CHAPER IV

DISCUSSION

4.1 Effect of nitrogen sources on nitrate reductase

The process of nitrate assimilation is of fundamental biological importance. It occurs in a wide variety of organisms including bacteria, yeast, fungi, algae and higher plants. The rate-limiting and regulated step of nitrate assimilation appears to be the initial reaction catalyzed by nitrate reductase. Thus, the nitrate reductase is the key enzyme for nitrate assimilation.

Most of the detailed investigations of nitrate reductase in bacteria have concerned the respiratory or dissimilatory nitrate reductase. Purification of the enzyme involves a solubilization of the enzyme from the membrane. Alcoholic acetones, various detergents of tertiary alcohol have been used to accomplish this task.

Nitrate reductase activity has been demonstrated in particulate fractions of *Nostoc muscorum* (Ortega *et al.*, 1976), *Anacystis nidulans* (Manzano *et al.*, 1976) and *Anabaeana cylindrica* (Hattori, 1970). The enzyme is particulate and although it has been released by detergent treatment of acetone precipitates (Hattori, 1970), there has been no description of molecular characteristics.

The effect of the nitrogen sources on the cellular activity of ferredoxin-nitrate reductase in different cyanobacteria, *Anacystic nidulans*, *Anabaena* Sp.strain 7119, *Nostoc* sp.strain 6719, was examined by in situ assay (Herrero *et* al., 1981; Manzono *et* al., 1976). In the unicellular species *A. nidulans*, nitrate reductase was repressed in the presence of ammonium. Nitrate reductase in *A. nidulans* was freed from ammonium repression by L-methionine-D,L-sulfoximine (MSX), an irreversible inhibitor of glutamine syathetase. Ammonium-promoted represssion appears therefore to be indirect; ammonium has to be metabolized through glutamine syhthetase to be effective in the repression of nitrate reductase. In the filamentous cyanobacteria *Anabaena* sp. strain 7119 and *Nostoc* sp. strain 6719 (both nitrogen fixers), nitrate

exhibits a positive effect on the regulation of nitrate reductase. Nitrogenase, another enzyme system involved in the assimilation of inorganic nitrogen, appears to be subjected to repression by ammonium in a similar indirect way, since inactivation of glutamine synthetase also alleviates the inhibitory effect of ammonium on nitrogenase synthesis. Ammonium repression on nitrate reductase sysnthesis in *Anabaena* and *Nostoc* species cannot be ruled out absolutely. It is interesting that nitrate plays a positive role in regulating nitrate reductase in those cells that N_2, whereas in *A. nidulans* which cannot fix N_2, the ammonium repression seems more important in controlling enzyme level. In this work the effect of nitrogen-sources on nitrate reductase of *Aphanothece halophytica*, showed maximum specific activity occured when a nitrate was present as nitrogen-source for growth and living. Changes in levels of nitrate reductases have been shown in response to changes of environmental conditions such as light, nitrogen source, water potential and other factors. These changes usually influence the capacity of the organisms to assimilate nitrate.

The presence of ammonium chloride or glutamine as nitrogen-source resulted in a decreased of nitrate reductase activity due to ammonium chloride and glutamine can directly conversion to an intermediate which is the precursor of nitrogen utilization of the cell. The result indicate that the presences of nitrate reductase dependent on the amount of nitrate which as substrate.

Nitrate plays important role as potential inducer to enhance nitrate reductase levels. Nitrate reductase is an inducible enzyme. The addition of enzyme inducers is usually very effective for maximum harvestable enzyme level. Feedback inhibition of the enzymes of the nitrate-reducing pathway by the reaction products (Vennesland and Guerrero, 1979) or by metabolites involved in ammonium assimilation (Flores *et al.*, 1980) may also have a regulatory role. The antagonistic effect of ammonium on nitrate metabolism with regard to the synthesis of the enzyme 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) and fungi (Garrett and Any, 1978)

In the mold *Neurospoora*, results from recent studies using immunological methods (Amy and Garran, 1979) and density labeling (Bahns and Garren, 1980) indicate that no major stable form or component of nitrate reductase is present in ammonium-grown mycelia. These results strongly suggest that prevention by ammonium of nitrate reductace synthesis affects an early stage of the process, either transcription or translation, thus impeding the appearance of enzyme of enzyme precursor proteins. In fact, present evidence supports the view that this control is exerted at the transcriptional level in the case of fungi (DunnColeman *et al.*, 1979; Garrett and Amy, 1978; Premakumar *et al.*, 1978, 1979). A transcriptional control of nitrate reductase synthesis by ammonium has also been thought to operate in unicellular blue-green algae (Herrero *et al.*, 1981). It is interesting that for both groups of organisme, blue-geen algae and fungi, not ammonium itself but rather an organic nitrogenous compound C whose generation from ammonium requires the participation of glutamine synthetase appears to be the real repressor of nitrate reductes synthesis. Glutamine has been suggested as the putative repressor of nitrate reductase in *Neurospora* (Dunn-Coleman et al., 1979; Premakumar *et al.*, 1979)

Premakumer *et al.*, have shown that glutamine prevents the synthesis of the short half-life mRNA for nitrate reductase, mRNA which is missing in ammonium grown *Neurospora*. Glutamine is also affected on nitrate reductase activity levels of *A. halophyti*ca. In the presence of glutamine as nitrogen-source supports to growth of *A. halophytica* but has affected to absent of nitrate reductase activity. Thus, ammonium or certain amino acids have been prevented the nitrate promoted increased in nitrate reductase activity by ammonium is inhibited nitrate uptake (Wangsupa, 2001). In the unicellular non-nitrogen fixing cyanobacterioum *Syneohococcus* sp. strain PCC 7942 and in the filamentous non heterocystous nitrogen-fixing cyanobacterium *Plectonerna boryanum* (Kikuchi *et al.*, 1996) found that nitrate and nitrite do activate the transcription of the nitrate assimilation operons, while ammonium inhibits transcription through its fixation into glutamine (Gln). Nitrate inducing transcription of nitrate assimilation genes has also been reported in higher plants (Crawford and Gilbert, 1993), fungi (Marzluf, 1993) and bacteria (Cali *et al.*,

1989; Goldman *et al.*, 1994), and nitrite has been shown to be an alternative inducer in the fungus *Aspergillus nidulans* (Hawker *et al.*, 1992; Unkles *et al.*, 1991) and the bacterium *Klebsiellla pneumoniae* (Goldman *et al.*, 1994). On the other hand, fixation of ammonium promotes negative regulation of nitrate transport into the cells (Lara *et al.*, 1987), which would lower the intracellular nitrate and nitrite concentration. Several amino acid, purines and pyrimidines, and the major end products of inorganic nitrogen metabolism, are prime candidates for effectors in the regulation of nitrate reductase in *Neurospora crassa* (Premakumar *et al.*, 1979). Ammonia and numerous amino acids have been reported to inhibit the transport of nitrate into *Neurosopra.* Arginine, tryptophan, valine and threonine were reported to inhibit the transport of nitrate maximally, where as glutamine was reported not to inhibit the uptake of nitrate (Schloemer and Garrett; 1974). Glutamine prevented the induced apperance of nitrate reductase.

4.2 Effect of salinity on nitrate reductase

In this research, the effect of salinity on nitrate reductase was investigated. We found that nitrate reductase activity in normal condition was higher than in salt-stress condition, according to the growth of *A.halophytica* in normal and salt-stress conditions. The decrease of nitrate reductase activity was resulted from the lower nitrate uptake under salt stress condition (Jutakae, 2001). Thus, the lower nitrate uptake under salt-stress condition reduced the growth and nitrate reductase activity. The molecular mechanisms involved in the adaptation to changes in environmental ionic strength are mainly concerned with the cytoplasmic accumulation of ions (Na^+, K^+) and/or organic osmolytes (compatible solutes). The responses can be rapid (by adjusting the concentration of cations at both sides of the membrane) or slow (by inducing a metabolic pathway for the synthesis of a compatible solute such as proline, glycine betaine or ectoine). It has been reported that inorganic nitrogen metabolism is strongly inhibited in saline environments. In the cyanobacterium *Anabaena* sp., nitrogen fixation ceases in the presence of moderate amounts of salt (Fernandes *et al.*, 1993) and in the phototrophic bacterium *Rhodobacter capsulatus*, both nitrogen fixation and nitrate assimilation are inhibited by 0.2 M NaCl. These effects are due to

the short-term inhibition of nitrogenase activity and nitrate uptake, and both are relieved by the addition of glycine betaine (Igeno *et al.*, 1995). *Rhodococcus* sp. RB1 is able to grow well up to 0.9 M NaCl and the cells remained completely viable even at 3 M salt. The influence of salinity on assimilatory nitrate reductase was also observed. The mechanisms for adapting to sudden changes in environmental salinity involves a rapid adjustment of monovalent cation concentration at both sides of the membrane with the values of cytosolic K^+ and Na^+ concentration observed in *Rhodococcus* sp. RB1. Also, nitrate reductase showed maximal activity at 0.5 M NaCl or KCl and a significant decrease was observed above this concentration, although the enzyme was active even at 2.0 M salt. Both the growth rate and nitrate uptake were decreased.

The salt tolerance of *A. halophytica* showed that this organism could adapt to a broad range of salt concentrations from 0.5 to 2.0 M NaCl (Fig.3.2). Choline and glycine betaine functioned as efficient osmoprotectants (Aphichart, 2001).

4.3 Localization of nitrate reductase in *A. halophytica* cells.

Nitrate reduction can be performed with three different purpose the utilization of nitrate as a nitrogen source for growth (nitrate assimilation), the generation of metabolic energy by using nitrate as a terminal electron acceptor (nitrate respiration), and the dissipation of excess reducing power for redox balancing (nitrate dissipation). The capacity for nitrate utilization in different organisms has been shown to vary in respones to changes in environmental conditions such as light, temperature, pH, CO_2 and oxygen levels, nitrogen source, and other factor (Beevers and Hageman, 1972; Garrett and Amy, 1978; Hewitt et al., 1976; Losada and Guerrero, 1979) ,

A. halophytica was grown photoautotrophically in normal medium containing sudium nitrate to grow and determined the type of nitrate reduction. *A. halophytica* was harvested and determined nitrate reductase activity by preparing cytoplasmic fraction, membrane fraction and periplasmic fraction from *A. halophytica* cells. We found that almost all nitrate reductase activity was localized in cytoplasmic fraction. It indicates a nitrate-assimilatory function which nitrate is important nitrogen source for survival. In the general assimilatory pathway, nitrate is converted via nitrite to

ammonia, which is then assimilated into nitrogen metabolism. Also, the level of nitrate reductase is high in organisms grown on nitrate and low if grown on ammonium. Nitrate reductases behave as adaptive enzyme. Physiological studies revealed that assimilatory nitrate reductase is repressed by ammonium and is insensitive to oxygen according to the characteristic of nitrate reductase of *A. halophytica*. Whereas, the enzymes of nitrate respirations are only formed where conditions for dissimilatory nitrate reduction are present. Various bacteria have the possibility of growing anaerobically in the presence of nitrate (NO^-_3). Under these conditions, NO^-_3 functions as a terminal electron acceptor instead of O_2. Dissimilatory nitrate reductases have been studied from a variety of bacteria. The enzymes of bacteria are membrane-bound enzymes which are induced in the presence of nitrate and anoxia or low oxygen concentrations. The role of the dissimilatory nitrate reduction system has been investigated extensively in enteric and denitrifying bacteria. It is known best from *Escherichia coli*.

4.4 Purification of nitrate reductase from *A.halophytica*.

The aim of a protein purification process is the removal of unwanted contaminants and the concentration of the desired protein. The principal properties of enzyme that can be exploited in separation methods are size, charge, solubility and the possession of specific binding site. Most purification protocols require more than one step to achieve the desired level of protein purity. Each step in the process will cause some loss of product.

Nitrate reductase was purified 406 fold with a 15.7 % yield from the crude extract of *A.halophytica* by a procedure involving ultracentrifugation, ammonium sulfate precipitation followed by column chromatography including DEAE-Toyopearl and Bio-Gel hydroxyapatite, respectively.

The first step in the purification of a protein is the preparation of crude extract containing the protein in a soluble form and extraction procedures should be selected according to the source of the protein. The nitrate reductase from *A.halophytica* is localized in cytoplasmic. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein prior to purification.

Ultrasonication or high pressure sound waves, which causes cell breakage by cavitations and shear forces, was used in this work. However, several potential problems may be consequent on distruption, due to the destruction of intracellular compartmentation and nitrate reductase activity can be lost for a variety of reason. Therefore, it is essential to consider strategies for protection of the enzyme activity. In this work, benzanidine and ethylenediamine tetraacetic acid (EDTA) were used in the extraction buffer as proteinase inhibitor and metalloprotease inhibitor, respectively, because the control of metabolic regulation mechanisms is lost when the cell is disrupted. Thus, the desired protein may be degraded by nature catabolic enzyme such as preteolytic enzyme (Eisenthal and Danson, 1992; Cooper, 1997; Harris and Angal, 1989).

In addition, the protein will encounter an oxidizing environment after distruption that may cause inactivation, denaturation or aggregation. Addition of a reagent containing a thiol group such as dithiothreitol (DTT) and also a chelating agent such as EDTA to chelate metal ions in the extraction buffer will minimize the oxidation damage. Also salt supplement, 0.5 M NaCl, can improve stability of the desired protein for activity assay.

Mechanical cell distruption may cause local heating with consequent denaturation of protein. To maximize recovery of active enzyme, the extract and equipment, therefore, were pre-chiled and several pauses of distruption used instead of one long continuous sonication because short interval of distruption will also minimize foaming and shearing, thereby minimizing denaturation (Chambers and Rickwod , 1993; Janson and Ryden, 1998).

Crude homogenates containing organelles or fragments can be fractionated by differential centrifugation processes in which based upon the differences in the sedimentation rate of particles of different size and density. Isolation of components from the homogenate is usually done by differential centrifugation. The organelles are usually separated by centrifugation at increasing speeds. Ultracentrifugation at high speed can separate subcellular organelles. The resulting supernatant represents the fraction of nitrate reductase activity. The ultracentrifugation step resulted in about half of the proteins being removed.

Solubility differences in salts are frequently exploited to separate proteins in the early stages of purification protocols. Salt fractionation is frequently carried out suing ammonium sulphate and was used in this work because it has many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Bollag *et.al.*, 1996). In the ammonium sulfate precipitation step, about one third of the proteins were removed but about 30% of the enzyme activity was lost. The loss of significant protion of nitrate reductase activity may be caused by the speed at which salt was added to an enzyme solution and the efficiency of stirring is improving factors. Dissolved air may come out of solution and cause frothing, which causes surface tension effects on protein caught in bubbles resulting in denaturation.

Moreover, care must be taken to monitor the pH because ammonium sulfate is slightly acidic; therefore, an appropriate buffer should be used to maintain a pH between 6.0-7.5. In this work, Tris-HCI buffer, pH 7.5 was used as the working buffer and also contained glycerol to stabilize protein, a reducing agent (dithiotreitol) to prevent protein oxidation and a chelating agent (EDTA) to remove possible traces of heavy metal cations in the ammonium sulfate which may be detrimental to the enzyme.

In some case, it is advantageous to carry out a selective heat denaturation as an early step in purification if the enzyme interest is more stable than the other proteins under the conditions of heat treatment. In the purification of nitrate reductase from *Plectonema boryanum* (Mikami and Ida, 1984) heat treatment step has been successfully used as another promising way to get rid of other bulk proteins form crude enzyme solution before ammonium sulfate precipitation step because impurity proteins in the crude extract are mostly denaturing during the treatment. Preliminary experiment on heat treatment was also done in this work; nevertheless, it was found that there was great loss of nitrate reductase activity. Thus, it was decided that this procedure was not the suitable step for purification of nitrate reductase from *A. halophytica* and was not further used in this work.

Most purification involves some forms of chromatography which is essential tool in every experiment where protein purification is needed. Ion exchange

chromatography separates proteins with differences in charge to give a very high resolution with differences loading capacity.

DEAE-Toyopearl is anion exchanger and used for purification of nitrate reductase from other sources (Mikami and Ida, 1984). About 50 % of the other bulk proteins were eliminated. Moreover, the result from chromatogram also indicates that net charge of the enzyme in working buffer, pH 7.5 was negative because our enzyme could bind to the column in this purification process (net charge of DEAE ligand was positive). Proteins with the same charge as the resin pass through the column to waste, after which bound proteins, containing the protein of interest, are selectively released from the column by gradually increasing the strength of salt ions in the buffer passing through the column. This ion competes with the protein for binding to the resin, the more weakly charged protein being eluted at the lower salt strength and the more strongly charged protein being eluted at higher salt strengths. Feature of the different charged groups found in protein is the fact that most proteins will differ in their isoelectric points. This can be implied that pI of our enzyme was less than pH 7.5. This difference in pI can be exploited using chromatofocusing.

In DEAE-Toyopeal column, linear gradient elution by graduate increasing salt was achieved for eliminating oher proteins. In this column, our enzyme was eluted by about 0.1 M NaCl solution.

The last step of purification, Bio-Gel hydroxyapatite chromatography the calcium phosphate that is crystalline. The crystalline particles in the column adsorb proteins on their surface but the adsorptive capacity is limited; indeed, a monolayer of a typical protein over the surface of spheres 0.1 mm in diameter amounts to no more than 0.1 $mgcm^{-3}$ (Bernardi, 1971) due to crevices and cracks within the particles giving much higher effective surface area. The limited capacity of crystalline calcium phosphate makes its use in enzyme purification mainly a late-stage procedure, often the final one after other techniques have failed to result in homogeneity. Adsorbed at low potassium phosphate concentration, all proteins could be eluted by increasing phosphate concentration. In this work, the purified enzyme from DEAE-Toyopearl column was desalted with dialysis in the same equilibrating buffer of Bio-Gel hydroxyapatite column. The use of linear gradient elution by increasing potassium phosphate concentration can provide more refined separation to remove other bulk

proteins. From the experiment, it seems that our enzyme is basic protein that could adsorb to the column in this purification system, in working buffer of 50 mM potassium phosphate buffer, pH 7.5.

The chromatogram (Fig 3.8) shows that two peaks of proteins were eluted after introducing a linear gradient in the range of 50 mM to 500 mM potassium phosphate buffer pH 7.5. The first peak was nitrate reductase peak while another one was unwanted proteins. In consequent of the result, about 80% of nitrate reductase activity was retained and purified to 406 fold. Moreover, efficient purification of nitrate reductase from cyanobacterium *P.boryanum* (Mikami and Ida, 1984) had been previously reported using hydroxyapatite column as the purification procedure after DEAE-Toyopearl and Sephadex G.150 column with purification of 19200 fold. The lower purification fold in this work was probably due to inappropriate of pH adjustment suitable for nitrate reductase from *A.halophytica.*

The ability to use ferredoxin as electron donor of *A. halophytica* nitrate reductase was also tested. According to previous studies (Candau, 1979; Hatori, 1970; Manzano et al., 1976; Ortega et al., 1976) nitrate reductase from almost all cyanobacteria including *Anabaena cylindrical*, *Anacystis nidulans* and *Nostoc muscorum* cannot accept electrons directly from NAD(P) H but is rather dependent on reduced ferredoxin as the physiological electron donor. Sequence analysis of the *Synechococcus* sp. PCC7942 assimilatory nitrate reductase predicts that it comprises an ~70 kDa polypeptide that binds only the bis-molybdopterin guanine dinucleotide (bis-MGD) cofactor and an iron-sulphur protein. The likely electron donor is reduced ferredoxin, generated by photosystem I during photosynthesis. According to the ability to use ferredoxin as physiological electron donor is found in most cyanobacteria. This was also the case for *A.halophytica* probably genes encoding most in nitrate metabolisms are homologous between different cyanobacteria.

4.5 The inhibitory effects of various reagents.

Chemical modification studies have suggested that a number of specific amino acid residues are required for nitrate-reducing activity of nitrate reductase. The possible participation of sulthydryl groups in the transfer of reducing equivalents between NAD(P)H and FAD was initially proposed for *Neurospora* nitrate reductase (Amy *et al.*, 197). In this work, the purified nitrate reductase from *A. halophytica* was tested for the inhibitory effect of a thiol-specific reagents e.g. *p*-chloromercuribenzoate, iodoacetamide and N-ethylmaleimide. Table 3.3, shows the thiol –specific reagents at 0.1 mM inhibited more than 80% of nitrate reductase activity from *A.halophytica.* This result, the inhibitory effect of the thiol-reagents, agrees with the assimilatory nitrate reductase from *P.boryanum* (Ida and Mikami, 1983) and nitrate reductase catalyzed NAD(P)H reduction in many organisms. The results suggested that cystein residue may be essential for ferredoxin – nitrate reductase activity in *A. halophytica.*

Substrate analog is one of inhibitors of the enzyme. ClO^-_3 acting as substrate analog inhibited the *A.halophytica* NR. Inhibitory effects of ClO^-_3, which was not substrate of the enzyme, was also reported for purified nitrate reductase where substrate acting as competitive inhibitor of the reduction of the others (Noton and Hewitt, 1979; Vennesland and Guerreru, 1979).

Cyanide is also a potent inhibitors of nitrate reductase activity and forms a stable complex with the reduced enzyme (Lorimer *et al.*, 1974). This complex has been proposed to have an important role in modulating in vivo nitrate reductase activity (Lorimer *et al.*, 1974; Solomonson and Spehar, 1979). Solomonson suggested that cyanide forms a complex with a reduced form of molybdenum, thereby preventing further catalytic turnover of the enzyme. EPR evidence also suggested that cyanide reacts with the Mo-pterin center (Solomonson *et al.*, 1984)

The purified nitrate reductase from *A.halophytica* was also inhibited by cyanide because it contains of molybdenum molybdopterin as cofactor binding at the active site.

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 enzyme of *Chlorella*, *Aspergillus* and *Rhodotorula* (Guerrero and Gutierrez, 1977; McDonald and Coddington, 1974; Vennesland and Guerrero, 1979). Nevertheless, nitrite does not act as competitive inhibitor of the purified nitrate reductase of *A. halophytica.*

In the presence of nitrate and nitrite at equal concentration (40mM), nitrite caused inhibitory effect only 5% of nitrate reductase activity. Azide, caused no inhibition of nitrate reductase of *A. halophytica*. On the other hand, azide caused potent inhibition of the diaphorase moiety of assimilatory NAD(P) H-nitrate reductase.