organism (Bagchi et al. 1990). Evidently in this case the electrons from oxidative pentose pathway were chanelled through FNR to nitrate reduction. Since the habitats harbouring these forms are generally rich in organic matter and not well illuminated, such shift in the mechanism, is expected in nature (see Foy & Smith 1980).

*Regulation by Inorganic and Organic Nitrogen Nutrients*

Phototrophic cyanobacteria are capable of growing on a variety of inorganic and organic nitrogen sources. Although majority of them are utilized through the most common GS-GOGAT pathway, there exists a competition between the different forms. This is predominantly at the level of expression of the structural genes responsible to operate the corresponding pathway. Ammonia, for example, will not let the cells to utilize molecular nitrogen, nitrate or nitrite. Among cyanobacteria tested, ammonium-inhibition of nitrate reduction was observed in *Anabaena cylindrica* (Hattori 1962, Ohmori & Hattori 1970), *Anacystis nidulans, Anabaena* sp. PCC 7119 and *Nostoc* sp. PCC 6719 (Herrero et al. 1981), *Agmenellum quadruplicatum* (Stevens & Van Baalen 1974). *Anabaena variabilis* and *Synechocystis* sp. (Herrero et al. 1985), *Nostoc muscorum* (Bagchi & Singh 1984), *Anabaena cycadeae* (Bagchi et al. 1985a) and *Calothrix* sp. 7101 (Martin-Nieto et al. 1989).

The ammonium-inhibition of nitrate metabolism could be attributed to ammonium itself or to the assimilatory intermediates. This problem was partly overcome by introducing MSX a GS inhibitor, so that the effects caused by product(s) was avoided. Stewart and Rowell (1975) proposed that ammonium metabolism is necessary to execute the ammonia-promoted repression on cyanobacterial nitrogenase. When MSX was added to nitrate assimilating *A. nidulans* cells, almost 85 to 90% of nitrate reduced was found released in the external medium in the form of $NH_4^+$ (Ramos et al. 1982b). Investigations were carried out to ascertain the exact mechanism of $NH_4$ mediated inhibition of cyanobacterial nitrate reduction. Initial work (Herreo et al. 1981) revealed that MSX could effectively reverse the ammonium-

effects on nitrate reduction in *A. nidulans*, *Anabaena* sp. 7119 and *Nostoc* sp. 6719, indicating the importance of ammonium metabolism in nitrate reduction. Further studies on other cyanobacteria like *A. variabilis*, and *Synechocystis* sp. (Herrero et al. 1985) confirmed these observations. A rapid increase in cellular glutamine/glutamic acid ratio on addition of $NH_4^+$ and its reversal due to MSX addition suggest that glutamine is the putative inhibitor for the process (Flores et al. 1980). Effect of different amino acids on MSX-treated *A. nidulans* nitrate assimilation was also examined (Romero et al. 1985a). Several L-amino acid viz. glutamine, isoleucine, leucine, methionine and asparagine, but not the corresponding D-isomers, inhibited nitrate utilization to variable extent. Glutamine alone cannot inhibit nitrate utilization as seen in *Anacystis* where azaserine, an inhibitor of glutamine amide transferases, lead to the accumulation of glutamine inside the cells without any concomitant inhibition of nitrate incorporation (Flores et al. 1983c). This suggests that some other nitrogenous metabolite(s) derived from glutamine as amido N donor may also be involved together with glutamine, for the charactristic inhibition. Asparagine may be one of such metabolite (Romero et al. 1985a, Cook & Anthony 1978). A report from Singh et al. (1983), claiming that MSX not only inhibits GS activity but also causes a strong inactivation of ammonium transport system in *Anabaena cycadeae*, created some confusion in understanding the role of $NH^{4+}$ played in nitrogenase and NR expression. In this case, MSX-caused reversal of ammonium repression could very well be due to prevention of ammonium intake, rather than its metabolism. Identical reports on bacterial (Kleiner & Castroph 1982) and cyanobacterial (Bergman 1984) systems further substantiated above hypothesis. On the basis of an elaborate study on a glutamine auxotroph (GS-strain, incapable of assimilating ammonia; Singh et al. 1983) of *A. cycadeae*, Bagchi et al. (1985) proposed that $NH^{4+}$ is itself the potent inhibitor of nitrate assimilating enzymes.

Expression of NR in variety of cyanobacteria (Bagchi & Singh 1984, Herrero et al. 1981) required *de novo* protein synthesis. This, together with the observation that ammonia does not inactivate cell-free enzyme activity, indicates that the inhibition by ammonia is basically of repression type. Further, as observed with *N. muscorum* (Bagchi et al. 1985b), ammonium-mediated repression of NR involved only the apoprotein moeity of the enzyme, while the molybdenum cofactor was relaxed from this control. Once repressor molecule is withdrawn, as observed with *A. cylindrica* (Hattori 1962, Ohmori & Hattori 1970), *Anabaena* 7119 and *Nostoc* 6719 (Herrero et al. 1981), *Agmenellum quadruplicatum* (Stevens & Van Baalen 1974), *A cycadeae* (Bagchi et al. 1985a), *A. variabilis* and *Synechocystis* sp. (Herrero et al. 1985) and *Calothrix* sp. 7601 (Martin-Nieto et al. 1989), development of NR required presence of nitrate.

Heterocystous cyanobacteria, in absence of combined nitrogen would fix atmospheric nitrogen, leading to the generation of intracellular ammonia, which may affect the nitrate and nitrite reductase activities. Therefore, mere removal of external ammonia may not be enough to release the $NH_4$-repression. To avoid this problem, non-nitrogen fixing mutants of *A. variabilis* were used in place of the wild type. Ammonia could not be generated from $N_2$-fixation. Inspite of this the NR and NiR activities were negligible, indicating that these enzymes are not derepressed in the absence of ammonia (Martin-Nieto et al. 1989). On the contrary, expression of NR did not require nitrate, as tested in *A. nidulans* (Herrero et al. 1981) and *N. muscorum* (Bagchi et al. 1985b), suggesting derepressible nature of the enzyme. Avissar (1985) reported that the NR activity in $N_2$ or $NH_4$ cultures of *A. variabilis* was

sufficiently high to maintain nitrate reduction. Absence of an active nitrate uptake system prevented nitrate entry. In presence of nitrate and absence of ammonia, nitrate transport process was activated, requiring no new protein synthesis. Subsequently, NR activity was induced, requiring *de novo* protein synthesis to an elevated level. Nitrate uptake system was also nitrate activating in *N. muscorum* (Bagchi & Singh 1984).

Relatively little work is done on the regulation of NiR in cyanobacteria. Like NR, NiR was also ammonia repressible (Herrero & Guerrero 1986). In the absence of ammonia, while nitrate or nitrite was required for the expression of this enzyme in dizaotrophic cyanobacteria (Ohmori & Hattori 1970, Martin-Nieto et al. 1989), no such inducer was necessary for *A. nidulans* (Herrero & Guerrero 1986) and *Phormidium laminosum* (Arizmendi et al. 1987).

Similar studies on *P. uncinatum* (Palod et al. 1990) indicated distinctive modes of ammonium-repression on NR; NiR. Ammonia by itself and its assimilatory products control NiR and NR activities respectively, which are otherwise derepressed in the absence of ammonia.

## Molecular Basis of Ammonium-promoted Regulation

Ammonia is not only a repressor of the reductases but also of the 48 kDa nitrate transport protein, GS and ammonium-(methylamine)-transport. Mutants of *Synechococcus* $R_2$ defective in NR and NiR, exhibited complete collapse of ammonium-regulation of the above processes. A gene, *ntc* (nitrogen control) A, in a 3.1 Kb DNA (genomic) fragment from the wild type could transform the mutants in such a way that ammonium-repression was restored (Vega-Palas et al. 1990). A much smaller fragment (*ca.* 0.4 Kb) within Bam HI-Hind III restriction sites, was also shown to be able to transform (Vega-Palas et al. 1992). A protein (Ntc A) with molecular weight 24817 Da, which belongs to the family of bacterial transcriptional activator, viz. Fnr, Cys R and Crp, was deduced from the open reading frame of this sequence. This protein is proposed to perform as transcriptional activator of genes subject to ammonium-control. Therefore, by function *ntc* A gene is analogous to *ntr* genes (Stock et al. 1989) known to regulate nitrogen assimilation in prokaryotes.

**Acknowledgements**

The author thanks the Head, Dept. Biol. Sciences, R.D. University, Jabalpur for lab. facilities and the CSIR, New Delhi for financial assistance in the form of two projects vide Nos. 9/331/91 EMR II and 38/598/86 EMR II.

## References

Arizamendi J M, Fresnedo O, Marinez Bilbao M, Alana A and Serra J L 1987 Inorganic nitrogen assimilation in the non-$N_2$-fixing cyanobacterium *Phormidium laminosum*. II. Effect of the nitrogen source on the nitrite reductase levels; *Physiol. Plant.* **70** 703-707

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

Azuara M P and Aparicio A 1984 Effects of light quality, $CO_2$ tension and $NO^{3-}$ concentration on the inorganic nitrogen metabolism of *Chlamydomonas reinhardii; Photosynth. Res.* **5** 97-103

Bagchi S N, Chauhan V S and Palod A 1990 Heterotrophy and nitrate metabolism in a cyanobacterium *Phormidium uncinatum; Curr. Microbiol.* **21** 53-57

——, Ernst A and Boger P 1991 The effect of activated oxygen species on nitrogenase of *Anabaena variabilis; Z. Naturforsch.* **46c** 407-415

—— and Kaloya P 1987 Cyanophage LPP-1 induced changes in the synthesis and stability of *Phormidium uncinatum* nitrate reductase; *Proc. Indian Nal. Sci. Acad.* **B53** 461-464

——, —— and Bisen P S 1987a Effect of a cyanophage N-1 infecion on the synthesis and stability of *Nostoc muscorum* nitrate reductase; *Curr. Microbiol,* **15** 61-65

——, Palod A and Chauhan V S 1989 Photosynthetic control of nitrate metabolism in *Phormidium uncinatum*, a cyanobacerium; *Curr. Microbiol.* **19** 183-188

——, ——— and ——— 1992 Sustained nitrate metabolism by a chlorate-resistant mutant of the cyanobacterium, *Phormidium uncinatum; J. Plant Physiol.* **139** 764-766

——, Rai U N, Rai A N and Singh H N 1985a Nitrate metabolism in the cyanobacterium *Anabaena cycadeae* : Regulation of nitrate uptake and reductase by ammonia; *Physiol. Plant.* **63** 322-326

——, Rai A N, Singh H N 1985b Regulation of nitrate reductase in cyanobacteria. Repression control of nitrate reductase apoprotein in the cyanobacerium *Nostoc muscorum; Biochim. Biophys. Acta* **838** 370-373

——, Sherman T D and Funkhouser E A 1987b Biochemical characterization of molybdenum-cofactor in a cyanobacterium, *Nostoc muscorum; Plant Cell Physiol.* **288** 1411-1419

—— and Singh H N 1984 Genetic control of nitrate reduction in cyanobacterium *Nostoc muscorum; Molec. Gen. Genet.* **193** 82-84

Bergman B 1984 Photorespiratory ammonium release by the cyanobacterium *Anabaena cylindrica* in the presence of methionine sulfoximine; *Arch. Microbiol.* **137** 21-25

Bisen P S, Bagchi S N and Audholia S 1986 Nitrate reductase activity of a cyanobacterium *Phormidium uncinatum* after cyanophage LPP-1 infection; *FEMS Microbiol. Lett.* **33** 69-72

Bothe H 1977 in *Encyclopeadia of Plant Physiology*, New series Vol 5 (Eds Trebst A and Avron M) 217-221 (Berlin : Springer-Verlag)

Candau P, Manzano C and Losada M 1976 Biconversion of light energy into chemical energy through reduction with water of nitrate to ammonia; *Nature* **262** 715-717

——, ——, Guerrero M G and Losada M 1980 Ferredoxin-dependent enzymatic reduction of nitrate with a deazaflavin photosystem; *Photobiochem. Photobiophys.* **1** 167-174

Cook R J and Anthony C 1978 Regulation by glutamine of ammonia transport in *Aspergillus nidulans; J. Gen. Microbiol.* 275-286

Fernandez E and Cardenase J 1981 *in vitro* complementation of assimilatory NAD(P)H-nitrate reductase from mutants of *Chlamydomonas reinhardii; Biochim. Biophys. Acta* **657** 1-10

Flores E, Guerrero M G and Losada M 1980 Short-term ammonium inhibition of nitrate utilization by *Anacystis nidulans* and other cyanobacteria; *Arch. Microbiol.* **128** 137-144

——, Ramos J L, Herrero A and Guerrero M G 1983a Nitrate assimilation by cyanobacteria. Photosynthetic prokaryotes: Cell differentiation and function, eds G C Popageorgious and L Packer pp 363-387 (New York: Elsevier Science Publication)

——, Guerrero M G and Losada M 1983b Photosynthetic nature of nitrate uptake and reduction in the cyanobacterium *Anacystis nidulans; Biochim. Biophys. Acta* **722** 408-416

——, Romero J M, Guerrero M G and Losada M 1983c Regulatory interaction of photosynthetic nitrate uptake and carbondioxide fixation in the cyanobacterium *Anacystis nidulans; Biochim. Biophys. Acta* **725** 529-532

Foy R H and Smith R V 1980 The role of carbohydrate accumulation in the growth of planktonic *Oscillatoria* species; *Br. Phycol. J.* **15** 139-150

Guerrero M G, Manzano C and Losada M 1974 Nitrite photoreduction by a cell-free preparation of *Anacystis nidulans; Plant Sci. Lett* **3** 689-699

—— and Lara C 1987 in *The Cyanobacteria* eds P Fay and C Van Baalen pp 163-186 (Amsterdam: Elsevier)

——, Vega J M and Losada M 1981 The assimilatory nitrate reducing system and its regulation; *Ann. Rev. Plant Physiol.* **43** 169-204.

Hattori A 1962 Adaptive formation of nitrate of reducting system in *Anabaena cylindrica; Plant Cell Physiol.* **3** 371-377

—— and Myers J 1967 Reduction of nitrate and nitrite by subcellular preparations of *Anabaena cylindrica*. II. Reduction of nitrate to nitrite; *Plan Cell Physiol.* **8** 327-337

Herrero A, Flores E and Guerrero M G 1981 Regulation of nitrate reductase levels in the cyanobacteria *Anacystis nidulans, Anabaena* sp. strain 7119, and *Nostoc* sp. 6719; *J. Bacteriol.* **145** 175-180 .

——, ——— and ——— 1984 Regulation of nitrate reductase level in *Anacystis nidulans*. Activity decay under nitrogen stress; *Arch. Biochem. Biophys.* **234** 454-459

—— and Guerrero M G 1986 Regulation of nitrate reductase in the cyanobacterium *Anacystis nidulans; J. Gen. Microbiol.* **132** 2463-2468

Hewitt E J 1975 Assimilatory nitrate-nitrite reduction; *Ann. Rev. Plant Physiol.* **26** 73-100

——, Hucklesby D P and Notton B A 1976 Nitrate Metabolism; in *Plant Biochemistry*, eds J Bonner and J E Warner, pp 633-681 (New York: Academic Press)

Ida S and Mikami B 1983 Purification and characterization of assimilatory nitrate reductase from

cyanobacterium *Plectonema boryanum; Plant Cell Physiol.* **24** 649-658

Ingram L O, Calder J A, Van Baalen C, Plucker F E and Parker P L 1973 Role of reduced exogenous organic compound in the physiology of the blue green bacteria (algae) : Photoheterotrophic growth of a 'heterotrophic' blue-green bacteria; *J Bacteriol.* **114** 695-700

Hetchum P A and Swarin R S 1973 *In vitro* formation of assimilatory nitrate reductase; presence of the constitutive component in bacteria; *Biochem. Biophys. Res. Commun.* **52** 1450-1456

Kleiner D and Castroph H 1982 Inhibition of ammonium (Methylammonium) transport in *Klebsiella pneumoniae* by glutamine and glutamate analogue; *FEBS Lett.* **146** 201-203

Kramer E and Schmidt A 1989 Nitrite accumulation by *Synechococcus* 6301 as a consequence of carbon - or sulphur-deficiency; *FEMS Microbiol. Lett.* **59** 191-196

Kuhlemeier C, Logtenberg T, Stoorvogel W, Van Heugten H A A, Borrais W E and Van Arkel G A 1984a Cloning of two nitrate reductase genes from the cyanobacterium *Anacystis nidulans; J. Bacteriol.* **159** 36-41

——, Teeuwsen V J P, Janssen M J T and Van Arkel G A 1984b Cloning of a third nitrate reductase gene from the cyanobacterium *Anacystis nidulans* $R_2$ using a shuttle Genee library; *Gene* **31** 109-116

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

Lara C, Romero J M, Flores E, Guerrero M G and Losada M 1984 Regulation of nitrate utilization by $CO_2$ fixation products in the cyanobactrium *Anacysts nidulans*; in *Advances in Photosynthesis Research* (Sybesma C ed.) pp 715-718 (Hague and Boston: Martinus Nijhoff Publishers)

Lee K Y, Pan S S, Erickson R and Nason A 1974 Involvement of molybdenum and iron in the *in vitro* assembly of assimilatory nitrate reductase utilizing *Neurospora* mutant nit-1; *J. Biol. Chem.* **249** 3941-3952

Laque I, Flores E and Herrero A 1993 Nitrite reductase gene from *Synechococcus* sp. PCC 7942 : Homology between cyanobacterial and higher-plant nitrite reductases; *Plant Mol. Biol.* **21** 1201-1205

——, Herrero A, Flores E and Madueno F 1992 Clustering of genes involved in nitrate assimilation in the cyanobacterium *Synechococcus; Molec. Gen. Genet.* **232** 7-11

Madueno F, Borrais W E, Van Arkel G A and Guerrero M G 1988a Isolation and characterization of *Anacystis nidulans* $R_2$ mutants affected in nitrate assimilation. Establishment of two new mutant types; *Molec. Gen. Genet.* **213** 223-228

——, Vega-Palas M A, Flores E and Herrero A 1988b A cytoplasmic membrane protein repressible by ammonia in *Synchococcus* $R_2$: Altered expression in nitrate-assimilation mutants; *FEBS Lett.* **239** 289-291

Manzano C, Candau P, Gomez-Moreno C, Relimpio A M and Losada M 1976 Ferredoxin dependent photosynthetic reduction of nitrate and nitrite by particles of *Anacystis nidulans; Mol. Cell. Biochem.* **10** 161-169

Martin-Nieto J, Herrero A and Flores E 1989 Regulation of nitrite reductases in dinitrogen fixing cyanobacteria and Nif$^-$ mutants; *Arch. Microbiol.* **151** 475-478

Marzulf G A 1981 Regulation of nitrogen metabolism and gene expression in fungi; *Microbiol. Rev.* **345** 437-461

Mendez J M, Herrero A and Vega J M 1981 Characterization and catalytic properties of nitrite reductase from *Anabaena* sp. 7119; *Z. Pflanzen-physiol.* **103** 305-315.

—— and Vega J M 1981 Purification and molecular properties of nitrite reductase from *Anabaena* sp. 7119; *Physiol. Plant.* **52** 7-14

Mikami B and Ida S 1984 Purification and properties of ferredoxin nitrate reductase form the cyano-bacterium *Plectonema boryanum; Biochim. Biophys. Acta* **791** 294-304

—— and —— 1986 Purification of nitrate reductase by methyl vilologen-bound CM sephadex C-50 from a cynaobacterium *Plectonema boryanum; Agricul. Biol. Chem.* **47** 1653-1654

Miller J B and Amy N K 1988 Molybdenum cofactor in chlorate resistant and nitrate reductase deficient insertion mutants of *Escherichia coli; J. Bacteriol.* **155** 793-801

Muller A J and Grafe R 1978 Isolation and characterization of cell lines of *Nicotiana tobaccum* lacking nitrate reductase; *Mol. Gen. Genet.* **161** 67-76

Ohmori M and Hattori A 1970 Induction of nitrate and nitrite reductases in *Anabaena cylindrica; Plant Cell Physiol.* **11** 873-878

Omata T M, Ohmori N A and Ogawa T 1989 Genetically engineered mutants of the cyano-bacterium *Synchococcus* PCC 7942 defective in nitrate transport; *Proc. Natl. Acad. Sci. USA* **86** 6612-6616

Ortega T, Castillo F and Cardenas J 1976 Photolysis of water coupled to nitrate reduction by *Nostoc muscorum* subcellular particles; *Biochem. Biophys. Res. Commun.* **71** 885-891

——, Rivas J, Cardanas J and Losada M 1977 Metabolic interconversion of ferredoxin-nitrate

reductase and NADP+1 reductase of *Nostoc muscorum; Biochem. Biophys. Res. Commun.* **7** 185-193

Palod A, Chauhan V S and Bagchi S N 1990 Regulation of nitrate reduction in a cyanobacterium *Phormidium uncinatum*: Distinctive modes of ammonium-repression of nitrate and nitrite reductases; *FEMS Microbiol. Lett.* **68** 285-288

Rajagopalan K V, Johnson J L and Hainline B E 1981 The pterin of the molybdenum cofactor; in *72nd Annual Meeting of the American Society of Biological Chemists,* (USA: St. Louis) pp.2608-2612

Ramos J L, Guerrero M G and Losada M 1982a Optimization of conditions for photoproduction of ammonia from nitrate by *Anacystis nidulans; Appl. Environ. Microbiol.* **44** 1013-1019

——, —— and —— 1982b Sustained photoproduction of ammonia from nitrate by *Anacystis nidulans; Appl. Environ. Microbiol.* **44** 1020-1025

Romero J M, Flores E and Guerrero M G 1985a Inhibition of nitrate utilization by amino acids in intact *Anacystis nidulans* cells; *Arch. Microbiol.* **142** 1-5

—— and Lara C 1987 Photosynthetic assimilation of $NO_3^-$ by intact cell of the cyanobacterium *Anacystis nidulans; Plant Physiol.* **83** 208-212

——, —— and Guerrero M G 1985b Dependence of nitrate utilization upon active $CO_2$ fixation in *Anacystis nidulans*: A regulatory aspect of the interaction between photosynthetic carbon and nitrogen metabolism; *Arch. Biochem. Biophys.* **237** 346-401

Serrano A, Guerrero M G and Losada M 1981 Nitrate and nitrite as *in vivo* quenchers of chlorophyll fluorescence in blue-green algae; *Photosynthesis Research* **2** 175-183

——, Rivas J and Losada M 1982 Changes in fluorescence spectra by nitrate and nitrite in a blue-green alga; *Photobiochem. Photobiophys.* **4** 257-264

Shearer G, Schneider J D and Kohl D H 1991 Separating the efflex and influx components of net nitrate uptake by *Synechococcus* $R_2$ under steady-state conditions; *J. Gen. Microbiol.* **137** 1179-1184

Singh H N, Rai U N, Rao V V and Bagchi S N 1983 Evidence for ammonia as an inhibitor of heterocysts and nitrogenase formation in the cyanobactrium *Anabaena cycadeae; Biochem. Biophys. Res. Commun.* **111** 180-187

——, Sonie K C and Singh H R 1977 Nitrate regulation of heterocyst differentiation and nitrogen fixation in a chlorate resistant mutant of blue-green alga, *Nostoc muscorum; Mutation Res.* **42** 447-452

——, Vaishampayan A and Singh R K 1978a Evidence for the involvement of a genetic determinant controlling functional specificity of group VI B elements in the metabolism of $N_2$ and $NO_3^-$ in the blue-green alga *Nostoc muscorum; Biochem. Biophys. Res. Commun.* **81** 67-74

——, —— and Soni K C 1978b Mutuation from molybdenum dependent growth to tungsten dependent growth and further evidence for a genetic determinent common to nitrogenase and nitrate reductase in blue-green alga *Nostoc muscorum; Mutation Res.* **50** 427-432

Stevens S E Jr and Van Baalen C 1974 Control of nitrate reductase in blue-green alga. The effect of inhibitors, blue light and ammonia; *Arch. Biochem. Biophys.* **161** 146-152

Steward W D P and Rowell P 1975 Effects 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

Stock J B, Ninfa A J and Stock A M 1989 Protein phosphorylation and regulation of adaptive responses in baceria; *Microbiol. Rev.* **53** 450-490

Syrett P J 1988 Uptake and utilization of nitrogen compounds; in *Biochemistry of the Algae and Cyanobacteria,* eds L J Roers and J R Gallon (Oxford: Clarendon Press)

Tischner R and Schmidt A 1984 Light mediated regulation of nitrate assimilation in *Synechococcus leopoliensis; Arch. Microbiol.* **137** 151-154

Ugalde A R, Imperial J, Shah K V and Brill J W 1985 Biosynthesis of the iron-molybdenum cofactor and the molybdenum cofactor in *Klebsiella pneumoniae*: Effect of sulfur source; *J. Bacteriol.* **164** 1081-1087

Vega J M, Cardenas J and Loasda M 1980 Ferredoxin-nitrite reductase; *Methods in Enzymol.* **69** 255-270

Vega-Palas M A, Flores E and Herrero A 1992 Ntc, nitrogen regulator from the cyanobacterium *Synechococcus* that belong to the Crp family of bacterial regulators; *Mol. Microbiol.* **6** 1853-1859

——, Madueno F, Herrero A and Flores E 1990 Identification and cloning of a regulatory gene for nitrogen assimilation in the cyanobacterium *Synechococcus* sp. strain PCC 7942; *J. Bacteriol.* **172** 643-647