

Regulated Nitrate Transport in the Cyanobacterium *Anacystis nidulans*

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Intracellular accumulation of nitrate, indicative of the operation of an active nitrate transport system, has been measured in intact cells of the cyanobacterium *Anacystis nidulans*. The ability of the cells to accumulate nitrate was effectively hindered by either ammonium addition or selective inhibition of CO₂ fixation by DL-glyceraldehyde, with the effect of either compound being prevented by previously blocking ammonium assimilation. The results support the contention that nitrate utilization in cyanobacteria is regulated at the level of nitrate transport through the concerted action of ammonium assimilation and CO₂ fixation.

The entrance of nitrate into the cell is the initial step in nitrate utilization, followed by nitrate reduction to ammonium and by subsequent ammonium incorporation to carbon skeletons yielding amino acids. Although there is a general belief in the involvement of a nitrate transport system in nitrate utilization, little is known of the biochemistry of nitrate transport in photosynthetic microorganisms and higher plants (1, 5, 6, 18, 21). In cyanobacteria, nitrate uptake has been studied by monitoring the disappearance of the anion from the outer medium, and the process has been shown to exhibit saturation kinetics with K_s values for nitrate below 50 μM (3, 6, 12, 20, 22). Nevertheless, a study of the entrance step separately from the subsequent metabolism of nitrate is lacking so far. It is well established, however, that nitrate utilization in cyanobacteria is inhibited by ammonium and stimulated by CO₂, with assimilation of these compounds required for their effects to be manifested (6). The target of this control has been suggested to be the nitrate entrance step (4), as it seems to be for the diatom *Phaeodactylum tricornutum* (18). To date, the existence of a nitrate transport system and its involvement in the control of the overall nitrate utilization process in cyanobacteria have remained unproven, mainly because of the difficulties found in estimating intracellular nitrate levels. These obstacles have been now overcome by using an improved chromatographic analysis of nitrate by ion-exchange high-pressure liquid chromatography after separation of the cells by silicone oil centrifugation. We report here results indicating an endergonic accumulation of nitrate in *Anacystis nidulans* cells and, hence, the operation of an active nitrate transport system. The nitrate transport activity was sensitive to the regulation exerted by products of both ammonium and CO₂ assimilation, thus providing evidence that photosynthetic nitrate assimilation in cyanobacteria is regulated at the level of substrate supply to the cell.

MATERIALS AND METHODS

Chemicals. DL-Glyceraldehyde (DLG) and L-methionine DL-sulfoximine (MSX) were purchased from Sigma Chemical Co., St. Louis, Mo. Versilube F-50 was a product of Aldrich Chemical Co., Steinheim, Federal Republic of Germany, and silicone 14615 was from Janssen Chimica,

Beerse, Belgium. All other chemicals were products of E. Merck AG, Darmstadt, Federal Republic of Germany.

Organism and culture conditions. *Anacystis nidulans* (*Synechococcus leopoliensis* 1402-1, from Göttingen University, Göttingen, Federal Republic of Germany), was grown photoautotrophically as previously described (8). Cells with low levels of nitrate reductase activity (referred to as tungstate-treated cells) were obtained by growing the cells on NH₄⁺-containing medium, followed by transfer to a NO₃⁻-containing medium in which molybdate had been replaced by tungstate by the procedure of Herrero and Guerrero (9). Nitrate reductase activity was routinely measured in mixed alkyltrimethylammonium bromide-permeabilized cells as described previously (8). Chlorophyll *a* was determined after methanol extraction (11).

Analytical procedures. Intracellular nitrate was determined in acid lysates of cells subject to silicone oil centrifugation (7). The upper layer (0.3 ml) contained 25 mM Tricine-NaOH-KOH buffer, pH 8.3, 10 mM NaHCO₃, 50 μM KNO₃, and a cell number equivalent to 10 μg of chlorophyll *a*. The silicone layer (80 μl) was a 2:1 mixture of versilube F-50 and silicone 14615-3. The lower layer (20 μl) was a 2 M solution of H₃PO₄. Assays were initiated by nitrate addition and illumination (1,400 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation) and stopped by rapid centrifugation (10,000 $\times g$, 1.5 min). Nitrate was analyzed in samples of the lower layer by ion-exchange high-pressure liquid chromatography (19), except that the mobile phase was a 50 mM solution of H₃PO₄ containing 2% (vol/vol) of tetrahydrofuran adjusted to pH 1.9 with NaOH. Under these conditions, nitrite, if present, is oxidized to nitrate (19), thus interfering with nitrate estimation. This interference is avoided by sulfamic acid treatment (2). In the present case, no nitrite was present in either normal untreated or tungstate-treated *Anacystis* cells. The lower limit of detection of the analytical method was 20 pmol of nitrate in a 10- μl injection volume, and the values obtained were highly reproducible.

The values presented have been corrected for extracellular nitrate carried by the cells through the silicone barrier in samples centrifuged immediately after nitrate addition. Intracellular concentrations have been calculated for an aqueous internal volume for *A. nidulans* cells of 100 μl per mg of chlorophyll *a* (17). Free energy changes of nitrate transport have been calculated according to the following equation:

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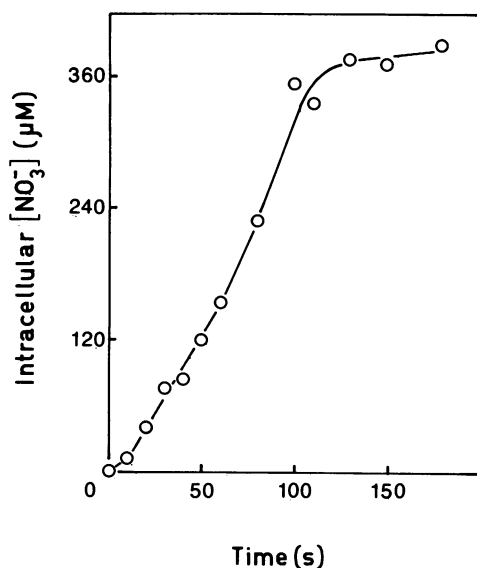


FIG. 1. Time course of intracellular nitrate accumulation in tungstate-treated cells of *A. nidulans*. Cells were incubated in the light in the presence of 50 μM KNO_3 . Nitrate estimated in extracts ranged from 30 pmol at time 0 to 213 pmol at 180 s in a 10- μl injection volume. Data are those of a representative experiment.

$\Delta G = RT \ln([\text{NO}_3^-]_i / [\text{NO}_3^-]_o) + ZF\Delta\Psi$, where R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the absolute temperature (298°K), $[\text{NO}_3^-]_i$ and $[\text{NO}_3^-]_o$ are, respectively, the internal and external concentrations of nitrate, Z is -1 , F is the Faraday constant ($96,487 \text{ J mol}^{-1} \text{ V}^{-1}$), and $\Delta\Psi$ is the membrane potential (in volts).

RESULTS AND DISCUSSION

The steady-state level of intracellular nitrate ultimately depends on the balance between the net rate of nitrate entrance and the velocity of its reduction by nitrate reductase. Internal nitrate concentrations of 20 to 30 μM have been estimated for intact nitrate-grown *A. nidulans* cells, which have a fully functional nitrate reductase, after incubation for 60 s in the presence of 50 μM nitrate. Intracellular nitrate accumulation is magnified under conditions that specifically lead to low levels of nitrate reductase activity. Molybdenum is a prosthetic group of cyanobacterial nitrate reductase with an essential role in catalysis (6, 13). Under conditions of molybdenum deprivation, tungsten can be incorporated into newly synthesized apoprotein in place of molybdenum, leading to the formation of an inactive nitrate reductase (9). In tungstate-treated *A. nidulans* cells, nitrate reductase activity was only 2 to 10% of that in normal nitrate-grown cells (data not shown). Concomitantly, intracellular nitrate levels after 60-s exposure to 50 μM nitrate ranged between 200 and 400 μM .

For a membrane potential of -100 mV , which can be considered an adequate value for illuminated *A. nidulans* under the conditions used here (-110 mV for illuminated cells at an external pH of 7.7 [14] and -95 mV for dark aerobic cells at an external pH of 8.3 [15]), the free energy changes of nitrate transport for maintenance of the intracellular nitrate concentrations found were positive (between $+13$ and $+14 \text{ kJ mol}^{-1}$ nitrate for tungstate-treated cells). Under conditions of coupled nitrate reduction, in which intracellular nitrate concentration was lower than that in the

outer medium, calculated free energy changes of nitrate accumulation are positive, even for normal cells ($+8 \text{ kJ mol}^{-1}$ nitrate). These results indicate the endergonic nature of nitrate entrance to *Anacystis* cells and, hence, the operation of an active nitrate transport system.

Figure 1 shows the time-dependent accumulation of nitrate in tungstate-treated cells. Intracellular nitrate concentrations rapidly increased with time to reach a plateau in about 2 min and, in some experiments, decreased slightly afterwards. The cessation of nitrate accumulation after a certain time suggests that accumulated nitrate (the product of transport) may induce a decrease in the rate of nitrate influx as the system approaches equilibrium or that thermodynamically favored nitrate efflux counteracts the nitrate transport activity.

A number of studies on nitrate uptake by cyanobacteria and other microalgae have established that the process is rapidly and effectively inhibited by the presence of ammonium in the outer medium, with the inhibition mediated by ammonium assimilation products (6, 18, 21). In addition, nitrate utilization by cyanobacteria exhibits a tight dependence upon the rate of CO_2 fixation (4, 10) and is inhibited by treatment of the cells with DLG, a selective inhibitor of CO_2 fixation (16). The negative effect of ammonium and the positive effect of CO_2 on nitrate utilization appear to be related, since ammonium assimilation inhibitors, such as MSX or azaserine, release nitrate uptake from both the ammonium inhibition and the CO_2 requirement (4, 16). As nitrate reductase is not affected in the short term by either ammonium addition or CO_2 deprivation, the earlier nitrate entrance step has been proposed to be the target of the effects of CO_2 fixation and ammonium assimilation (4). We have tested this hypothesis in tungstate-treated *Anacystis* cells. Ammonium, present in the cell suspensions together with nitrate, completely prevented intracellular nitrate accumulation (Table 1). Inhibition of ammonium assimilation by treatment of the cells with MSX resulted in the abolition of the negative effect of ammonium on nitrate transport, with the intracellular nitrate level reaching values similar to those of cells not exposed to ammonium (Table 1). Inhibition of CO_2 fixation by treatment of the cells with DLG caused complete suppression of nitrate accumulation, which, again, was prevented by previously inhibiting ammonium assimilation with MSX (Table 1). These results strongly indicate that primary regulation of the overall process of nitrate utilization by products of both ammonium and CO_2 assimilation is exerted on the transport of nitrate into the cell.

A previously proposed model for the regulation of nitrate

TABLE 1. Inhibition of nitrate transport in *A. nidulans* by ammonium and DLG and its prevention by MSX^a

Supplement	Mean intracellular nitrate concn ^b (μM)
None	208
MSX	225
NH_4^+	0
MSX and NH_4^+	211
DLG	0
MSX and DLG	196

^a Assays were performed on tungstate-treated cells exposed for 60 s to 50 μM KNO_3 . $(\text{NH}_4)_2\text{SO}_4$ (0.5 mM) was added to the cell suspensions 5 min before KNO_3 was added. MSX or DLG was added by preincubating the cells with 1 mM MSX for 15 min in the light or with 30 mM DLG for 15 min in the dark. In cells treated with both compounds, MSX preceded DLG.

^b Of three independent experiments.

utilization (4) states that the process is permanently under the feedback control exerted by organic nitrogenous compounds resulting from nitrate assimilation via ammonium. The level of these inhibitory compounds would be determined, on the one hand, by the rate of their generation from ammonium and, on the other hand, by the rate of their removal through combination with carbon compounds arising from CO₂ fixation. The nature of the regulatory organic nitrogenous compounds remains to be established. The molecular mechanism by which these effectors, whatever they are, modify the activity of the nitrate transport system is also a challenging question, currently being addressed in our laboratory.

It is worth mentioning that no accumulation of nitrate could be detected in ammonium-grown cells (data not shown). This suggests that the nitrate transport system of *Anacystis* is subject to nutritional repression by ammonium, as are the enzymes of the nitrate-reducing system, nitrate reductase (8) and nitrite reductase (9).

In summary, present results provide evidence on the operativity of an energy-requiring nitrate transport system in cyanobacteria. The activity of the system is sensitive to the control by ammonium assimilation and CO₂ fixation previously shown to regulate the overall process of nitrate utilization. These findings reinforce the importance of metabolic regulation at the level of substrate supply to the cell, an idea that is gaining consideration among biochemists and cell physiologists. Further research is needed to characterize the nitrate transport system and the mechanism by which the activity of the nitrate translocator is controlled.

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