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Involvement of Photosystem II in the ammonia metabolism of a heterotrophic cyanobacterium

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Herbicides that bind specifically to Photosystem II greatly increased ammonia liberation by a heterotrophic cyanobacterium incubated with methionine sulfoximine anaerobically in light. Comparison of cells incubated under argon and nitrogen indicated that about one-half of the liberated ammonia came from endogenous sources, as well as from dinitrogen fixation. Chromatography of cell extracts revealed a light-induced, general breakdown of cellular proteins in the presence of methionine sulfoximide. Cultures grown on ammonia, and hence free of heterocysts and nitrogenase, liberated ammonia in the dark in nitrogen-free media with methionine sulfoximine and this liberation was inhibited separately by herbicides or light. A combination of light and herbicide, however, also enhanced ammonia liberation by these cells. Herbicidal Photosystem II inhibitors strongly inhibited light-induced assimilation of the ammonia analog, [^{14}C]methylamine, by cyanobacteria. These results implicate Photosystem II directly in the ammonia metabolism of this cyanobacterium and suggest that herbicide-binding protein(s) of this system may regulate nitrogen assimilation coordinately with electron transport.

Introduction

The accumulation of ammonia by photosynthetic cells has been studied in both plants and green algae. When assimilation is interrupted by glutamine synthetase inhibitors, ammonia can be formed by photorespiration [1–3], degradation of intracellular proteins [4–6], or from added nitrate [7,8]. There is evidence for an interplay between these processes [6] that is dependent upon experimental conditions, particularly CO_2 and O_2 concentrations. In plants, ammonia formation requires light. In the cyanobacteria (blue-green algae), an additional source of ammonia is available from dinitrogen fixation occurring in the heterocyst [26], followed by transfer of this fixed nitrogen to vegetative cells of the cyanobacterial chains [27]. Furthermore, a close relationship between regulation of dinitrogen fixation and of ammonia uptake and assimilation has been suggested from genetic and biochemical studies of photosynthetic bacteria [24], cyanobacteria [25] and *Azotobacter* [31].

Cyanobacteria that are facultatively hetero-

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; atrazine, 2-chloro-4-ethylamino-6-isopropylamine-*S*-triazine; propanil, 3′,4′-dichloropropionanilide; dinoseb, 2,4-dinitro-6-*sec*-butylphenol; Mes, 4-morpholineethanesulfonic acid.

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trophic have alternative sources of reductant for growth. When such cyanobacteria are grown photoautotrophically, Photosystem II serves a principal electron donor; heterotrophically in light or dark, carbon sources can supply reductant to Photosystem I [16–18]. Earlier, it was shown that a heterotrophically grown cyanobacterium isolated from the fern *Azolla* possessed relatively high nitrogenase activity, and liberated ammonia into the medium when the glutamine synthetase inhibitor, methionine sulfoximine, was present [28]. Inhibition of Photosystem II, however, enhanced ammonia liberation by these cells without affecting nitrogen fixation (acetylene reduction) activity. The ratio of nitrogen fixed to ammonia released was changed by Photosystem II inhibition, and the internal pool of ammonia was depleted. These findings implicated Photosystem II in ammonia metabolism of this cyanobacterium, apart from its role as an electron donor to nitrogenase. The present experiments were designed to explore this phenomenon further. A variety of Photosystem II inhibitors, commonly used as herbicides, were found to enhance ammonia liberation and inhibit its assimilation, regardless of the origin of the ammonia available to the cell. Hence, these data further implicate Photosystem II in ammonia assimilation and/or retention by this organism, and suggest an action on nitrogen metabolism for herbicides that bind specifically to Photosystem II, as a consequence of their inhibition of electron transfer at this site.

Materials and Methods

Anabaena sp., a facultatively heterotrophic strain isolated from *Azolla caroliniana* [29], has been deposited as NRRL B-4374 in the ARS Culture Collection, Northern Regional Research Center. Cultures were grown as previously described [28] on BG-11 medium [30] without nitrate. For heterotrophic growth, 0.05 M fructose was added; ammonia-grown cultures contained 0.02 M ammonium chloride and 0.10 M Mes at pH 6.7. Cell growth was followed spectrophotometrically at 650 nm, and protein content was determined as described previously [9,28]. Acetylene reduction [10] and ammonia [11] were also determined by earlier methods.

[^{14}C]Methylamine assimilation was determined directly on samples of cultures to which $1 \cdot 10^{-4}$ M [^{14}C]methylamine ($2 \cdot 10^{6}$ cpm/μmol) was added. For incubation, 15 ml reaction flasks sealed with rubber septa were used, and 1.0 ml aliquots of cells were collected and washed on PHWP Millipore filters. The filters were counted directly with a liquid scintillation system.

Reverse-phase high-performance liquid chromatography (HPLC) was used to examine the total protein profile of the cyanobacterium. Culture samples (5.0 ml) were passed three times through a French pressure cell and centrifuged to remove cell debris, and the extracts were chromatographed directly on a Synchropak RP-P column, essentially as described by Bietz [12]. Solvent A, 15% acetonitrile/0.1% trifluoroacetic acid, and solvent B, 80% acetonitrile/0.1% trifluoroacetic acid, were used in a linear gradient from 10–67% solvent B in 70 min. The size of the sample chromatographed was selected to give a full-scale deflection among the major protein peaks, which would correspond to approx. 50 μg total protein added to the column, and to reveal a large enough number of proteins to serve as a typical profile for the cyanobacterial cells used.

Methionine sulfoximine was obtained from Sigma (St. Louis, MO, U.S.A.) and Diuron (DCMU) from DuPont (Wilmington, DE, U.S.A.). Atrazine, propanil and dinoseb were obtained from Chem Service Inc. (West Chester, PA, U.S.A.). [^{14}C]Methylamine was a product of Research Products International (Mount Prospect, IL, U.S.A.).

Results

Liberation of ammonia by cyanobacterium B4374

To assess the contribution of recently fixed nitrogen to ammonia liberated by the heterotrophically grown cyanobacterium treated with methionine sulfoximine, a comparison was made of ammonia released by cells incubated under atmospheres of argon and N_2. Data in Table I show that approximately half as much ammonia was released from cells in which the substrate, N_2, was absent as was released by nitrogen-fixing cyanobacteria. DCMU stimulated ammonia liberation under both conditions. Nitrogenase activity,

TABLE I

AMMONIA LIBERATION BY HETEROTROPHIC CYANOBACTERIA INCUBATED IN LIGHT WITH METHIONINE SULFOXIMINE UNDER ARGON AND NITROGEN ATMOSPHERES

Cultures grown aerobically in light on 0.05 M fructose, 48 h, to a density of 0.24 mg protein/ml. Methionine sulfoximine, $1 \cdot 10^{-4}$ M, DCMU, $1 \cdot 10^{-5}$ M when added. N fixation calculated from sum of acetylene reduction with time using mol N fixed = 2/3 mol ethylene reduced.

Incubation conditions	Time (h)	N fixation (calculated) (μmol/mg protein)	Ammonia in medium (μmol/mg protein)
Argon	8	16.0	0.00
	16	36.8	1.86
	24	48.8	2.67
Argon, DCMU	8	14.4	0.48
	16	33.6	4.54
	24	55.2	6.67
N_2	8	15.2	0.56
	16	34.0	2.55
	24	48.0	5.47
N_2, DCMU	8	15.0	2.08
	16	30.0	12.4
	24	52.8	15.8

TABLE II

EFFECTS OF PHOTOSYSTEM II INHIBITORS ON LIBERATION OF AMMONIA BY CYANOBACTERIA INCUBATED WITH METHIONINE SULFOXIMINE IN AN ATMOSPHERE OF ARGON

Cultures grown aerobically in light on 0.05 M fructose, 48 h, in N-free BG-11 medium to cell densities of 0.25 mg protein/ml. Methionine sulfoximine, $1 \cdot 10^{-4}$ M; other inhibitors, $1 \cdot 10^{-5}$ M when added.

Incubation conditions	Ammonia in medium (μmol/mg protein)		
Time (h):	8	16	24
Dark	0.06	0.02	0.12
Light	0.12	2.57	2.58
Dark, DCMU	0.12	0.00	0.16
Light, DCMU	0.57	4.34	5.23
Dark	0.08	0.16	0.16
Light	0.23	2.25	2.63
Dark, propanil	0.08	0.08	0.23
Light, propanil	0.70	4.55	6.45
Dark	0.00	0.00	0.05
Light	0.38	1.37	1.97
Dark, atrazine	0.03	0.04	0.04
Light, atrazine	0.81	2.34	4.68
Dark	0.08	0.16	0.16
Light	0.12	2.47	2.82
Dark, dinoseb	0.14	0.10	0.16
Light, dinoseb	1.14	3.75	5.25

assayed as acetylene reduction, remained the same in all cultures. Thus, these data show that a substantial fraction of the ammonia appearing in the medium was derived from endogenous sources, and this liberation was enhanced by DCMU. The ammonia liberated under argon constitutes a substantial amount of the total cellular nitrogen estimated in the culture; of 12 μmol total nitrogen estimated from 1 mg cell protein, 6.67 μmol or approx. 50% of the total cellular nitrogen was released in 24 h, suggesting extensive cellular breakdown under these conditions.

Phenylurea, triazine, acylanilide and phenolic types of Photosystem II inhibitor with herbicide activity were examined for their ability to stimulate ammonia liberation from endogenous sources by the cyanobacteria. These compounds, when titrated, were found to be effective at concentrations of $1 \cdot 10^{-5}$ M or lower, which inhibit oxygen evolution. Data in Table II show that all compounds tested enhanced liberation, whether classified as inhibitors (DCMU, atrazine) or inhibitory uncouplers (Propanil, Dinoseb). The ammount of ammonia liberated in the absence of Photosystem II inhibition generally reached a plateau at about 16 h when cells were incubated under argon atmospheres, suggesting a limit of intracellular degradation to about 20% of the total nitrogen; addition of Photosystem II inhibitors doubled the amount of ammonia released in this manner.

Cells grown on ammonia

Generally, when cyanobacteria are grown on media containing ammonia, nitrogen fixation ceases, and a population of cells devoid of heterocysts is obtained. In contrast to cultures containing heterocysts, when incubated with methionine sulfoximine, strain B4374, liberated ammonia in the dark, and this liberation was inhibited by light

TABLE III

LIBERATION OF AMMONIA BY HETEROTROPHIC CYANOBACTERIA GROWN ON AMMONIA AND INCUBATED IN A NITROGEN-FREE MEDIUM CONTAINING METHIONINE SULFOXIMINE

Cells were grown aerobically in light in BG-11 medium containing 0.05 M fructose and 0.02 M NH_4Cl to a concentration of 0.30 mg protein/ml, washed, and then incubated in N-free BG-11 medium containing 0.05 M fructose and $1 \cdot 10^{-4}$ M methionine sulfoximine. Atmosphere: argon. Concentration of herbicides, when added, $1 \cdot 10^{-5}$ M.

Incubation conditions	Time (h):	Ammonia in medium (μmol/mg protein) 8	16	24
Dark		0.00	1.08	1.61
Light		0.00	0.09	0.17
Dark, DCMU		0.02	0.09	0.21
Light, DCMU		0.67	1.37	2.00
Dark, propanil		0.20	0.08	0.19
Light, propanil		0.80	1.59	2.85

or herbicides. In the light, however, herbicides again enhanced ammonia liberation by these cells. These results suggest that, in contrast with nitrogen-fixing cells, the ammonia released in the dark was liberated passively from an intracellular pool. The data indicate that DCMU can affect ammonia metabolism in cells devoid of nitrogenase; they also reveal a unique effect of DCMU on the dark metabolism of the cyanobacteria (Table III).

Assimilation of [^{14}C]methylamine

Since all Photosystem II inhibitors enhanced light-induced liberation of ammonia by the heterotrophically grown cyanobacterium, these compounds were examined for their action on assimilation of the ammonia analog [^{14}C]methylamine. Fig. 1A illustrates that DCMU and atrazine at low concentrations both strongly inhibited light-induced [^{14}C]methylamine assimilation under conditions identical to those used to study ammonia liberation. These compounds were more effective in preventing ammonia assimilation than methionine sulfoximine (Fig. 1B), which has been demonstrated to inhibit ammonia assimilation in other systems [13,32]. Fig. 2 depicts a similar effect of propanil and dinoseb on [^{14}C]methylamine assimilation. Hence, all types of Photosystem II inhibi-

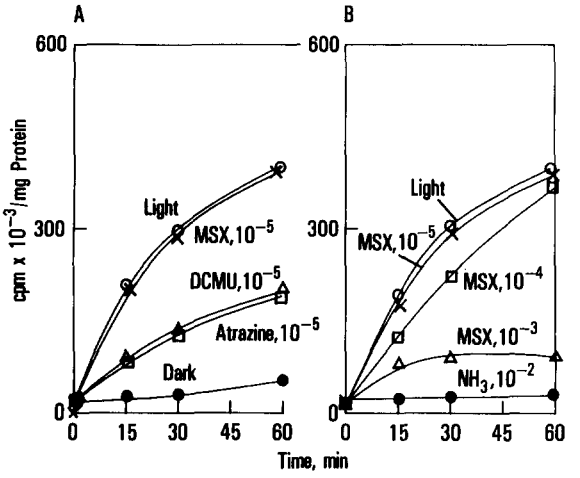

Fig. 1. Assimilation of [^{14}C]methylamine by heterotrophic cyanobacteria. Cultures contained $1 \cdot 10^{-4}$ M [^{14}C]methylamine ($2 \cdot 10^6$ cpm/μmol) and 0.5 mg cell protein/ml; atmosphere, N_2. (A) Effects of Photosystem II inhibitors. Cultures incubated in light (○); dark (●); with methionine sulfoximine (MSX), $1 \cdot 10^{-5}$ M (×); DCMU, $1 \cdot 10^{-5}$ M (△); atrazine, $1 \cdot 10^{-5}$ M (□). (B) Effect of methionine sulfoximine on uptake. Cultures incubated under N_2 in light. Control, no addition (○); with NH_4Cl, $1 \cdot 10^{-2}$ M (●); methionine sulfoximine, $1 \cdot 10^{-3}$ M (△); methionine sulfoximine, $1 \cdot 10^{-4}$ M (□); methionine sulfoximine, $1 \cdot 10^{-5}$ M (×). Samples were recovered and washed on Millipore filters for counting.

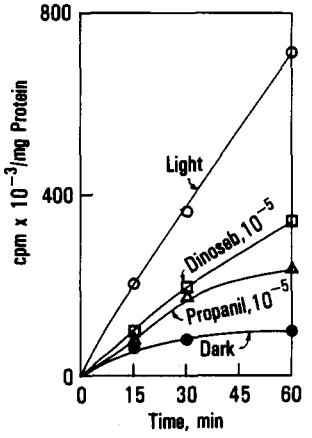

Fig. 2. Assimilation of [^{14}C]methylamine by heterotrophic cyanobacteria; effect of Photosystem II inhibitors. Cultures contained $1 \cdot 10^{-4}$ M [^{14}C]methylamine ($2 \cdot 10^6$ cpm/μmol) and 0.20 mg cell protein/ml. Atmosphere: argon. Dinoseb and propanil added at zero time, $1 \cdot 10^{-5}$ M. Samples (1.0 ml) recovered and washed on filters for counting at times indicated.

tor studied were potent inhibitors of ammonia assimilation, a finding consistent with their enhancement of ammonia liberation in light.

Origin(s) of the liberated ammonia

The large amount of ammonia liberated by this cyanobacterium incubated with methionine sulfoximine and DCMU in light seemed to rule out any specific intracellular source of nitrogen in this process. Cyanophycin [41,42], a nitrogen storage polypeptide, and phycobiliproteins [44] of these organisms have been shown to have specific proteinases responsible for their degradation, but these sources are not abundant enough in the heterotrophic cyanobacteria to account for the amounts of ammonia released. Turnover of intracellular proteins of cyanobacteria has been observed [43] and is induced by methionine sulfoximine. Consequently, the protein profiles of the cyanobacterium were examined by high-performance liquid chromatography of extracts from cells incubated under argon in light and dark with methionine sulfoximine and DCMU. These data, given in Fig. 3, show a general reduction in protein content of the cells incubated in light with methionine sulfoximine under argon (Fig. 3B) but no degradation in the dark with or without the inhibitor (Fig. 3C,D). Some growth occurred in the light in the absence of methionine sulfoximine during the experimental period (Fig. 3E). In gen-

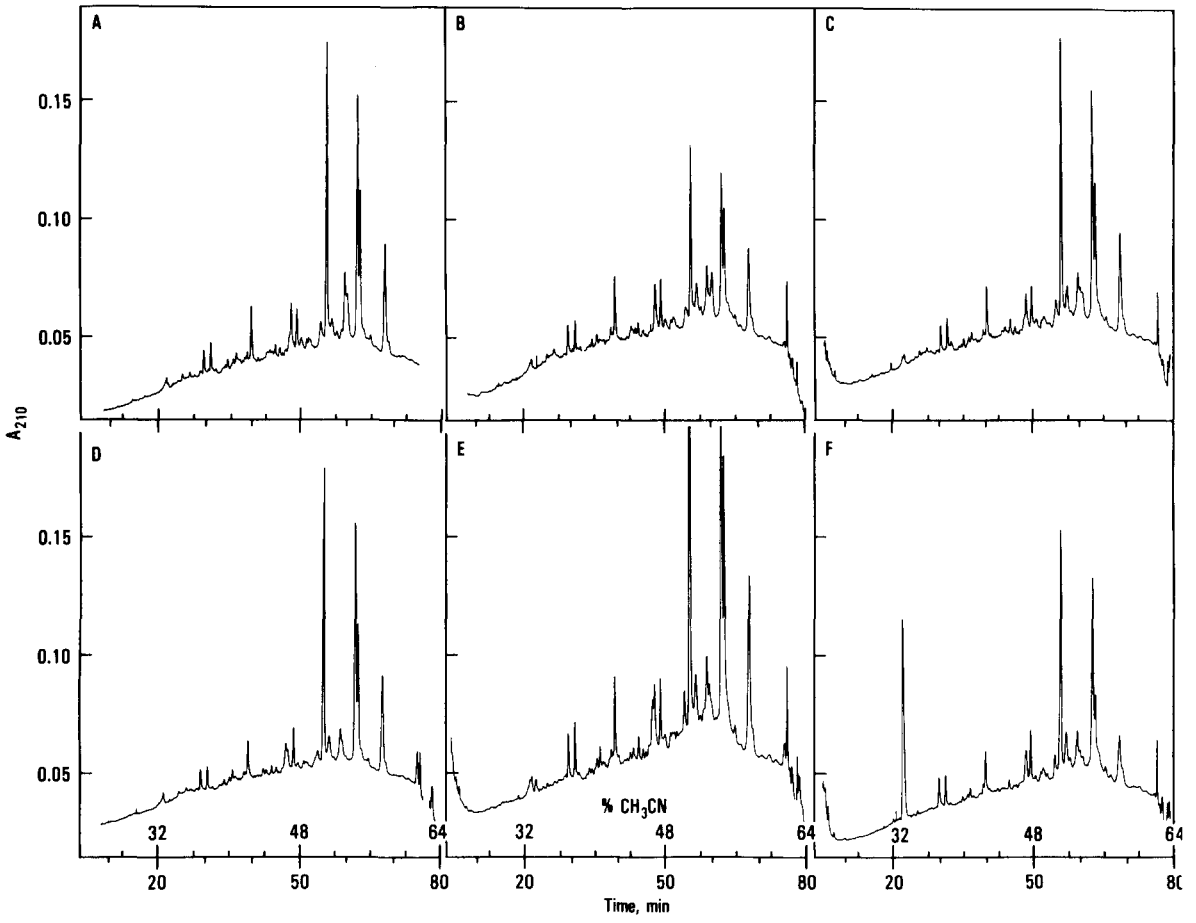

Fig. 3. Profile of algal proteins on HPLC column. Cultures contained 0.220 mg protein/ml at zero time and were incubated under argon 24 h; 0.15 ml extracts applied to column. (A) Initial culture; (B) incubated in light, $1 \cdot 10^{-5}$ M methionine sulfoximine; (C) dark incubation, $1 \cdot 10^{-4}$ M methionine sulfoximine; (D) dark incubation; (E) light incubation; (F) light, $1 \cdot 10^{-4}$ M methionine sulfoximine, $1 \cdot 10^{-5}$ M DCMU.

eral, DCMU had only slight effects on protein breakdown with methionine sulfoximine in light (Fig. 3F), which supports the idea that Photosystem II is involved in the metabolism of ammonia primarily after its release from intracellular sources. Endogenous ammonia production by cyanobacteria was observed by Bousseba et al. [22,23], who also suggested that the ammonia was derived from protein degradation. These investigators, studying autotrophic cells, noted that the breakdown of cellular constituents was energy dependent. Our data provide direct evidence for extensive protein catabolism and further suggest that Photosystem I can serve as energy donor for this breakdown.

Discussion

The binding of Photosystem II herbicides by strain B4374 treated with methionine sulfoximine causes massive ammonia liberation by the cells in the light, regardless of the source of the intracellular ammonia. Since these compounds are simultaneously very effective inhibitors of light-induced ammonia assimilation, their effect appears to be directional with regard to ammonia movement across the cyanobacterial membrane. Liberation of ammonia by this photoheterotrophic cyanobacterium differs experimentally from those photosynthetic systems previously studied, because a functional Photosystem II is not needed for growth [16,17]. Consequently, the effect of specific inhibition of Photosystem II could be examined in cells still capable of carrying out a full complement of light-activated metabolic processes. These circumstances permitted study of the relationship between activity of Photosystem II and ammonia metabolism in this organism, and seems to implicate this system specifically in ammonia uptake and/or retention by the cells. Alternatively, Photosystem II inhibition could also reduce the carbon supply for ammonia assimilation and disrupt the C : N balance, leading to ammonia liberation via a more generalized mechanism.

Several energy-dependent ammonium uptake [32] and liberation [23,28] systems have been described in cyanobacteria, and have been differentiated kinetically into transport and sustained uptake systems, the latter of which are inhibited by methionine sulfoximine. However, as with plant systems, these previous studies involved use of autotrophic cells in which inhibition of Photosystem II would be expected to eliminate all light-driven processes, and hence a specific role for this system could not be established. It is not clear what role light plays in ammonia liberation. In theory, ammonia formed intracellularly could leave the cells by passive diffusion [14]; it may accompany proton efflux from the cells [39,40] in a manner analogous to ammonia uptake by chloroplasts in light [15]. Ammonia release in the dark from ammonia-grown cells was inhibited by herbicides, but enhanced by illumination in their presence (Table III). Light was also required for breakdown of intracellular proteins (Fig. 3) and therefore may be required for intracellular ammonia formation. The data indicate, however, that binding of Photosystem II herbicides in this cyanobacterium locks the system into a state that permits ammonia liberation only upon illumination. This finding would be the converse of ammonia inhibition of the oxygen-evolving apparatus [19,20] and further suggests competition of water and ammonia at this site [21].

Many recent biochemical and genetic studies have identified proteinaceous constituents of the photosynthetic membrane system associated with electron transfer and herbicide binding [33–35]. The 32 kDa protein, in particular, coded by the psbA gene [37,38], serves as an essential constituent of Photosystem II, termed Qb, which accepts electrons in a bound quinone and specifically binds herbicides. This protein also turns over rapidly in light in photosynthetic cells [36], and may play a regulatory role in plant metabolism through its control of electron flow. The present work also implicates Photosystem II in ammonia metabolism and suggests that it may coordinate carbon and nitrogen flow in photosynthetic cells by serving as a gate for ammonia entrance into the metabolism of the cyanobacterium. It follows that nitrogen starvation may be an important immediate consequence of herbicide action.

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