6. GLUTAMATE UPTAKE AND METABOLISM IN ANABAENA 7120

6.1. INTRODUCTION:

Glutamate and glutamine are known to serve as nitrogen donors for biosynthetic reactions leading to the production of almost all cellular nitrogenous compounds. Enteric bacteria generate glutamine by glutamine synthetase and glutamate by glutamate dehydrogenase or glutamate synthase (Ninfa et al., 1986). Modulation of enteric bacterial N-metabolism as a function of N-source is known to operate under the well defined control system of nitr genes (Reitzer and Magasanik, 1986). In heterocystous cyanobacteria glutamate is produced by glutamate synthase and glutamine by glutamine synthetase (Haselkorn, 1978; Stewart, 1980). Repression-derepression or induction system of control is one known type of nitrogen control functioning in regulation of cyanobacterial nitrogen assimilation (Bagchi et al., 1985a; 1985b). The other known type of nitrogen control involved in modulating nitrogen dependent expression of gin A gene in Anabaena 7120 is mediated by a novel cyanobacterial RNA-polymerase sigma factor (Tumer et al., 1983). Much more studies on the possible range of cyanobacterial nitrogen sources are needed to understand clearly the nature and types of molecular control regulating cyanobacterial nitrogen assimilation.

There are very few studies on amino acids as sole N- sources and the findings are variable as well as conflicting (Neilson and Larson, 1980; Vaishampayan, 1982). Glutamate has been found growth
toxic in *Anabaena variabilis* (Chapman and Meeks, 1983) and growth stimulatory in *Anabaena cylindrica* PCC 7122 (Rawson, 1985). Glutamine synthetase mutants of *Anabaena cycadeae* (glutamine auxotrophs) are capable of utilizing glutamine as sole N-source (Singh et al., 1985b). In the present chapter, using a class of non N₂-fixing (Nif⁻), non heterocystous (Het⁻) mutant of *Anabaena* 7120 and its parent, the role of glutamine and glutamate as sole N-source was studied. The evidences show that glutamate is not metabolized like a N-source, but acts as an inhibitor of *in vivo* nitrogenase activity and diazotrophic growth, that NO₃⁻ availability eliminates growth inhibition by glutamate by inhibiting glutamate uptake, and that glutamine is utilized like a fixed N-source. Association of glutamate toxicity with nitrogenase activity leading to the inhibition of diazotrophic growth suggests this to be a possible reason for vegetative cell localization, and not heterocyst localization, of glutamate synthase, the enzyme catalyzing glutamate production during growth with N₂ as N-source.

6.2. MATERIALS AND METHODS:

6.2.1. Organisms and growth conditions:

Axenic cultures of *Anabaena* 7120 parent (Het⁺, Nif⁺) and Het⁻ Nif⁻ mutant were grown in 5 mmol.dm⁻³ NO₃⁻ supplemented BG-11₀ medium (Rippka et al., 1979), unless otherwise stated, at 28 ± 1°C and at a photon fluence rate of 50 μmol.m⁻².s⁻¹. Growth was measured by measuring O.D. at 663 nm.
6.2.2. Estimation of chlorophyll and nitrogenase activity:

Chl a concentration was measured according to Mackinney (1941). Nitrogenase activity was measured using acetylene reduction assay (Stewart et al., 1967).

6.2.3. Measurement of glutamate uptake:

Exponentially growing cells were centrifuged, washed and resuspended in 10 mmol.dm$^{-3}$ HEPES-NaOH buffer, pH 7, and equilibrated for 30 min at 28°C and at a photon fluence rate of 50 μmol.m$^{-2}$.s$^{-1}$. 14C-labelled glutamate was then added to a final concentration of 50 μmol.dm$^{-3}$ (specific activity 185 kBq.μmol$^{-1}$) and at time intervals 400 mm$^3$ samples were taken and cells separated from their bathing medium using oil microcentrifugation technique (Scott and Nicholls, 1980). 14C-incorporation was determined using Beckman Liquid Scintillation Spectrometer LS 1801. When needed NO$_3^-$ was added to a final concentration of 5 mmol.dm$^{-3}$ in the assay medium. Non-specific binding of 14C-glutamate was determined by measuring its incorporation in toluene treated cells as described by Rai et al. (1984).

6.2.4. Calculation heterocyst frequency:

Heterocyst frequency was calculated as percentage of total cells, by light microscope observation of the filaments of *Anabaena* 7120.

6.2.5. Estimation of glutamate dehydrogenase activity:

Glutamate dehydrogenase activity was measured as described by Stewart and Rowell (1977).
6.2.6. Estimation of protein concentration:

Protein was estimated by Lowry method (Lowry et al., 1951).

6.2.7. Chemicals:

$^{14}$C-glutamate was purchased from BARC, Bombay, India. Silicon oil DC 550 and dinonylphthalate were obtained from Fluka AC, Buchs, Switzerland. All other chemicals were obtained from Sigma Chemical Company, U.S.A.

6.3. RESULTS:

As shown in Table 6.1 parent strain produced heterocyst and nitrogenase activity in $N_2$-medium and also grew reasonably well at the expense of $N_2$ as sole N-source, while its mutant strain neither produced heterocyst and nitrogenase activity nor grew at the expense of $N_2$ under such conditions. Growth of parent strain in $NO_3^-$-medium was accompanied by absence of heterocyst and nitrogenase activity. The mutant strain grew nearly as well as its parent in $NO_3^-$-medium, thus suggesting the two strains to be almost similar in using nitrate as N-source. Both strains also grew almost equally well in glutamine-medium where the parental strain produced non heterocystous, non $N_2$-fixing filaments as it did in $NO_3^-$-medium. The ability of Het$^-$ Nif$^-$ mutant strain to grow in glutamine-medium provides evidence for glutamine to be utilized as a sole N-source for the cyanobacterial growth. The growth of the parent in glutamine-medium without producing heterocyst and nitrogenase is a further evidence for utilization of glutamine as N-source in *Anabaena* 7120. In contrast, glutamate failed to
Table 6.1.

Growth (O.D. at 663 nm after 6 days of inoculation), heterocyst frequency (%) and nitrogenase activity (nmol C₂H₂ reduced h⁻¹ μg⁻¹ Chl a) of parent (Het⁺ Nif⁺) and mutant (Het⁻ Nif⁻) strains of *Anabaena* 7120 in different nitrogen media.

<table>
<thead>
<tr>
<th>Nitrogen medium</th>
<th>Parent strain</th>
<th></th>
<th></th>
<th>Mutant strain</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Heterocyst</td>
<td>Nitrogenase</td>
<td>Growth</td>
<td>Heterocyst</td>
<td>Nitrogenase</td>
</tr>
<tr>
<td></td>
<td>frequency</td>
<td>frequency</td>
<td>activity</td>
<td>frequency</td>
<td>frequency</td>
<td>activity</td>
</tr>
<tr>
<td>N₂ medium (BG-11₀)</td>
<td>0.68</td>
<td>5-6</td>
<td>12.6</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>BG-11₀ + 5 mmol.dm⁻³ KNO₃</td>
<td>0.85</td>
<td>0.00</td>
<td>0.00</td>
<td>0.82</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>BG-11₀ + 2 mmol.dm⁻³ glutamine</td>
<td>0.75</td>
<td>0.00</td>
<td>0.00</td>
<td>0.78</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>BG-11₀ + 1 mmol.dm⁻³ glutamate</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>BG-11₀ + 5 mmol.dm⁻³ KNO₃ + 1 mmol.dm⁻³ glutamate</td>
<td>0.72</td>
<td>0.00</td>
<td>0.00</td>
<td>0.68</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
support the growth of Het\(^{-}\) Nif\(^{-}\) mutants thereby suggesting lack of the ability in the mutant strain to metabolize glutamate as N-source. Addition of NO\(_3^-\) to the glutamate-medium resulted in recovery of the cyanobacterial mutant growth to as good a level as that obtained in glutamine-medium. This further suggested that absence of growth of the mutant strain in the glutamate-medium was due to the cyanobacterial inability to metabolize glutamate as N-source. The implication of this observation is that cyanobacterial mutant strain lacks the catabolic glutamate dehydrogenase which is essential for utilization of glutamate as N-source. Indeed both parent and mutant had very low NADH-dependent glutamate dehydrogenase activity (<0.1 nmol product formed.min\(^{-1}\). mg\(^{-1}\) protein).

Parent strain showed inhibition of nitrogenase activity and diazotrophic growth in glutamate-medium however, availability of NO\(_3^-\) in the glutamate-medium helped the recovery of parental growth like that of mutant growth. Evidently glutamate inhibition of diazotrophic growth seems to result from glutamate inhibition of nitrogenase activity. The occurrence of parental growth in glutamate containing NO\(_3^-\)-medium suggest that NO\(_3^-\) relief of glutamate toxicity is the result of either NO\(_3^-\) mediated elimination of glutamate sensitive nitrogenase activity or NO\(_3^-\) inhibition of glutamate uptake or both. To check whether NO\(_3^-\) effects the glutamate uptake, \(^{14}\)C-glutamate uptake was studied in \(N_2^-\) and NO\(_3^-\)-grown cultures. As shown in Fig 6.1 both the parent and mutant strain showed active biphasic system of glutamate uptake under N-limited/starved growth conditions. In the \(N_2^-\) grown parent strain the \(^{14}\)C-glutamate uptake rates were 0.075 and 0.030
Fig 6.1 Glutamate uptake in *Anabaena* 7120 (a) and its Het⁻ Nif⁻ mutant strain (b). O, N₂-grown cells, ¹⁴C-glutamate uptake in absence of 5 mmol.dm⁻³ KNO₃; ●, N₂-grown cells, ¹⁴C-glutamate uptake in presence of 5 mmol.dm⁻³ KNO₃; △, NO₃⁻-grown cells, ¹⁴C-glutamate uptake in absence of 5 mmol.dm⁻³ KNO₃; ▲, NO₃⁻-grown cells, ¹⁴C-glutamate uptake in presence of 5 mmol.dm⁻³ KNO₃.

In Fig 1b, the rates of ¹⁴C-glutamate uptake in "N₂-grown cells" refers to the nitrate-grown cells which were subjected to N-starvation for 24 h before measuring ¹⁴C-glutamate uptake; this is because the mutant does not grow in N₂-medium.
nmol min\(^{-1}\)µg\(^{-1}\) Chl a during the initial- and second phase, respectively. Since the mutant strain did not grow in N\(_2\)-medium, for a comparable study, the NO\(_3^-\)-grown cultures of the mutant were N-starved for 24 hours and then \(^{14}\)C-glutamate uptake was measured. The \(^{14}\)C-glutamate uptake in such cells was 0.12 and 0.02 nmol min\(^{-1}\)µg\(^{-1}\) Chl a, during the first- and second phase of uptake, respectively. The observed \(^{14}\)C-glutamate uptake rates, in NO\(_3^-\)-grown cultures, were 0.0459 and 0.0115 nmol min\(^{-1}\)µg\(^{-1}\) Chl a in the parent; and 0.0864 and 0.0154 nmol min\(^{-1}\)µg\(^{-1}\) Chl a in the mutant, during first- and second phase, respectively. Thus, NO\(_3^-\)-grown cultures of both strains showed almost 50\% reduction in the level of glutamate uptake. Presence of NO\(_3^-\) in the reaction mixture did not inhibit significantly the process of glutamate uptake (Fig 6.1). It is therefore, concluded that the inhibitor of glutamate uptake is not NO\(_3^-\) itself but a metabolic product of it. Since the inhibitor of heterocyst formation and nitrogenase activity is also a product of NO\(_3^-\) metabolism (Bagchi and Singh, 1984) one is tempted to think of some physiological connection between NO\(_3^-\) mediated inhibition of heterocyst and nitrogenase and NO\(_3^-\) mediated inhibition of glutamate uptake. The glutamate toxicity to \(N_2\)-fixing culture is certainly further suggestive of non utilization of this amino acid as N-source. It is thus concluded that glutamate can not serve as N-source in this cyanobacterium and glutamate inhibition of nitrogenase activity is possibly the consequence of the sensitivity of the cyanobacterial \(N_2\)-fixing process. NO\(_3^-\) relief of glutamate inhibition appears to be the result of NO\(_3^-\) inhibition of glutamate uptake.
6.4. DISCUSSION:

During diazotrophic growth, heterocystous cyanobacteria assimilate N\(_2\) into glutamine by the sequential action of nitrogenase and glutamine synthetase within the heterocyst and the glutamine thus produced in heterocyst is then transported to adjacent vegetative cells where it is utilized for the synthesis of glutamate by glutamate synthase (Thomas et al., 1977; Rai et al., 1984). The physiological significance of localization of glutamate synthase in vegetative cells, and not in heterocysts, has so far remained largely unexplained. The present finding of glutamate inhibiting heterocyst located cyanobacterial nitrogenase activity leading to inhibition of diazotrophic growth suggests that localization of glutamate forming enzyme in vegetative cells away from heterocyst, the site of N\(_2\)-fixation, is the biological strategy involved to avoid glutamate inhibition of N\(_2\)-fixation in heterocystous forms. Cyanobacterial glutamine synthetase has two functions, one in assimilation of ammonia as N-source and the other associated with production of glutamine which has been found to function both as a sole N-source as well as a source of glutamine for protein formation (Bagchi and Singh, 1984). Growth of parent *Anabaena* 7120 in glutamine medium without producing heterocysts and nitrogenase activity and of Het\(^-\) Nif\(^-\) mutant strain clearly suggest that *Anabaena* 7120 can assimilate glutamine as sole N-source. Glutamate can be expected to serve as N-source provided it is degraded to ammonia by catabolic glutamate dehydrogenase needed for synthesis of glutamine by glutamine synthetase. Lack of this activity would preclude utilization of glutamate as N-source. These results suggest
possible lack of catabolic glutamate dehydrogenase to be the reason for non utilization of glutamate as N-source.

Since nitrogenase activity is the glutamate sensitive target and since NO₃⁻ assimilatory cultures do not show nitrogenase activity, NO₃⁻ inhibition of nitrogenase activity appears to be one reason for the observed NO₃⁻ elimination of glutamate toxicity. But since NO₃⁻ metabolism also results in 50% reduction in glutamate uptake, it could as well be that inhibition of glutamate toxicity in the mutant is the result of reduction in glutamate uptake by NO₃⁻ metabolizing cultures. It could also be possible that NO₃⁻ inhibition of nitrogenase and NO₃⁻ inhibition of glutamate uptake both contribute to the observed NO₃⁻ elimination of glutamate toxicity in this cyanobacterium.