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REGULATORY INTERACTION OF PHOTOSYNTHETIC NITRATE UTILIZATION AND CARBON DIOXIDE FIXATION IN THE CYANOBACTERIUM *ANACYSTIS NIDULANS*

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The rate of photosynthetic nitrate utilization in *Anacystis nidulans* is strongly influenced by the availability of carbon dioxide. This dependence can be relieved by inhibiting the metabolism of the ammonium derived from nitrate reduction. Nitrate uptake seems to be modulated through a sensitive regulatory system integrating the photosynthetic metabolism of carbon and nitrogen, with CO_2 fixation products antagonizing the inhibitory effect of ammonium derivatives.

Nitrate assimilation is a metabolic process essential in the acquisition of nitrogen for a variety of plants and microorganisms [1]. The utilization of nitrate by green cells is strongly dependent upon the availability of carbon dioxide, a fact early recognized [2] and repeatedly corroborated at the phenomenological level [3–9]. The mechanistic aspects, however, have been virtually unknown [1,9]. As is the case for the assimilation of CO_2, it is by now well established that nitrate utilization in cyanobacteria (blue-green algae) is a process photosynthetic in nature [1,9–11]. A study aimed at the elucidation of the way whereby carbon and nitrogen metabolism are integrated in the green cell and, more specifically, to clarify the role played by CO_2 on nitrate utilization has been undertaken in the cyanobacterium *Anacystis nidulans.*

As is the case for other photosynthetic organisms [2–9], the utilization of nitrate by cells of *A. nidulans* exhibits a stringent requirement for CO_2. When CO_2 was excluded from *Anacystis* cell suspensions by various procedures (sparging with either argon or CO_2-free air, or shaking in a Warburg vessel containing KOH in the center well), values for the rate of nitrate utilization were always lower than 10% of those found in the presence of saturating amounts of CO_2. The effect of the concentration of available CO_2 on the rate of nitrate uptake by *Anacystis* cells is shown in Fig. 1. The results obtained fit within a hyperbolic staturation curve, and a value of 3.7 μM was calculated for the concentration of CO_2 that allowed nitrate utilization to proceed at half-maximum rate. This curve matched that obtained for the substrate dependence of photosynthetic CO_2-fixation, as also shown in Fig. 1. The calculated apparent K_m value for CO_2 of the latter process was 4 μM. The rate of nitrate utilization seems thus to be closely dependent on the CO_2-fixation rate. The effect of CO_2 on nitrate uptake might thus well be mediated by CO_2-fixation products, a proposal that is also supported by results obtained with *A. nidulans* cells previously subjected to nitrogen starvation. These cells, which display symptoms of nitrogen deficiency and are rich in carbohydrates [12], did not exhibit in the short-term a CO_2 requirement for nitrate utilization (Table I). The CO_2-fixation products accumulated in the nitrogen-starved cells can apparently replace

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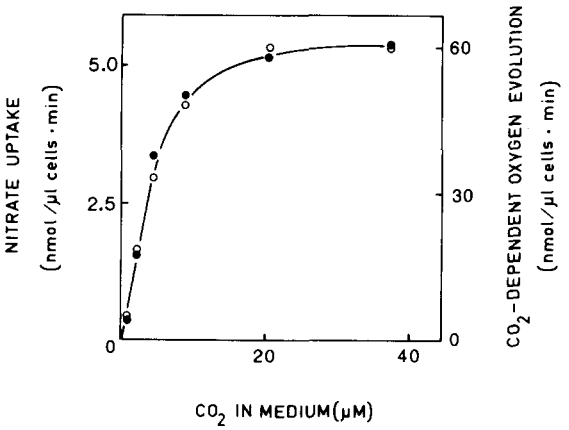


Fig. 1. Effect of the concentration of CO_2 on nitrate uptake and on CO_2-fixation by *A. nidulans*. Cells of *A. nidulans* (strain L 1402-1 from the University of Göttingen) grown photoautotrophically at 40°C with nitrate as the nitrogen source [16] were used. The experiments were carried out in closed Warburg vessels containing 3 μl of packed cell volume (1 μl cells contained 7–8 μg chlorophyll *a* and about 150 μg protein [13]), in 3 ml 0.1 M $NaHCO_3/Na_2CO_3$ buffer of the appropriate pH to obtain the desired CO_2 concentration [17], and supplemented with 0.5 mM KNO_3 only in the nitrate uptake experiments. The assays were carried out, at 40°C, for 30 min (nitrate uptake) or 60 min (CO_2-fixation) under illumination (100 $W \cdot m^{-2}$, white light) with continuous shaking and were started by switching the light on. CO_2-fixation was estimated as the light- and CO_2-dependent evolution of oxygen. Oxygen was determined manometrically, and nitrate was determined in aliquots of the cell suspensions, after rapid removal of the cells by filtration (Millipore HA 0.45 μm pore size filter) by absorption at 210 nm in acid solution [18]. It is relevant to mention that the nitrate uptake rate was virtually constant along the pH range used in these experiments (9.6–10.7), as tested in separate experiments with CO_2 at about 8 μM and 25 mM glycine-NaOH buffers of different pH values. ○, Nitrate uptake; ●, CO_2-dependent oxygen evolution.

CO_2 in its stimulating effect on nitrate uptake.

Contrary to the situation with normal cells, *Anacystis* cells treated with the glutamate analog L-methionine DL-sulfoximine are able to take up nitrate and reduce it to ammonium in the absence of CO_2 [10]. Results in Table I also show that the rate of nitrate uptake in L-methionine DL-sulfoximine-treated cells was indeed virtually the same regardless of the presence or absence of CO_2 in the cell suspension. L-Methionine DL-sulfoximine is a highly specific glutamine synthetase inactivator, that effectively hampers ammonium assimilation in *A. nidulans* [11,13] without any side-effect on photosynthetic CO_2 fixation (unpublished data). Thus, prevention of ammonium incorporation into carbon skeletons releases nitrate uptake from the CO_2 requirement. The role played by the CO_2-fixation products in nitrate utilization by *A. nidulans* appears therefore to be regulatory and not just that of mere substrates needed for the assimilation of the ammonium generated from nitrate reduction.

We previously showed that treatment of *A. nidulans* cells with L-methionine DL-sulfoximine released nitrate uptake from its inhibition by ammonium, the eventual product of nitrate reduction,

TABLE I

RELIEF OF THE CO_2 REQUIREMENT FOR NITRATE UTILIZATION IN *A. NIDULANS* CELLS BY EITHER NITROGEN STARVATION OR TREATMENT WITH L-METHIONINE DL-SULFOXIMINE

Experiments were carried out in closed Warburg vessels with either CO_2-free or CO_2-enriched air as the gas phase. These atmospheres were established by placing in the center well 0.25 ml of either 20% KOH or 0.5 M $NaHCO_3/NaCO_3$ buffer (pH 9.6), respectively. The cells (2.7–5 μl packed cell volume – containing about 5 μg chlorophyll *a* per μl N-starved cells and about 7 μg chlorophyll *a* per μl normal cells) were resuspended in 2.75 ml 25 mM Tricine-NaOH buffer (pH 8.3) and placed in the main compartment. After 20 min preincubation with the vessels closed, the assays were started by the addition of 2 μmol KNO_3 from the side-arm to the main compartment and switching the light on. Nitrate was determined in aliquots of the cell suspensions after rapid removal of the cells. Nitrogen-starved cells were prepared by incubating nitrate-grown cells under culture conditions in a medium lacking any added nitrogen source for 5.5 h. After this time the cells had lost a significant fraction of phycocyanins, as evidenced by the absorbance decrease at 628 nm of the cell suspension, an indication of nitrogen deficiency [12]. The term MSX-treated cells refers to nitrate-grown cells which prior to the experiment were pretreated, at 40°C, with 1 mM L-methionine DL-sulfoximine for 15 min in the light in air-opened conical flasks. The level of glutamine synthetase activity [19] in such cells was negligible throughout the experiment. Other experimental conditions and methods were as those quoted in Fig. 1 and in Refs. 10 and 13.

Type of cells	Nitrate uptake (nmol/μl cells per 30 min)	
	minus CO_2	plus CO_2
Normal untreated	10	154
N-starved	127	180
MSX-treated	177	171

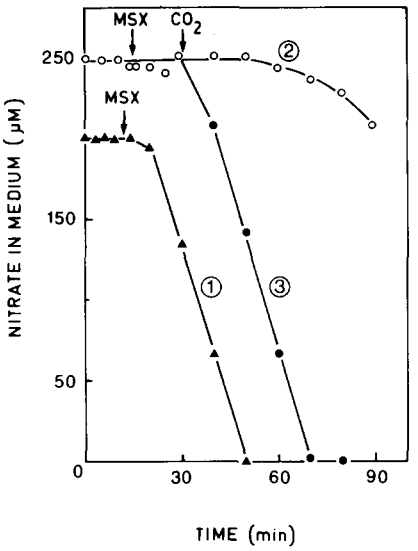


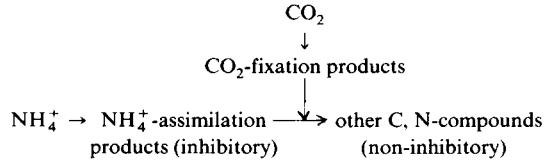
Fig. 2. Effect of CO_2 on the L-methionine DL-sulfoximine-promoted release of the inhibition by ammonium of nitrate uptake in *A. nidulans* cells. Cell suspensions containing 1 μl cells (equivalent to 8 μg chlorophyll *a* and 150 μg protein) per ml of 25 mM Tricine-NaOH buffer (pH 8.3) were preincubated for 30 min in the dark and sparged either with argon (○) or with a 95 : 5 (v/v) argon/CO_2 mixture (▲). At $t = 0$, NH_4Cl to give a final concentration of 0.3 mM and KNO_3 to give the indicated concentration were added, and the light (100 $W \cdot m^{-2}$, white light) was switched on. L-Methionine DL-sulfoximine (1 mM final concentration) was added at the time indicated by the first arrow. The second arrow indicated the time at which the suspension incubated with argon (○) was split into two halves, one of which was then sparged with a 95 : 5 (v/v) argon-CO_2 mixture (●). Cellular glutamine synthetase (transferase activity [19]) estimated in cells permeabilized by treatment with 75% (v/v) ethanol was 2.15 units per mg protein prior to MSX addition and became 0.08 units per mg protein 15 min after addition of the inhibitor.

thus indicating that products of ammonium assimilation, rather than ammonium itself, are active as negative effectors of nitrate uptake [11,13]. It appears, moreover, that the rate of nitrate utilization in *Anacystis* is permanently under the control exerted by the ammonium, internally generated from nitrate [11,13]. The simultaneous release by L-methionine DL-sulfoximine both of the stimulation by CO_2 – through CO_2-fixation products – and of the inhibition by ammonium – through ammonium assimilation products – of nitrate uptake establishes a link between these two aspects of the regulation of nitrate utilization.

L-Methionine DL-sulfoximine is not only able to prevent nitrate uptake from the inhibition caused by either ammonium addition or absence of CO_2, but also to reverse such inhibition once it has taken place. The positive effect of CO_2 on the L-methionine DL-sulfoximine-promoted restoration of nitrate uptake after its inhibition by ammonium (Fig. 2) strongly suggests a role for CO_2 as an antagonist of ammonium in its effect on nitrate uptake. The time required for L-methionine DL-sulfoximine (1 mM) to cause 90–95% inactivation of *A. nidulans* glutamine synthetase [11] after addition of the inhibitor to a cell suspension was of 10–15 min, both in presence and absence of CO_2. When CO_2 was present, the ability to utilize nitrate by cells previously exposed to added ammonium was regained just about 10 min after L-methionine DL-sulfoximine addition (Fig. 2, curve 1). Nevertheless, the time lapse between L-methionine DL-sulfoximine addition and the restoration of nitrate uptake lasted about 40–50 min in the case of cell suspensions maintained in the absence of CO_2 (Fig. 2, curve 2). Note that this time interval is far longer than the 10–15 min required for substantial inactivation of glutamine synthetase. This might suggest that nitrate uptake remains inhibited by accumulated negative effectors, the removal of which – once their further synthesis has been prevented by inactivation of glutamine synthetase – is sluggish when CO_2 is absent, but very fast in its presence (compare curves 1 and 2 in Fig. 2). CO_2 is moreover able to counteract rapidly the negative effect on nitrate uptake of such a sustained inhibitory condition after the latter has been established. This is clearly illustrated by the immediate restoration of nitrate uptake observed following the provision of CO_2 just 15 min after L-methionine DL-sulfoximine addition to a cell suspension which had been kept exposed to ammonium in the absence of CO_2 (Fig. 2, curve 3).

Altogether, the above results can be interpreted in terms of the involvement of some CO_2-fixation product(s) as part of a sensitive regulatory system controlling nitrate uptake, a system which would also include the participation of ammonium assimilation products. While the ammonium derivatives clearly play the role of negative effectors [11,13], the CO_2-derivatives have a stimulatory effect on nitrate uptake. The stimulating effect of

the CO_2-derivatives might be interpreted in terms of their direct positive action on nitrate uptake. The lack of stimulation by CO_2 of nitrate uptake in cells unable to generate the inhibitory ammonium derivatives (cells pretreated with L-methionine DL-sulfoximine in the presence of CO_2, Table I) does not favor this view, however. The results support instead the contention of an indirect effect of the CO_2-fixation products, which through combination with the negative effectors would remove the latter compounds, thus counteracting the inhibitory effect of the ammonium derivatives on nitrate uptake. Such an interaction can be diagrammatically represented as follows:



The identification of the nature of the regulatory metabolites and of their actual site of action, which might well be the nitrate transport system [11,13,14], is presently being pursued.

The present interpretation of a regulatory role for CO_2 on nitrate utilization provides a rationale for the interpretation of the effect of CO_2 on nitrate metabolism in different algae [2–9,15]. The regulation of nitrate assimilation through a system such as that described herein, which includes products of the metabolism of both carbon and nitrogen, can help in the understanding of an outstanding aspect of cellular control, namely the way whereby nitrogen and carbon metabolism are integrated and interact with each other to balance these elements in the cell.

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