Sustained Photoproduction of Ammonia by the Nitrogen-fixing Blue-green Alga *Nodularia harveyana* (Thw.) Thuret

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(Received 21 May 1986: after revision 29 September 1986)

The addition of the glutamate analogue L-methionine-DL-sulphoximine (MSX) to illuminated nitrogen-fixing cultures of a local isolate of *Nodularia harveyana* caused a rapid inactivation of cellular glutamine synthetase, prevention of cell growth and release of ammonia into the medium. With optimal initial concentration of MSX (4 nmol/μg chl-a), the ammonia production lasted for about 28 hr, but its effectiveness was lost thereafter. The maximum rate of ammonia production (5.5 μmol/mg chl-a/hr) was reached about 12 hr after the addition of MSX and did not decrease over the next 16 hr period. When MSX was added every 16 hr, the maximum rate of ammonia production was sustained for about 90 hr. After this time, the production of ammonia slowed down till it ceased. The process could not be reinitiated by addition of MSX. The effective period of ammonia production could be further extended by intercalating 6 hr recovery periods in the absence of MSX after every 30 hr period of production in the presence of the inhibitor. With this procedure, a steady generation of ammonia lasting for 10 days with a net production of 11 mM ammonia has been attained.

Key Words: *Nodularia harveyana*, L-methionine-DL-sulphoximine, Glutamine synthetase, Ammonia

Introduction

The light-dependent reduction of molecular nitrogen with the consequent generation of ammonia by photosynthetic diazotrophs is a process of great interest keeping in view the high cost of energy inputs required for chemical dinitrogen fixation. Nitrogen-fixing blue-green algae are unique in that they can synthesize ammonia from atmospheric nitrogen and water at the expense of light energy. In these organisms the metabolism of ammonia is regulated primarily by the glutamine synthetase (GS)—glutamate synthase (GOGAT) enzymatic pathway and GS is the first and the most active enzyme involved in ammonia assimilation (Dharmawardene et al. 1972, Haystead et al. 1973, Wolk et al. 1976). In the presence of glutamate analogues, such as L-methionine-DL-sulphoximine (MSX), hydroxyllysine (HYL) or phosphinotricin (PT) which inhibit GS, dinitrogen fixation proceeds freely whereas the key aminating pathway remains blocked and, consequently, ammonia is liberated extracellularly (Stewart & Rowell 1975, Ladha et al. 1978, Lea et al. 1984). An efficient and continued production of ammonia by MSX- and PT-treated filaments of a marine strain of *Anabaena* has been reported (Ramos et al. 1984).

This communication reports the achievement of sustained photoproduction of ammonia by a local isolate of the nitrogen-fixing blue-green alga *Nodularia harveyana*.

Materials and Methods

*N. harveyana* was isolated from the surface of moist garden soil and grown axenically in BG-11, combined nitrogen-free medium (Rippka et al. 1979) under a continuous light intensity of approximately 3000 lux at 26°C. The culture medium was buffered with 5 mM N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), pH 7.6.

For ammonia production experiments, filaments from 4-day-old exponentially growing cultures, containing about 4–5 μg of chl-a per ml, were used. The filaments...
were harvested by centrifugation, washed with culture medium, and finally resuspended in the same medium to a cell density equivalent to 6.5–8 \mu g of chl a per ml. The cultures were then supplemented with 3.8–4 nmoles of MSX per \mu g of chl-a. All experiments were run in triplicate series and repeated twice.

Ammonia was determined by the phenol-hypochlorite method (Solorzano 1969). Chlorophyll-a was measured spectrophotometrically after extraction with methanol, employing the extinction coefficient given by MacKinney (1941).

In situ activity of GS was determined in toluene-treated filaments using transferase assay (Shapiro & Stadtman 1970). GS (transferase) activity units correspond to \mu mol \gamma-glutamyl hydroxamate formed per min. MSX, supplied by Sigma Chemical Co., USA, was freshly prepared in distilled water as required and sterilized by filtration.

**Results**

The addition of 26 \mu M MSX to illuminated nitrogen-fixing cultures of *N. harveyana* (about 6.5 \mu g chl-a per ml medium) caused a rapid inactivation of cellular GS with prevention of cell growth, and promoted the release of ammonia to the outer medium (figure 1). The process continued to be operative for about 28 hr. The accumulation of ammonia at detectable levels occurred about 4 hr after the addition of MSX when about 99% inhibition of the total GS activity had taken place. The rate of ammonia production reached a maximal value of 5.5 \mu mol/mg chl-a/hr about 12 hr after the addition of MSX and remained constant over the next 16 hr. After this time, GS activity suddenly recovered and the production of ammonia ceased. These changes were accompanied with resumption of cell growth and utilization of ammonia accumulated in the medium. The cessation of ammonia production about 28 hr after the addition of MSX was perhaps due to the metabolism of the analogue by the cells since under the experimental conditions MSX solution remains active and does not undergo spontaneous degradation for at least 40 hr.

In the attempt to lengthen the period of ammonia production fresh MSX (4 nmoles/\mu g chl-a) was added to suspensions of *N. harveyana* in which the inhibitor had lost its effect and GS activity had recovered. This treatment resulted again in inhibition of GS activity and accumulation of ammonia in the medium. When MSX was added every 16 hr, the maximal rate of ammonia production (5.5 \mu mol/mg chl-a/hr) was maintained for about 90 hr (figure 2). During this period, GS remained almost fully inactive. After 90 hr the production of ammonia decreased progressively till it ceased although the GS activity did not recover. At this stage further addition of MSX did not result in the recovery of ammonia production process. Death and lysis of the cells ensued shortly afterwards.

The effective phase of ammonia production could be prolonged by allowing MSX-treated filaments to recover by alternating treatment with MSX with periods in which the GS inhibitor was removed (figure 3). With recurrent addition of 32 \mu M MSX to suspensions (about 8 \mu g chl-a per ml medium) of the alga every 30 hr followed by 6-hr periods in the absence of the inhibitor, a sustained ammonia production lasting for 10 days with a net production of about 11 mM ammonia was achieved. The maximal rate of ammonia production (5.6 \mu mol/mg chl-a/hr) was continuously maintained during 30-hr periods in the presence of MSX whereas there was no ammonia production during the 6-hr periods in the absence of MSX. During the recovery periods, GS activity increased from 0.3 U to 1.8–3 U/mg chl-a (6–10% of the level in untreated controls) and slight growth occurred (from 8 to 8.3 \mu g chl-a per ml in 6 hr).

After 10 days of steady generation of ammonia the sequential treatment no longer resulted in ammonia production due to the fact that the filaments developed resistance to MSX. It then became necessary to increase the concentration of MSX up to 96–120 \mu M (12–15 nmoles per \mu g chl-a) in order to bring about a significant inactivation of cellular GS.

**Discussion**

Ammonia production rates reported by different researchers vary considerably. Stewart and Rowell (1975) reported that in the presence of 1 \mu M MSX, illuminated *Anabaena cylindrica* cell suspensions excreted ammonia at a rate of about 3 \mu mol/mg chl a/hr. Subsequently, Ladha et al. (1978) recorded a maximum rate of 5.75 \mu moles of ammonia per mg of chl-a per hr in the same alga by treatment with 75 \mu M HYL. In contrast, Ramos et al. (1984) reported high rates of ammonia production (25–30 \mu mol/mg chl-a/hr) lasting for more than 2 weeks with the marine *Anabaena* strain 33047 by alternating treatments with MSX and PT, and intercalating 8-hr recovery periods in the absence of the inhibitors. The rate of ammonia production by MSX-treated *N. harveyana* filaments is comparable to that reported for *A. cylindrica*.

Genetically altered nitrogen-fixing blue-green algae could be of great interest for the photoproduction of ammonia. Glutamine auxotrophs of *Anabaena cyladeae* have been described, which lack active GS and produce ammonia from dinitrogen (Singh et al. 1983). Efforts are being made in our laboratory to
Figure 1: Time-course of the effect of MSX on ammonia production (●), cellular GS activity (Δ), cell density (○) and rate of ammonia production (○) in *N. harveyana*. Filament suspensions were supplemented with MSX at zero time.

Figure 2: Continuous production of ammonia by *N. harveyana*. MSX was added at zero time and read every 16 hr.

Figure 3: Sustained ammonia production by *N. harveyana* by recurrent addition and removal of MSX. Filament suspensions were supplemented with MSX at zero time. After 30 hr, the filaments were harvested, washed thoroughly with fresh medium lacking MSX and resuspended in the same medium. After 6 hr in the absence of MSX the cell density was adjusted to about 8 μg chl a per ml and fresh MSX was readded. The arrows indicate the recovery periods.
develop mutants with low levels of GS activity and significant derepression of nitrogenase. Nitrogen-fixing blue-green algae clearly have a potential to produce ammonia from dinitrogen in a light-dependent reaction. Effective in vivo ammonia-generating systems could be developed by the use of immobilisation techniques.

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Acknowledgements

The authors are grateful to Dr P V Sane, Director, National Botanical Research Institute, Lucknow, for constant encouragement, and to the Council of Scientific and Industrial Research for the award of a Senior Research Fellowship to one of them (AD).