8. GENERAL DISCUSSION: NITROGENASE REGULATION AND TRANSPORT OF AMMONIUM, GLUTAMINE AND GLUTAMATE

8.1. Ammonium transport:

Ammonium is a preferred source of inorganic nitrogen in cyanobacteria. It can be taken up by the cell through a transport system. The ammonium transport system (ATS) has been characterized in various bacteria and cyanobacteria using an ammonium analogue, $^{14}$CH$_3$NH$_3^+$, as a probe (Boussiba et al., 1984b; Rai et al., 1984; 1986b; Kleiner, 1985a; Singh et al., 1987; Boussiba, 1988). This is mainly due to the fact that, CH$_3$NH$_3^+$ uses the same transport system as NH$_4^+$ in these organisms. The NH$_4^+/CH$_3$NH$_3^+$ uptake studies presented in the chapters 4 and 5 in Anabaena 7120 and Nostoc ANTH also indicated that CH$_3$NH$_3^+$ can be used as a probe to characterize ATS.

The ATS studies in Anabaena 7120 and Nostoc ANTH indicated that two energy-dependent transport systems are involved in uptake of external ammonium into the cells. One of the transport systems is MSX-insensitive while the other is MSX-sensitive. The former operates at a faster rate than the latter. Both these transport systems are NH$_4^+$-repressible and are derepressed in N$_2^-$ and NO$_3^-$ grown cells (see chapter 4 and 5). Since ATS is repressed in ammonium grown cells, the N-needs of the cell is served by the diffusible NH$_3$ (Kleiner, 1985b). The two cyanobacterial strains examined indicate existence of two intracellular ammonium pools — one in thylakoids and the other in cytoplasm (see chapter 4 and 5;
discussion section). The MSX-insensitive ATS serves the thylakoid pool while the MSX-sensitive ATS serves the cytoplasmic pool. The latter is assimilated by the cell via GS. Because of these characteristics of the two ATS, the pattern of $\text{NH}_4^+ / \text{CH}_3\text{NH}_3^+$ uptake in these cells is biphasic: a fast initial phase, lasting about 60 s, due to MSX-insensitive ATS and a slower second phase due to MSX-sensitive ATS. Hence, the second phase of $\text{NH}_4^+ / \text{CH}_3\text{NH}_3^+$ uptake is sensitive to MSX. MSX causes immediate inhibition of the second phase of ATS and hence the second phase of uptake. In longer term MSX also inhibits GS thereby blocking $\text{NH}_4^+ / \text{CH}_3\text{NH}_3^+$ assimilation.

Both the ATS reported here show affinity modulation in response to external $\text{CH}_3\text{NH}_3^+ / \text{NH}_4^+$ concentration (see chapter 4 and 5). At high external concentration of $\text{NH}_4^+ / \text{CH}_3\text{NH}_3^+$, the transport systems show low affinity while at low external substrate concentration the affinity increases. This provides a novel mechanism to control ammonium metabolism in cyanobacteria by regulating its entry into the cell.

In addition, *Nostoc* ANTH shows a capability to utilize $\text{CH}_3\text{NH}_3^+$ as N-source (see chapter 3). For this a distinct $\text{CH}_3\text{NH}_3^+$ transport system was found to be induced only in *Nostoc* ANTH cells (see chapter 5). This transport system was not shared by $\text{NH}_4^+$, was insensitive to MSX and specific for $\text{CH}_3\text{NH}_3^+$.

### 8.2. Ammonium transport and nitrogenase regulation:

In all free living diazotrophs nitrogenase synthesis/activity is regulated by $\text{NH}_4^+$ and other combined nitrogen compounds
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(Stewart and Lex, 1970; Rippka and Stanier, 1978; Jones and Monty, 1979; Brill, 1980; Stewart, 1980; Thomas et al., 1982; Singh et al., 1983b; Turpin et al., 1984; Reich et al., 1986; 1987; Stewart et al., 1987). Ammonium, has been shown to cause two types of effects on nitrogenase in diazotrophs: a short term effect (within minutes) on nitrogenase activity and a long term effect on nitrogenase synthesis (Haaker et al., 1980; Stewart, 1980; Hallenbeck, 1987).

In *Rhizobium leguminosarum* and *Azotobacter vinelandii*, Haaker et al. (1980) have shown that a minimum of -100 mV Δψ is needed for optimum nitrogenase activity. Δψ is involved in reverse electron flow from NADPH to ferredoxin. The latter is the e⁻ donor for nitrogenase. At Δψ values below -90 mV, reverse e⁻ flow is blocked. NH₄⁺ uptake causes Δψ to collapse and therefore nitrogenase activity is severely inhibited, within minutes, due to blockage of e⁻ flow to nitrogenase. In cyanobacteria also it has been shown that Δψ and nitrogenase activity are directly correlated. A minimum of -70 mV Δψ is necessary for optimum nitrogenase activity in cyanobacteria (Hawkesford et al., 1980; 1981). However, at physiological pH, NH₄⁺ does not cause short term inhibition of nitrogenase (fig 8.1). As shown in fig 8.2 NH₄⁺ addition to N₂-fixing cultures of *Nostoc* ANTH, at pH 7 lowered Δψ from -110 mV to -97.5 mV. However, it is clear that the residual Δψ was still above the value required for optimum nitrogenase activity in cyanobacteria (-70 mV). This may explain why NH₄⁺ does not cause quick inhibition of nitrogenase activity in cyanobacteria. Short term effect of NH₄⁺ on nitrogenase has been noted at high pH 10 (Reich et al., 1986; 1987). However, this may
Fig 8.1 Effect of NH₄Cl and MSX on nitrogenase activity in N₂-grown *Nostoc* ANTH filaments, at pH 7. 100% nitrogenase activity = 5.83 nmol C₂H₂ reduced h⁻¹ μg⁻¹ Chl a. O, NH₄Cl (3 mmol.dm⁻³) added at zero time; ●, NH₄Cl (3 mmol.dm⁻³) + MSX (10 μmol.dm⁻³) added at zero time; Δ, NH₄Cl (3 mmol.dm⁻³) added at zero time to cultures preincubated with 10 μmol.dm⁻³ MSX for 2 h.
Fig 8.2 Effect of NH$_4$Cl (3 mmol.dm$^{-3}$) on $\Delta\psi$ in N$_2$-grown *Nostoc* ANTH filaments, at pH 7. $\Delta\psi$ values were measured, at pH 7, as described in chapter 2.
be due to uncoupling effect of NH$_4^+$ because at high pH excessive intracellular ammonia accumulation would occur by diffusion of external NH$_3$ and its entrapment in the cell by protonation. This would be consistent with the ammonium transport studies at pH 9 (see chapter 4 and 5).

NH$_4^+$ has been shown to cause nitrogenase repression in cyanobacteria (Stewart, 1980; Hallenbeck, 1987). This is exemplified by a slow inhibition of in vivo nitrogenase activity on ammonium addition (Fig 8.1). NH$_4^+$ caused total inhibition of nitrogenase activity over a period of 4 h. In MSX preincubated cells, however, NH$_4^+$ did not cause such an inhibition. Stewart and Rowell (1975), from similar experiments on Anabaena cylindrica, concluded that since MSX inhibits GS and thereby ammonium assimilation, the results may be taken to indicate that a product of ammonia assimilation, rather than NH$_4^+$ per se, was the actual repressor of nitrogenase in cyanobacteria. However, subsequently it was challenged. Singh et al. (1983b) and Turpin et al. (1984) suggested that MSX may also cause inhibition of ammonium uptake and therefore lack of nitrogenase inhibition in MSX preincubated cells may not necessarily be due to blockage of ammonia assimilation. It may be equally possible that NH$_4^+$ per se was the repressor but since MSX blocked entry of ammonium in the cells, the NH$_4^+$ effect on nitrogenase was not observed. Indeed our studies on ATS (chapters 4 and 5) show that MSX does block the second ATS which serves the cytoplasmic pool used by GS. However, if the above reasoning of Singh et al. (1983b) and Turpin et al. (1984) was correct, then NH$_4^+$ should have affected nitrogenase activity in the cells having been preincubated with MSX as well as
in the cells where MSX was added simultaneously with NH$_4^+$. As seen in fig 8.1 this is not so. In MSX-preincubated cells where GS was fully inhibited NH$_4^+$ did not cause nitrogenase inhibition but in cells where MSX was added together with NH$_4^+$, nitrogenase inhibition occurred during the first 1 h, after which no further inhibition was noted. It should be noted here that MSX (10 μmol.dm$^{-3}$) causes immediate inhibition of the second ATS while the GS inhibition is progressive and takes about 1 h for total inhibition in *Nostoc ANTH* (data not shown). Thus, the partial inhibitory effect of NH$_4^+$, in presence of MSX, noted in fig 8.1 correlates with GS activity rather than inhibition of second ATS by MSX. The results are consistent with the view that nitrogenase repression by ammonia is caused by a product of ammonia assimilation. The fact that CH$_3$NH$_3^+$ is not metabolized beyond methylglutamine in *A. variabilis* and *Anabaena 7120* (*Nostoc muscorum*) (Rai et al., 1984; Rai and Prakasham, 1989; and also see chapter 3) and yet it represses nitrogenase activity (Singh et al., 1983a; and see chapter 3) suggests that, most likely, the repressor of nitrogenase is glutamine.

It should be further emphasized that MSX does not affect the first ATS. If NH$_4^+$ *per se* was the repressor then in the presence of MSX, nitrogenase inhibition should have continued rather than stop after 1 h when GS activity is fully inhibited. In addition, the fact that NH$_4^+$ represses ATS in the long term (Rai et al., 1986a; see also chapter 4 and 5) and yet nitrogenase is repressed by assimilation of diffusible NH$_3$ species, argues against ATS inhibition by MSX being the explanation for MSX alleviating NH$_4^+$ repression of nitrogenase.
8.3. Glutamine & glutamate transport and regulation of nitrogenase

Addition of glutamine to N₂-fixing *Nostoc* ANTH filaments caused a complete inhibition of nitrogenase activity within 6 h (Fig 8.3). Such results are similar to those observed in other N₂-fixing organisms where nitrogenase activity inhibition is observed in the presence of glutamine (Arp and Zumft, 1983). In the presence of MSX, glutamine, however, caused only a partial inhibition of nitrogenase activity during the first 1 h; thereafter no further inhibition was observed (Fig 8.3). The lack of glutamine effect on nitrogenase, beyond 1 h, in presence of MSX may be either due to the inhibition of glutamine uptake by MSX or MSX, being structurally similar to glutamine, may compete for the glutamine binding site thereby preventing nitrogenase inhibition/repression. The former possibility is ruled out since glutamine uptake studies in this cyanobacterium, presented in chapter 7, showed that MSX does not inhibit glutamine transport. Arp and Zumft (1983) have given a similar argument for observations on *Rhodopseudomonas palustris*.

Glutamate (see chapter 6) caused inhibition of diazotrophic growth and was toxic to the cyanobacteria studied (*Anabaena* 7120 and its Het⁻ Nif⁻ strain). This toxicity was related to the mode of N-nutrition; where NO₃⁻ was provided as N-source, glutamate toxicity was not observed. This was found to be due to partial inhibition of glutamate uptake by NO₃⁻.
Fig 8.3 Effect of glutamine and MSX on nitrogenase activity in N$_2$-grown *Nostoc* ANTH filaments, at pH 7. 100% nitrogenase activity = 5.83 nmol C$_2$H$_2$ reduced.h$^{-1}$.µg$^{-1}$ Chl a. O, glutamine (2 mmol.dm$^{-3}$) added at zero time; •, glutamine (2 mmol.dm$^{-3}$) + MSX (10 µmol.dm$^{-3}$) added at zero time.
8.4. Some biotechnological implications:

Cyanobacteria have long been recognized as having enormous potential for use in biotechnology, especially in agriculture. In fact, use of cyanobacteria in rice fields is being popularized at present. This is mainly because,

1. Cyanobacteria are simple photosynthetic prokaryotes which have simple growth requirements and which use a cheap source of reductant, i.e. water. This gives them an edge over other photosynthetic bacteria.

2. Many cyanobacteria combine photosynthesis and N<sub>2</sub>-fixation. This gives them an edge over other eukaryotic photosynthetic organisms.

Current use of cyanobacteria in rice fields (Singh, 1961; Venkatraman, 1980; Stewart et al., 1987) has serious limitations. Normal cyanobacteria use much of their fixed-N for their own growth, releasing only a small amount in the field. Furthermore, presence of nitrogen fertilizers in the field adversely affects N<sub>2</sub>-fixation and cyanobacteria, instead of fixing N<sub>2</sub>, start using the nitrogen available in the field. Thus, they become like weed for the crop. Despite these problems cyanobacterial inoculation in the field has been shown to be beneficial for rice crops, saving considerable amount of expenditure which has to be incurred on chemical fertilizers. It is obvious therefore, that if we produce suitably modified strains the benefits could be increased several fold. To achieve this goal some of the strategies are discussed below:

First thing, of course, is to maximize N<sub>2</sub>-fixation. For this, a modification of cyanobacterial metabolism resulting in
diversion of more energy for N$_2$-fixation, and less for ammonia assimilation and biomass growth, is necessary. Some of the target points for this have been shown in Fig 8.4. The best modification point, in the above scheme, is the level of GS. It should be emphasized that these mutants should have 5-10% of GS activity left; otherwise the mutant would become a glutamine auxotroph and its survival would require glutamine supply from outside. Reduction in GS level would restrict utilization of ATP, reductant and carbon skeletons for ammonia assimilation and biomass growth thus, leading to diversion of more energy for N$_2$-fixation. It may also lead to an increase in heterocyst frequency as in the case of symbiotic cyanobacteria (Rai, 1990). Reduction in GS level would also lead to accumulation of ammonia since ammonia assimilation would be restricted. This would lead to ammonia release, at larger scale than in normal cyanobacteria, since ATS would be unable to cope with the recycling requirements over and above the maintenance of normal internal concentration (Rai and Prakasham, 1989). At the same time, the residual GS activity would ensure some ammonia assimilation necessary for the survival of the cell.

An alternative strategy for generating suitable cyanobacterial strains for ammonia liberation is to manipulate its ATS. ATS plays an important role in cyclic retention of ammonia within the cell (Kleiner, 1985b). It has been calculated that nitrogenase derived ammonia can diffuse across the plasma membrane in bacteria six times before being captured by GS (Kleiner, 1985b; see Fig 1.1). So, if ATS is abolished, or made inefficient, much of the nitrogenase derived ammonia would escape out since cyclic retention by ATS will not take place or would take place at a
Fig 8.4 Targets for modification of cyanobacterial cellular metabolism for photobiological production of ammonia. Expected outcome of such modifications include: 1) diversion of more photosynthate for nitrogen fixation and less for biomass production; 2) increased nitrogen fixation and reduced ammonia utilization. *, targets of modification.
slower rate (Fig 1.1). Secondly, in such a strain nitrogenase will not be repressed by the presence of ammonium or nitrate fertilizers in the field since these exogenous sources will not cause ammonium accumulation in the cell in absence of ATS. This will not only ensure continued N₂-fixation but also ensure that cyanobacteria do not use of the nitrogen fertilizer meant for the crop. Thirdly, absence of ATS will result in less assimilation of nitrogenase-derived ammonia by the cyanobacterium (since much of it would escape). Therefore, less energy would be utilized in ammonia assimilation and biomass growth; i.e. more energy would be available for N₂-fixation. Some of the consequences of the absence of ATS are depicted in fig 8.5.

The ATS-deficient or the GS-deficient strains of the kind discussed above are obviously better suited for rice field application since they would liberate more ammonia and would fix N₂ at a higher rate. In addition, these strains can also be used in laboratory for photobiological production of ammonia. Currently, normal cyanobacteria, after immobilization, are being used for such a purpose (Stewart et al., 1983; 1987) by inhibiting endogenous ammonia assimilation using MSX (a GS inhibitor). Such a system has serious drawback since total inhibition of GS renders the cyanobacterium totally dependent on provision of exogenous glutamine for its survival. Moreover, the MSX is poisonous and expensive. Therefore, the use of ATS-deficient or GS-deficient strains would be far superior for such an application.

The ATS-deficient and GS-deficient strains can also be used for establishment of artificial symbiosis with crop plants. Earlier attempts in this direction have not been successful but
Fig 8.5 Modification of ammonium transport system: Consequences for a diazotrophic cyanobacterium. Expected outcome of such a modification includes inefficient nitrogen-assimilation, slow growth, less photosynthate for biomass growth, higher energy availability for nitrogen-fixation and inefficient nitrogen control of nitrogenase.
with the increased knowledge about the symbiotic cyanobacteria it is worthwhile to continue the attempt. Recent work of Gusev and his colleagues have shown encouraging results (Gusev and Korzhenevskaya, 1990).

At present, the production of various amino acids or other compounds are obtained from cyanobacteria by increasing the permeability of plasmamembrane (Fukui and Ishida, 1972; Clement et al., 1984) or by making a transitory loss of plasmalemma integrity (Reed et al., 1986). This is achieved by using specific detergents. The constant use of detergents causes damage to the cellular metabolic mechanism and consequently growth is affected (Reed et al., 1986). Moreover, separation of liberated compounds from the detergent is another difficult problem. Kleiner (1985a) has suggested that amino acid transport systems may have a role in maintaining intracellular pools of amino acids and that abolition of these transport systems may cause liberation of amino acids from the cells. Indeed the two cyanobacterial strains studied here do possess specific transport systems for glutamine and glutamate (see chapters 6 and 7). Further detailed studies on amino acid transport systems followed by their manipulation may yield amino acid transport-defective mutants which can be used for photobiological production of amino acids. Such strains would have an advantage over detergent affected strains in production of amino acids without disturbing the cellular integrity and/or metabolism. They also show a constant growth as well as diversion of more energy to produce that particular compound.