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Liberation of ammonia during nitrogen fixation by a facultatively heterotrophic cyanobacterium

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Nitrogen fixation (acetylene reduction) and ammonia liberation were studied in a facultatively heterotrophic cyanobacterium. Autotrophically grown cells lost acetylene reduction activity when incubated under anaerobic conditions; the activity was maintained in the presence of methionine sulfoximine; or by pretreatment of the cells with a carbon supply. Heterotrophically grown cells maintained acetylene reduction activity anaerobically in the absence of methionine sulfoximine. Both cell types required light for maintenance of activity. The data indicate that methionine sulfoximine preserves the intracellular pool of reductant needed for nitrogenase. Autotrophs and heterotrophs both liberated ammonia when treated with methionine sulfoximine under nitrogen-fixing conditions. However, on treatment with methionine sulfoximine under anaerobiosis, heterotrophs also accumulated large amounts of intracellular ammonia in a pool which was diminished by the Photosystem II inhibitor, 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). DCMU enhanced ammonia liberation without affecting acetylene reduction activity, and hence changed the ratio of acetylene reduced to ammonia formed by the heterotrophs. These data suggest a role for Photosystem II in ammonia liberation by the cyanobacteria.

Introduction

Many nitrogen-fixing bacteria liberate ammonia when assimilation of fixed nitrogen is inhibited by the glutamine synthetase inhibitor, methionine sulfoximine [1,2]. In the cyanobacteria (blue-green algae), reductant supply for ammonia formaiton from N_2 can be derived from glycogen and the pool of fixed carbon intermediates which provide electrons via Photosystem I to nitrogenase in the heterocyst [3]. When this supply of reductant is reduced by experimental manipulation, nitrogenase activity declines, but cn be restored by supplying an exogenous electron donor, such as molecular hydrogen,. to nitrogenase [4]. Ammonium transport systems have been studied in a variety of nitrogen fixing bacteria [5–7] with a view to understanding mechanisms of nitrogen transfer from symbiont to host plant. Recent studies with free living and symbiotic cyanobactaeria have demonstrated energy-dependent systems responsible for uptake of ammonium by these cells. In *Anabaena variabilis*, ammonium transport was biphasic and an initial incorporation into the cell followed by a sustained metabolic uptake, sensitive to methionine sulfoximine, could be differentiated [8]. In *Anacystic nidulans*, sustained uptake

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Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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was also methionine sulfoximine-sensitive, and it was suggested that uptake was regulated by the internal pool of metabolites [9].

Anabaena sp., strain 2B (NRRL-B4374), is a facultatively heterotrophic cyanobacaterium isolated from the symbiotic nitrogen fixing fern, *Azolla caroliniana* [10]. During studies on the nitrogen metabolism of this cyanobacterium, it was observed that methionine sulfoximine eliminated the decline of nitrogenase activity caused by anaerobiosis. Our data indicate that, in addition to allowing liberation of ammonia in this cyanobacterium, methionine sulfoximine stabilizes the level of intermediates which permit maintenance of nitrogenase activity. Manipulation of the metabolic condition of the cells can result in either enhanced intracellular ammonia accumulation or release by this cyanobacterium. Furthermore, our data suggest a role for Photosystem II in light-activated ammonia liberation.

Materials and Methods

Anabaena sp., strain 2B, a facultatively heterotrophic strain originally isolated from *Azolla caroliniana* [10] has been deposited in the Northern Regional Research Center Culture Collection and is designated as NRRC strain B-4374. All cultures were grown on the BG-11 medium of Stanier et al. [11] without nitrate. For photoautotrophic growth, cultures were incubated in 100 ml of this N-free medium in 250 ml Erlenmeyer flasks on a rotary shaker in a light chamber with cool white fluorescent lamps (10 W/m^2) at 25°C. For heterotrophic growth in the dark, fructose was added to a concentration of 0.05 M and the flasks were wrapped in aluminium foil. To change gas atmospheres the flasks were fitted with rubber stoppers containing outlet tubes, evacuated and refilled with appropriate gases for incubation. Growth was followed spectrophotometrically at 650 nm after dilution of cultures to read 0.5–1.0 absorbance units (A). Protein content of cultures was determined using the method of Spector [12] on dilute sonic extracts of cells at different growth stages and correlated with optical density: one A_{650} unit corresponded to 0.075 mg cell protein per ml using this assay; the relationship was linear at this concentration.

Assays for nitrogenase (acetylene reduction) were performed on 5 ml aliquots of cultures in 15 ml reaction flasks sealed by rubber septa and evacuated and filled with argon/10% acetylene. The vessels were incubated under illumination (30 W/m^2) on a rotary shaker and samples of the gas phase were removed for determination of ethylene [13]. The molar amount of N-fixed was calculated as equivalent to 2/3 of the ethylene formed. Ammonia in aliquots of culture supernatant liquids wa determined by the micro hypochlorite-nitroprusside method of Ternberg and Hershey [14].

For determination of intracellular pool ammonia, cells were removed by centrifugation, washed with water and the cell pellets, generally containing about 1.5 mg cell protein, suspended in a 0.5 ml water and boiled for 2–3 min. Cold citrate buffer (0.5 ml) was then added and the samples were centrifuged. Ammoia in the extracts was determined using an automatic amino acid analyzer.

Methionine sulfoximine was obtained from Sigma (St. Louis, U.S.A.), and Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), and DCMU from DuPont (Wilmington, DE, U.S.A.).

Results

Effect of anaerobiosis on nitrogenase activity

Fig. 1A illustrates the effect of incubation under N_2 on acetylene reduction activity of *Anabaena* sp. strain 2B grown autotrophically in light. There is a rapid loss of activity in light and dark. However, addition of $1 \cdot 10^{-4}$ M methionine sulfoximine at the onset of anaerobic incubation in light completely prevents this decline of nitrogenase. It should be noted that light is required to maintain acetylene reduction of cells incubated anaerobically in the presence of methionine sulfoximine.

When this cyanobacterium is grown heterotrophically on fructose in the dark, the specific activity of acetylene reduction rises several-fold [10]. These cells also lose acetylene reduction upon anaerobiosis, as illustrated in Fig. 1B, but the decline of acetylene reduction activity in heterotrophically grown cells takes place only under anaerobic conditions in the dark; cells in light maintain activity, even in the absence of

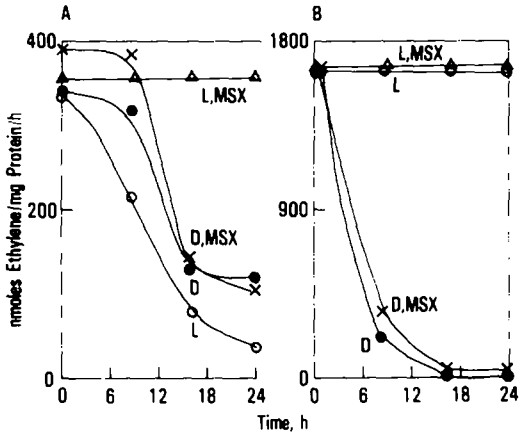

Fig. 1. Comparison of effect of anaerobiosis (N_2) and methionine sulfoximine (MSX) ($1 \cdot 10^{-4}$ M) treatment on acetylene reduction in autotrophically and heterotrophically grown cyanobacteria. (A) Autotrophs incubated in dark (●———●) and light (○———○) and in the presence of methionine sulfoximine in dark (×———×) and light (△———△). (B) Heterotrophs in dark (●———●) and light (○———○) and with methionine sulfoximine in dark (×———×) and light (△———△).

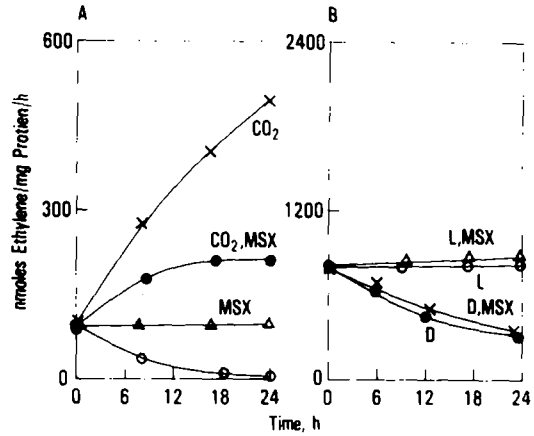

Fig. 2. Effect of carbon source addition on acetylene reduction in autotrophically grown cyanobacteria incubated anaerobically under N_2 in light. (A) CO_2, 1.0% added to gas phase at zero time (×———×); CO_2 plus $1 \cdot 10^{-4}$ M methionine sulfoximine (MSX) (●———●); methionine sulfoximine added (△———△) and no addition (○———○). (B) Autotrophically grown cells preincubated with 0.05 M fructose 24 h, washed, resuspended in N-free BG-11 medium and incubated under N_2, in dark (●———●); with $1 \cdot 10^{-4}$ M methionine sulfoximine in dark (×———×)) in light (○———○) and with methionine sulfoximine in light (△———△).

TABLE I

COMPARISON OF N FIXED WITH INTERNAL AND EXTERNAL AMMONIA DURING INCUBATION OF AUTOTROPHIC CYANOBACTERIA WITH METHIONINE SULFOXIMINE

Autotrophically grown cultures contained 0.2 mg cell protein/ml; $1 \cdot 10^{-4}$ M methionine sulfoximine. Sum of acetylene reduction with time using the relationship; μmol N fixed = 2/3 ethylene.

Incubation conditions	Time (h)	N fixed (calculated) (μmol/ml)	Ammonia	
			medium (μmol/ml)	cells (μmol per mg protein)
Aerobic, dark	8	0.021	0.000	0.044
	16	0.021	0.000	0.042
	24	0.021	0.010	0.039
Aerobic, light	8	0.245	0.130	0.035
	16	0.490	0.280	0.063
	24	0.735	0.360	0.070
N_2 dark	8	0.140	0.000	0.028
	16	0.236	0.000	0.056
	24	0.278	0.010	0.049
N_2 light	8	0.300	0.140	0.067
	16	0.600	0.280	0.063
	24	0.900	0.380	0.045

methionine sulfoximine. Further, methionine sulfoximine did not eliminate the decline of nitrogenase in either autotrophs or hetrotrophs incubated anaerobically in the dark.

These data suggested that N_2 incubation depleted endogenous reductants in the cyanobacteria and that methionine sulfoximine can maintain the levels of these metabolites by preventing carbon incorporation into cell material. Consequently, autotrophically grown cells were incubated with added CO_2 [15] or preincubated with fructose and the subsequent effect of anaerobiosis on acetylene reduction was examined. These results are illustrated in Fig. 2. Both treatments increased acetylene reduction activity of the cells, and N_2 incubation did not reduce activity below the enhanced levels obtained by these treatments; the cells responded as heterotrophs (Fig. 2B). The data, therefore, support the view that incubation under N_2, which would deprive the cells of both CO_2 and oxygen, was depleting endogenous reductants for nitrogenase, a condition prevented by MSX in autotrophically grown cells. Presumably, heterotrophically grown cells have a large enough supply of carbon to maintain nitrogenase activity

for a substantial period of time in the absence of methionine sulfoximine. It should be noted, however, that with both cell types light was required for maintenance of nitrogenase activity.

Internal and external ammonia formation

The mechanism of inhibition of nitrogenase under N_2 could entail either a depletion of endogenous reserves necessary for reductant supply to the enzyme, or alternatively an increase of inhibitory compoundl(s) which are released by cells treated with methionine sulfoximine. To examine these alternate hypotheses, the level of internal and extracellular ammonia was examined under various conditions of nitrogenase inhibition and release by methionine sulfoximine and light. Data in Table I are results of assays of autotrophically grown cells for N fixed calculated from acetylene reduction assays, medium ammonia and intracellular ammonia under aerobic and anaerobic conditions in the presence of methionine sulfoximine. These data show that maximal nitrogenase activity and ammonia liberation require light under both aerobic and anaerobic conditions, and that the levels of intracellular ammonia were not significantly altered by treatment with methionine sulfoximine under these experimental conditions. These results also confirm the findings of Stewart and Rowell [2] that as much as one half of the nitrogen fixed by autotrophic *Anabaena* sp. treated with methionine sulfoximine in light is released as ammonia.

Data obtained thus far indicated that light was required for both maintenance of nitrogenase activity and liberation of ammonia anaerobically in the presence of methionine sulfoximine. Because these cells would normally evolve oxygen upon illumination, DCMU, an inhibitor of Photosystem II, was used to eliminate oxygen production during the anaerobic incubation of cyanobacteria in the light. For these experiments heterotrophically grown cells were used, and the levels of nitrogenase, internal pool ammonia and medium ammonia were determined. As indicated in Table II, heterotrophically grown cells showed patterns of activity very different from those of the autotrophs. These cells show initial acetylene reduction rates much higher than autotrophically grown cyanobacateria, and also release ammonia in the light when methionine sulfoximine is added. However, this ammonia liberation is accompanied by increased intracellular ammonia which is diminished by DCMU. Simultaneously, DCMU enhanced ammonia release. These findings appear to rule out oxygen as essential for ammonia formation. It should be emphasized that although more total ammonia is

TABLE II

LEVELS OF INTERNAL AND EXTERNAL AMMONIA IN HETEROTROPHIC CYANOBACTERIA TREATED WITH METHIONINE SULFOXIMINE AND DCMU UNDER N_2

Cultures grown aerobically in the dark on 0.05 M fructose contained 0.15 mg cell protein/ml; methionine sulfoximine $1 \cdot 10^{-4}$ M, DCMU when added, $1 \cdot 10^{-6}$ M. Sum of acetylene reduction with time using the relationship; μmol N fixed = 2/3 ethylene.

Incubation conditions	Time (h)	N fixed (calculated) (μmol/ml)	Ammonia	
			medium (μmol/ml)	cells (μmol/mg protein)
Dark, methionine sulfoximine	8	4.48	0.000	0.057
	16	7.18	0.000	0.057
	24	7.68	0.000	0.049
Light, methionine sulfoximine	8	4.95	0.010	0.054
	16	9.90	0.500	0.108
	24	14.8	0.900	0.308
Light, methionine sulfoximine, DCMU	8	4.50	1.00	0.065
	16	9.50	1.40	0.066
	24	14.4	2.00	0.103

TABLE III

LEVELS OF INTERNAL AND EXTERNAL AMMONIA IN HETEROTROPHIC CYNAOBACTERIA TREATED WITH METHIONINE SULFOXIMINE AND DCMU AEROBICALLY

Culture grown aerobically in dark, 0.05 M fructose to 0.15 mg per protein per ml; methionine sulfoximine added to $1 \cdot 10^{-4}$ M, when added DCMU concentration was $1 \cdot 10^{-6}$ M. Sum of acetyene reduction with time using the relationship; μmol N fixed = 2/3 ethylene

Incubation conditions	Time (h)	N fixed (calculated) (μmol/ml)	Ammonia	
			medium (μmol/ml)	cells (μmol/mg protein)
Dark, methionine sulfoximine	8	4.00	0.100	0.040
	16	7.44	0.400	0.035
	24	9.00	0.680	0.035
Light, methionine sulfoximine	8	4.00	0.800	0.056
	16	8.00	1.00	0.051
	24	11.20	3.00	0.175
Dark, methionine sulfoximine, DCMU	8	3.40	0.120	0.035
	16	5.85	0.650	0.084
	24	7.88	0.600	0.091
Light, methionine sulfoximine, DCMU	8	3.52	0.900	0.045
	16	5.87	1.05	0.056
	24	9.47	1.10	0.070

liberated by the heterotrophs, the percentage of N fixed which appears as ammonia is much lower than with autotrophs, because of the relatively high specific activity of acetylene reduction in the heterotrophs. In addition, DCMU had no effect on nitrogenase activity, which indicates that the major source of reductant for N fixation was being supplied by the reductant pool through Photosystem I under these conditions. Consequently, DCMU enhancement of ammonia production was not caused by an increase in nitrogenase, but was the result of a change in the ratio of ammonia formed to acetylene reduction activity.

Since strain 2B can also be grown on fructose aerobically in the dark, it was of interest to compare its responses to methionine sulfoximine and DCMU under these conditions, where an oxidative metbolism replaces light for growth. These results are given in Table III. Under these conditions light also enhanced both acetylene reduction and ammonia liberation, and was accompanied by a smaller rise in internal ammonia, diminished by DCMU. DCMU also reduced both the levels of nitrogenase and external ammonia, indicating that reductant for N fixation was being provided by both photosystems in the heterotrophs incubated aerobically in light.

Discussion

In the cyanobacteria, liberation of ammonia appears to require either oxidative metabolism or light. With autotrophic cells using Photosystem II as principal reductant, the effects of methionine sulfoximine treatment seem straightforward and require little further discussion. However, heterotrophically grown cells, which have alternate choices of energy generation for nitrogenase and ammonia liberation, present an opportunity to isolate these processes for study. These cells can be grown photosynthetically in light, oxidatively in dark or by a combination of pathways aerobically in light. Thus, heterotrophs can fix nitrogen and liberate ammonia under growth conditions aerobically in the dark or light, or as resting cells under N_2 in light. Under an N_2 atmosphere, ammonia formation requires light and is enhanced by DCMU; under growth conditions in the dark with oxygen present DCMU does not increase ammonia liberation.

DCMU has been used extensively as an inhibitor of electron transfer from Photosystem II in plants [17] and in studies of growth and nitrogenase synthesis in cyanobacteria [18,19,20]. Since DCMU can increase ammonia liberation by cells in the absence of any measurable effect on acetylene reduction activity, an additional role for DCMU more directly in ammonia liberation is indicated. The data, which show enhanced ammonia liberation, with a lowering of the intracellular pool of ammonia indicate that the inhibitor is allowing more release of newly fixed nitrogen by the cell. That exogenous ammonia can equilibrate readily with recently fixed nitrogen in nitrogen fixing bacteria was demonstrated in early studies using $^{15}N_2$ [21]. Ammonia is a potent inhibitor of nitrogen fixation in all systems studied, though whether it may function as an intracellular regulatory molecule has been open to question [22–24]. Our data also support the earlier findings of Stewart and Rowell [2] which show that a substantial rise in intracellular ammonia can accompany high nitrogenase activity in cyanobacteria treated with methionine sulfoximine. However, this pool can be depleted by the presence of DCMU leading to enhanced liberation of ammonia.

Ammonia as the unprotonated base can traverse plant cell membranes [25], uncouple photophosphorylation [26,27] and inhibit the oxygen-evolving apparatus [28,29] on the oxidizing side of Photosystem II. The close chemical similarity between water and ammonia suggests that they may compete with each other at this site [30], and affords a mechanism for enhanced ammonia liberation by DCMU in the cyanobacteria.

Crofts [31] studied uptake of ammonium ions by illuminated chloroplasts and postulated a mechanism whereby unprotonated ammonia equilibrated across the membrane and eliminated the light-generated proton gradient. Inhibition of oxygen evolution by ammonia has characteristics similar to those of uncoupling of photophosphorylation [27]. A variety of evidence has supported a close relationship between Photosystem II, ammonia and ion transport in photosynthetic systems. Our data, which indicate that in cyanobacteria a higher percentage of ammonia is formed by nitrogenase in the presence of DCMU than in its absence, indicate that the efficiency of ammonia production from N_2, as well as its liberation is enhanced by DCMU. This suggests that DCMU may also facilitate anaerobic electron flow for nitrogen reduction in the cyanobacteria, resulting in an alteration of ratio of acetylene reduction activity to ammonia formed. Laane et al. [32] have provided evidence for the hypothesis that electron transfer to nitrogenase is regulated by the electrical component of the proton-motive force generated in nitrogen fixing cells, and that uptake of ammonium lowers this membrane potential with consequent switching off of the flow of reducing equivalents to nitrogenase. A relationship between membrane potential and nitrogenase was also observed by Hawkesford et al. [33] in *Anabaena variabilis*, and supported by other observations [34]. Our studies, which indicate that inhibition of Photosystem II can enhance liberation of ammonia in the cyanobacteria, lend further support for the general hypothesis of membrane involvement in ammonia formation by nitrogenase.

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