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PHOTOPRODUCTION OF AMMONIA FROM NITRATE BY *ANACYSTIS NIDULANS* CELLS

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The addition of some glutamate analogs to illuminated cell suspensions of the cyanobacterium *Anacystic nidulans* in nitrate-containing medium promoted the release of significant amounts of ammonia. Best results were obtained with micromolar concentrations of the glutamine synthetase inactivator L-methionine-DL-sulfoximine (MSX). Inactivation of cellular glutamine synthetase by MSX was followed by prevention of cell growth and by a significant increase in the rates of uptake and reduction of nitrate. Between 85 and 90% of the ammonia resulting from the photosynthetic reduction of nitrate by the cells was found to accumulate in the outer medium. High rates of ammonia production (25–30 μmol per mg chlorophyll per h) were sustained for longer than 24 h, without interferences of the accumulated ammonia on the process. The MSX-treated cells actively producing ammonia did not appear to suffer of a general nitrogen deficiency, but they rather exhibited a specific deficiency in glutamine and abnormally high levels of carbohydrates. The results indicate the feasibility of using whole cyanobacterial cells for the photoproduction of significant amounts of ammonia from nitrate at the expense of light energy, in a simple version of photobiological energy transduction and storage.

Introduction

The bioconversion of solar energy into suitable redox energy through photosynthesis of the water-splitting type is of outstanding interest and significance, and a major effort is presently under way to use this process to generate 'biofuels' on a renewable basis (see, for example, Ref. 1). The fact that some photosynthetically generated metabolites are highly reduced compounds of immediate practical interest has, in general, been overlooked till now, however. Such is the case of ammonia, the eventual end-product of assimilatory nitrate reduction (and N_2-fixation) that, in addition to be a valuable fertilizer, is an excellent and powerful fuel [2,3].

The assumulatory reduction of nitrate to ammonia as it occurs in green cells is a light-driven process, being a particularly simple example of photosynthesis in the case of the cyanobacteria (blue-green algae) [3–5]. For these organisms it has conclusively been shown that reduced ferredoxin resulting from water photolysis directly supplies the electrons required for the sequential reduction of nitrate, first to nitrite in a two-electron reduction catalyzed by nitrate reductase, and then to ammonia in a six-electron reduction catalyzed by nitrite reductase [3–6].

The light-dependent reduction of nitrate with the consequent generation of ammonia seems to have possibilities as a photobiochemical energy transduction and storage process. Previous attempts to use reconstituted systems for the photoproduction of ammonia frrom nitrate [3,7] have been hampered mainly because of the stability problems inherent to these in vitro systems. We feel that whole cyanobacterial cells, if conveniently manipulated, are better candidates for achieving an efficient and prolonged

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; MSF, L-methionine sulfone; MSX, L-methionine-DL-sulfoximine.

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production of ammonia. The accomplishment of such a goal requires to prevent the assimilation of ammonia into organic nitrogen to take place. Interferences of the accumulated product, ammonia, with the operation of the generation process should also be avoided. Previous results of short-term experiments aimed to the study of regulatory aspects of nitrate utilization by cyanobacteria [8,9] have indicated the possibility of simultaneously fulfulling both of these requisites by chemically inhibiting the incorporation of ammonia into carbon skeletons.

The present paper deals with the study and characterization of the steady photoproduction of ammonia from nitrate by the cyanobacterium *Anacystis nidulans*, following the treatment of the cells with some glutamate analogs (especially MSX) that cause inhibition of ammonia assimilation. A preliminary report of this investigation has been presented at the 2nd Meeting of the Federation of European Societies of Plant Physiology [10].

Materials and Methods

Materials. ADP, Hepes, γ-benzyl-L-glutamate, L-glutamic dehydrogenase (type II, from bovine liver), δ-hydroxylysine, L-methionine-DL-sulfoxide, MSX, MSF, *N*-methyl-DL-glutamic and *S*-carbamoyl cysteine were purchased from Sigma (St. Louis); L-glutamic-γ-hydrazine from Senn (Dielsdorf, Switzerland): NAD^+, NADH and NADPH from Boehringer (Mannheim); other chemicals were products from Merck (Darmstadt).

Growth of cells. Anacystis nidulans strain L 1402-1 from the Göttingen University Algal Culture Collection was grown photoautotrophically under continuous fluorescent illumination (25 W/m^2, white light) at 40°C on a synthetic medium containing nitrate as the nitrogen source [8], in a stream of 2% CO_2 in air (v/v), which was bubbled through the culture at a flow rate of 1 litre per litre cell suspension per h.

Ammonia production experiments. Cells from 2-day-old cultures (containing about 20–25 μg chlorophyll/ml) were used. The cells were harvested by centrifugation, washed with culture medium and finally resuspended in the same medium, unless than otherwise stated, to a cell density equivalent to 8–10 μg chlorophyll per ml. The cell suspensions were then incubated under standard culture conditions with the indicated modifications.

Extraction of cellular metabolites. This was achieved by treatment of the cells with perchloric acid [9].

Assays of enzyme activities. For the estimation of in situ activities of both nitrate reductase and glutamine synthetase, toluene-treated cells were used [8]. Aliquots of the preparation of permeabilized cells containing 2–3 μg chlorophyll were added to the corresponding reaction mixtures. Other conditions for the determination of nitrate reductase activity were as previously described [8]. One unit of nitrate reductase activity corresponds to the formation of 1 μmol nitrite per min. The assay mixture (1.0 ml final volume) for the estimation of glutamine synthetase (transferase) activity was as in [11], except that 50 mM Hepes buffer (pH 7.3) replaced the imidazol buffer. Incubation was at 30°C, and the γ-glutamyl hydroxamate formed was determined after 10 min. Glutamine synthetase (transferase) activity units corresponds to μmol γ-glutamyl hydroxamate formed per min.

Analytical methods. Nitrate was determined as nitrite after enzymatic reduction (Flores, E., personal communication) with a preparation of nitrate reductase from *A. nidulans* purified according to Ref. 12. Nitrite was estimated as described by Snell and Snell [13] after elimination of the dithionite oxidation products by treating an aliquot of the reaction mixture with 1 vol. of 0.5% (w/v) formaldehyde [14] for 3 min. Ammonia was determined as described by Solorzano [15]. γ-Glutamyl hydroxamate was estimated after its reaction with $FeCl_3$ in acid medium [11]. Glutamate was measured with glutamate dehydrogenase [16], and glutamine was estimated as glutamate after acid hydrolysis [9].

Packed cell volume was estimated by centrifugation of the cell suspensions in calibrated hematocrit tubes; cellular protein by the Lowry procedure as modified by Bailey [17], pretreating the cells with 10% (w/v) trichloroacetic acid; total nitrogen by the Kjehldal procedure; carbohydrates by the phenol-sulfuric acid method [18]; chlorophyll *a* with methanol, employing the extinction coefficient given by McKinney [19]. Measurements of the absorbance values at 628 and 680 nm of cell suspensions for estimation of the phycocyanins/chlorophyll ratio were performed by placing the sample-containing cuvette in the turbid-samples' compartment of a Pye Unicam

SP 1750 spectrophotometer. Light intensity measurements were done with a YSI-Kettering model 65A radiometer.

Results

The glutamine synthetase-glutamate synthase enzyme pathway represents the main route for the assimilation of ammonia by the strain of *Anacystis* used in this work (Ref. 20, and Flores, E. and Ramos, J.L., unpublished data). Some glutamate analogs that can interfere with the operation of either or both enzymes of the pathway have been tested with regard to their effectivity in inhibiting the conversion of ammonia into organic nitrogen by intact *A. nidulans* cells. With nitrate as the nitrogenous substrate, such an interference should lead in principle to excretion to the medium of the ammonia resulting from the photosynthetic reduction of nitrate by the cells. This was indeed the result obtained when *A. nidulans* cell suspensions in nitrate medium were treated with either MSX, MSF or δ-hydroxylysine, whereas L-methionine-DL-sulfoxide, L-glutamic-γ-hydrazine, *N*-methyl-DL-glutamic acid, γ-benzyl-L-glutamate and *S*-carbamoyl cysteine were ineffective to this end. Representative results are shown in Table I for the case of those analogs that promoted ammonia release by *A. nidulans* cells. The highest rates of ammonium release were always recorded with MSX, MSF being less effective in this respect, although still more efficient than was hydroxylysine. Moreover, whereas millimolar concentrations of the latter compounds were required for the induction of ammonia production by *A. nidulans*, maximal rates of ammonia release were already obtained with concentrations of MSX in the range 10–20 μM. As also shown in Table I, the glutamate analogs, the action of which gave rise to ammonia production, also exhibited inhibitory effects on cell growth. This inhibition was about 25% for 10 mM hydroxilysine and 65% for 1 mM MSF, while MSX concentrations lower than 5 μM already sufficed to arrest fully the cell growth of *A. nidulans*.

The process of ammonia production by *A. nidulans* cells treated with MSX has been characterized in a greater detail. The MSX-promoted ammonia release did not take place in the absence of nitrate, indicating that ammonia accumulated in the outer medium represents the product of nitrate reduction, and excluding therefore the possibility that it might originate from the degradation of nitrogenous cell components. A strict requirement for light was also evident since no accumulation of ammonia could be detected under dark conditions (data not shown).

Fig. 1. shows the time course of some of the main events following the addition of 10 μM MSX to a suspension of *A. nidulans* cells in nitrate medium. A correlation between the inactivation by MSX of cellular

TABLE I

EFFECT OF DIFFERENT GLUTAMATE ANALOGS ON AMMONIA PRODUCTION AND CELL GROWTH

Suspensions of *A. nidulans* cells containing 8.5 μg chlorophyll per ml culture medium were supplemented with the corresponding glutamate analogs at the indicated final concentrations. Incubation time, 28 h. Other conditions as described under Materials and Methods

Analog	Ammonia formed (μmol/ml)	Cell density (μg chlorophyll/ml)
None	0.0	32.6
δ-Hydroxylysine, 10 mM	0.9	26.5
MSF, 1 mM	3.6	16.4
MSX, 0.01 mM	4.5	8.5

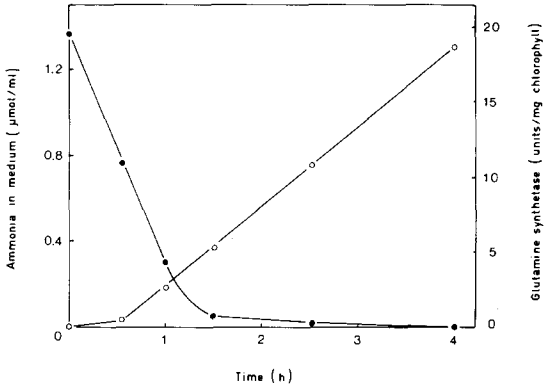

Fig. 1. Time course of the effect of MSX on ammonia production (○) and cellular glutamine synthetase activity (●) in *A. nidulans*. A cell suspension with a density of 10 μg chlorophyll per ml culture medium was incubated for 4 h in the light under standard culture conditions. MSX (10 μM final concentration) was added at zero time.

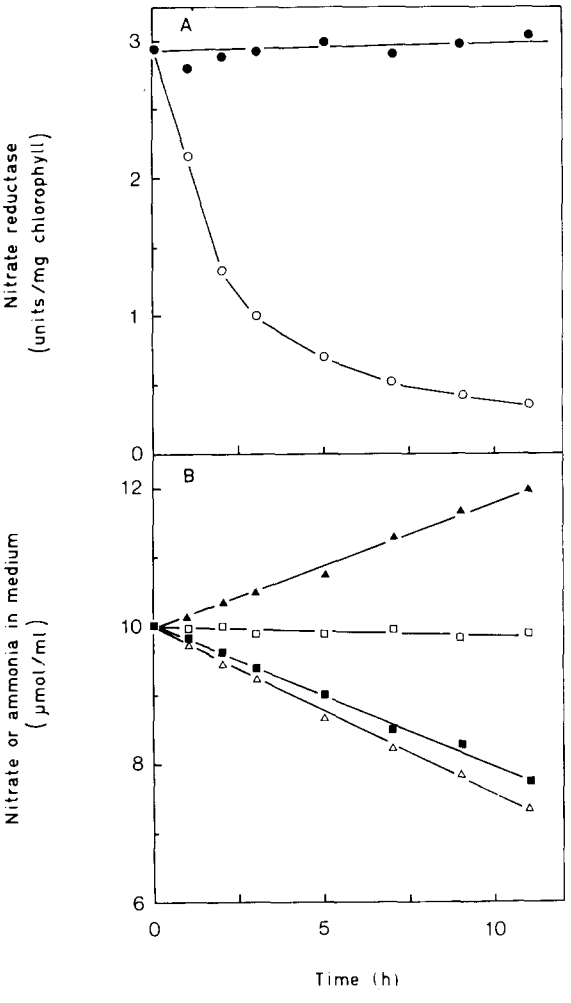

Fig. 2. Prevention by MSX of the antagonistic effects of ammonia on the utilization of nitrate by *A. nidulans*. Nitrate-grown cells were harvested, washed, and resuspended in culture medium containing 10 mM NH_4NO_3, at a final density of 9 μg chlorophyll per ml. The resulting cell suspension was divided into two halves, one of which was supplemented with MSX to reach a final concentration of 10 μM (closed symbols), whereas no MSX was added to the remaining aliquot (open symbols). At the times indicated, cellular nitrate reductase activity (circles), and nitrate (squares) and ammonia (triangles) in the medium, were estimated.

glutamine synthethase and the production of ammonia is apparent from these data. In fact, the accumulation of ammonia at detectable levels occurred about 30 min after the addition of MSX, when a significant inactivation of glutamine synthetase (about 50% of the total activity) had taken place. Once a major proportion of the enzyme had been inactivated, the rate of ammonia production reached a maximal value, which kept constant till the end of the experiment. Mean values for ammonia production by *A. nidulans* cell suspensions containing about 10 μg chlorophyll per ml nitrate medium, in the presence of 10 μM MSX, were in the range of 25–30 μmol/mg chlorophyll per h.

The accomplishment of a continued photoproduction of ammonia from nitrate by whole *Anacystis* cells demands potential antagonistic effects of the accumulated reaction product to be circumvented. In fact, under normal metabolic conditions ammonia effectively inhibits nitrate uptake and causes repression of the synthesis of the nitrate-reducing enzyme system. Fortunately for our purposes, both of these negative effects of ammonia appear to be indirect, arising as a result of its metabolism through glutamine synthetase [8,9].

The results in Fig. 2 support this proposal as they clearly show that, over a long-term period, the negative effects of ammonia on nitrate uptake and nitrate reductase synthesis, evident for the case of untreated *A. nidulans* cells, are both prevented by the presence of the glutamine synthetase inhibitor MSX. In these experiments, nitrate-grown cells were transferred to media containing 10 mM ammonium nitrate to mimic the situation expected to arise after a few hours of active ammonia production by cells originally suspended in a medium containing nitrate as the sole nitrogen source. Under these conditions a fast decline in the nitrate reductase activity level of normal untreated cells took place (Fig. 2A), ammonia but not nitrate being taken up by the cells (Fig. 2B). Nitrate was, however, actively used by the MSX-treated cells (at a rate about 2-fold higher than that of normal cells in nitrate medium, as previously shown in short-term experiments [9]) the ammonia resulting from nitrate reduction being excreted to the outer medium, where it accumulated over the ammonia originally added, which could not be used by the MSX-treated cells. On the other hand, in the presence of MSX the cellular level of nitrate reductase activity did not decrease as in the control cells, but it kept constant or even increased slightly over its original value (Fig. 2A). Similar results were obtained with ammonia concentrations of up to 30 mM (data not included). These results establish, therefore, the fea-

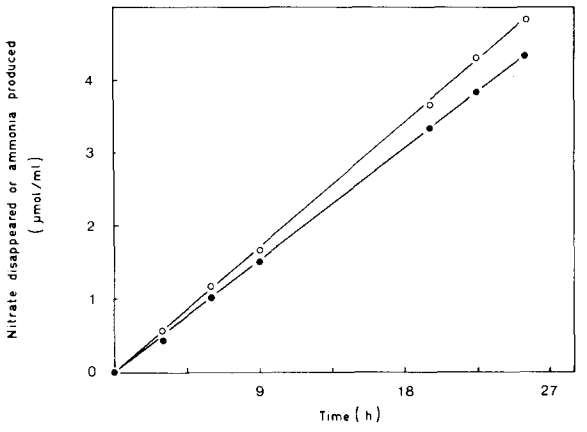

Fig. 3. Sustained uptake of nitrate (○) and concomitant ammonia production (●) by *A. nidulans* in the presence of MSX. The complete system consisted of a suspension of *A. nidulans* cells containing 9 μg chlorophyll per ml of nitrate-containing medium supplemented with 10 μM MSX. The cell suspension was incubated for 26 h in the light.

sibility of the system to generate considerable amounts of ammonia on a long-term basis, without interference from the accumulated reaction product with the generation process.

Fig. 3 shows the time course of nitrate uptake and ammonia production over a period longer than 24 h by *A. nidulans* cells suspended in a nitrate-containing medium supplemented with MSX. It can be seen that the rate of ammonia production remained constant throughout this period and that most of the nitrate taken up could be accounted for by the ammonia accumulated in the medium (90% for the experiment in Fig. 3, and between 85 and 90% for a number of similar experiments). It seems therefore that a small fraction (about 10%) of the nitrate taken up is being assimilated by the MSX-treated cells. This could mean that, in addition to the glutamine synthetase-glutamate synthase pathway, a minor route for ammonia assimilation is also operative in *A. nidulans*. Measurable levels of NAD(H)-specific alanine dehydrogenase activity have been detected in cell-free preparations of the strain of *A. nidulans* used in this work, and the aminative activity of this enzyme could actually account for the observed rates of inorganic nitrogen assimilation under conditions of MSX-inactivated glutamine synthetase (Ramos, J.L., unpublished data).

The activity of such a secondary pathway could be

TABLE II

EFFECT OF MSX ON THE LEVELS OF DIFFERENT CELL COMPONENTS AND METABOLITES

A suspension of *A. nidulans* containing 1.45 μl cells per ml culture medium was divided into two halves, to one of which MSX was added at $t = 0$ to reach a final concentration of 10 μM. After 22 h incubation under standard growth conditions the cell density values reached were 3.48 and 1.48 μl cells/ml for the MSX-free and MSX-containing suspensions, respectively

	$t = 0$	$t = 22$ h	
		no MSX	10 μM MSX added
A_{628}/A_{680}	1.0	1.0	1.0
Nitrogen [a]	15.0	17.3	15.6
Protein [a]	93.1	106.8	93.8
Chlorophyll [a]	6.0	6.1	5.9
Free glutamate [b]	17.6	17.0	11.2
Free glutamine [b]	3.2	3.5	0.0
Carbohydrates [a]	6.2	6.6	17.3

[a] Values are in μg/μl cells.
[b] Values are in nmol/μl cells.

responsible for the fact that no symptoms of nitrogen starvation are apparent in MSX-treated cells actively producing ammonia. This is illustrated by the results in Table II, where it can be seen that the relative value of the phycocyanins-to-chlorophyll ratio (A_{628}/A_{680}, often taken as an index of the general nitrogen status in cyanobacteria) in MSX-treated *A. nidulans* cells remained comparable to those of normal untreated cells, indicating no loss in phycocyanin(s) and, correspondingly, no general deficiency in nitrogen. The values of the estimates of total nitrogen and protein contents were also very close to those of normal cells growing on nitrate media, as also did those of the chlorophyll levels. The main differences found between MSX-treated and untreated *A. nidulans* were those regarding the cellular levels of glutamine, glutamate and carbohydrates. As a consequence of the inactivation by MSX of cellular glutamine synthetase, the level of free glutamine dropped to zero in cells which had been exposed to the action of the inhibitor for 22 h. A moderate decrease in the level of free glutamate was also observed in the MSX-treated cells, reaching a value of about 65% of that recorded

for untreated control cells. More striking was the increase in the carbohydrate content of the cells kept in the presence of the inhibitor, where carbohydrate levels about 3-fold higher than those recorded for normal cells were present after 22 h of treatment with MSX (Table II).

This increase in the cellular level of carbohydrates appears to be a result of the maintenance of normal CO_2 fixation rates in the MSX-treated cells, under the special conditions otherwise established by the presence of the inhibitor, i.e., drastic impediment of the incorporation of ammonia to carbon skeletons and absence of measurable cell growth. The reported lack of effect of MSX on the rate of photosynthetic CO_2 fixation (estimated as light- and CO_2-dependent oxygen evolution) by *A. nidulans* in short-term experiments [9,21] has been confirmed by us for cells subjected to the action of the inhibitor for longer than 20 h. We have also evaluated the proportion of the photosynthetic electron flow contributing to the reduction of nitrate under conditions of simultaneous reduction of CO_2 and nitrate, both in normal untreated and MSX-treated cells. Whereas for normal *A. nidulans* cells only 15% of the total oxygen evolution is nitrate-dependent, the proportion of the extra-oxygen evolution corresponding to the reduction of nitrate to ammonia in MSX-treated cells reaches values of about 30% of the total (data not shown). This is well in agreement with the above described enhancement (about 2-fold) in nitrate uptake and reduction resulting from the treatment of the cells with MSX.

Discussion

The fact that in the presence of MSX, a specific irreversible inhibitor of the enzyme glutamine synthetase [22], a major fraction of the ammonia generated through nitrate reduction is exported to the medium indicates a key role of glutamine synthetase in the assimilation of ammonia by *A. nidulans.* This is in support of our previous suggestions [9,20,21] that the glutamine synthetase-glutamate synthase pathway represents the main route for the incorporation of ammonia to carbon skeletons in this cyanobacterium and is contrary to the claims of other workers [23] who have suggested a major role of glutamate dehydrogenase in the assimilation of ammonia by *A. nidulans.* In this regard, it is worth mentioning that all of our attempts aimed at detecting any glutamate dehydrogenase activity in cell-free extracts of this cyanobacterium have been without success (unpublished results). The reasons for MSX to behave as the most efficient among a variety of glutamate analogs tested in inhibiting ammonia assimilation in *A. nidulans,* causing therefore the release to the outer medium of most of the synthesized ammonia, are most probably due to the peculiar deactivation of glutamine synthetase caused by this compound [24]. Differences in the relative permeability of the cell membrane to the various analogs may also contribute to differences in efficiency with regard to the inhibition of ammonia assimilation in whole cell systems.

Inhibition by MSX of the assimilation of ammonia through glutamine synthetase leads to a general avoidance of the antagonistic effects of ammonia on the utilization of nitrate by *A. nidulans.* This confirms and extends for the case of long-term experiments the validity of the conclusions raised recently that the ammonia-promoted inhibition of nitrate uptake [9] and of nitrate reductase synthesis [8] are indirect. Ammonia metabolism through glutamine synthetase appears to be required for the negative effects of ammonia on nitrate utilization to be expressed (Refs. 8, 9; see also Ref. 5 for a general discussion including other groups of organisms).

The relevance of the effects caused by MSX with regard to the achievement of efficient cell systems able to photoproduce ammonia is therefore not limited to the fact that this compound promotes the release to the medium of the ammonia synthesized by the cells. In addition, its action ensures the protection of the processes involved in the generation of ammonia against negative effects of the accumulated product. Moreover, as a consequence of the efficiency of MSX in inhibiting the conversion of ammonia into organic nitrogen, cell growth remains fully arrested. This ensures that if a critical cell density established to be optimal for ammonia photoproduction purposes (8–10 μg chlorophyll per ml medium under the standard conditions used for the experiments herein described) is settled at the beginning of the experiment, it will remain unchanged as long as the MSX is exerting its effects. Such a system will not suffer of constraints such as decreasing production rates with time due to light limitation caused by the

increased turbidity of the cell suspension. On the other hand, as only low amounts of the reaction product are required for maintenance of the system, practically all (about 90%) of the produced ammonia remains unused by the cells and available for collection.

Although the MSX-treated *A. nidulans* cells do not grow, cells in this system should not be regarded as belonging to the 'resting' type, since they exhibit otherwise a very active metabolism, similar to that of normal cells in exponential phase of growth, exception being made for the functioning of the metabolic pathway specifically affected by the inhibitor, and other related reactions. This is particularly true for what concerns the operation of photosynthetic reactions and CO_2-fixation, the continued operation of which at normal rates in cells that do not grow leads to remarkably high levels of accumulated carbohydrates in the MSX-treated cells, which cannot use these carbohydrates as ammonia acceptors in the main route that under normal conditions takes care of ammonia assimilation. Besides, an increased flow of photosynthetically generated reducing power, specifically channelled to the reduction of nitrate to ammonia, occurs in the MSX-treated cells suspended in nitrate-containing medium. These modified cells can thus be considered as 'microfactories', the activity of which is addressed mainly to the photoproduction of ammonia.

The biochemical characterization of some relevant parameters in the MSX-treated cells, e.g., the maintenance in the levels of phycocyanins – which are considerably decreased in cells subjected to nitrogen-starvation as happens under conditions of lack of an available nitrogen source [25] – reveals that a specific deficiency in glutamine, but not a general nitrogen starvation which could irreversibly damage the operativity of the system, is (together with the increased carbohydrate levels) the most relevant difference found with regard to normal untreated *A. nidulans* cells (Table II).

The photoproduction of ammonia by MSX-treated *A. nidulans* cells represents an effective photobiological solar energy conversion and storage process, in which a significant fraction of the sunlight energy reaching the system is converted to chemical energy stored in the ammonia molecule. For each 16 quanta of photochemically active radiation, 360 kJ are used for the conversion of nitrate to ammonia [2–4], according to the equation:

$$HNO_3 + 4\ H_2O \xrightarrow[8e^-]{16\ h\nu} NH_3 + 2\ O_2 + 3\ H_2O$$

$(\Delta E'_0,\ \text{pH } 7 = -0.47\ \text{V};\ \Delta G'_0,\ \text{pH } 7 = +360\ \text{kJ} \cdot \text{mol}^{-1})$

The oxidation to nitrate of the resulting ammonia, in a highly exergonic reaction:

$$NH_3 + 2\ O_2 \xrightarrow[8e^-]{} HNO_3 + H_2O$$

$(\Delta E'_0,\ \text{pH } 7 = +0.47\ \text{V};\ \Delta G'_0,\ \text{pH } 7 = -360\ \text{kJ} \cdot \text{mol}^{-1})$

would, in conjunction with the above stated photoreduction of nitrate to ammonia, close a simple cycle transducing solar energy into another readily usable form of energy.

The photoproduction of ammonia from nitrate by the system consisting of MSX-treated cells here described, with 10 μM MSX (final concentration) added at the beginning of the experiment, is operative for about 30 h. After this time, the production of ammonia suddenly slows down till it virtually ceases. This is accompanied by recovering of glutamine synthetase activity, with resumption of cell growth, ammonia assimilation through glutamine synthetase starting to be fully active again. Beginning at this point, the antagonistic effects of ammonia on nitrate utilization come again into play. Several means of maximizing the extent to which the production of ammonia takes place and of prolonging the effective period of ammonia photoproduction are currently being investigated and will be reported elsewhere.

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