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Inorganic nitrogen regulation of glutamate uptake in the cyanobacterium *Nostoc muscorum*

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In *Nostoc muscorum* (*Anabaena* ATCC 27893) glutamate was not metabolised as a fixed nitrogen source, rather it functioned as an inhibitor of growth. The latter effect was nitrogen source specific and occurred in N_2-fixing cultures but not in cultures assimilating nitrate or ammonium. NO_3^--grown cultures lacked heterocysts and nitrogenase activity and showed a nearly 50% reduction in glutamate uptake rates, as well as in the final extent of glutamate taken up, compared to N_2-fixing or nitrogen-limited control cultures. NH_4^+-grown cultures showed a similar response, except that the reduction in glutamate uptake rates and the final extent of glutamate taken up was over 80%. The present results suggest a relation between nitrate/ammonium nitrogen-dependent inhibition of glutamate uptake, probably via repression of the glutamate transport system, and glutamate toxicity.

Key words – *Anabaena* ATCC 27893, cyanobacteria, glutamate uptake, growth, NH_4^+-grown, N_2-fixation, NO_3^--grown, *Nostoc muscorum*.

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Introduction

Considerable work has been done on regulation of N_2-fixation and of NO_3^-- and NH_4^+-assimilation, by various nitrogen sources, in diazotrophic cyanobacteria (Haselkorn 1978, Stewart 1980, Tumer et al. 1983, Bagchi et al. 1985a,b). However, there are only few studies on amino acids as sole nitrogen sources for heterocystous cyanobacteria, and the findings are variable as well as conflicting (Nielson and Larson 1980, Vaishampayan 1982). Glutamate is toxic for growth in *Anabaena variabilis* (Chapman and Meeks 1983) and stimulatory in *A. cyclindrica* PCC 7122 (Rawson 1985). GS mutants of *A. cycadeae* (glutamine auxotrophs) are capable of utilizing glutamine as sole nitrogen source but not glutamate (Singh et al. 1983). Studies on the nature of amino acid transport systems in heterocystous cyanobacteria have recently started, and a common transport system for glutamine and glutamate may occur in them (Chapman and Meeks 1983, Flores and Muro-Pastor 1988). However, the role of fixed inorganic nitrogen sources, like nitrate and ammonium, in the control of amino acid transport has not been studied in cyanobacteria.

We have studied the role of nitrate and ammonium nitrogen sources in regulating the uptake/metabolism of glutamate in the *Nostoc muscorum* parent strain and its nonN_2-fixing (Nif^-), nonheterocystous (Het^-) mutant. Here, for the first time, we present evidence that glutamate is toxic for the growth of N_2-assimilating cultures but not for cultures assimilating nitrate and ammonium; and that both nitrate and ammonium assimilating cultures, as compared to N_2-assimilating controls, show a drastic decrease in the rate and final extent of glutamate uptake (>50% reduction in NO_3^--grown cultures and >80% reduction in NH_4^+-grown cultures). We suggest that, due to decreased glutamate uptake, cultures assimilating nitrate and ammonium may overcome the toxic effect of glutamate by eliminating glutamate sensitive processes.

Abbreviations – GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase.

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Materials and methods

Axenic cultures of *Nostoc muscorum* (*Anabaena* ATCC 27893) parent strain (Het^+Nif^+) and its Het^-Nif^- mutant, were grown in BG-11_0 medium (Rippka et al. 1979) supplemented with 5 m*M* KNO_3, unless otherwise stated, at 25°C and a photon fluence rate of 50 μmol m^{-2} s^{-1} (white fluorescent light; Philips India Ltd.). Growth was measured by absorbance at 663 nm.

Chlorophyll *a* was measured according to Mackinney (1941). Nitrogenase activity was measured by acetylene reduction (Stewart et al. 1967).

Glutamate uptake was assayed for 30 min, using L-(U-^{14}C)glutamic acid. Exponentially growing cells were harvested by centrifugation (10000 *g* for 10 min), washed and resuspended in 10 m*M* HEPES-NaOH buffer, pH 7. After equilibrating for 30 min at 25°C and a photon fluence rate of 50 μmol m^{-2} s^{-1}, L-(U-^{14}C) glutamic acid was added to a final concentration of 50 μ*M* (specific activity 9.25 kBq ml^{-1} or 185 GBq mol^{-1}). At suitable time intervals, 400 μl samples were taken and the cells separated from their bathing medium by silicon oil microcentrifugation (Scott and Nicholls 1980). ^{14}C-incorporation was determined using a Beckman Liquid Scintillation Spectrophotometer LS 1801. When needed, 5 m*M* KNO_3 or 1 m*M* NH_4Cl (final concentrations) were added to the assay medium.

Nonspecific binding of L-(U-^{14}C)glutamic acid was determined by measuring its incorporation in toluene-treated cells as described by Rai et al. (1984). All data were plotted after substracting the respective values for toluene treated cells to eliminate background due to nonspecific binding. Heterocyst frequency was calculated as percentage of total number of cells, determined by light microscopy. Glutamate dehydrogenase activity was measured in cell-free extracts by following the oxidation of NADH at 340 nm as before (Stewart and Rowell 1977). Protein was estimated by the method of Lowry et al. (1951).

L-(U-^{14}C)glutamic acid was purchased from BARC, Bombay, India. All other chemicals were obtained from Sigma Chemical Company, USA.

Results

As shown in Tab. 1, the parent strain produced heterocysts and showed nitrogenase activity in N_2-medium, and it also grew well at the expense of N_2 as sole nitrogen source. In contrast, the mutant strain neither produced heterocysts nor did it show nitrogenase activity, and it did not grow with N_2 alone. Growth of the parent strain in NO_3^-- or NH_4^+-medium was accompanied by absence of heterocysts and nitrogenase activity. The mutant strain grew nearly as well as its parent in NO_3^-- or NH_4^+-medium, thus suggesting that the two strains had similar abilities to utilise nitrate and ammonium as nitrogen sources. Both strains also grew almost equally well in glutamine medium, although they did not form heterocysts or show nitrogenase activity, suggesting that glutamine can be metabolised and used as a fixed nitrogen source.

Glutamate inhibited nitrogenase activity as well as diazotrophic growth of the parental strain, and the Het^-Nif^- mutant did not grow in glutamate containing medium (Tab. 1). The latter observation thus suggests an inability of the mutant strain to utilise glutamate as fixed nitrogen source. Catabolic glutamate dehydrogenase (NADH dependent GDH) is normally considered to be the enzymic mechanism of utilisation of glutamate as a fixed nitrogen source. NADH-dependent GDH activity was extremely low in the parent as well as in the mutant strain (<0.1 nmol product formed mg^{-1} protein min^{-1}), which may explain the lack of glutamate utilisation by these strains. Cultures of either strain assimilating nitrate or ammonium grew well with glutamate in the growth medium. To explain this lack of glutamate toxicity, the effect of NO_3^- and NH_4^+ on glutamate uptake was examined.

Both N_2- and NO_3^--grown cultures of *N. muscorum*, showed a biphasic pattern of glutamate uptake. The

Tab. 1. Growth (absorbance at 663 nm 6 days after inoculation), heterocyst frequency (%) and nitrogenase activity (nmol C_2H_2 reduced μg^{-1} Chl *a* h^{-1}) of parent (Het^+Nif^+) and mutant (Het^-Nif^-) strains of *Nostoc muscorum* in media containing various nitrogen sources. Immediately after inoculation (at zero time) the absorbance at 663 nm was 0.1. Experiments were done in triplicate with two readings taken from each replicate. Thus, the data given are average mean (±SEM) from 6 readings for each treatment. ND – not detectable.

Nitrogen medium	Parent strain			Mutant strain		
	A_{663}	Heterocyst frequency	Nitrogenase activity	A_{663}	Heterocyst frequency	Nitrogenase activity
N_2-medium (BG-11_0)	0.68±0.07	5–6	12.6±1.15	0.10±0.03	0	ND
BG-11_0 + 5 m*M* KNO_3	0.85±0.09	0	ND	0.82±0.07	0	ND
BG-11_0 + 1 m*M* NH_4Cl	0.58±0.05	0	ND	0.63±0.07	0	ND
BG-11_0 + 2 m*M* glutamine	0.75±0.07	0	ND	0.78±0.09	0	ND
BG-11_0 + 1 m*M* glutamate	0.10±0.03	0	ND	0.10±0.04	0	ND
BG-11_0 + 1 m*M* glutamate + 5 m*M* KNO_3	0.72±0.06	0	ND	0.68±0.05	0	ND
BG-11_0 + 1 m*M* glutamate + 1 m*M* NH_4Cl	0.51±0.03	0	ND	0.56±0.04	0	ND

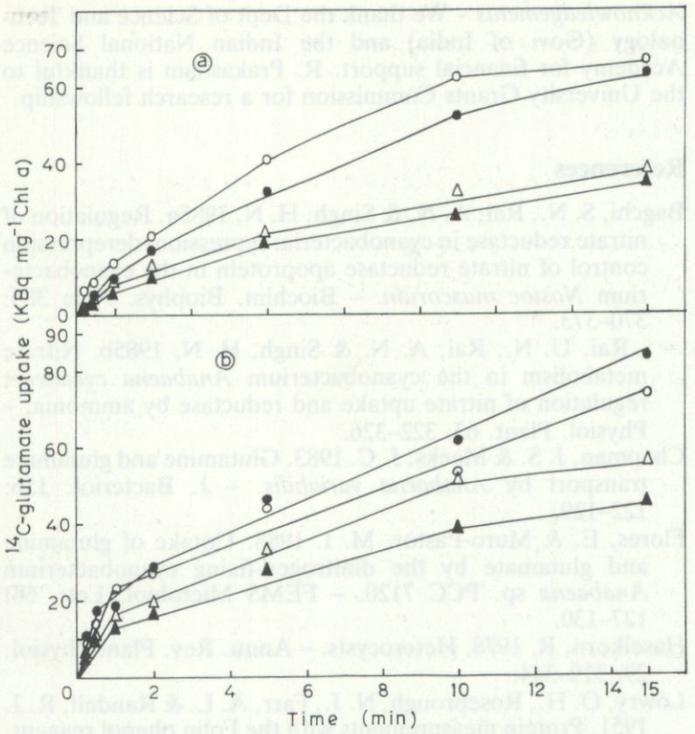

Fig. 1. Glutamate uptake in *Nostoc muscorum* (a) and its Het$^-$Nif$^-$ mutant strain (b) as influenced by NO_3^-. ○, N_2-grown cells, ^{14}C-glutamate uptake measured in the absence of KNO_3; ●, N_2-grown cells, ^{14}C-glutamate uptake measured in the presence of 5 m*M* KNO_3; △, NO_3^--grown cells, ^{14}C-glutamate uptake measured in the absence of KNO_3; ▲, NO_3^--grown cells, ^{14}C-glutamate uptake measured in the presence of 5 m*M* KNO_3. In Fig. 1b and 2b, the rates of ^{14}C-glutamate uptake in "N_2-grown cells" refer to NO_3^--grown cells, which were subjected to N-starvation for 24 h before measuring ^{14}C-glutamate uptake; this is because the mutant does not grow in N_2-medium. In both Fig. 1 and Fig. 2, the experiments were done in triplicate and two readings were taken from each replicate. The data presented are means. Variation range was 5–10%. Specific activity of ^{14}C-glutamate in the uptake medium was 185 GBq mol^{-1}.

initial fast rate of uptake became progressively slower and by 15 min, the uptake totally ceased (Fig. 1a). The rate of glutamate uptake (measured during the initial 30 s) by NO_3^--grown cultures, and the final extent of glutamate taken up, was nearly 50% lower than that by N_2-grown cultures. The mutant strain showed a similar effect (Fig. 1b; data not shown beyond 15 min).

Glutamate uptake in NH_4^+-grown cultures of the parent and the mutant strains also showed a biphasic pattern (Fig. 2a,b). In such cultures, glutamate uptake, and the final extent of glutamate taken up, were >80% lower than that in N_2-grown cultures. Furthermore, glutamate uptake ceased within 5 min. These observations indicate a stronger inhibitory effect of NH_4^+, as compared to NO_3^-, on glutamate uptake since 1 m*M* NH_4Cl produced a stronger effect than 5 m*M* KNO_3.

The cessation of glutamate uptake within minutes in all the cultures, and the inability of the strains to utilise glutamate for growth, indicate lack of glutamate metabolism by *N. muscorum*. The reason for this could be the lack of catabolic GDH. Thus, the final extent of glutamate taken up (i.e. up to the time when uptake ceases) indicates the level of glutamate accumulation in the cells.

Short-term effects of NO_3^- and NH_4^+, on glutamate transport, were studied to see whether they acted as repressors or inhibitors of the glutamate transport. Addition of NO_3^- during glutamate uptake by N_2- and NO_3^--grown cultures of the parent strain, did not cause any significant inhibition of glutamate uptake (Fig. 1a). In a similar experiment, NO_3^- did not cause any significant effect on the initial reates of glutamate uptake by nitrogen-starved and NO_3^--grown cultures of the mutant strain (Fig. 1b), although it led to a 12% increase and an 18% decrease in glutamate accumulation (final extent of glutamate taken up) in nitrogen-starved and NO_3^--grown cultures, respectively (Fig. 1b). In short term experiments, NH_4^+ also showed no significant effect on glutamate uptake and accumulation (data not shown). These data indicate that neither NO_3^- nor NH_4^+ itself directly inhibits glutamate transport system and that the reduction of glutamate uptake and accumulation in NO_3^-- and NH_4^+-grown cultures is due to the repression of the glutamate transport system.

Discussion

Heterocystous cyanobacteria assimilate N_2 into glutamine by the sequential action of nitrogenase and GS within heterocysts. Subsequently, glutamine is transferred to vegetative cells, where it is converted to glutamate by GOGAT (Thomas et al. 1977, Rai et al. 1984).

As the present growth results (Tab. 1) suggest, glutamate is not utilised as a source of fixed nitrogen, pos-

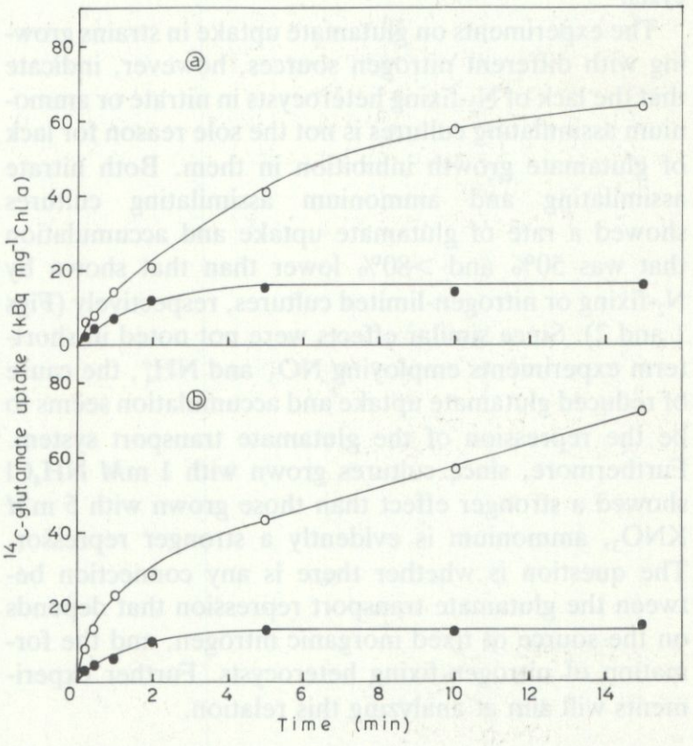

Fig. 2. Glutamate uptake in *Nostoc muscorum* (a) and its Het$^-$Nif$^-$ mutant strain (b) as influenced by NH_4^+. ○, ^{14}C-glutamate uptake in N_2-grown cells; ●, ^{14}C-glutamate uptake in 1 m*M* NH_4^+-grown cells.

sibly due to a negligible level of catabolic GDH. However, it acts as an inhibitor of growth, which is nitrogen-source specific and occurs under diazotrophic conditions but not under nitrate or ammonium assimilating conditions.

Since catabolic GDH activity remains negligible in cultures grown with N_2, NO_3^- or NH_4^+ as nitrogen source, the lack of glutamate growth inhibition in cultures assimilating nitrate or ammonium is evidently due to factors other than the catabolic GDH. Indeed, a large reduction in glutamate uptake and accumulation in NO_3^-- and NH_4^+-grown cultures (Figs 1 and 2), together with the fact that NO_3^- and NH_4^+ were available as N-sources in such cultures, seems to be the real reason for the lack of growth inhibition by glutamate in such cultures.

Glutamate treated, nitrogen-fixing cultures showed inhibition of nitrogenase activity (Tab. 1). There was no inhibitory effect of glutamate on growth in cultures assimilating nitrate or ammonium as nitrogen source. This finding suggests that nitrogen-fixing heterocysts are the glutamate sensitive target for the observed growth inhibition under diazotrophic conditions. In heterocystous N_2-fixing filaments, the glutamate producing enzyme, GOGAT, is predominantly localized in vegetative cells, and not in heterocysts (the site of N_2-fixation). This spatial separation of glutamate formation and N_2-fixation is of physiological significance in view of the observed glutamate inhibition of N_2-fixation. Thus, the compartmentalization of GOGAT in vegetative cells may be a novel strategy in heterocystous cyanobacteria to avoid glutamate inhibition of N_2-fixation in heterocysts.

The experiments on glutamate uptake in strains growing with different nitrogen sources, however, indicate that the lack of N_2-fixing heterocysts in nitrate or ammonium assimilating cultures is not the sole reason for lack of glutamate growth inhibition in them. Both nitrate assimilating and ammonium assimilating cultures showed a rate of glutamate uptake and accumulation that was 50% and >80% lower than that shown by N_2-fixing or nitrogen-limited cultures, respectively (Figs 1 and 2). Since similar effects were not noted in short-term experiments employing NO_3^- and NH_4^+, the cause of reduced glutamate uptake and accumulation seems to be the repression of the glutamate transport system. Furthermore, since cultures grown with 1 m*M* NH_4Cl showed a stronger effect than those grown with 5 m*M* KNO_3, ammonium is evidently a stronger repressor. The question is whether there is any connection between the glutamate transport repression that depends on the source of fixed inorganic nitrogen, and the formation of nitrogen-fixing heterocysts. Further experiments will aim at analyzing this relation.

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