The role of glutamine synthetase activity in ammonium and methylammonium transport in *anacystis nidulans* R-2

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1. INTRODUCTION

Glutamine synthetase plays a key role in ammonia incorporation in many microorganisms, including cyanobacteria [1–3]. Methionine sulfoximine (MSX) inhibits glutamine synthetase irreversibly by a well-documented mechanism [4,5], and has been widely used to probe the relationship between glutamine synthetase activity and other critical features of nitrogen metabolism, including nitrogenase derepression [6].

Transport systems that mediate the intracellular accumulation of ammonium and its analog methylammonium have now been detected in many microorganisms, including free-living and symbiotic diazotrophs [7–12]. In most, internal $^{14}CH_3NH_3^+$ is rapidly converted to the methyl analog of glutamine [13–16]; for example the radioactivity taken up by *Anacystis nidulans* R-2 after the first 50–60 s of incubation with $^{14}CH_3NH_3^+$ is accounted for by accumulation of this product, while intracellular [$^{14}CH_3NH_3^+$] remained unchanged [16]. It is therefore important in inhibitor studies to distinguish clearly between effects on the translocation of a substrate and on its subsequent metabolic conversion. Recent reports have suggested that MSX may inhibit the transport of $^{14}CH_3NH_3^+$ directly [17–19]. The short duration experiments described here suggest that MSX affects $^{14}CH_3NH_3^+$ accumulation only as a consequence of its inhibitory action on glutamine synthetase. The reduction of transport is a secondary effect, and may be caused by intracellular NH_4^+ production when amidation is blocked.

2. MATERIALS AND METHODS

2.1. *Organism and culture conditions*

Anacystis nidulans R-2 was grown as described [21].

2.2. *$^{14}CH_3NH_3^+$ transport*

Washed cells from exponential cultures, resuspended in 20 mM K phosphate: 10 mM $NaHCO_3$ (pH 7.2) were prepared and preincubated as described [16]. Rates were calculated from radioactivity taken up between 5 and 60 s after addition of 9 μM $^{14}CH_3NH_3Cl$ (spec. act. 56 mCi·mmol^{-1}).

2.3. *Glutamine synthetase activity*

The transferase assay was used on cells mixed vigorously with 0.02 vol. toluene as in [20]. Activi-

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ty was the same in cells broken by passage through a French press as after toluene treatment.

2.4. *Ammonium uptake*

Residual NH_4^+ in filtrates from suspensions to which 50 μM NH_4Cl had been added was determined chemically [21].

3. RESULTS AND DISCUSSION

3.1. *Effect of MSX on $^{14}CH_3NH_3^+$ transport*

Methionine sulfoximine (up to 1 mM) added at the same time as 9 μM $^{14}CH_3NH_3^+$, did not affect the uptake of radioactivity over the standard 60 s assay. Thus, even high concentrations of MSX did not interfere immediately with $^{14}CH_3NH_3^+$ entry or accumulation. However, this transport was reduced by incubation in the presence of MSX before the start of the transport assay. Table 1 shows that although glutamine synthetase was inactivated more rapidly than $^{14}CH_3NH_3^+$ transport, which was barely affected after 10 min, both activities were eliminated after 30 min before the start of the assay. In parallel experiments, NH_4^+ uptake was also abolished when glutamine synthetase was 70–80% inhibited (not shown).

3.2. *Effect of dark preincubation with MSX on transport and glutamine synthetase*

When suspensions were mixed with 100 μM MSX in darkened tubes, and then samples transferred to light for transport assays, or taken for enzyme activity measurements, inhibition developed more slowly than in illuminated cells (table 2); the activities were unaffected by dark incubation in the absence of MSX. These experiments suggest that energy is needed for MSX inhibition to be exerted, and that access of inhibitor to its target in darkness may be limited by the low respiration rate of *A. nidulans*.

3.3. *Effect of glutamine on inhibition by MSX*

A common transport system for glutamine, methionine and MSX has been identified in another cyanobacterium [22]. The possibility that the rate at which MSX entered the cells might govern the onset of inhibition was therefore tested by examining the effect of competition with glutamine. Unlike methylammonium uptake in *Klebsiella pneumoniae* which is reported to be inhibited by high concentrations of glutamine [17], $^{14}CH_3NH_3^+$ transport in *A. nidulans* showed at most a 30% reduction if measured 30 s after adding 5 mM glutamine; thereafter, even after

Table 1

Inhibition of glutamine synthetase and $^{14}CH_3NH_3^+$ accumulation by MSX in light, effect of glutamine

Preincubation time (min)	+ MSX (100 μM)		+ MSX (100 μM) and glutamine (5 mM)	
	$^{14}CH_3NH_3^+$ transport[a]	Glutamine synthetase[b]	$^{14}CH_3NH_3^+$ transport[a,c]	Glutamine synthetase[b]
0	7.4	160	6.9	145
10	7.0	48	6 5	150
20	3.5	20	6.8	165
30	0	0	6 6	160

[a] nmol·min^{-1} mg protein^{-1}

[b] nmol hydroxamate formed min^{-1} mg protein^{-1}

[c] Control with glutamine alone unchanged after 30 min

Samples (0.3 ml) of suspension containing 130 μg cell protein·ml^{-1} were kept for 15 min in light at 30°C. Glutamine (5 mM) was added to one portion, and 2 min later 100 μM MSX was added to both $^{14}CH_3NH_3^+$ transport was measured at the times indicated over 1-min periods after addition of 9 μM $^{14}CH_3NH_3Cl$ (56 mCi·mmol^{-1})

Samples for glutamine synthetase were treated as described in the text

Table 2

Inactivation of glutamine synthetase and of $^{14}CH_3NH_3^+$ transport by MSX; preincubation in light or darkness

Preincubation (min)	Relative glutamine synthetase activity		Relative $^{14}CH_3NH_3^+$ transport	
	Light	Dark	Light	Dark
0	100[a]	100[b]	100[c]	100[d]
10	30	40	95	96
20	13	26	47	47
30	0	20	0	36

[a] 100% = 220 nmol hydroxamate formed $min^{-1} \cdot mg$ $protein^{-1}$

[b] 100% = 100 nmol hydroxamate formed $\cdot min^{-1} \cdot mg$ $protein^{-1}$

[c] 100% = 7 2 nmol $^{14}CH_3NH_3^+$ accumulated $min^{-1} \cdot mg$ $protein^{-1}$

[d] 100% = 7 4 nmol $^{14}CH_3NH_3^+$ accumulated $min^{-1} \cdot mg$ $protein^{-1}$

A suspension containing 150 μg protein $\cdot ml^{-1}$ was incubated with 100 μM MSX in light or in foil-wrapped tubes Portions were removed for transport and enzyme activity measurements at the times indicated

30 min further incubation, counts taken up during the standard assay were as high in the presence of glutamine as in its absence. The most probable explanation for the transient reduction of $^{14}CH_3NH_3^+$ transport is trace contamination of the freshly made glutamine solution with NH_4^+, since the transport system has a much higher affinity for NH_4^+ than for $CH_3NH_3^+$ [16], and as little as 1–2 μM NH_4^+ causes a delay in the start of $^{14}CH_3NH_3^+$ uptake (not shown). Methionine (5 mM) had no effect on the rate of $^{14}CH_3NH_3^+$ accumulation.

When glutamine (5 mM) was present during incubation with MSX in light, glutamine synthetase was protected from the effects of the inhibitor, and the ability to take up $^{14}CH_3NH_3^+$ also remained as high as at the start of the experiment (table 1). Similar protection was seen when the MSX concentration was increased to 1 mM. Methionine addition (5 mM) also eliminated MSX inhibition (not shown). Since the amino acids had no direct effect on $^{14}CH_3NH_3^+$ transport, their protection effect in MSX is most probably due to competitive inhibition of MSX entry into the cells.

3.4. *Methylammonium uptake in an MSX-resistant mutant*

Several MSX-resistant colonies were obtained by plating *A. nidulans* R-2 mutagenized with EMS [23] on medium containing 50 μM MSX. One of these, selected for further study, grew as fast as the parent in nitrate or ammonium medium, with or without 50 μM MSX. Glutamine synthetase transferase activity in permeabilized or broken cells averaged only 10–15% that of the parent, but was insensitive to the presence of 1 mM MSX in the assay mixture. The MSX resistance was therefore not due to failure of the inhibitor to enter the cells, as found in one type of mutant of *Anabaena* [22]. Ammonium uptake occurred at only about half to two thirds the rate characteristic of the parent, but $^{14}CH_3NH_3^+$ transport rates were comparable. The uptakes of both compounds were unaffected by preincubation in light with MSX for 30 min (table 3).

These experiments show that MSX treatment caused loss of ability to concentrate $^{14}CH_3NH_3^+$ in wild-type *Anacystis nidulans* only when glutamine synthetase activity was reduced to below 30% of its initial value. Any conditions which delayed onset of inhibition of glutamine synthetase also protected transport ability. The mutant was able to transport $^{14}CH_3NH_3^+$ at the same rate as the parent, but both glutamine synthetase and transport were unaffected by MSX. The two activities thus show parallel behavior in both mutant and parent, and suggest that MSX has a single target. Release of NH_4^+ formed by NO_3^- reduction

Table 3

Effect of MSX on NH_4^+ and $^{14}CH_3NH_3^+$ uptakes by a mutant of *A nidulans* resistant to MSX

Preincubation (min)	Uptake[a]	
	$^{14}CH_3NH_3^+$	NH_4^+
0	7.4	17.5
10	8.2	15
20	7.0	20
30	7.3	18

[a] Rate units: nmol $\cdot min^{-1} \cdot mg$ $protein^{-1}$

MSX (100 μM) was added to suspensions kept in light at the start of the preincubation period

[24] or through protein degradation [20] on addition of MSX has been demonstrated; under the conditions used in the experiments described here, extracellular NH_4^+ becomes detectable chemically when glutamine synthetase activity is reduced to about 10% of its initial value. The simplest explanation for the effect of MSX on $^{14}CH_3NH_3^+$ accumulation is therefore that the transport system becomes fully occupied with attempts to recycle intracellularly produced NH_4^+, and $^{14}CH_3NH_3^+$ can no longer compete for entry. This explanation is strengthened by the finding that the MSX-resistant mutant does not release NH_4^+ during growth on NO_3^-, nor during incubation with MSX in the absence of nitrogen source. These experiments do not rule out the existence of further targets for MSX, which has been considered a very specific inhibitor of glutamine synthetase, but they do suggest strongly that the primary target is in fact this enzym, and that the effect on methylammonium transport, at least in *Anacystis nidulans*, is secondary.

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REFERENCES

[1] Brown, C N , MacDonald-Brown, D.S and Meers, J.L. (1974) in: Advances in Microbial Physiology (Rose, A.H. and Tempest, W. eds) pp.1–52, Academic Press, London, New York

[2] Stewart, W.D.P (1980) Annu Rev. Microbiol. 34, 497–536.

[33] Orr, J. and Haselkorn, R. (1982) J. Bacteriol. 152, 626–635

[4] Meister, A (1974) in. The Enzymes (Boyer, P D ed.) 3rd edn, vol.10, pp 699–754, Academic Press, New York.

[5] Shapiro, B M. and Stadtman, E R (1970) Annu. Rev. Microbiol. 24, 501–524

[6] Stewart, W.D.P and Rowell, P (1975) Biochem Biophys Res. Commun. 65, 846–856

[7] Kleiner, D. (1981) Biochim. Biophys Acta 639, 41–52

[8] Hackette, S L , Skye, G.E , Burton, C U and Segel, H.I (1970) J. Biol. Chem 245, 4241–4250

[9] Stevenson, R. and Silver, S. (1977) Biochem Biophys Res. Commun. 75, 1132–1139.

[10] Kleiner, D and Fitzke, E (1981) Biochim Biophys. Acta 641, 138–147

[11] Weigel, J. and Kleiner, D. (1982) FEMS Microbiol Lett. 15, 61–63

[12] Glenn, A.R. and Dilworth, M J. (1984) J Gen. Microbiol. 130, 1961–1968

[13] Barnes, E M. and Zimniak, P. (1981) J Bacteriol. 15, 616–623

[14] Barnes, E M., Zimniak, P. and Jayakamar, A. (1983) J. Bacteriol. 156, 752–757.

[15] Gober, J.W. and Kashket, E.R. (1983) J Bacteriol. 155, 1196–1201.

[16] Boussiba, S., Dilling, W. and Gibson, J (1984) J Bacteriol., in press

[17] Kleiner, D. and Castorph, H. (1982) FEBS Lett 146, 201–203.

[18] Singh, H.N., Rai, U.N., Rao, V.V and Bagchi, S.N (1983) Biochem. Biophys Res. Commun. 111, 180–187.

[19] Turpin, D H , Edie, S.A and Canvin, D.T. (1984) Plant Physiol. 74, 701–704.

[20] Boussiba, S , Liu, X.-Q. and Gibson, J. (1984) Arch. Microbiol. 138, 217–219

[21] Boussiba, S , Resch, C. and Gibson, J (1984) Arch. Microbiol 138, 287–292

[22] Chapman, J.S. and Meeks, J.C (1983) J Bacteriol 156, 122–129

[23] Herdman, M , Delaney, S.F and Carr, N.G (1980) Arch. Microbiol 124, 178–184

[24] Ramos, J L., Guerrero, M.G. and Losada, M (1982) Appl. Environ. Microbiol. 44, 1013–1017.