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A Common Transport System for Methionine, L-methionine-DL-Sulfoximine (MSX), and Phosphinothricin (PPT) in the Diazotrophic Cyanobacterium *Nostoc muscorum*

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Abstract We present evidence, for the first time, of the occurrence of a transport system common for amino acid methionine, and methionine/glutamate analogues L-methionine-DL-sulfoximine (MSX) and phosphinothricin (PPT) in cyanobacterium *Nostoc muscorum*. Methionine, which is toxic to cyanobacterium, enhanced its nitrogenase activity at lower concentrations. The cyanobacterium showed a biphasic pattern of methionine uptake activity that was competitively inhibited by the amino acids alanine, isoleucine, leucine, phenylalanine, proline, valine, glutamine, and asparagine. The methionine/glutamate analogue-resistant *N. muscorum* strains (MSX-R and PPT-R strains) also showed methionine-resistant phenotype accompanied by a drastic decrease in ^{35}S methionine uptake activity. Treatment of protein extracts from these mutant strains with MSX and PPT reduced biosynthetic glutamine synthetase (GS) activity only in vitro and not in vivo. This finding implicated that MSX- and PPT-R phenotypes may have arisen due to a defect in their MSX and PPT transport activity. The simultaneous decrease in methionine uptake activity and in vitro sensitivity toward MSX and PPT of GS protein in MSX- and PPT-R strains indicated that methionine, MSX, and PPT have a common transport system that is shared by other amino acids as well in *N. muscorum*. Such information can become useful for isolation of methionine-producing cyanobacterial strains.

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Introduction

Cyanobacteria comprise a phylogenetically coherent ubiquitous group of photosynthetic eubacteria. Some of them are diazotrophic in nature. Additionally, they utilize amino acids as nitrogen sources with different growth-promoting activities [8, 26, 27]. Glutamate, a growth-inhibitory amino acid in *Anabaena variabilis* [6], and *Nostoc muscorum* [17] behave differently in *Anabaena cylindrica* [19]. On the other hand, inorganic nitrogen compounds protect cyanobacteria from toxic amino acids [23]. Amino acid like–proline has an additional role as an osmoprotectant [22]. The reasons for nutritional discrimination of amino acids are not well understood.

Amino acid uptake systems participate in recapturing leaked amino acids in addition to extracellular amino acids [14, 15]. Application of mutant strains has shown the existence of amino acid transport systems with fairly broad specificity in *Synechocystis* sp. strain 6803 [11]. Three high-affinity transport systems have been described in *Anabaena* PCC 7120: one for basic amino acids [10] and two for neutral amino acids [14]. Another study involving nine cyanobacteria has shown occurrence of at least a neutral amino acid transport system and some strains also bearing specific transport systems for basic or acidic amino acids [15]. Besides high affinity [5], cyanobacteria bear a low-affinity transport system for basic- [10] and acidic amino acids [14]. Certain amino acids may share a transport system for their entry into cells [6, 9, 25].

No information is available on the uptake regulation of methionine transport system in cyanobacteria except for *Prochlorococcus* spp. [28]. Kinetics of methionine uptake in bacteria has been studied in detail [2, 7]. In this paper, we report, probably for the first time, the occurrence of a

transport system in diazotrophic filamentous cyanobacterium *Nostoc muscorum* that is shared by methionine, L-methionine-DL-sulfoximine (MSX), phosphinothricin (PPT), and a host of other amino acids. MSX and PPT are glutamate analogue and irreversible inhibitors of glutamine synthetase activity. The inclusion of these two inhibitors in the study is justified by the fact that they are also methionine analogues [4].

Materials and Methods

Culture Growth Conditions

The *Nostoc muscorum* strain used in the present study is a fresh water isolate and has been reported extensively in various research publications [17, 22, 23]. Axenic batch cultures of parent *N. muscorum* and its L-methionine-DL-sulfoximine resistant (MSX-R) and phosphinothricin-resistant (PPT-R) mutant strains were routinely grown and maintained in combined nitrogen-free medium (BG 11_0) under a photon fluence rate of 50 μmole m^{-2} S^{-1} and at a temperature of 28 ± 2°C [20]. When required, 5 mM KNO_3 or 1 mM NH_4Cl or 1 mM amino acids were added to BG 11_0 media buffered with 10 mM HEPES-NaOH buffer (pH 7.5).

Isolation of Spontaneous Mutant Strains

The method of Singh et al. [22] was followed for isolation of MSX-R and PPT-R mutant strains of *N. muscorum* using actively growing cultures. A concentration of 5 μM of MSX or PPT was found to be 100% lethal dose to parent type *N. muscorum* strain in liquid media. Spontaneously occurring MSX-R and PPT-R strains were obtained at a frequency of 3.5×10^{-6} and 2.1×10^{-6}, respectively, at 10 μM concentrations of MSX and PPT in nutrient agar prepared in BG 11_0 medium.

Growth and Protein Content Determination

Growth was measured as changes in concentration of phycocyanin [3] and Chl *a* [13]. Protein content was determined according to [12].

Enzyme Assays

Acetylene reduction assay was used for nitrogenase activity measurement [24], and glutamine synthetase activity was measured by following the method of Sampio et al. [21].

Methionine Uptake Assay

Methionine uptake activity was performed according to Rai et al. [18] by following incorporation of ^{35}S-labeled methionine in the cyanobacterial cells. Six-day-old cells in exponential phase were harvested, washed, and resuspended in uptake media containing 10 mM HEPES-NaOH buffer (pH 7.0) to a concentration of 7–8 μg Chl *a* mL^{-1} and equilibrated for 30 min at a photon fluence rate of 50 μmol m^{-2} s^{-1} at 25°C. Methionine uptake activity was initiated by adding 60 μM ^{35}S-methionine (specific activity 370 kBq μmol^{-1}) in the uptake reaction mixture. Nonspecific binding of radioactivity determined obtained in toluene-treated cells was always subtracted from the value obtained for toluene-untreated cells. For determining competitive inhibition of methionine uptake by inorganic and organic nitrogen compounds, ^{35}S-methionine was added in parent *N. muscorum* suspension simultaneously along with 500 μM nitrate or 500 μM ammonium or 60 μM amino acids or 10 μM MSX or PPT. The intracellular level of ^{35}S-methionine was determined in samples collected 2 min after addition of ^{35}S-methionine. From such values, nonspecific binding of radioactivity was always subtracted.

Results

Isolation of Glutamine Synthetase Inhibitor Resistant Spontaneous Mutant Strains

Glutamate structural analogues MSX and PPT are irreversible inhibitors of glutamine synthetase (GS) activity. Spontaneous MSX-R and PPT-R mutant strains of *N. muscorum* were isolated by growing cyanobacterium in the presence of these GS inhibitors in 1% agar prepared in BG 11_0 media. Three weeks after incubation mutant colonies from MSX- and PPT-containing plates were picked up and raised in bulk in liquid medium.

Determination of GS Enzyme Activity

In order to understand the mechanism of resistance to these inhibitors, effects of MSX and PPT on GS (biosynthetic) activity in wild type and its MSX- and PPT-R strains were determined in vivo and in vitro. As shown in table 1, in vivo GS activity of wild type strain grown in medium without MSX or PPT was 72.5 nmol NADH oxidized mg^{-1} protein min^{-1} which was inhibited drastically in presence of either of the inhibitors at 10 μM concentrations and approached nearly zero within 24 h. The inhibition was 90% within first 6 h. This activity remained almost unaffected in MSX-R strain under MSX treated and untreated

conditions and in PPT-R strain under PPT treated and untreated conditions. GS activity in mutant strains showed a slight inhibition when MSX-R strain was treated with 10 μM PPT and PPT-R strain was treated with 10 μM MSX for 24 h (Table 1). This inhibition was 23.5% in MSX-R strain and 19.7% in PPT-R strain in comparison to their respective untreated cultures. On the other hand, in vitro treatment of enzyme extracts with 10 μM MSX or PPT led to a drastic decrease in GS activity. Activity inhibition in the enzyme extracts from all the strains ranged from 14.5 to 11.2 nmol NADH oxidized mg^{-1} protein min^{-1} (Table 1).

Effect of Methionine on Growth and Nitrogenase Activity

2 mM of methionine was lethal for wild-type strain of *N. muscorum*. At this concentration, nitrogenase activity approached zero after 3 days. Interestingly, at lower concentrations ranging from 0.06 mM to 0.1 mM, methionine induced nitrogenase activity significantly with 100% increase at 0.1 mM concentration (nitrogenase activity was 12.61 nmol C_2H_4 produced μg^{-1} chl *a* h^{-1} and 25.02 nmol C_2H_4 produced μg^{-1} chl *a* h^{-1} in combined N-free and in the presence of 0.1 mM methionine-supplemented media). Further increase in methionine concentration in medium led to decrease in nitrogenase activity that approached approximately at 2 mM methinone concentration. Since nitrogenase activity was influenced in a concentration-dependent manner, the effect of inorganic and organic nitrogen compounds on methionine toxicity in wild-type strain was studied. Combined nitrogen sources as 5 mM nitrate or 1 mM ammonium or 1 mM amino acids, namely, alanine, isoleucine, leucine, phenylalanine, proline, valine, glutamine, and arginine in the medium protected *N. muscorum* from methionine's toxic effect to a varied extent. Arginine, glutamine, and nitrate supported maximum growth followed by alanine, proline, leucine, phenylalanine, ammonium, isoleucine, and valine in deceasing order (Table 2). Interestingly, presence of ammonium in media showed as high growth protection against methionine toxicity as arginine, glutamine, and nitrate. Complete inhibition of nitrogenase activity was observed in nitrate, ammonium, arginine, glutamine, alanine, and praline-fortified media. However, nitrogenase activity was only partially repressed in presence of isoleucine, leucine, phenylalanine, and valine. Contrary to wild type, MSX-R and PPT-R strains grew well at 2 mM methionine concentration with normal heterocyst frequency and nitrogenase activity (data not shown), thus indicating a relation between MSX- and PPT-R phenotype and methionine transport system.

Table 1 Glutamine synthetase (biosynthetic) activity (nmol NADH oxidized mg^{-1} protein min^{-1}) in wild-type *Nostoc muscorum* and its L-methionine-DL-sulfoximine (MSX)- and phosphinothricin-resistant (PPT-R) strains after in vivo treatment of glutamine synthetase (GS) protein in intact cells or in vitro treatment of enzyme extract with MSX and PPT

In vivo treatment of GS protein in intact cells with MSX or PPT			
Culture growth condition	Wild-type strain	MSX-R strain	PPT-R strain
$BG11_0$ medium	72.5	68	66.1
$BG11_0$ + 10 μM MSX	0.69	67.2	53.1
$BG11_0$ + 10 μM PPT	0.87	52.0	65.3
In vitro treatment of enzyme extract with MSX or PPT			
10 μM MSX	14.5	13.6	11.2
10 μM PPT	13.3	12.2	12.4

For in vivo treatment, 6-day -old cultures of parent *N. muscorum* and its MSX- and PPT-R strains grown in BG 11_0 medium were harvested, washed, and resuspended in the same growth medium with and without 10 μM concentrations of MSX or PPT. Such cultures after 24-h incubation were used for GS biosynthetic activity assay. In case of in vitro treatment, enzyme extract obtained from 6-day-old culture were incubated for 1 h with 10 μM concentrations of MSX or PPT before GS activity assay. Each reading is an average of three independent experiments done in duplicate

Methionine Uptake Activity

Sixty micromolar methionine was found to be saturating concentration for methionine transport activity. To understand the mechanism of nitrogen sources protection against methionine toxicity in cyanobacterium under study, methionine uptake was measured in wild-type strain in the presence of equimolar concentrations (60 μM) of individual amino acids and in the presence of 10 μM each of MSX and PPT in uptake reaction mixture. Inhibition of methionine uptake was found to be by MSX (80%), PPT (84%), alanine (77%), isoleucine (57%), leucine (68%), phenylalanine (64%), proline (53%), valine (62.5%), glutamine (78.3%), and arginine (79%). Inorganic nitrogen compounds nitrate and ammonium did not inhibit methionine uptake activity at all.

The methionine transport system was biphasic, with a fast first phase lasting for a period of about 1 min followed by a slower second phase. Methionine uptake in nitrate- and ammonium-grown cells was comparable to uptake in BG 11_0-grown cells. The rates of first-phase and second-phase activity in the parent strain were 130 $\pm$ 5 nmol/min and 41 $\pm$ 3 nmol/min, respectively, measured in either BG 11_0 grown cells or NO_3^- or NH_4^+ grown cells. Methionine uptake activity in MSX- and PPT-R strains measured in BG 11_0 grown cells showed about fivefold reductions in uptake activity in comparison to the parent strain. This activity in MSX- and PPT-R strains remained unaffected by inorganic nitrogen sources.

Table 2 Effect of different nitrogen sources on chlorophyll *a* and phycocyanin contents and nitrogenase activity of *Nostoc muscorum*

Growth Conditions	Phycocyanin concentration		Chlorophyll *a* concentration		Nitrogenase activity	
	+Met	−Met	+Met	−Met	+Met	−Met
N_2 medium	2.85	16.73	0.20	1.86	1.69	12.61
NO_3^-	30.93	36.34	3.93	3.98	0.0	0.0
NH_4^+	12.55	13.01	1.44	1.46	0.0	0.0
Alanine	29.02	31.37	3.37	3.41	0.0	0.0
Isoleucine	9.0	26.17	1.47	2.31	1.72	3.68
Leucine	14.16	25.41	1.88	2.6	1.41	2.88
Phenylalanine	12.6	24.11	1.11	2.54	0.49	3.72
Proline	25.2	26.87	2.31	2.87	0.0	0.0
Valine	8.92	19.15	1.26	2.05	1.17	5.34
Glutamine	40.83	42.81	4.11	4.49	0.0	0.0
Arginine	47.69	52.34	4.55	4.67	0.0	0.0

The chlorophyll *a*, phycocyanin, and nitrogenase activity were measured in 6-day-old cells grown in BG 11_0 medium with and without 1 mM methionine and 5 mM NO_3^- or 1 mM NH_4^+ or amino acids

Discussion

Diazotrophic cyanobacteria are independent for their nitrogen supply but can utilize different inorganic and organic nitrogen sources whenever these are present in the surroundings. However, these nitrogenous compounds have differential effect on cyanobacterial metabolism [6, 19, 23]. The present study revealed a concentration-dependent effect of methionine in *N. muscorum* stimulating its nitrogenase activity at lower concentrations and inhibiting the same at increased concentrations, suggesting a regulatory role of methionine and/or its metabolized product on nitrogen metabolism. This conclusion was supported by results of cyanobacterial growth and nitrogenase activity in the presence of combined nitrogen sources in methionine-supplemented media. Inorganic (nitrate and ammonium) and organic (amino acids) nitrogen sources in methionine-supplemented media protected cyanobacterium from growth-inhibitory action exerted by methionine (Table 2). It seems that methionine toxicity is exhibited through its influence on internal nitrogen status mainly by affecting nitrogenase activity of the organism. Amino acids that could act as a nitrogen source were also able to protect cyanobacterium against methionine toxicity. However, unlike nitrate and ammonium, nitrogenase activity inhibition by amino acids varied, and the extent of growth protection by amino acids was determined by their ability to act as nitrogen sources (Table 2). The best utilizable nitrogen sources such as nitrate and amino acids arginine, glutamine, alanine, and proline supported maximum growth and inhibited nitrogenase activity completely followed by other nitrogen sources. Contrary to nitrate and ammonium, amino acids seems to provide protection at two levels, first at the level of entry of methionine into cells and second by meeting intracellular nitrogen requirement. That both mechanisms operate in case of amino acids became clear by following methionine uptake and nitrogenase activity in the presence of nitrate, ammonium, and amino acids in the medium. Nitrogenase activity was inhibited in all combined nitrogen sources containing media, though to varied degrees. However, methionine uptake activity inhibition occurred only in the presence of amino acids (Tables 2 and 3). No influence of inorganic nitrogen compounds on methionine uptake activity suggested a methionine transport system independent of nitrate or ammonium transport.

As shown in Figure 1, the methionine-resistant nature of MSX- and PPT-R strains of *N. muscorum* was due to defect in their methionine transport activity. On examination, these strains showed a drastic reduction in methionine uptake. MSX and PPT are glutamate/methionine analogues and are irreversible inhibitors of GS enzyme both in vivo and in vitro. In the case of MSX- and PPT-R strains, the in vitro sensitivity of GS protein to MSX and PPT from both strains indicated that MSX- and PPT-R phenotypes were a result of defect in their MSX and PPT transport activity and not due to mutation in their GS protein (Table 1). This defect in transport system would deny accessibility of MSX and PPT to intracellular GS protein. A simultaneous methionine-resistant phenotypic nature of MSX- and PPT-R strains concomitant with a drastic reduction in their methionine uptake activity suggested the existence of a transport system common for MSX, PPT, and methionine. A study conducted on the methionine transport system in bacterium *Salmonella typhimurium* showed occurrence of a transport system common for MSX, methionine, and other amino acids [4]. However, ours is the first report of its kind presenting evidence for sharing of a transport system by

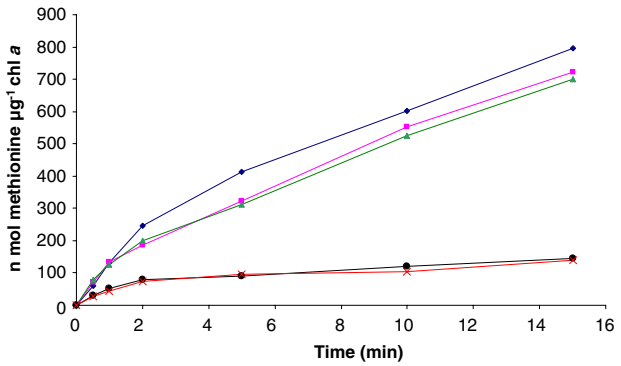

Fig. 1 ^{35}S-methionine uptake in *Nostoc muscorum* and its L-methionine-DL-sulfoximine-resistant (MSX-R) and phosphinothricin-resistant (PPT-R) mutant strains. Six-day-old exponentially grown cells were harvested and used for estimating methionine uptake activity in N_2 grown (♦), NO_3^- grown (■), and NH_4^+ grown (▲) parent *N. muscorum* and N_2 grown MSX-R (•) and PPT-R (x) strains. The readings are an average of two independent experiments

methionine, MSX, and PPT in *N. muscorum*. Analyses of methionine analogue α-methylmethionine-resistant strains of *Salmonella typhimurium* have confirmed a role of two closely linked genes, *met P* and *gln P*, in methionine transport, glutamine transport activity, and MSX resistance [1, 16]. Mutant strains resistant to α-methylmethionine and methionine sulfoximine have been shown to be severely defective in methionine permease activity in *Salmonella typhimurium* [2]. Occurrence of amino acid transport systems with specific and broad specificity has been demonstrated in cyanobacteria as well [11, 15]. A common transport system for MSX, glutamine, and glutamate has been reported in *Anabaena variabilis* [6], showing that glutamate and glutamine inhibit transport of each other and when included in the growth medium, protect cyanobacterium from MSX toxicity. As shown in Table 1, in vitro sensitivity of GS protein from MSX- and PPT-R strains to MSX and PPT treatments suggested that the two mutant phenotypes arose as a result of a defect in their MSX and PPT transport. Inhibition of methionine uptake activity in wild-type *N. muscorum* in the presence of equimolar (60 μM) concentrations of individual amino acids or 10 μM MSX or PPT in methionine transport reaction mixture and only in vitro sensitivity of GS proteins from MSX- and PPT-R strains to MSX or PPT suggested the occurrence of a transport system, which is common for amino acids used in this study and the glutamate analogues. These findings are in concurrence with observations of Chapman and Meeks [6]. The methionine-resistant nature of MSX- and PPT-R strains may imply that methionine and GS inhibitors MSX and PPT act as analogues only at the transport level and not at the biochemical activity level. Characterization of MSX and PPT transport system(s) in cyanobacteria at the molecular level may provide much-needed information for their biotechnological application in photobiological production of nitrogenous compounds including amino acids, especially methionine.

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