CHAPTER 1

INTRODUCTION

1.1 Nitrogen metabolism

Nitrogen is a quantitatively important bioelement which is incorporated into the biosphere through assimilatory processes carried out by microorganisms and plants. Numerous nitrogen containing compounds can be used by different organisms as source of nitrogen. Nitrogen is available to all living cells in the three basic forms: free nitrogen gas, combined inorganic or organic compounds (Figure1.1).

1.1.1 Nitrogen fixation

Cyanobacteria are able to fix molecular nitrogen gas (dinitrogen) and can grow in the absence of a source of combined nitrogen. This process is found only among the usually prokaryotes and exclusively in the photosynthetic cyanobacteria. Nitrogen fixation is concerned with symbiotic associations of plants and microorganisms functioning in nitrogen fixation. The best-characterized association is the legume/Rhizobium system.

Nitrogen fixation can be also carried out by free-living blue-green algae, though the organisms are able to carry out the process in symbiotic associations. The best characterized systems are the association of fungi and blue-green algae in lichens. Some filamentous cyanobacteria (e.g., those of the genera *Anabaena* and *Nostoc*) have similar functional associations between the Pteridophyte *Azolla* and the blue-green algae *Anabaena* (Peters and Mayne, 1974) and between small cycads and blue-green algae *Nostoc* or *Anabaena* (Halliday and Pate, 1976) .

Nitrogen fixation is the conversion of gaseous nitrogen (N_2, N_2O) to ammonia (NH_3). It requires nitrogenase, the enzymatic complex performing nitrogen fixation

which is extremely oxygen sensitive. The genera *Anabaera* and *Nostoc,* therefore, confine nitrogenases is heterocysts, differentiated cells specialized in nitrogen fixation.

1.1.2 Ammonium assimilation

Ammonium is the sole form of nitrogen which is incorporated directly into organic compounds. Ammonium from the soil solution or produced as a result of nitrate reduction or symbiotic nitrogen fixation is taken up and incorporated into organic compounds inside the cell. This conversion occurrs through the activities of the enzyme glutamine synthetase-glutamate synthase system. All except a few bacteria and plants are able to utilize ammonia. Many are able to use nitrate, urea or other nitrogenous compounds.

1.1.3 Nitrate metabolism

Nitrate is a major source of nitrogen for photosynthetic organisms including cyanobacteria, algae and plants. Nitrate reduction plays a key role in the nitrogen cycle and has important agricultural, environmental, and public health implications. Nitrate reduction can be performed with three different purposes:

i) the utilization of nitrate as a nitrogen source for growth, called nitrate assimilation,

ii) the generation of metabolic energy by using nitrate as a terminal electron acceptor, called nitrate respiration, and

iii) the dissipation of excess reducing power for redox balancing, called nitrate dissimilation.

The enzyme nitrate reductase is important in nitrate metabolism. It is the first enzyme that catalyzes the conversion of nitrate (NO_3^-) into nitrite (NO_2^-).

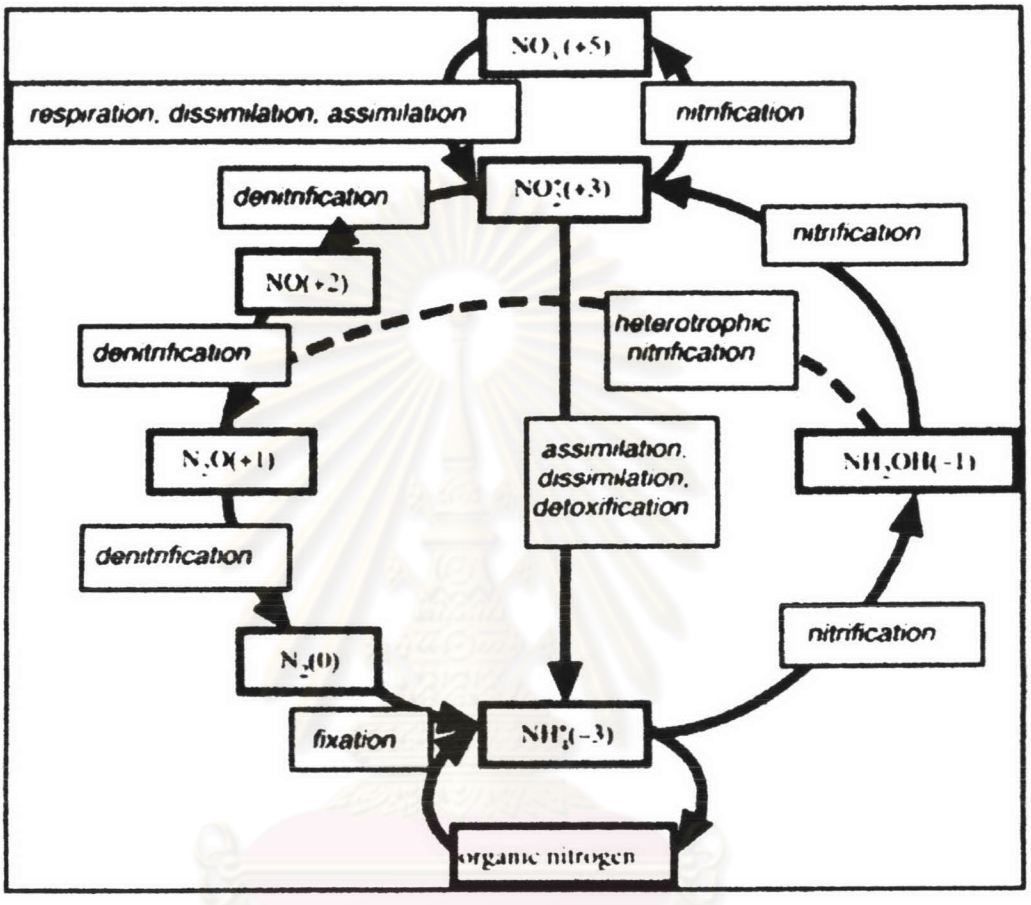


Figure 1.1: The biological nitrogen cycles.

1.1.4 Nitrification

Nitrification is usually defined as the biological oxidation of ammonium to nitrate with nitrite as an intermediate in the reaction sequence. There is no bacterium which is able to convert ammonium directly to nitrate; the oxidation is dependent on the cooperation of two different groups of bacteria, the ammonium oxidizers, recognizable by their genus name Nitroso- (*Nitrosomonas*, *Nitrosococcus*, etc.) and the nitrite oxidizers, recognizable by their genus name Nitro- (*Nitrobacter*, *Nitrococcus*). A combination of which are called nitrifier. The nitrifiers derive energy from the oxidation of ammonium and/ or nitrite. These organisms require O_2 during ammonium oxidation to nitrite and nitrite oxidation to nitrate. Oxidation of ammonium to nitrate is a two-step process:

$$NH_4^+ + 2\,O_2 \rightarrow NO_3^- + 2\,H^+ + H_2O$$

The first step, the oxidation of ammonia to nitrite, is executed by strictly chemolithotrophic bacteria (obligate chermolithotrophs) which are entirely dependent on the oxidation of ammonia for the generation of energy for growth. The probable reaction sequence for the oxidation of ammonium to nitrite by Nitroso group bacteria is:

Ammonia	→	hydroxylamine	→	nitroxyl	→	nitrohydroxylamine	→	nitrite
(NH_3/NH_4)		(NH_2OH)		(NOH)		($NO_2.NH_2OH$)		(NO_2^-)

The second step in the process of nitrification, the oxidation of nitrite to nitrate, is performed by facultative chemolithotrophic bacteria which can also use organic compounds, in addition to nitrite, for the generation of energy for growth .

1.1.5 Denitrification

The first anaerobic oxidation process occured after oxygen depletion is the reduction of nitrate to molecular nitrogen or ammonia. Denitrification is a respiratory process with nitrate as electron acceptor; nitrate is reduced to nitrogen via dinitrogen oxide. Many aerobic bacteria are able to denitrify and generate biochemical energy by electron transport phosphorylation under anaerobic conditions when oxygen is absent and either nitrate, nitrite, or dinitrogen oxide are provided.

All denitrifiers can as well live with oxygen; there is no obligate denitrifier so far. They contain a complete respiratory system: the enzyme system required for denitrification, nitrate reductase and nitrite reductase, are membrane-bound and only formed in the absence of oxygen.

The ability of many denitrifying bacteria to grow with nitrite or dinitrogen oxide indicates that the function of the dissimilatory nitrite reductase is coupled to oxidative phosphorylation.

Denitrification is most commonly defined as the biochemical reduction of NO_2^- or NO_3^- to N_2 or gaseous N oxides. Denitrification is a bacterial process in which N oxides (in ionic and gaseous forms) serve as terminal electron acceptors for respiratory electron transport.

Denitrification is illustrated by following equation:

$$6\,(CH_2O) + 4\,NO_3^- \rightarrow 6\,CO_2 + 2\,N_2 + 6\,H_2O$$

This reaction is irreversible, and occurs in the presence of available organic substrate only under anaerobic or oxygen-free conditions where nitrogen is used as an electron acceptor in place of oxygen.

1.2 Nitrate Metabolism

1.2.1 The nitrate assimilation.

The assimilatory reduction of nitrate is a fundamental biological process in which a highly oxidized form of inorganic nitrogen is reduced to ammonia. This ammonia in turn combines with carbon skeletons to form the different biological nitrogenous compounds. Not only algae, cyanobacteria and higher plants but also a variety of bacteria and fungi have the ability to assimilate nitrate. The assimilatory nitrate reducing system consists of two metalloproteins, namely assimilatory nitrate reductase (Nas) and nitrite reductase, which catalyze the stepwise reduction of nitrate to nitrite and ammonia:

$$NO_3^- \xrightarrow[\text{Nitrate reductase}]{2e^-} NO_2^- \xrightarrow[\text{Nitrite reductase}]{6e^-} NH_4^+$$

Ammonia, the end product of nitrate reduction, is incorporated into organic compounds.

According to the specificity for the electron donor, two main types of Nas can be distinguished:

1.2.1.1 Ferredoxin – dependent nitrate reductase

Ferredoxin – dependent nitrate reductase is typically present in cyanobacteria (blue-green algae) and presumably in chemoergonic and photosynthetic bacteria. According to present evidence (Candau, 1979; Hattori, 1970; Manzano *et al.*, 1976; Ortega *el al.*, 1976), nitrate reductase from cyanobacteria *Anabaena cylindrica*, *Anacystic nidulans*, and *Nostoc muscorum* cannot accept electrons directly from NAD(P)H but is rather dependent on reduced ferredoxin as the physiological electron donor. The reaction catalyzed by cyanobacterial nitrate reductase can thus be written as:

$$NO_3^- + 2\,Fd_{red} + 2\,H^+ \xrightarrow{2e^-} NO_2^- + 2\,Fd_{ox} + H_2O$$

Also flavodoxin, a low molecular weight flavoprotein which physiologically substitutes for ferredoxin under conditions of iron starvation, can act as an electron source for the reduction of nitrate to nitrite catalyzed by *Anacystis* nitrate reductase when reduced photosynthetically (Candau, 1979; Manzano, 1977) .

These enzymes are active with reduced viologens, but they cannot use reduced pyridine nucleotides as reductant.

Ferredoxin-nitrate reductase of *Anacystis* (Manzano *et al.*, 1978) has been purified recently to homogeneity and partly characterized . The enzyme is a molybdoprotein having only one polypeptide chain with a molecular weight of 75 kDa.

The cyanobaterial ferredoxin – Nas is single subunit of 58 to 85 kDa (Mikami *et* al., 1984; Rubio, 1996) whereas the flavodoxin - Nas of *Azotobacter vinelandii* is a polypeptide of 105 kDa (Gangeswaran *et al.*, 1996; Gangeswaran *et al.*, 1993). The purified Nas protein of *A.vinelandii* and *Plectonema boryanum* contain one Mo, four Fe and four acid-labile S atoms per molecule (Gangeswaran *et al.*, 1993; Mikami, 1984). Ferredoxin – Nas is also present in *Azotobacter chroococcum* (Tortolero *et al.*, 1975), *Clostridium perfringens* and *Ectothiorhodospira sphaposhnikovii* (Guerrero *et al.*, 1981).

Cyanide and *p*- hydroxymercuribenzoate (p HMB) are powerful inhibitors of *Anacystis* nitrate reductase (Manzano *et al.*, 1976). The inhibition of this enzyme by iron-binding agents and the apparent lack of heme in the enzyme molecule suggest the participation of nonheme iron in its catalytic activity (Candau, 1979). Nitrate reductases from the aerobic chemoergonic bacteria *Azotobacter chroococcum* and *Acinetobacter calcoaceticus* have been characterized partly and shown to be molybdoproteins of about 100 kDa whose activity is inhibited by cyanide and *p*HMB and stimulated by cyanate (Guerrero and Vega, 1975; Guerrero *et al.*, 1973; Villalobo *et al.*, 1977).

1.2.1.2 NAD(P)H - Nitrate reductase

Nitrate reductases of green algae, higher plants and fungi catalyzes the reduction of nitrate to nitrite by reducing pyrimidine nucleotides (Losada and Guerrero, 1979; Vennesland and Guerrero, 1979) according to the equation:

$$NO_3^- + NAD(P)H + H^+ \xrightarrow{2e^-} NO_2^- + NAD(P)^+ + H_2O$$

According to the specificity for NADH or NADPH, three subclasses of pyrimidine nucleotide-dependent nitrate reductases have been distinguished:

i) EC 1.6.6.1 is specific for NADH and corresponds to the enzymes from photosynthetic eukaryotes (Beevers and Hagernan, 1969; Beevers and Hagernan, 1972; Kuo *et al.*, 1980) and in some species of green algae such as *Chlorella* (Solomonson, 1979; Syrett and Morris, 1963) show a certain preference for NADH.

ii) EC 1.6.6.2 found in other green algae such as *Ankistrodesmus braunii* (Ahmed and Spile, 1976; Diez *et al.*, 1977) and yeast (Guerrero and Gutierrez, 1977; Rivas *et al.*, 1973) can use either of the reduced nucleotides with about the same effectiveness. Accordingly, these nitrate reductase have been classified as NAD(P)H - dependent.

iii) EC 1.6.6.3 is specific for NADPH and appears to be a better reductant for fungal nitrate reductases such as nitrate reductases of the molds *Aspergillus nidulans* and *Neurospora crassa* (McDonald *et al.*, 1974; Pan and Nason, 1978).

Nitrate assimilation process and comparison of the utilization of electron donor shows in Figure 1.2.

In addition to catalyzing the normal physiological reaction, i.e. reduction of nitrate by reduced pyrimidine nucleotides, NAD(P)H - nitrate reductase exhibits two other activities, which can be assayed separately and selectively inhibited to involve only partial functions of the overall electron transport chain of the enzyme complex.

The diaphorase activity represents the function of the first (NAD(P)H-activating) moiety of the complex. Its action results in the reduction by NAD(P)H of a variety of 1- or 2-electron acceptors. The so-called terminal nitrate reductase constitutes the second (nitrate - activating) moiety of the complex and is expressed as a pyridine nucleotide-independent activity which results in the reduction of nitrate by reduced flavins or viologens. Both moieties participate jointly and sequentially in the transfer of electrons from NAD(P)H to nitrate (Losada and Guerrero, 1979).

The pH optima, Km for substrates and molecular properties of some of the highly purified pyrimidine nucleotide nitrate reductases from different sources are summarized in Table 1.

The general pattern which can be inferred from the available data is that NAD(P)H - nitrate reductase is an oligomeric enzyme (molecular weight from 197 to 460 kDa) composed of a variable number of apparently identical subunits.

Table 1.1: Properties of highly purified assimilatory nitrate reductases.

	Anacystis nidulans	*Chlorella vulgaris*	*Ankistrodesmus braunii*	*Spinacia oleracea*	*Neurospora crassa*	*Rhodotorula glutinis*
Molecular weight (kdaltons)	75	280	460	197	228	230
Subunits	1	3	8	2 + 2 ?	2	2
$s_{20,w}$(S)	5.4	10.0	10.9	8.1	8.0	7.9
Stokes radius (nm)	3.2	6.3	9.8	6.0	7.0	7.0
Electron donor	Fd	NADH	NAD(P)H	NADH	NAD(P)H	NAD(P)H
Specific activity (U/mg)	875[a]	93	61[b]	24	125[b]	148[b]
Turnover number (s^{-1})	1094[a]	434	468[b]	79	475[b]	567[b]
Optimum pH	10.5[a]	7.6	7.5	7.5	7.5	7.5
K_m NO_3^- (μM)	690	84	150[b]	180	200[b]	125[b]
K_m electron donor (μM)	13	nr[c]	13[b]	4.6	62[b]	20[b]
Prosthetic groups (mol/mol)						
FAD	—	2.6	nr*[c]	nr*	nr*	nr*
cyt *b*-557	—	2.5	4	nr*	1.5	1
Mo	nr*	2.0	nr*	nr*	0.9	nr*
E_{m7} cyt *b*-557 (mV)	—	nr	−73; n = 1	−60; n = 1	nr	nr
Spectral properties						
Absorption maxima	273	279; 413 (ox) 423; 527; 557 (red)	280; 416 (ox) 424; 527; 557 (red)	280; 413 (ox) 424; 527; 557 (red)	280?; 413 (ox) 423; 528; 557 (red)	278; 412 (ox) 423; 527; 557 (red)
A_{280}/A_{Soret}	—	1.7	3.1	4.6	nr	nr
ϵ (Soret) ($mM^{-1} \cdot cm^{-1}$)	—	280	460	nr	nr	124

[a] Dithionite-reduced methyl viologen as reductant.
[b] NADPH as reductant.
[c] Definitions: nr, not reported; nr*, present, but no quantitative data available.

The NADH - nitrate reductase of *Klebsiella pneumoniae* and *Rhodobacter capsulatus* are heterodimers of a 45 kDa FAD-containing diaphorase and a 95 kDa catalytic subunit with MGD (*bis*-molybdopterin guanine dinucleotide) cofactor and a putative N-terminal [4Fe-4S] center.

From the available data on highly purified NAD(P)H - nitrate reductase from different sources, it appears that FAD, cytochrome *b-557* and molybdenum are ubiquitous prosthetic groups. Nitrate reductase of the mold *Neurospora* appears to contain one molybdenum atom and about two cytochrome *b-557* molecules per enzyme molecule (Pan and Nason, 1978). Also, only one molecule of cytochrome *b* is thought to be present in the enzyme of the yeast Rhodotorula (Guerrero and Gutierrez, 1977). The NADH - dependent enzyme of *Chlorella* (molecular weight 280 kDa) has been show to contain about three molecules of FAD, a molecule of cytochrome *b* and two molybdenum atoms, and make up of three similar subunits of about 90 kDa (Giri and Ramadosss, 1979). In contrast, NAD(P)H - nitrate reductase from *Ankistrodesmus* exhibits a higher molecular weight (460 kDa) and appears to be composed of eight similarly sized (58 kDa) subunits with an overall content of at least four cytochrome *b* molecules (Ahmed and Spiler, 1976; De la Rosa, 1980).

Assimilatory NAD(P)H - nitrate reductases are generally inhibited by *p*–HMB, cyanide, azide and cyanate. The diaphorase moiety of the enzyme complex is very sensitive to the action of sulfhydryl-binding reagents, whereas azide and cyanate inhibit competitively with respect to nitrate (Vennesland and Guerrero, 1979). Cyanide is a potent inhibitor of nitrate reductase activity and forms a stable complex with the reduced enzyme (Lorimer *et al.*, 1974). Nitrite, the reaction product, has been reported to act as an inhibitor of NAD(P)H - nitrate reductase. The inhibition is of the competitive type with respect to nitrate in the enzymes of *Chlorella*, *Aspergillus*, and *Rhodotorula* (Guerrero and Gutierrez, 1977; McDonald and Coddington, 1974; Vennesland and Guerrero, 1979). Chlorate can replace nitrate as a substrate for purified nitrate reductase or as competitive inhibitor of the reduction (Notton and Hewitt, 1979; Vennesland and Guerrero, 1979).

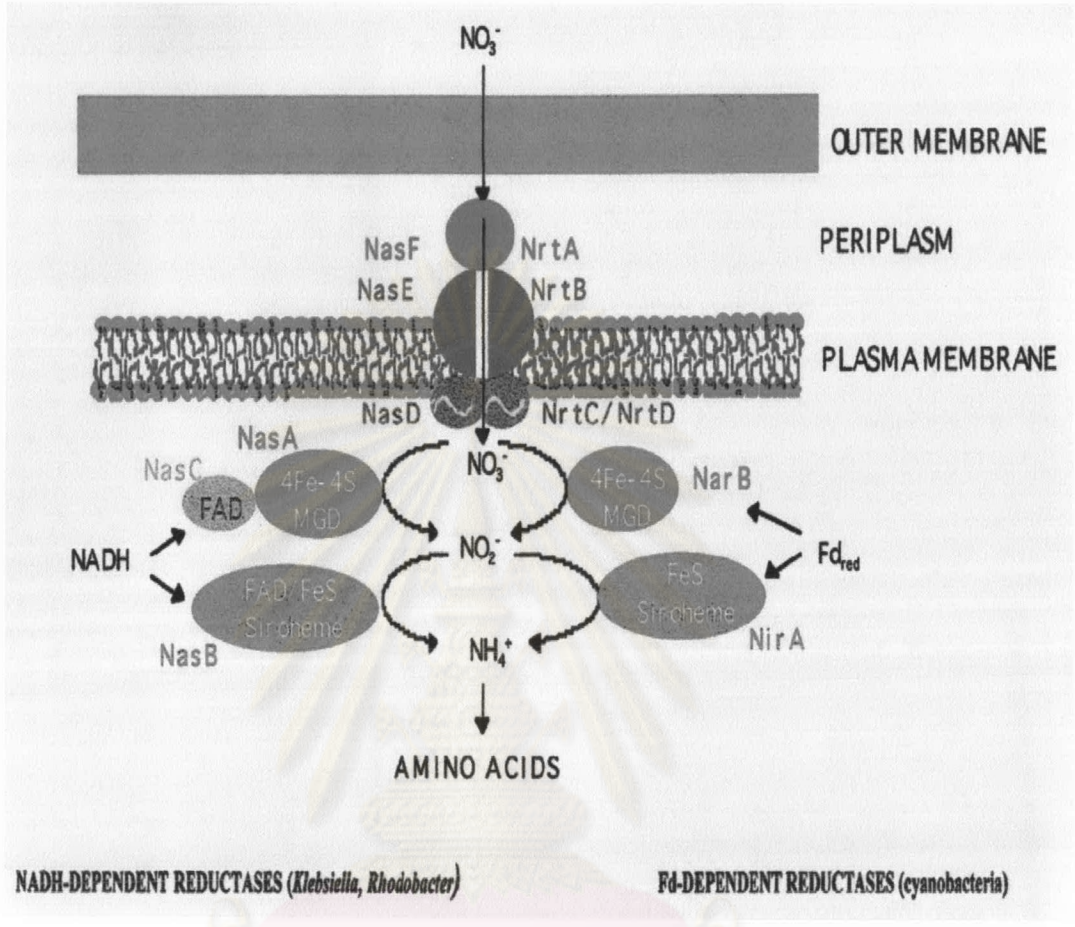


Figure 1.2: Nitrate assimilation and comparison of the utilization, the scheme shows the cyanobacterial ferredoxin (Fd)-dependent assimilatory nitrate and nitrite reductase (right) and the NADH-dependent nitrate and nitrite reductases from *Klebsiella* and *Rhodobacter* (left) (Moreno-Vivian *et al.*, 1999).

The pathway of electrons from NAD(P)H to nitrate through nitrate reductase from eukaryotes may thus be depicted as:

$$NAD(P)H \longrightarrow (\ FAD \longrightarrow \text{cyt } b\text{-}557 \longrightarrow Mo \longrightarrow NO_3^-\ )$$

Regulation of nitrate reductase by changing the activities of the enzymes of the nitrate-reducing pathway is important in the control of the overall process of nitrate assimilation. The reduction of nitrate to nitrite, rather than the further reduction of nitrite to ammonia, appears therefore more likely to be a rate-controlling step in nitrate reduction. Beevers & Hageman (1969) concluded that nitrate reductase was the logical point to effect regulation of the input of reduced nitrogen for the organism. In fact, nitrate reductase levels have been shown to fluctuate in response to changes of environmental conditions such as light, temperature, pH, CO_2 and oxygen tensions, nitrogen source, water potential, and other factors (Beevers and Hageman, 1969, 1972; Hageman, 1979; Hewitt, 1975; Hewitt *et al.*, 1976; Losada and Guerrero, 1979; Losada *et al.*, 1981; Marris, 1974; Srivastava, 1980; Syrett and Leftley, 1976; Vennesland and Guerrero, 1979). These changing that usually also influence the capacity of the organisms to assimilate nitrate. In general, the level of nitrate reductase synthesis is high in organisms grown on nitrate and low in organisms grown on ammonium. The antagonistic effect of ammonium on nitrate metabolism with regard to the synthesis of the enzymes of the nitrate-reducing system is evident in many organisms. The situation appears particularly clear for lower plants where ammonium usually overrides any stimulating effect of nitrate, enzyme activity being totally absent or present only at basal levels both in ammonium and in ammonium nitrate containing medium. This is indeed the case for some chemoergonic bacteria (Villalobo *et al.*, 1977), unicellular blue-green algae (Herrero et al., 1981), eukaryotic algae (Diez *et al.*, 1977; Herrera *et al.*, 1972; Losada *et al.*, 1970; Rigano *et al.*, 1974; Syrett and Hipkin, 1973; Syrett and Leflley, 1976; Vega *et al.*, 1971) and fungi (Garrett and Amy, 1978).

1.2.2 The nitrate respiration

The ability to respire nitrate is widely spread among bacteria that can dissimilate the produced nitrite to gaseous compounds (denitrification) or to ammonia (dissimilatory nitrate reduction to ammonia). Denitrification is the major biotic process leading to the loss of fixed nitrogen from the environment as well as removal of excess nitrate from surface and wastewaters, accumulated mainly as a result of agricultural. The major of dissmilatory nitrate reductase is respiratory mambrane-bound nitrate reductase (Nar). Membrane-bound nitrate reductase are associated with denitrification and anaerobic nitrate respiration (Figure 1.3). The membrane-bound nitrate reductase is widely spread among enteric bacteria (Hochstein and Tomlinson, 1988; Knowles, 1981; Tiedje, 1988; Zumft, 1992, 1997), however, characterised best in *E.coli* (Garland *et al.*, 1975; Bnnefoy-Orth, 1981; Chauldry *et al.*, 1983; Blasco *et al.*, 1992). This enzyme couples the reduction of nitrate to the formation of a transmembrane proton gradient, which generates a transmembrane proton motive force (PMF) allowing ATP synthesis. In *E. coli,* there are two different membrane-bound isoenzymes: NRA(nitrate reductase A), which is expressed under anaerobiosis in the presence of nitrate and represents 90% of total activity, and NRZ(nitrate reductase Z), which is expressed constitutively (Blasco *et al.*, 1990; Bonnefoy and De Mogs, 1994). Nar enzymes are composed of three subunits: a catalytic α subunit (Nar G) of 112 to 140 kDa with MGD (*bis*-molybdopterin guanine dinucleotide) cofactor, a soluble β subunit (Nar H) of 52 to 64 kDa with one [3Fe-4S] and three [4Fe-4S] centers, and a 19 to 25 kDa membrane biheme *b* quinol oxidizing γ subunit (Nar I). Nar GHI is arranged in two domains with the α- and β- subunits constituting the cytoplasmic domain and the γ -subunit constituting the membrane domain required for attachment of the α- and β-subunits to the cytoplasmic side of the inner membrane (MacGregor, 1975, 1976; Sodergren *et al.*, 1988). Dissimilatory nitrate reductase in membrane fractions from bacteria has been shown to be capable of utilizing a variety of respiratory intermediates and reduce pyridine nucleotides for nitrate reduction (Cole and Wimpeny, 1968; Knook *et al.*, 1973; Burke and Lascelles, 1975; Enoch and Lester, 1975).

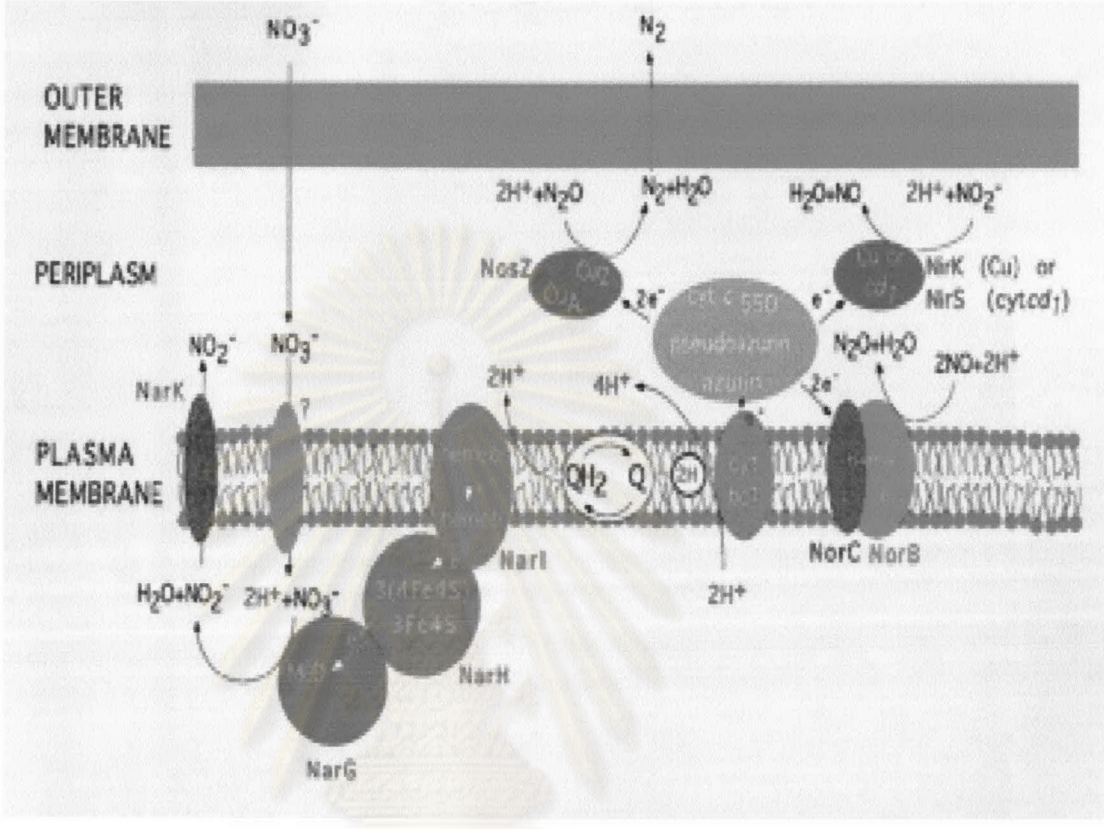


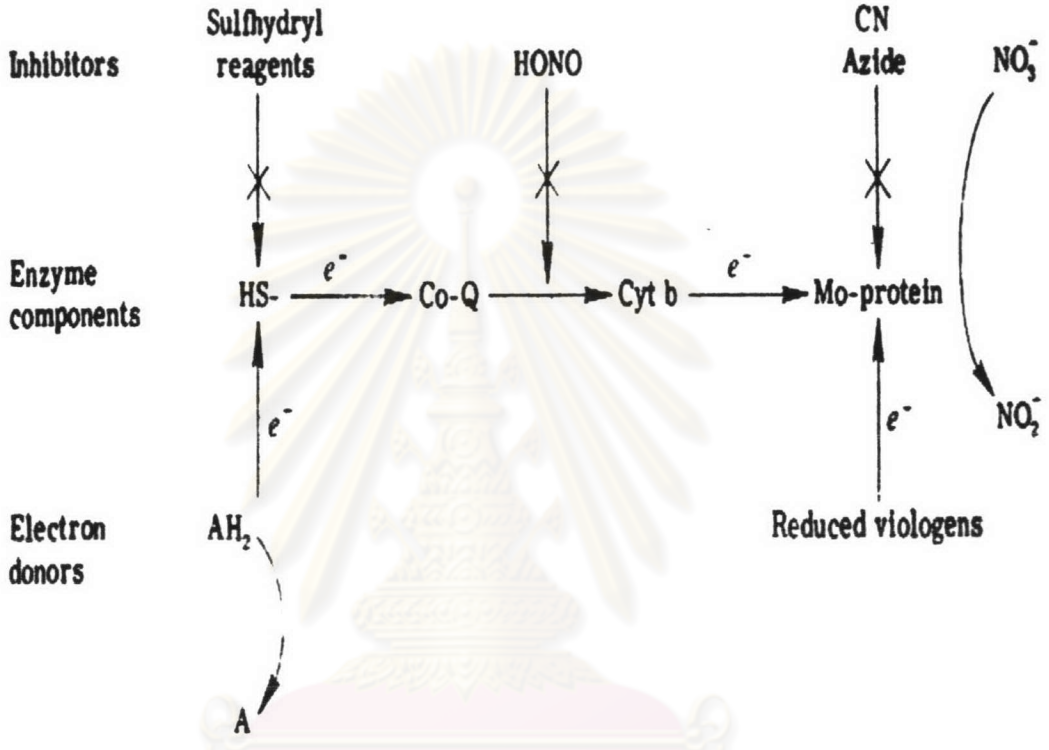
Figure 1.3: Nitrate respiration and denitrification pathways in bacteria. The quinone pool is indicated by the white oval labeled $QH_2 \rightleftarrows Q$. cyt , cytochrome (Moreno-Vivian *et al.*, 1999).

Reduction of nitrate by the membrane fractions, when respiratory substrates or pyridine nucleotides serve as reductant, is generally inhibited by azide, cyanide and *p*-chloromercuribenzoate. Nitrate reduction, mediated by respiratory substrates, could be inhibited by *n*-heptylhydroxyquinoline-N oxide (HONO) or dicoumoral (Ruiz-Herrera and DeMoss, 1969; Knook *et al.*, 1973; Burke and Lascelles, 1975). Extraction of ubiquinone with pentane eliminates the nitrate reducing capacity of membrane particles from *Klebsiella aerogenes* (Knook and Planta, 1971). The activity could be restored by incorporation of ubiquinones. When particular preparation from bacteria grown anaerobically in the presence of nitrate are incubated with respiratory substrates or reduced pyridine nucleotides, it is observed that the cytochromes become reduced (Knook *et al.*, 1973; Lam and Nicholas, 1969; Ruiz-Herrera and DeMoss, 1969; Vila *et al.*, 1977). Because the cytochrome *b* component is reoxidized when nitrate is added, it appears that the Mo protein (nitrate reductase) transfers eletrons from cytochrome *b* to nitrate. These observations on dissimilatory nitrate reduction by bacteria are summarized in the following scheme (Figure 1.4).

Electrons are transferred by the respiratory electron chain to a cytochrome *b* which then donates electrons to the Mo-protein (nitrate reductase). The sensivity of nitrate reduction to inhibition by cyanide is considered to be due to association of the inhibitor with molybdenum residues in the nitrate reductase (Enoch and Lester, 1975). Cytochrome *b* involved in nitrate reduction does not bind cyanide.

For nitrate transport in dissimilatory membrane-bound nitrate reductase system, nitrate has to be transported into the cells before it is reduced, and nitrite is usually excreted to the periplasm by specific nitrite extrusion system. The respiratory nitrate uptake is poorly understood, although it is clear that the nitrate porter is highly specific for nitrate and is inhibited by oxygen (Denis *et al.*, 1990). Oxygen inhibition of nitrate transport seems to be caused by an indirect mechanism (i.e., the diversion of electrons to oxygen), rather than causing conformational changes in the porter system (Denis *et al.*, 1990). Regulation of respiratory membrane-bound nitrate reductases, in *E.coli*, Nar protein are synthesized during anaerobic growth in the presence of nitrate

or nitrite. Synthesis of Nar enzymes is unaffected by ammonium (Berk *et at.*, 1995; Hochstein and Tomlinson, 1988; Stewart, 1994; Zumft, 1997).



where A=pyridine nucleotides or respiratory substrates and appropriate enzynes .

Figure 1.4: The scheme of electron transfer and the effect of inhibitor on dissimilatory nitrate reductase.

1.2.3 Periplasmic nitrate reductase (Nap)

Periplasmic nitrate reductases were first reported for phototrophic and denitrifying bacteria, but they are widespread among gram-negative bacteria. Different physiological functions have been proposed for this enzyme. The Nap activity seems not to be primarily involved in nitrate assimilation or anaerobic respiration, although the nitrite generated by Nap can be used as a nitrogen source or as a substrate for anaerobic respiration depending on the organism. The Nap enzyme, as a consequence of its periplasmic location, does not directly contribute to the generation of a PMF. The Nap system is also independent of the energy- conserving cytochrome bc_1 complex, but it is likely linked to the generation of a PMF when the electrons from NADH are passed through the proton-translocation NADH dehydrogenase (Berk *et al.*, 1995; Richardson and Watmough, 1999). However, this seems to be insufficient to support anaerobic growth on nitrate in *Rhodobacter sphaeroides* in the dark (Kerber and Cardenas, 1982; Moreno-Vivian and Ferguson, 1998). Also, the anaerobic growth rate on nitrate of a *T. pantotropha* $NarH^-$ mutant overexpressing Nap activity is decreased threefold on account of the reduced energy conservation by Nap relative to Nar during denitrification (Bell *et al.*, 1993). However, in *Pseudomonas* sp. strain G-179, the Nap enzyme catalyzes the first step of denitrification in an energy generating process, although the mechanism used by Nap to gain energy is unclear (Bedzyk *et al.*, 1999). Thus, the physiological role of the Nap system may vary in different organisms even in the same bacterium under different metabolic conditions. There are clear evidences that Nap is a dissimilatory enzyme used for redox balancing (Berks *et al.*, 1995; Moreno-Vivian and Ferguson, 1998; Richardson *et al.*, 1988; Roldan *et al.*, 1994; Sears *et al.*, 1997). Maintenance of an appropriate redox balance can be necessary for optimal bacterial growth under some physiological conditions, particularly during fermentative processes in enteric bacteria, oxidative metabolism of highly reduced carbon substrates in aerobic heterotrophs, or anaerobic photoheterotrophic growth in photosynthetic bacteria. In addition, since oxygen primarily inhibits denitrification at the level of nitrate transport (Denis *et al.*, 1990) and the Nap system does not require this step, some denitrifiers perform aerobic denitrification coupling the Nap enzyme

to the nitrite and N-oxid reductases (Bell *et al.*, 1990; Berks *et al.*, 1995). Aerobic denitrification can be a valuable feature for organisms growing under microaerobic conditions or in environments rapidly changing between aerobic conditions and anoxia. Other proposed roles for Nap are the adaptation to anaerobic metabolism after transition from aerobic conditions, the utilization of alternate reductants (Siddiqui *et al.*, 1993), or even a self-defense mechanism forming high nitrite levels to inhibit the growth of potential competing bacteria (Kerber and Cardenas, 1982). Nap systems have been studied at the biochemical and genetic level in *Alcaligenes eutrophus* (*Ralstonia eutropha*), *T. pantotropha* (*P. denitrificans*), *E. coli*, and *Rhodobacter* species (Figure1.5).

The enzyme is a heterodimer containing a 90-kDa catalytic subunit (NapA) with MGD cofactor and an N-terminal [4Fe-4S] center and a 15-kDa biheme c cytochrome (NapB), which receives electrons from NapC, a membrane bound tetraheme cytochrome c of 25 kDa (Berks *et al.*, 1994, 1995; Reyes *et al.*, 1996, 1998). Activity with reduced viologens as electron donors decreases when the NapB subunit is lost during the purification of the *R. capsulatus* enzyme (McEwan *et al.*, 1987). Evidence for a role of NapC in the electron transfer to the periplasmic enzyme complex has been presented by the results of mutational analysis in *R. sphaeroides* (Reyes *et.al.*, 1996, 1998). NapC homologues are involved in electron transfer between the membrane quinol pool and several soluble periplasmic reductases. Electron transfer by the NapC family appears to be cytochrome bc_1 independent and is not coupled to proton translocation. The spectroscopic characterization of a soluble form of NapC expressed as a periplasmic protein has been recently reported (Roldan *et.al.*, 1994).

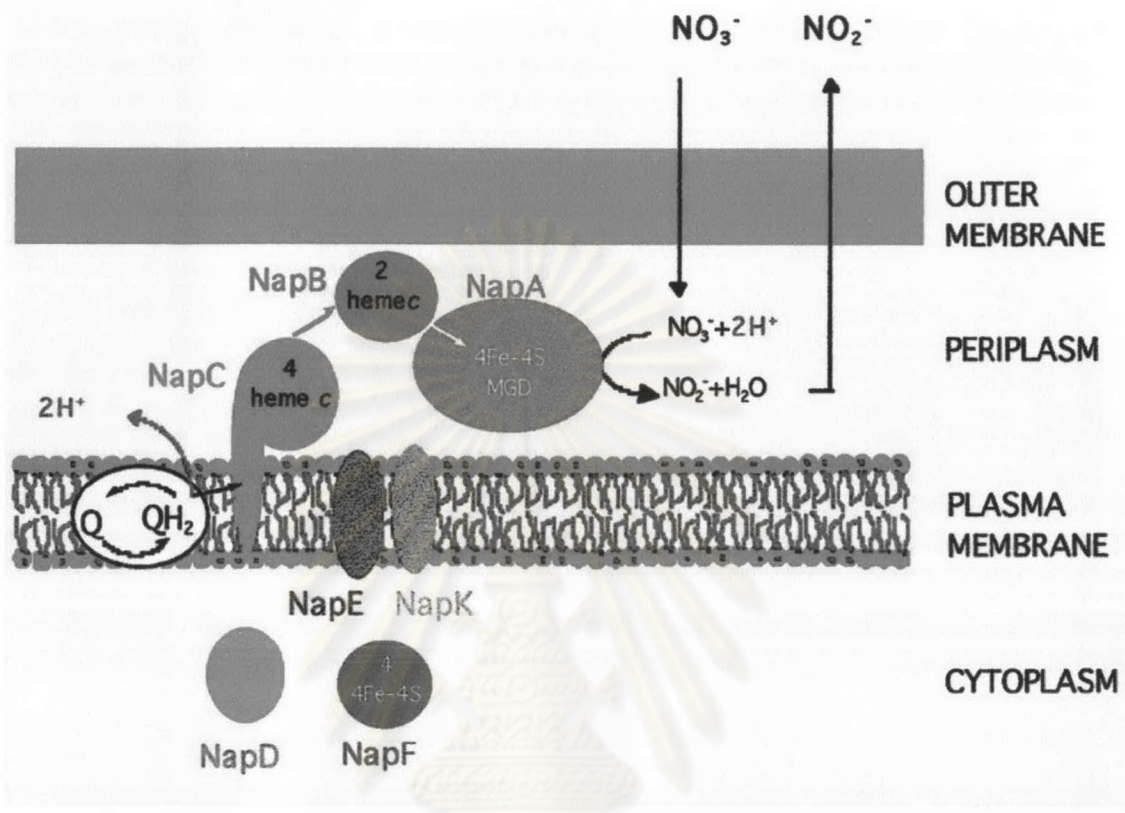


Figure 1.5: Periplasmic nitrate reductase of *R.sphaeroides* (Moreno-Vivian *et al.*, 1999).

The crystal structure of the *Desulfovibrio desulfuricans* Nap protein, a single-subunit form that lacks the biheme cytochrome c subunit (NapB), has been recently determined at a resolution of 1.9 A (Dias *et.al.*, 1999). The Nap protein is folded into four domains, all of them involved in MGD cofactor binding, and its structure is more similar to that of formate dehydrogenase than to that of dimethylsulfoxide reductase. In the catalytic site, a single Mo atom is coordinated to two MGD molecules, a Cys residue, and a water/hydroxo ligand, and an electron transfer pathway through bonds connects the molybdenum and the [4Fe-4S] center (Dias *et. al.*, 1999). This structure suggests a monooxo/desoxo catalytic cycle, although a di-oxo/mono-oxo cycle has also been proposed for the *T.pantotropha* enzyme (Bennett *et al.*, 1996). The results of electron paramagnetic resonance and X-ray absorption spectroscopy analyses suggest that the Mo environments in the soluble Nas and Nap enzymes are similar to each other but distinct from that in the membrane-bound Nar (Bennett *et al.*, 1996; Breton *et.al.*, 1994; Gangeswaran *et al.*, 1993). Nap and Nar are also catalytically distinct; Nap is less sensitive to inhibition by cyanide, does not use chlorate as substrate, and is slightly stimulated by thiocyanate and azide (Berks *et al.*, 1995; Hochstein and Tomlinson, 1988). However, in *R. sphaeroides*, Nap activity is competitively inhibited by chlorate. Both nitrate and chlorate stimulate the phototrophic growth in the wild-type strain, but not in a NapA$^-$ mutant. This Nap-dependent chlorate or nitrate stimulation of bacterial growth has been explained in terms of redox balancing; the dissipation of excess photosynthetic reducing power allows optimal growth (Moreno-Virian *et al.*, 1994; Roldan *et al.*, 1994).

Regulation of dissimilatory periplasmic nitrate reductases have been investigated. Although there are some differences in the *nap* gene expression depending on the organisms, the Nap systems are normally unaffected by ammonium or oxygen. In phototrophic bacteria, the Nap activity is present under aerobic and anaerobic conditions and is unaffected by ammonium or by the intracellular C and N balance. In addition, Nap activity is stimulated by nitrate, although a basal activity is also observed in the absence of nitrate (Dobao *et al.*, 1994; Reyes *et al.*, 1996). In *P. denitrificans*, the Nap activity is observed in aerobically grown cells even in the absence of nitrate. Activity is maximally expressed during growth on highly reduced carbon sources, such as butyrate, suggesting a Nap regulation in response to the redox

state of the bacterium (Sears *et al.*, 1997). Similarly, the Nap system is not induced by nitrate in *A. eutrophus*, and maximal expression is observed under aerobic conditions at the stationary phase (Siddiqui *et al.*, 1993).

The Nap has now been purified from various bacteria including enterobacteria, denirifiers and non-sulfur photosynthetic bacteria, such as *Rhodobacter capsulatus* (McEwan *et al.*, 1987; Richardson *et al.*, 1990), *Thaiosphaera pantotropha* (reclassified as a strain of *Paracoccus denitrificans* by Ludwing *et al.*; Bell *et al.*, 1990; Sears, 1993; Berks *et al.*, 1994) and *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (Siddiqui *et al.*, 1993; Warnecke-Eberz and Friedrich, 1993).

Periplasmic and membrane-bound nitrate reductases have distinct structures as shown by the existence of only two subunits in Nap. These two subunits, termed NapA (63-93 kDa) (Siddiqui *et al.*, 1993; Berks *et al.*, 1994; Warnecke-Eberz and Friedrich , 1993) and Nap B (13-19 kDa) (Reyes *et al.*, 1996; Berks *et al.*, 1994; Grove *et al.*, 1996).

1.3 Nitrate Uptake

Nitrate uptake is generally believed to be the rate-limiting step in nitrate assimilation, and the overall rate of nitrate assimilation is tightly regulated at the nitrate uptake step by the metabolic carbon / nitrogen status of cells (Flores and Herrero, 1994). It has been known for a long time that ammonium suppresses nitrate uptake in many algae species and also in various higher plants (Ohmori *et al.*, 1977). It was demonstrated many times that in the presence of both ammonium and nitrate in the medium, the NH_4^+ is assimilated first, and only when it has gone is NO_3^- utilized (Shen, 1969). Preferential uptake of NH_4^+ has been shown often for both marine and freshwater algae (Hewitt *et al.*, 1980). However, there have been few reports showing preferential assimilation of nitrate; this phenomenon has been reported for *Pandorina* and *Haematococcus*. There are several reasons for this preferential assimilation of ammonium. Active nitrate reductase is not formed in the presence of NH_4^+ nor is the NO_3^- uptake system. And even if active nitrate reductase and NO_3^- uptake system are present, the addition of NH_4^+ can lead to a rapid cessation of NO_3^-

utilization (Losada *et al.*, 1970). There are at least three mechanisms by which nitrate reductase activity can disappear from cells. These include two sorts of reversible inactivation phenomena and irreversible loss of enzyme due, presumably, to degradation (Losada *et al.*, 1970). The effect of addition of NH_4^+ to cells assimilating NO_3^- is complex. The first effect appears to be an inhibition of NO_3^- uptake and this is followed by loss of nitrate reductase (and nitrite reductase) activities. The loss of nitrate reductase activity will be partly due to reversible inactivation , and partly due to irreversible loss of enzyme with the rate of proteolytic breakdown of nitrate reductase possibly being greater in the presence of NH_4^+ (Morris, 1974). At the same time addition of NH_4^+ stops the synthesis of nitrate reductase. The nitrate reductase formation is repressed in the presence of NH_4^+.

Nitrate uptake can be stimulated by light, leading to indirect activation of nitrate reductase (Tisher *et al.*, 1978). The enzyme nitrate reductase can also be directly activated by light; light can furthermore induce nitrate reductase synthesis (Tisher *et al.*, 1978). Nitrate reductase activity was induced both by red and blue light pulses and the effect was partially reversed by far red light in the green marine alga *Ulva lactuca*. Continuous blue light generally is more effective than continuous red light at stimulating nitrate uptake and metabolism . Light also controls nitrate reductase activity in *Chlorella* sp (Tisher *et al.*, 1978). Most authors have not observed inhibition of high light intensities.

1.3.1 Nitrate uptake in cyanobacteria

The first unequivocal demonstration of gene responsible for nitrate transport and of its essential role in nitrate assimilation is the cyanobacterium *Synechococus* sp. PCC7942 (Omata *et al.*, 1989), and the subsequent works have shown the structure of the nitrate transporter of the cyanobacteria (Omata *et al.*, 1989). Clusters of genes involved in cyanobacterium *Synechococus* sp.PCC7942 nitrate assimilation has been mapped. They are in an operon that is regulated by nitrogen availability and are cotranscribed. The operon consists of two nitrogen regulated genes *nirB* and *ntcB*, one structural gene *nirA* for nitrite reductase, four genes for nitrate transport (*nrtA*, *nrtB*, *nrtC* and *nrtD*) , and one structure gene *narB* for nitrate reductase. Nitrate

transport is essential for the growth of the cyanobacterium at physiological concentration of nitrate and has been shown to be involved in the active transport of nitrate as well. The deduced amino acid sequences of the NrtB, NrtC, NrtD proteins indicate that the transport is a member of the ABC (ATP-binding cassette) superfamily of active transport. A 4 kb fragment of the marine diazotrophic, filamentous cyanobacterium *Trichodesmium* sp. strain WH9601 contains gene sequences encoding the nitrate reduction enzymes, nirA and narB. A third gene positioned between *nirA* and *narB* encodes a putative membrane protein with similarity to the nitrate permeases of *Bacillus subtilis* (NasA) and *Emericella nidulans* (CrnA). The gene was shown to functionally complement a Delta *nasA* mutant of *B. subtilis* and was assigned the name napA (nitrate permease). NapA was involved in both nitrate and nitrite uptake by the complemented *B. subtilis* cells. NapA is distinct from the *nrt* genes that encode the nitrate transporter of freshwater cyanobacteria.

The activity of the nitrate-assimilation system of cyanobacterium is regulated transcriptionally in response to changes in nitrogen availability; biosynthesis of the NrtA protein, expression of nitrate-transporting ability, and expression of NR and NiR activities are all subjected to nutritional repression by ammonium (Herrero *et al.*, 1981). In *Synechococus* sp.PCC7942, the concerted regulation of the activities related to nitrate assimilation is ascribed to cotranscription of gene *nirA-nrtABCD-narB* as a single operon (Suzuki *et al.*, 1992, 1993). Ammonium, either added to the medium or generated internally by reduction of nitrate, negatively regulates the transcription of the *nir A* operon (Suzuki, 1993). Nitrate assimilation by cyanobacteria is subjected not only to transcriptional regulation but also post-translational regulation (Guerrero and Lara, 1987). Addition of ammonium to medium causes prompt cessation of nitrate uptake in nitrate-utilizing cells of *Synechococus* sp. PCC6301 (*Anacystis nidulans*), a strain closely related to the strain PCC7942. By determining the intracellular concentration of nitrate, Lara *et al.* (1987) showed that ammonium inhibits active transport of nitrate. As in the regulation of the *nir A* operon, fixation of ammonium to Glu is required for the negative effects of ammonium to prevail on the activity of nitrate transport (Lara *et al.*, 1987). It is supposed that a metabolite(s) of Glu acts as a negative effecter of nitrate transport.

Nitrate transport by *Synechococus* sp. PCC 7942 cells is inhibited by ammonium and inhibitors of CO_2 fixation. Ammonium assimilation inhibitors, such as L-methionine D,L-sulfoximine (MSX), are known to prevent the negative effects of ammonium and inhibitors of CO_2 fixation on nitrate uptake, leading to the suggestion that CO_2 fixation is required to counteract the feed back inhibition of nitrate assimilation. In NR-loss mutants, L-methionine D, L-sulfoximine prevents the negative effects of ammonium on nitrate transport, but not always prevents those inhibiting CO_2 fixation. The mutant strain NC2, constructed by deleting 3 portion of *nrtC*, shows high nitrate transport activity insensitive to ammonium but sensitive to inhibitors of CO_2 fixation. These findings indicate that the C-control and N-control of nitrate transport are independent at both the physiological and the molecular level (Rodriguez *et al.*, 1998). In the unicellular cyanobacterium *Synechococcus* PCC 7942 that does not fix molecular nitrogen, interactions between N assimilation and C assimilation occur through the signal transducer P-II and the global nitrogen regulator NtcA. Under high CO_2 concentration, P-II liganded to ATP and bound to 2-oxoglutarate becomes phosphorylated and negatively controls the high affinity transport for bicarbonate. In contrast, under low CO_2, P-II being only liganded to ATP becomes dephosphorylated and negatively controls the nitrate/nitrite active transport system.

The presence of NaCl in the nutrient solution promotes nitrate uptake in parent *Anabaena* sp. PCC 7210 mutants SP7 (defective in nitrate reductase activity) and SP17 (partially defective in nitrate reductase activity), but not in the mutant SP9 (defective in nitrate transport and reduction). Nitrate reductase activity of the parent and mutant SP17 is increased with increasing concentration of nitrate in saline medium, while mutants SP7 and SP9 do not respond to the altered salinity (Ashwani and Tiwari, 1999).

The effect of low temperature on nitrate assimilation was examined in the cyanobacterium *Synechococcus* sp. PCC6301 to determine the factor that limits growth. The rate of nitrate consumption by cells as a function of temperature decreases with decreasing temperature. Cells can not actively take up nitrate at 15^0C, although nitrate reductase and nitrite reductase are still active.

1.3.2 Enhancement of cyanobacteria salt tolerance by combined nitrogen

Salt overloading in soils is by far the major hindrance for plant growth, and a crucial problem for agriculture. Each organism displays a salinity tolerance range, which includes its optimal growth conditions, though larger ranges of salt concentrations (resistance range) may still be compatible with cell survival. Crop plants are particularly limited in their tolerance range, whereas other photosynthetic organisms, including microalgae and cyanobacteria, may display wider acclimation capacities. Exposure of cells to salt concentrations that are physiologically above those present intracellular threatens them via two deleterious effects, increase of both the osmotic pressure and the ion concentration. The water potential is decreased, leading to loss of water by the cells, and simultaneously influx of ions into the cytoplasm. Stress from NaCl, in particular, causes a dramatic increase in the concentration of inorganic ions.

Cyanobacteria, the only prokaryotes performing oxygenic photosynthesis and probable ancestors of chloroplasts, constitute valuable models for the study of the molecular mechanisms involved in tolerance to high salinity, or to its corollary, drought, a major agricultural problem. The critical demands of cyanobacteria exposed to high salinity, i.e., accumulation of osmoprotectors and extrusion of sodium ions, are met through immediate activation and/or long term (protein synthesis-dependent) adaptation of various processes: (i) uptake and endogenous biosynthesis of osmotica, the nature and amount of which are strain and salt concentration dependent (ii) probable modifications of membrane lipid composition (iii) increased energetic capacity, at the level of cyclic electron flow around photosystem I (through routes induced under these conditions) and cytochrome c oxidase, and (iv) enhancement of H^+- ATPase activity and active extrusion of sodium ions.

Extrusion of sodium ions phenomena has an important influence on the halotolerance of various organisms. Cyanobacteria do not accumulate Na^+ although a transient net Na+ uptake may occur in response to hypersaline upshock (Reed *et al.*, 1985). It was shown further that the ability to curtail Na^+ influx can also be induced by certain environmental factors like alkaline pH or presence of combined nitrogen in the form of nitrate or ammonium in the growth medium (Apte *et al.*, 1985).

The presence of certain nitrogenous compounds in the growth medium significantly enhanced the salt tolerance of freshwater cyanobacterium *Anabaena* sp. strain L-31 as well as the brackish water cyanobacterium *Anabaena torulosa*. Among these, nitrate, ammonium, and glutamine are most effective followed by glutamate and aspartate. These nitrogenous compounds also inhibit Na^+ influx in both *Anabaena* sp. with the same order of effectiveness as that observed for protection against salt stress.

The previously studied halotolerant cyanobacterium *Aphanothece halophytica* (Jutakae, 2001) of the effect of NH_4^+ on nitrate uptake showed that the ability of the cells to take up nitrate was effectively reduced by either ammonium addition or selective inhibition of CO_2 fixation by DL- glyceraldehyde. The effect of salinity on nitrate uptake showed that the nitrate uptake in salt stress condition (2.0 M NaCl) were lower than normal condition (0.5 M NaCl), thus causing the low level of nitrate uptake. However, this study indicated that nitrate uptake in *A. halophytica* in normal and salt stress conditions are indifferent to the extracellular sodium concentrations. Also, the result of Cl^- and PO_4^{3-} showed no inhibition of nitrate uptake in both normal and stress conditions. Thus, the next work is the characterization study of the nitrate reductase enzyme, the first enzyme to convert nitrate into nitrite, of *A. halophytica*.

In this work, the unicellular *Aphanothece halophytica* (this organism is classified into *Chroococcales* order, *choococcacean* cyanobacteria subgroup (Geitler, 1932; Stanier *et al.*, 1971) was used to study (a) the enzyme nitrate reductase activity in normal and salt stress conditions, (b) the effect of other nitrogen-sources on nitrate reductase activity, (c) the localization of nitrate reductase in *A. halophytica* cells, and (d) the purification and some biochemical properties of the enzyme nitrate reductase from *A. halophytica*.