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PHOTOSYNTHETIC NATURE OF NITRATE UPTAKE AND REDUCTION IN THE CYANOBACTERIUM *ANACYSTIS NIDULANS*

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The photosynthetic nature of the initial stages of nitrate assimilation, namely, uptake and reduction of nitrate, has been investigated in cells of the cyanobacterium *Anacystis nidulans* treated with L-methionine DL-sulfoximine to prevent further assimilation of the ammonium resulting from nitrate reduction. The light-driven utilization of nitrate or nitrite by these cells results in ammonium release and is associated with concomitant oxygen evolution. Stoichiometry values of about 2 mol oxygen evolved per mol nitrate reduced to ammonium and 1.5 mol oxygen per mol nitrite have been determined in the presence of CO_2, as well as in its absence, with nitrate or nitrite as the only Hill reagent. This indicates that in *A. nidulans* water photolysis directly provides, without the need for carbon metabolites, the reducing power required for the in vivo reduction of nitrate and nitrite to ammonium, processes which are besides strongly inhibited when the operation of the photosynthetic noncyclic electron flow is blocked. Evidence indicating the participation of concentrative transport system(s) in the uptake of nitrate and nitrite by *A. nidulans* is also presented. The operation of these energy-requiring systems seems to account for the sensitivity to ATP-synthesis inhibitors exhibited by nitrate and nitrite utilization in L-methionine DL-sulfoximine-treated cells. The utilization of nitrate by *A. nidulans* cells, concomitant with oxygen evolution, can therefore be considered as a genuinely CO_2-independent photosynthetic process that makes direct use of photosynthetically generated assimilatory power.

Introduction

Nitrate is the major source of nitrogen for most photosynthetic organisms in their natural environment. Little information is available about the entrance of nitrate into the cell, the first event in nitrate assimilation, especially with regard to microorganisms [1,2]. Once nitrate enters the cell, and prior to its incorporation into organic nitrogen, it must be converted into ammonium. This represents an eight-electron reduction which proceeds in two steps, nitrate being first reduced to nitrite in a two-electron reaction catalyzed by nitrate reductase, and nitrite being then reduced to ammonia in a six-electron reaction catalyzed by nitrite reductase [2,3]. Although for green cells and tissues the photosynthetic nature of the latter reaction is at present universally accepted, the dependence upon photosynthesis of the uptake of nitrate and its reduction to nitrite, as well as the interconnection with CO_2 metabolism, still remain a matter of controversy [2–4].

Stimulation by light of nitrate utilization has

Abbreviations: Chl, chlorophyll; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Tricine, *N*-tris(hydroxymethyl)methylglycine.

been reported for a range of algal species, including representatives of the blue-green algae (cyanobacteria) [1,2]. The latter group of organisms represents a valuable material for in vivo studies of the relationships between photosynthesis and nitrate assimilation. Previous in vitro and in situ studies carried out with the blue-green alga *Anacystis nidulans* have shown that in this obligate photoautotroph both nitrate reductase and nitrite reductase are ferredoxin-dependent enzymes, which appear to be structurally and functionally linked to photosynthetic membranes [5,6].

Under normal physiological conditions, the ammonium resulting from nitrate reduction is promptly incorporated to carbon skeletons. The occurrence of such a tight coupling imposes a serious limitation to the performance of in vivo studies of the early steps of the nitrate assimilation process, namely, entrance and reduction of nitrate, separately from the subsequent metabolism of ammonium. We have recently reported [7] that the treatment of *A. nidulans* cells with the glutamine synthetase inactivator L-methionine DL-sulfoximine provides a slightly modified cell system where nitrate uptake and reduction remain fully active, ammonium assimilation being severely hampered, however.

The present report deals with the use of L-methionine DL-sulfoximine-treated *A. nidulans* cells for the study of in vivo nitrate utilization. The results indicate a CO_2-independent close relation of nitrate reduction to photosynthesis with regard to the generation of the reductant for the reactions involved. Besides, the data suggest the involvement in nitrate utilization of an active transport system for the entrance of nitrate into the cell.

Materials and Methods

Organism and culture conditions. A. nidulans (strain L 1402-1 from Göttingen University's Algal Culture Collection) was grown photoautotrophically at 39°C with nitrate as the nitrogen source on the medium previously described [8].

Experimental Procedures. Cells from 1- or 2-day-old cultures (density about 2–3 μl cells per ml) were harvested by filtration. After washing with 25 mM Tricine-NaOH buffer, pH 8.3, the cells were resuspended in the same buffer to a density of about 1 μl cells (7 μg Chl) per ml. Assays were carried out with continuous shaking under illumination (100 $W \cdot m^{-2}$, white light), at 40°C, in air-opened conical flasks or, where indicated, in closed Warburg vessels containing either CO_2-free or CO_2-enriched air as the gas phase; this was achieved by placing in the center well 20%KOH or 0.5 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.6, respectively. The experiments were started by the addition of KNO_3 or KNO_2 (0.1–0.7 mM, final concentration) to cell suspensions which had been preincubated for 10 min under the above conditions in the presence of 1 mM L-methionine DL-sulfoximine. Nitrate or nitrite disappearance or ammonium release was determined by estimating the concentration of the corresponding ion in the medium in aliquots of the cell suspension after rapid removal of the cells by filtration (Millipore HA 0.45 μm pore size filter). DCCD, DCMU and FCCP were prepared as ethanolic solutions. When these inhibitors were added to the cell suspensions, precautions were taken to ensure that the final ethanol concentration did not exceed 0.4% (v/v), a concentration which did not affect the processes under consideration.

Analytical methods. Nitrate was determined by optical absorption at 210 nm in acid solution [9]; nitrite was estimated by the method of Snell and Snell [10]; ammonium was determined with glutamate dehydrogenase [11]; and oxygen was estimated manometrically. Chlorophyll, protein and packed cell volume were determined as previously described [7]. 1 μl cells contained 7–8 μg Chl and 150–175 μg protein [7]. In situ nitrate reductase activity assays were carried out using toluene-treated cell suspensions as previously described [8].

Chemicals. Tricine, ADP, L-methionine DL-sulfoximine and L-glutamic dehydrogenase (type II, from bovine liver) were purchased from Sigma Chemical Co., St. Louis. FCCP and NADPH were from Boehringer, Mannheim, and DCMU from Serva, Heidelberg. Other chemicals were products of Merck, Darmstadt.

Results

Light-dependent reduction of nitrate to ammonium

Illuminated suspensions of L-methionine DL-

sulfoximine-treated *A. nidulans* cells take up nitrate (and nitrite) at substantial rates, 3–4-fold higher than those corresponding to nitrate-grown cells which have not been treated with this glutamine synthetase inactivator [7]. Contrary to the normal untreated cells, which do not release ammonium at detectable levels, amounts of ammonium corresponding to about 90% of that of the nitrate or nitrite taken up by the L-methionine DL-sulfoximine-treated cells accumulated in the outer medium (Fig. 1). In the absence of the nitrogenous substrates, only basal levels of ammonium were detected, indicating that the ammonium release does not result from the degradation of cellular organic nitrogenous compounds, but that it originates from the reduction to the end product, ammonium, of the nitrate and nitrite taken up by the cells. With nitrate as the substrate no nitrite could be detected in the outer medium, which indicates that nitrite reduction is not limiting and that the nitrate taken up is quantitatively reduced to the term of ammonium. The L-methionine DL-sulfoximine treatment of the cells thus provides a simplified experimental system amenable to the study of the early steps of nitrate utilization, namely, nitrate uptake and reduction, without further interference, caused by the resulting ammonium.

As is also the case for untreated cells, the utilization of both nitrate and nitrite by L-methionine DL-sulfoximine-treated *A. nidulans* is light dependent. Fig. 2 shows that either process proceeded steadily under illumination, being immediately halted upon switching off the light, and recovering its linear rate immediately after restoring the illumination. Addition of DCMU, an inhibitor of photosynthetic noncyclic electron flow, also resulted in a prompt effect, similar to that obtained by switching off the light, on nitrate and

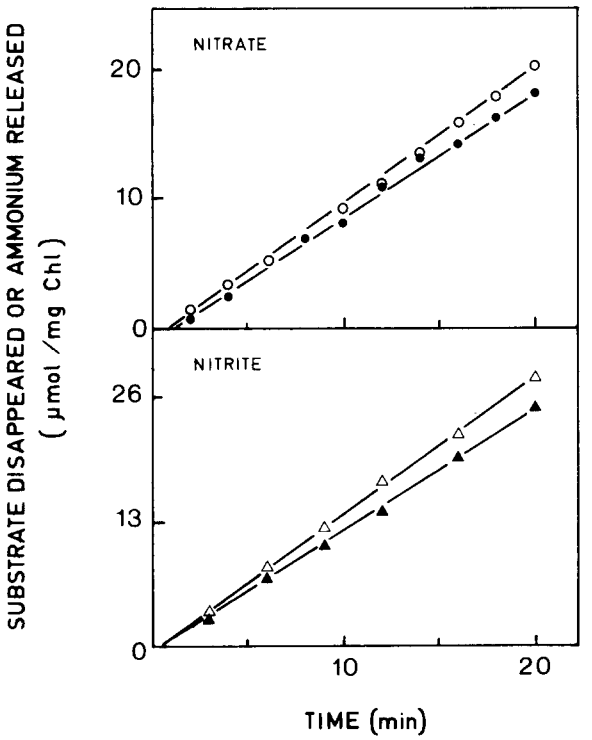

Fig. 1. Nitrate- and nitrite-dependent ammonium release by L-methionine DL-sulfoximine-treated *A. nidulans* cells. The experiments were carried out in the light under an atmosphere of air. After 10 min preincubation with 1 mM L-methionine DL-sulfoximine, the assays were started by addition of KNO_3 or KNO_2 to reach a final concentration of 0.25 mM. (○) Nitrate; (△), nitrite; (●, ▲), ammonium.

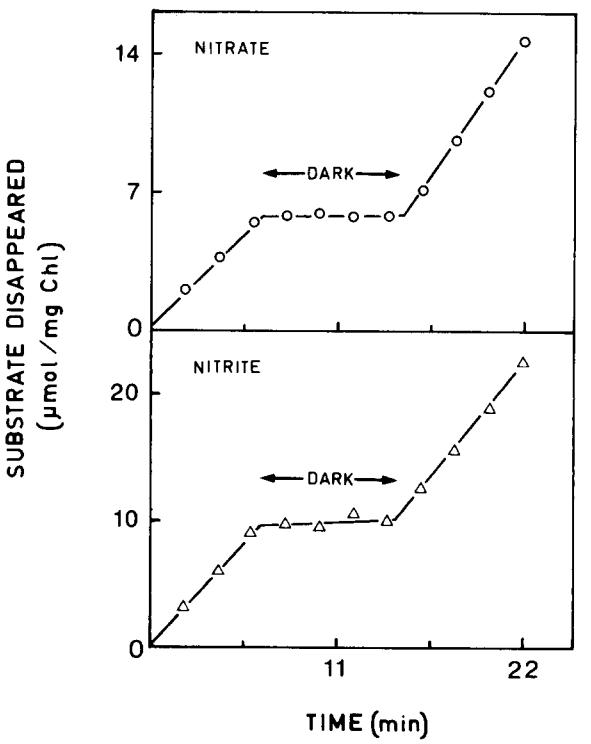

Fig. 2. Light requirement for nitrate and nitrite utilization by L-methionine DL-sulfoximine-treated *A. nidulans* cells. Conditions as in Fig. 1, except that the light was switched off during the indicated time period.

nitrite consumption, with immediate inhibition of either process and of the accompanying ammonium release. With 10 μM DCMU, consumption of nitrate and nitrite by *A. nidulans* cells was inhibited by 90 and 82%, respectively. The above results indicate that the in vivo conversion into ammonia of both nitrate and nitrite by *A. nidulans* are light-driven processes, which make use of photosynthetically generated reducing power for the corresponding reductive reactions to proceed.

Stoichiometric oxygen evolution coupled to nitrate reduction

A. nidulans cells evolved oxygen at greater rates during nitrate and nitrite utilization in the light than under otherwise analogous conditions in the absence of these anions. In the presence of CO_2 at saturating concentrations, values of oxygen-evolution rates in L-methionine DL-sulfoximine-treated cells are, as for untreated cells, in the range 350–400 μmol/mg Chl per h. The addition of either nitrate or nitrite to the cell suspensions resulted in increased rates, 25–50% over the above-mentioned figures. When the extent of the extra oxygen evolution was compared with that of nitrate consumption, stoichiometry values quite close or equal to 2 mol oxygen per mol nitrate were found (Fig. 3). Values for the stoichiometry with nitrite as the substrate were typically equal to 1.5 mol oxygen per mol nitrite (Fig. 3).

It is worth remembering that ammonium assimilation is severely inhibited in the L-methionine DL-sulfoximine-treated cells and that, accordingly, ammonium is found to accumulate in the outer medium in amounts very close to those of nitrate or nitrite taken up by the cells. The stoichiometry values found for the L-methionine DL-sulfoximine-treated cells can thus be considered to correspond solely to the photoreduction to ammonium of nitrate (eight electrons) or nitrite (six electrons), water acting as the ultimate electron donor for these reductive reactions.

Contrary to the case with normal untreated cells, L-methionine DL-sulfoximine-treated *A. nidulans* cells are able to utilize nitrate or nitrite under conditions in which the presence of CO_2 is excluded [12]. As the results in Fig. 4 show, when L-methionine DL-sulfoximine-treated *A. nidulans* cell suspensions placed in a CO_2-free atmosphere

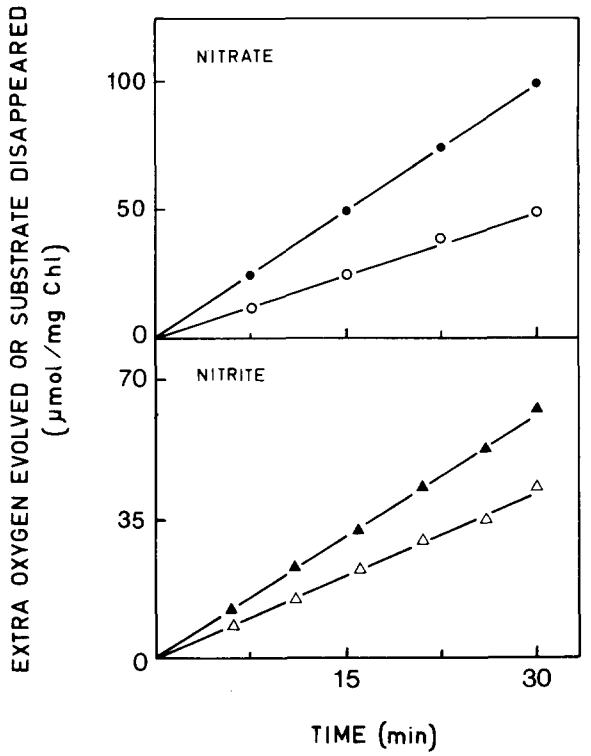

Fig. 3. Stoichiometric oxygen evolution associated with nitrate and nitrite utilization by L-methionine DL-sulfoximine-treated *A. nidulans* cells in the presence of CO_2. The experiments were carried out in Warburg vessels, in the light and under CO_2-enriched air (see Material and Methods). Values of extra oxygen evolution (closed symbols) have been calculated by subtracting the amounts of oxygen evolved in the control vessels without any nitrogenous substrate from the values recorded for the vessels containing added nitrate or nitrite. Initial concentration of KNO_3 or KNO_2 was 0.5 mM. Open symbols correspond to the consumption of nitrate (○) or nitrite (△).

and in the absence of any other added electron acceptor were illuminated, no oxygen evolution took place (except for a basal release of oxygen which was observed during the first 10–20 min and stopped thereafter). The addition of nitrate at this stage resulted, however, in an immediate evolution of oxygen at a considerable rate. Analogous results were obtained upon nitrite addition (not shown). These results demonstrate the occurrence of photosynthetic reduction of nitrate or nitrite in the absence of any other added electron acceptor, i.e., with either of these nitrogenous compounds representing the only Hill reagent.

Also, in the absence of CO_2 the existence of a

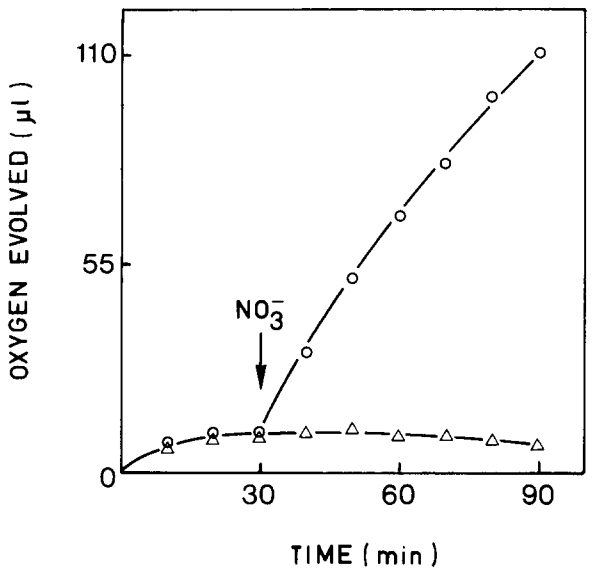

Fig. 4. Nitrate-dependent oxygen evolution by L-methionine DL-sulfoximine-treated *A. nidulans* cells in the absence of CO_2. The experiments were carried out under CO_2-free air in Warburg vessels containing KOH in the center well. Suspensions containing 7.5 μl cells (55 μg Chl) in 2.5 ml buffer supplemented with 1 mM L-methionine DL-sulfoximine were incubated in the light. At the time indicated by the arrow, 5 μmol KNO_3 were added from the side arm to one of the cell suspensions (○) but not to the control vessel (△).

TABLE I

STOICHIOMETRY BETWEEN OXYGEN EVOLUTION AND NITRATE OR NITRITE REDUCTION TO AMMONIUM BY L-METHIONINE DL-SULFOXIMINE-TREATED *A. NIDULANS* CELLS, IN THE ABSENCE OF CO_2

Conditions were as in Fig. 4, except that the amounts of KNO_3 or KNO_2 added were as indicated. Values of total oxygen evolved were corrected with those of appropriate controls lacking the nitrogenous substrate.

Substrate added (μmol)	Oxygen evolved (μmol)	O_2/NO_3^-	O_2/NO_2^-
Nitrate			
0.5	0.91	1.83	–
1.0	1.95	1.95	–
1.5	2.98	1.99	–
Nitrite			
0.5	0.78	–	1.56
1.0	1.50	–	1.50
1.5	2.29	–	1.53

stoichiometric relationship between oxygen evolution and the utilization of nitrate or nitrite was evident, with about 2 mol oxygen being evolved per mol of nitrate used up by the cells and about 1.5 for the case of nitrite. This is illustrated by the results in Table I, summarizing a series of experiments analogous to those in Fig. 4, but in which known limiting amounts of nitrate or nitrite were added, the corresponding concomitant oxygen evolution being followed until it ceased as a result of the exhaustion of the added nitrate or nitrite.

Active transport into the cell of nitrate and nitrite

The reductive reactions involved in the conversion of nitrate, via nitrite, to ammonium do not exhibit any requirement for ATP. Both reactions are of exergonic nature and they freely proceed in the presence of the corresponding enzymes and reductants, without any apparent requirement for metabolic energy. This applies to a variety of in vitro and in situ systems [2,3], including purified enzymes and membrane preparations of *A. nidulans* [5,6]. Nevertheless, the utilization of either nitrate or nitrite by L-methionine DL-sulfoximine-treated *A. nidulans* cells was effectively hindered by low concentrations (10 μM) of the uncoupler FCCP or of the ATPase inhibitor DCCD (Fig. 5). These compounds are effective inhibitors of ATP-

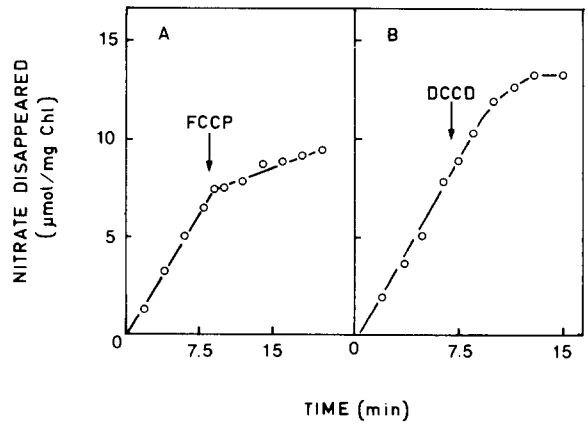

Fig. 5. Time course of the inhibition by FCCP and DCCD of nitrate utilization in L-methionine DL-sulfoximine-treated *A. nidulans* cells. At the times indicated by the arrows, FCCP (A) or DCCD (B) to reach a final concentration of 10 μM was added to L-methionine DL-sulfoximine-treated cell suspensions actively using nitrate. Other conditions as in Fig. 1.

requiring reactions in *Anacystis* and, at the concentrations used, caused complete inhibition of the photosynthetic fixation of CO_2, a process that was routinously estimated as the CO_2-dependent oxygen evolution in the light.

Inhibition by FCCP of nitrate and nitrite utilization was immediate (Fig. 5A), with 10 μM FCCP inhibiting nitrate consumption by 80–90%. Nitrite consumption was slightly less sensitive to this inhibitor, with inhibitions ranging from 70 to 75% for 10 μM FCCP. FCCP inhibited somewhat nitrate reductase activity (30–70%). Nevertheless, no apparent correspondence could be found between the extent of the inhibition of nitrate reductase and that of nitrate uptake, indicating that the effect of FCCP on nitrate uptake cannot be accounted for solely by its inhibitory effect on nitrate reductase activity. FCCP also caused an inhibition of the operation of the photosynthetic electron flow, although this is an effect which develops slowly with time and is by far not as prompt as the inhibition caused on nitrate or nitrite uptake.

DCCD at 10 μM concentration caused absolute cessation of nitrate uptake, nitrite uptake being inhibited severely although not completely (85–90%) by the same concentration of the inhibitor. The inhibitory effect of DCCD was not expressed immediately after its addition to the cell suspension, but took about 5 min to develop (Fig. 5B). Neither the enzymes of the nitrate-reducing system nor the photosynthetic electron flow, the operation of both of which is essential for nitrate reduction, were significantly affected by the action of DCCD.

From the results of the above experiments with FCCP and DCCD it becomes evident that the utilization of nitrite by L-methionine DL-sulfoximine-treated *A. nidulans* cells is somewhat less sensitive than that of nitrate to the action of these inhibitors. This differential sensitivity is even more evident in cells not subjected to treatment with L-methionine DL-sulfoximine, as shown by the results in Fig. 6, corresponding to experiments in which the effect of incubating normal cells with increasing concentrations of DCCD on the utilization of both nitrate and nitrite was tested. DCCD at concentrations lower than 10 μM caused similar inhibition of both nitrate and nitrite utilization.

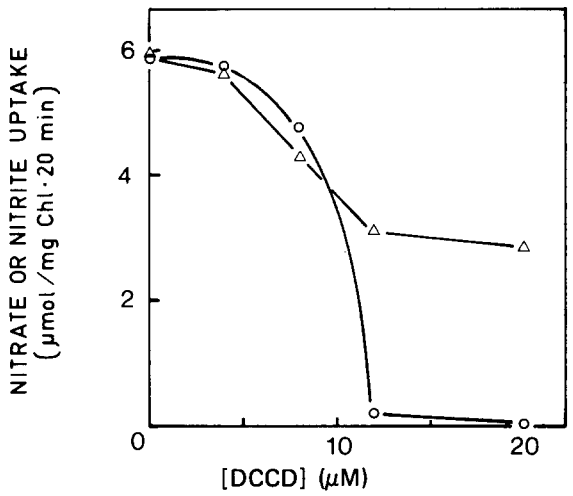

Fig. 6. Effect of the concentration of DCCD on the utilization of nitrate and nitrite by *A. nidulans* cells. Cell suspensions containing 16 μg Chl per ml of 25 mM Tricine-NaOH buffer, pH 8.3, were preincubated for 10 min in the light, at 40°C, with DCCD at the concentrations indicated prior to the addition of nitrate (○) or nitrite (△).

Increasing the concentration of DCCD above 10 μM resulted in full inhibition of nitrate uptake whereas that of nitrite was inhibited only by 50% (Fig. 6). The extent of the inhibition of nitrite uptake did not increase even if the concentration of DCCD was raised to 100 μM. It is worth noting that at DCCD concentrations of 20 μM and higher, all of the nitrite taken up by the cells was reduced and could be quantitatively found in the outer medium as ammonium, as the latter compound could not be assimilated by the cells, most probably because of the unavailability of ATP for the operation of glutamine synthetase.

The lower sensitivity of nitrite utilization, as compared to that of nitrate, to the action of the ATP-synthesis inhibitors could be accounted for by the occurrence of a passive influx of nitrous acid ($pK_a = 3.4$) into the cell simultaneous to an ATP-requiring active transport of NO_2^-.

The participation of a nitrate-transport system acting prior to the nitrate reduction step is also sustained by another piece of evidence. The rate of nitrate utilization by *A. nidulans* cells stays constant at different values of nitrate concentration within the range 0.01–1 mM, as shown by estimations of the nitrate uptake rate (measured as ammonium release) of L-methionine DL-sulfoximine-

treated cells at different nitrate concentrations (results not shown) and by experiments in which the utilization of nitrate by the cells was followed until exhaustion of the substrate in the outer medium (Fig. 7A). These results indicate that the half-saturation constant for nitrate of the uptake process is below 10 μM. The K_m (NO_3^-) of *A. nidulans* nitrate reductase has been estimated from both in vitro and in situ assays to be 0.7 mM, however [13]. It thus seems that the high affinity for nitrate of nitrate utilization is not determined by nitrate reductase itself, as might happen if nitrate entry into the cell were by diffusion, and the participation of a high-affinity nitrate-transport system is therefore suggested to be involved in nitrate utilization by *A. nidulans* cells. Analogous considerations can be applied to the case of nitrite uptake,

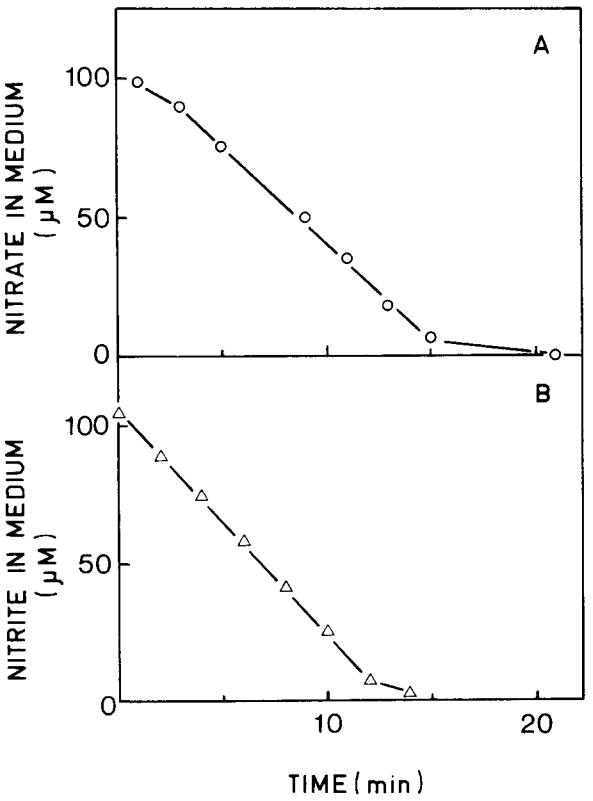

Fig. 7. Utilization of limiting amounts of nitrate and nitrite until their exhaustion in the outer medium by L-methionine DL-sulfoximine-treated *A. nidulans* cells. Conditions as in Fig. 1, except that the initial concentrations of KNO_3 and KNO_2 were as indicated. The suspensions contained 1 μl cells (7 μg Chl) per ml.

with an estimated half-saturation constant for nitrite also below 10 μM (Fig. 7B), whereas the K_m (NO_2^-) value of *A. nidulans* nitrite reductase is about 70 μM [5,14]. This also suggests the involvement of a high-affinity transport system in nitrite utilization.

Discussion

The strict requirement of an operative glutamine synthetase for ammonium assimilation in *A. nidulans* to proceed and the specific and effective inhibition of the enzyme caused by L-methionine DL-sulfoximine [15] are the essential facts that have enabled us to perform a basic characterization of the first stages of nitrate assimilation in whole cyanobacterial cells. Not only the constraints imposed by the simultaneous occurrence of the reactions involved in ammonium metabolism are avoided, but also the various regulatory antagonistic effects of ammonium on nitrate uptake and reduction are prevented in L-methionine DL-sulfoximine-treated cells [7,8].

The reported results clearly show the dependence upon light of nitrate and nitrite utilization in *A. nidulans*. Photosynthetically generated reducing power is used for the in vivo reduction of nitrate to the end product, ammonium, and oxygen is evolved in amounts stoichiometric to those of nitrate (or nitrite) reduced to ammonium. The obtained stoichiometry values, determined both in the presence and absence of CO_2, match those expected for the photosynthetic reduction of nitrate with electrons derived from water photolysis:

$$NO_3^- + H_2O \rightarrow NO_2^- + H_2O + 0.5O_2$$
$$NO_2^- + 3H_2O + 2H^+ \rightarrow NH_4^+ + 2H_2O + 1.5O_2$$
$$NO_3^- + 4H_2O + 2H^+ \rightarrow NH_4^+ + 3H_2O + 2O_2$$

The ability of the L-methionine DL-sulfoximine-treated cells to reduce nitrate with concomitant oxygen evolution in the absence of CO_2 unequivocally shows that CO_2 is actually not required for in vivo nitrate (and nitrite) reduction by *A. nidulans*. The compulsory requirement of recent products from CO_2 fixation as intermediates in nitrate reduction in photosynthetic cells and tissues – a view currently shared by many workers in the field of nitrate metabolism

[2,4] – clearly does not apply to the case of this cyanobacterium. Our evidence rather shows that in *A. nidulans* both nitrate and nitrite act in vivo as direct Hill reagents, with water as the terminal reductant.

These findings confirm for the case of intact cells the suggestions in previous reports about the photosynthetic nature of nitrate reduction in cyanobacteria. These include studies at the subcellular level [5,6] and experiments showing chlorophyll fluorescence quenching upon nitrate or nitrite addition to suspensions of slightly sonicated cells of filamentous cyanobacteria [16].

The close relationship between nitrate uptake and nitrate reduction has led to the suggestion that both processes are mechanistically linked, being functions of the same molecule, i.e., nitrate reductase [17]. The affinity for nitrate of the utilization process in L-methionine DL-sulfoximine-treated cells cannot, however, be just explained in terms of nitrate reductase itself being responsible for the entrance of nitrate into the cell. Furthermore, the uptake system involved exhibits a requirement for metabolic energy (as shown by the inhibitory effect of both FCCP and DCCD on nitrate utilization) which is not shared by the nitrate-reduction process, suggesting the involvement of an active transport system for the entrance of nitrate into the cell.

Considering together the anionic character of the substrate, the relatively low affinity of nitrate reductase for nitrate, and the amount of this enzyme in *Anacystis* [7], an active nature of the nitrate-transport system has again to be postulated in order to explain the observed rates for in vivo nitrate reduction at low nitrate concentrations in the outer medium. The intracellular nitrate level has therefore to be maintained against a large electrochemical gradient at the expense of metabolic energy. The existence of an active system for nitrate transport in *Anacystis* is in agreement with proposals of other workers about the participation of such systems in the uptake of nitrate by different algae [1,18–21], including filamentous cyanobacteria [22,23].

Presently available information does not allow one to draw conclusions about the mechanism of energy coupling to the transport process. The inhibition by DCCD of nitrate utilization indicates the participation of ATP in the link between energy metabolism and nitrate uptake. It remains, however, to be established whether nitrate transport is driven directly by an ATP-dependent pump [24] or by an ion or proton gradient built up through the action of an ATPase [25].

Arguments analogous to those considered above for nitrate transport can also be raised with regard to the uptake of nitrite, with the only exception being that in the latter system a passive component corresponding to the influx of HNO_2 into the cell [20] also has to be taken into account.

Acknowledgements

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