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Endogenous ammonia production by *Anacystis nidulans* R-2 induced by methionine sulfoximine

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Abstract. *Anacystis nidulans* R-2 produced ammonia from endogenous sources for at least 6 h when illuminated without external nitrogen source but with CO_2 in the presence of 50 μM methionine sulfoximine. The onset of ammonia release coinciding with complete inhibition of glutamine synthetase. The total quantity of ammonia which could be released exceeded the nitrogen content of small molecule pools, and suggested protein degradation as the most likely source of the nitrogen. Ammonia release was not accompanied by leakage of carbon compounds from the cells. Methionine sulfoximine-induced ammonia release was energy requiring, and was barely detectable under dark anaerobic conditions, or in the presence of 10 μM carbonyl cyanide m-chlorophenylhydrazone in light. Phenyl methyl sulfonylfluoride, an inhibitor of serine proteases, eliminated ammonia release, and the rate of release was reduced to one-third of control values, after a lag, in the presence of 50–75 μg/ml chloramphenicol. The rate of NH_4^+ release was maximal (1.4 nmol · min^{-1} · mg^{-1} protein) if suspensions were bubbled with 100% O_2, but could not be reduced below 0.6 nmol · min^{-1} · mg^{-1} protein in air:CO_2, suggesting that release was at most only partly due to photorespiration.

Key words: Ammonia production – Protein breakdown – *Anacystis nidulans*

Cyanobacteria produce ammonia from externally supplied nitrogen sources when illuminated in the presence of L-methionine D,L-sulfoximine, an irreversible inhibitor of glutamine synthetase (Ronzio et al. 1969). Heterocyst-containing cultures of *Anabaena cylindrica* released ammonia formed by N_2 reduction in the presence of the inhibitor (Stewart and Rowell 1975). Ramos et al. (1982a) characterized the production of NH_4^+ from nitrate in *Anacystis nidulans*, and worked out conditions for sustained release (Ramos et al. 1982b). These findings emphasize the role of glutamine synthetase in ammonia assimilation, and also show that nitrate or dinitrogen reduction is not inhibited when ammonia incorporation is blocked.

During the course of an investigation into the uptake and retention of ammonia in *Anacystis nidulans* R-2, we observed that the addition of 50 μM MSX led to the sustained production of NH_4^+ even in the absence of an external nitrogen source. The extent of this production suggested that it resulted from protein degradation. Ammonia release was reduced in the presence of a protease inhibitor or chloroamphenicol, and was energy dependent.

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Abbreviations used: MSX, L-methionine D,L-sulfoximine; PMSF, phenylmethylsulfonylfluoride; CAP, chloramphenicol; CCCP, carbonyl cyanide-m-chlorophenyl hydrazone

Materials and methods

Anacystis nidulans R-2 was obtained from Dr. John Williams, Boyce Thompson Institute, Cornell University. Cultures were grown in BG-11 (Rippka et al. 1979) at 30°C in an illuminated water bath; cells were collected for experiments when the cell density was between 0.05–0.1 mg cell protein · ml^{-1}. The culture was centrifuged and the cell pellet washed once with BG-11_0 (BG-11 with omission of $NaNO_3$) but supplemented with 20 mM K phosphate buffer, pH 7.2. Suspensions containing 0.3–0.5 mg cell protein · ml^{-1} were incubated at 30°C for following ammonia release. Samples were centrifuged in an Eppendorf microcentrifuge, and ammonia determined in the supernatant solution by Solorzano's method (1969) using a total reaction volume of 0.82 ml. Phycocyanin was estimated from absorbance at 620 nm (Myers and Kratz 1955) after passage of suspensions through a French press. Total protein was estimated as described by Lowry et al. (1951).

Glutamine synthetase was estimated as the transferase activity in cells from 10 ml suspension, collected and resuspended in 2 ml 50 mM Tris · HCl, pH 7. The concentrated sample was shaken with 2% toluene and then kept in ice for 15 min before addition to the assay mixture described by Sampaio et al. (1979). Total ammonia pools were estimated in cells from 20 ml of the suspension as described (Boussiba et al. 1984).

Results and discussion

Anacystis nidulans R-2 released ammonia when incubated in light in the presence of MSX without added nitrogen source, although only at about one-tenth the rate observed in the presence of added nitrate (Table 1). The onset of this release coincided with inactivation of glutamine synthetase (Fig. 1). Release continued linearly for at least 6 h; in one experiment, ammonia production continued steadily for 24 h. Changes in the preexisting pools of ammonia or amino acids inside the cells were insufficient to account for the total MSX induced ammonia release. Over 300 nmol NH_4^+ · mg^{-1} cell protein was produced in 6 h (Table 1) which amounted to approximately 4% of the total cell nitrogen. The ammonia

Table 1. Ammonia release by *A. nidulans* R-2. Ammonia release was determined as described in the text. Cell densities: Experiment 1: 0.52 mg protein · ml^{-1}. Experiment 2: 0.35 mg protein · ml^{-1}

Additions	NH_4^+ released (nmol · min^{-1} · mg^{-1} protein)
Experiment 1	
None	N.D.
50 μM MSX; light	0.85
50 μM MSX; dark	0.35
50 μM MSX; dark, anaerobic	0.1
50 μM MSX; 10 μM CCCP; light	0.02
50 μM MSX + 5 mM NO_3^-; light	13.5
Experiment 2	
50 μM MSX; light	0.92
50 μM MSX; 75 μg/ml CAP; light	0.33[a]
50 μM MSX; 1 mM PMSF; light	0.32
50 μM MSX; 4 mM PMSF; light	N.D.

N.D. – Not detected (< 2 μM)
[a] Calculated from linear release rate from 60–80 min

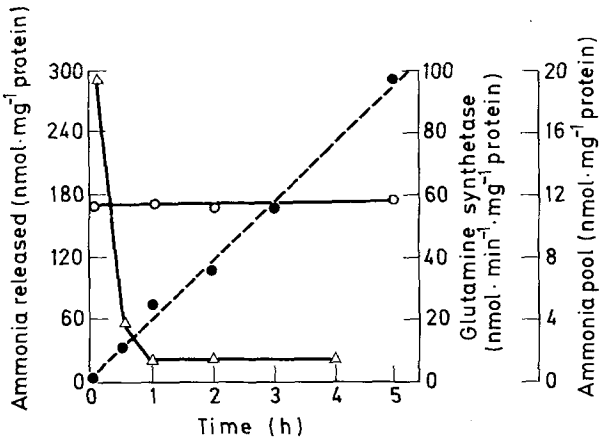

Fig. 1. Ammonia pool and glutamine synthetase activity during ammonia release in the presence of MSX. Exponentially growing cells (0.1 mg cell protein · ml^{-1}) were washed and incubated without external N-source and in the presence of 50 μM MSX in light as described in the text. Samples were taken for determining intracellular ammonia pool, ammonia released and glutamine synthetase activity at the times indicated. ●——● Ammonia released; pH 7.0 and pH 8.5; ○——○ Ammonia pool; pH 7.0; in other experiments no difference in pool was observed in cells maintained at pH 7.0 or 8.5 (Boussiba et al., accompanying paper); △——△ Glutamine synthetase activity

pool of 12 nmol · mg^{-1} protein remained constant during incubation with MSX (Fig. 1), and the total amino acid pool, although larger (230 nmol · mg^{-1} protein) was also unchanged. Cells which had been grown with 2 mM NH_4Cl as nitrogen source released as much NH_4^+ during incubation with MSX as those grown on NO_3^-, making it improbable that a large intracellular pool of NO_3^+ was involved. The storage polymer cyanophycin (Simon 1971) can also be discounted, since ammonia release by cells taken from cultures which had reached cell densities equivalent to only 20 μg protein · ml^{-1} before starting the incubation with MSX was as great as that by cells whose growth rate was light-limited. The cyanophycin content of another unicellular cyanobacterium (*Aphanocapsa* 6308) was less than 1% of the total dry weight of cells taken from low density cultures grown under comparable conditions (Allen et al. 1980). The most probable source of the released ammonia was therefore protein breakdown, and the effect of the serine protease inhibitor PMSF in reducing ammonia production (Table 1) is consistent with this suggestion. Whether the significantly decreased release rate after the first hour which was observed when chloroamphenicol was added at the same time as MSX implied induction of a new protease, or indicates that a protease with a short half-life is present during normal growth, is not at present clear.

The induction of a specific phycocyanin degrading protease has been observed during nitrogen starvation in other cyanobacteria (Foulds and Carr 1977; Boussiba and Richmond 1980; Wood and Haselkorn 1980). It has been difficult to demonstrate a consistent small decrease in phycocyanin content during the 6 h experiments carried out in this study. The ammonia release may therefore come from a wider range of cellular proteins than phycocyanin alone.

Ammonia release was energy-dependent, and was not observed in the presence of a protonophore or under dark anaerobic conditions (Table 1). There are at least two possible reasons for this energy requirement. In addition to the needs of protein synthesis suggested by the chloroamphenicol effect (Table 1), energy may be needed either for degradation of protein as in mammalian cells (Goldberg and St. John 1976) or for the release of ammonia from the cell. In *Anabaena cyclindrica,* there was a doubling in intracellular ammonia when cultures were incubated for long periods with low concentrations of MSX under nitrogen fixing conditions (Stewart and Rowell 1975). In *Anacystis,* however, we have been unable to observe any increase in intracellular NH_4^+ either preceding or accompanying its release from endogenous sources induced by MSX, and the rate of release was the same in suspensions incubated at pH 7 or at pH 8.5 (Fig. 1). Nonspecific leakage from the cells thus seems improbable, but the mechanism of ammonia release when glutamine synthetase is blocked is not at present understood. This release is in sharp contrast to the retention of the ammonia pool in cells incubated without MSX (Boussiba et al. 1984).

Ammonia release by eukaryotic algae and higher plants appears to be intimately linked with photorespiration (Keys et al. 1978; Cullimore and Simms 1980), and the rate of its production in the presence of MSX can be greatly varied by alterations in the ratio of O_2:CO_2 in the environment. In *Chlamydomonas* ammonia release was barely detectable in air:CO_2, but increased to 1.35 nmol · min^{-1} · mg^{-1} protein in pure O_2 (Cullimore and Sims 1980). In *A. nidulans* the ammonia release rate could be varied by a factor of only two by bubbling the suspensions either with air: 3% CO_2 or with pure O_2, when a maximal rate of 1.44 nmol NH_4^+ · min^{-1} · mg^{-1} protein was observed (Table 2). The ability of the organisms to transport bicarbonate actively (Miller and Colman 1980; Kaplan et al. 1980), and thus maintain a relatively high concentration of CO_2 in the immediate vicinity of ribulose bisphosphate carboxylase may limit the extent of photorespiration in the cyanobacterium.

Table 2. Effect of varying CO_2:O_2 on rate of ammonia release. Ammonia release from suspensions incubated in light + 50 μM MSX was followed as described in the text. Cell density: 0.36 mg protein · ml^{-1}

Incubation conditions	Ammonia released (nmol · min^{-1} · mg^{-1} protein)
Bubbled with air	1.0
Bubbled with air: 3% CO_2	0.66
Bubbled with O_2	1.44

Cells uniformly labelled by growth in $^{14}CO_2$ released approximately 3% of the total cellular radioactivity as acid stable counts within 90 min of transfer into unlabelled medium without added nitrate. No further release was observed over as much as 24 h. The presence of MSX during the incubation did not affect either the rate or the extent of carbon compound release. Cell carbon therefore appears to be retained or recycled within the cells during protein turnover even when the nitrogen is lost as ammonia.

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