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Nitrite uptake and its regulation in the cyanobacterium *Anacystis nidulans*

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Two different components seem to participate in the uptake of nitrite by the cyanobacterium *Anacystis nidulans*, namely a transport system sensitive to *N,N'*-dicyclohexylcarbodiimide and a passive influx. The relative contribution of each component depended on the pH of the medium, that of the active system being prevalent at high pH values. The active transport of nitrite appears to be mediated by a high-affinity system, whereas the affinity for nitrite of the passive system is lower, similar to that of nitrite reductase. The utilization of nitrite was inhibited by products of the assimilation of ammonium via glutamine synthetase, apparently acting at the level of the active component involved in nitrite uptake.

Introduction

Most cyanobacteria are able to use nitrate, nitrite or ammonium as the sole nitrogen source for growth [1]. Whereas several studies concerning the uptake of nitrate [2–7] and ammonium [6,8–10] by different strains of cyanobacteria have appeared, the published information about nitrite uptake is scarce [4,11].

The proposal has been raised that two components, namely, active transport and passive diffusion, contribute to nitrite uptake in *Anacystis nidulans* [4]. In this report evidence is presented in support of the operation of both systems in nitrite acquisition by *A. nidulans* cells. Some features of the energy-requiring system have been characterized. The active component of nitrite uptake is regulated by ammonium and carbon dioxide availability, to which it responds in a similar way to that previously described for the nitrate uptake system [3,5].

Materials and Methods

Anacystis nidulans (strain L 1402-1 from the Göttingen University Algal Culture Collection; also known as *Synechococcus leopoliensis* or *Synechococcus elongatus* [12]) was grown photoautotrophically at 39°C with nitrate as the nitrogen source on a medium previously described [13]. Cellular chlorophyll was determined in methanolic extracts [14].

The cells were harvested and washed by filtration through Milipore filters (type HA-0.45 μm). When a treatment with DCCD or MSX was carried out before the uptake experiments, the cells were suspended in 30 mM Tricine-NaOH buffer (pH 8.1) (unless otherwise indicated) and incubated in air-opened conical flasks at 40°C in the light (100 $W \cdot m^{-2}$, white light). Nitrite uptake

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Mops, 4-morpholinepropanesulfonic acid; MSX, L-methionine-D,L-sulfoximine; MTA, mixed alkyltrimethylammonium bromide; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine.

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was studied with washed cells at 40°C in the light (as above) under the particular conditions described for each experiment. Nitrite uptake was followed as nitrite disappearance from the medium; the cells were removed by filtration and nitrite in the medium determined by the method of Snell and Snell [15]. Alternatively, aliquots of the cell suspension were directly added to the nitrite determination assay, removing the cell debris by low-speed centrifugation. Similar results were obtained with any of the two methods. Ammonium was determined in the filtrates with glutamate dehydrogenase [16].

The K_m (NO_2^-) of nitrite reductase was determined in cells made permeable to small molecules by the detergent mixed alkyltrimethylammonium bromide (MTA). *A. nidulans* cells (2 μg chlorophyll) were added to a reaction mixture containing in a final volume of 1 ml: MTA, 750 μg; Mops-NaOH buffer (pH 7.2), 25 μmol; methyl viologen, 5 μmol; 20 μmol of $Na_2S_2O_4$ in 0.1 ml of 0.3 M $NaHCO_3$; and KNO_2 at the indicated concentration. After incubation at 30°C for 5 min, nitrite was determined [15]. Activity units correspond to μmol of nitrite disappeared per min. (Mixed alkyltrimethylammonium bromide did not interfere with the assay for nitrite determination.)

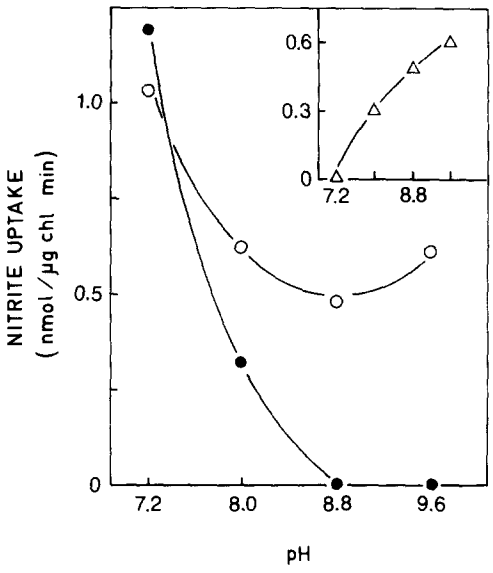

Fig. 1. Effect of extracellular pH on nitrite utilization by DCCD-treated *Anacystis nidulans* cells. The cells (8 μg chlorophyll/ml) were preincubated for 15 min in the light, at 40°C, suspended in 30 mM Tricine-NaOH buffer (pH 8.1) supplemented (●) or not (○) with 10 μM DCCD. After washing by filtration, the cells were suspended (8 μg chlorophyll/ml) in 10 mM Mops/10 mM Tricine/10 mM glycine-NaOH buffer of the indicated pH value and, after addition of nitrite (0.5 mM KNO_2, final concentration), nitrite utilization was assayed for 30 min. The insert shows the pH dependence of the DCCD-sensitive nitrite uptake (nitrite utilization by untreated cells minus nitrite utilization by DCCD-treated cells).

Results and Discussion

Two different components in nitrite uptake

Nitrate-grown cells of the cyanobacterium *Anacystis nidulans* exhibit high activities of nitrite utilization in the light, in the range of 1–3 nmol · μg^{-1} (chlorophyll) · min^{-1}. Nitrite uptake in *Anacystis* is sensitive to DCCD [4], an inhibitor of bacterial ATPases [17], suggesting the involvement in nitrite uptake of an active transport system and the participation of ATP in the link between energy metabolism and the uptake process [4]. The extent to which nitrite utilization was inhibited by DCCD-treatment of the cells depended on the pH of the medium. Treatment of the cells with DCCD did not affect nitrite utilization at neutral pH, whereas a negative effect was manifest at higher pH values, the degree of inhibition increasing as the pH raised (Fig. 1). Increasing the pH would reduce the concentration of available nitrous acid (pK_a NO_2^-/HNO_2, 3.4), which suggests that diffusion of uncharged nitrous acid might account for the passive nitrite influx. On the other hand, a DCCD-sensitive activity of nitrite uptake is manifest at the higher pH values tested. Consequently, DCCD can be used to evaluate the relative contribution of diffusion and active transport to the net nitrite uptake by *A. nidulans* cells. Interestingly enough, the pH dependence of the DCCD-sensitive uptake (Fig. 1, insert) is very similar to the pH dependence of nitrate uptake in *A. nidulans* [18], which takes place in an active way [4].

Under conditions in which active nitrite uptake is operative, *Anacystis* cells exhibit a high affinity for nitrite, the K_m (NO_2^-) being below 10 μM [4]. However, when contribution of the active component was low (pH 7.2), a biphasic pattern was apparent in the response of nitrite utilization by

whole *A. nidulans* cells to changes in nitrite concentration (Fig. 2). For the low concentration range, a K_m (NO_2^-) of about 6 μM (V_{max} about 0.6 nmol $NO_2^- \cdot \mu g^{-1}$ (chlorophyll) $\cdot$ min^{-1}) could be calculated, whereas for the higher nitrite concentration range, the calculated K_m (NO_2^-) was higher (about 450 μM), the V_{max} being about 3.5 nmol $NO_2^- \cdot \mu g^{-1}$ (chlorophyll) $\cdot$ min^{-1}. This can be interpreted in terms of the contribution of two systems with different affinities for the substrate to nitrite utilization by *Anacystis*. The high-affinity component might correspond to the active (DCCD-sensitive) system, scarcely active at pH 7.2, whereas the low-affinity component probably reflects passive influx of nitrous acid. As a matter of fact, the K_m (NO_2^-) of nitrite uptake by DCCD-treated cells was estimated to be 400 μM (Fig. 3).

Active uptake of nitrite by whole cells of *A. nidulans* may well be mediated by a permease with a high affinity for the substrate. On the other hand, under conditions in which nitrite uptake takes place mainly as a passive process, the affinity for nitrite exhibited by the cells might be determined by nitrite reductase, the first enzyme involved in the assimilation of nitrite. To test this

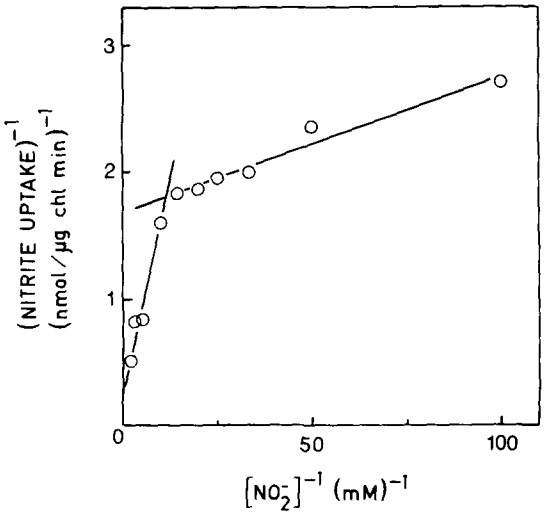

Fig. 2. Lineweaver-Burk plot of the effect of the concentration of nitrite on the rate of nitrite utilization by *A. nidulans* at pH 7.2. The assays were started by the addition of washed cells (3 μg chlorophyll/ml, final concentration) to solutions containing different concentrations of KNO_2 in 25 mM Mops-NaOH buffer (pH 7.2).

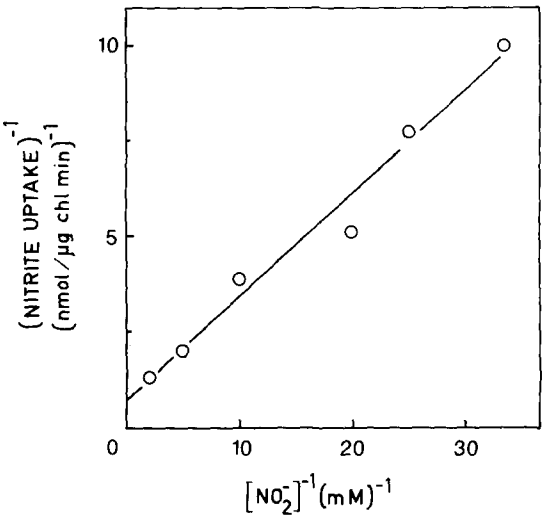

Fig. 3. Lineweaver-Burk plot of the effect of the concentration of nitrite on the rate of nitrite utilization at pH 7.2 by DCCD-treated *A. nidulans*. A cell suspension (10 μg chlorophyll/ml) in 30 mM Tricine-NaOH buffer (pH 8.1) was treated with 30 μM DCCD for 15 min. After washing, the cells were added (4 μg chlorophyll/ml, final concentration) to solutions containing different concentrations of KNO_2 in 25 mM Mops-NaOH buffer (pH 7.2), and the nitrite uptake rates determined.

possibility, the K_m (NO_2^-) of nitrite reductase has been estimated under in situ conditions, in *Anacystis* cells made permeable to small molecules by the detergent alkyltrimethylammonium bromide, and a value of about 120 μM was found (Fig. 4). This compares well to the K_m (NO_2^-) of intact untreated cells under conditions favoring passive uptake of nitrite (about 450 μM; Fig. 2) or to that of DCCD-treated cells (400 μM; Fig. 3).

Because cyanobacteria generally have been found in alkaline natural waters and exhibit optimal rates of growth and photosynthesis at an alkaline pH [19–22], the active transport of nitrite probably contributes significantly to nitrite-dependent growth, especially at the low concentrations of nitrite usually found in Nature [23] which are below the value of the K_m (NO_2^-) of the active system.

Regulation of nitrite uptake

Both ammonium and CO_2 have a prominent role in the regulation of nitrate utilization in *A. nidulans* [3,5,7]. The effect of these two com-

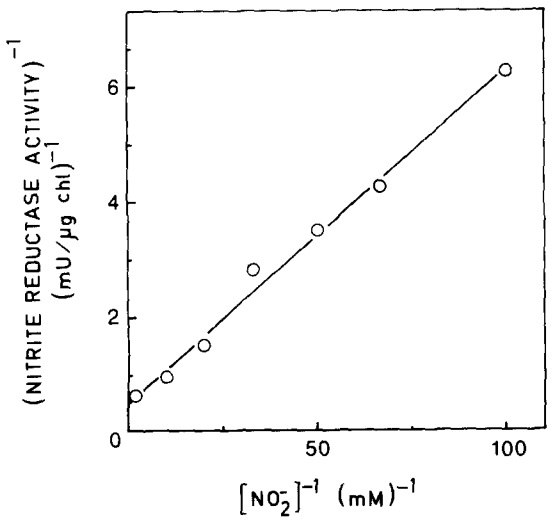

Fig. 4. Lineweaver-Burk plot of the effect of the concentration of nitrite on nitrite reductase activity of *A. nidulans* estimated in situ. See Materials and Methods for the experimental procedure.

pounds on nitrite uptake has been tested. Nitrite uptake was inhibited by ammonium and stimulated by CO_2, the magnitude of the effect of either compound being pH-dependent (Table I). Both the inhibition by ammonium and the positive effect of carbon dioxide on nitrite utilization were in fact more pronounced at the higher pH values tested (Table I), conditions under which the contribution to nitrite uptake of the active component is prevalent. The active component of nitrite uptake, rather than its passive counterpart or any intracellular component involved in nitrite assimilation, seems thus to be the target of the effects of ammonium and CO_2 on nitrite utilization.

The nitrite taken up in the absence of CO_2 was quantitatively recovered as ammonium in the outer medium (Table II). This can be expected from the requirement of CO_2-fixation products as substrates for ammonium assimilation, which is also evident for the uptake of exogenously supplied ammonium (Table II; see also Ref. 8).

The glutamine synthetase/glutamate synthase pathway is the main route for ammonium assimilation by the strain of *A. nidulans* used throughout this work [6], the incorporation of ammonium into carbon skeletons being drastically hampered [4,6] by L-methionine-D,L-sulfoximine (MSX), a gluta-

TABLE I

EFFECT OF AMMONIUM AND CARBON DIOXIDE ON NITRITE UTILIZATION BY *A. NIDULANS* AT DIFFERENT VALUES OF EXTRACELLULAR pH

Suspensions containing 7 μg chlorophyll per ml of 15 mM Tricine/15 mM glycine-NaOH buffer of the indicated pH were used. Experiment 1 was carried out in air-opened conical flasks and, where indicated, the suspensions were supplemented with 0.5 mM NH_4Cl. Experiment 2 was carried out in closed Warburg vessels, in which the cell suspension was placed in the main compartment, 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 assays were started by the addition of 0.5 mM KNO_2 (final concentration).

Expt.	pH	Nitrite taken up (nmol/μg chlorophyll per 30 min)		Ratio
1		$-NH_4^+$	$+NH_4^+$	$+NH_4^+/-NH_4^+$
	7.5	29.7	27.7	0.93
	8.5	12.1	9.9	0.81
	9.5	19.1	5.0	0.26
2		$+CO_2$	$-CO_2$	$+CO_2/-CO_2$
	7.5	34.7	20.6	1.69
	8.5	27.6	11.3	2.44
	9.5	26.3	8.3	3.17

TABLE II

EFFECT OF CARBON DIOXIDE ON NITRITE OR AMMONIUM UPTAKE AND ON NITRITE-DEPENDENT AMMONIUM RELEASE BY *A. NIDULANS*

The experiment was carried out in closed Warburg vessels containing in the main compartment cell suspensions (7 μg chlorophyll/ml) in 25 mM Tricine-NaOH buffer (pH 8.3). CO_2-free or CO_2-enriched air as the gas phase was achieved as described in Table I. The assays were started by the addition of 0.4 mM KNO_2 or NH_4Cl (final concentration). n.d., not determined.

Substrate added	Conditions	Substrate taken up (nmol/μg chlorophyll per 30 min)	Ammonium released (nmol/μg chlorophyll per 30 min)
Nitrite	$+CO_2$	27.6	0.0
	$-CO_2$	6.6	6.4
Ammonium	$+CO_2$	25.9	n.d.
	$-CO_2$	0.0	n.d.

mate analog that inactivates glutamine synthetase [24]. The inhibition by ammonium of nitrite utilization was prevented by treatment of the cells with L-methionine-D,L-sulfoximine (Table III). Thus, in analogy to what happens with nitrate uptake [3], ammonium must be assimilated via glutamine synthetase in order to inhibit nitrite uptake. Results in Table III also show that treatment of *Anacystis* cells with L-methionine-D,L-sulfoximine allowed increased rates of nitrite utilization. This may result from the prevention by MSX of the assimilation of the ammonium resulting from nitrite reduction. Thus, the regulatory system modulating nitrite uptake via the active transport system seems to involve products of the assimilation of ammonium via glutamine synthetase.

If the inhibitory metabolites resulting from ammonium assimilation specifically modulate the active component of nitrite uptake, L-methionine-D,L-sulfoximine should affect the active uptake of nitrite but not its passive utilization. As shown in Table IV, this was actually the case, since nitrite utilization by DCCD-treated cells was not affected at all by MSX, whereas MSX promoted an increase in nitrite uptake by untreated cells.

The results presented in this paper show that uptake of nitrite in *A. nidulans* is subject to regulation by a system similar to that modulating nitrate uptake [4,5,6], involving products of ammonium assimilation via glutamine synthetase as negative effectors. No direct evidence is available about the nature of the target of the regulation system in nitrate assimilation, although the nitrate transport system appears to be a good candidate [6]. The above results indicate that the control of nitrite utilization is exerted at the level of the active transport of nitrite. Analogously, the nitrate transport system might well be the target of the regulatory system modulating nitrate utilization in *Anacystis*.

TABLE III

RELEASE BY MSX OF THE AMMONIUM-PROMOTED INHIBITION OF NITRITE UTILIZATION IN *A. NIDULANS*

The cells (7 μg chlorophyll/ml) were preincubated for 15 min in the light, at 40°C, in 30 mM Tricine-NaOH buffer (pH 8.1) supplemented or not with 1 mM L-methionine-D,L-sulfoximine (MSX). After washing by filtration, the cells were resuspended (7 μg chlorophyll/ml) in 30 mM glycine-NaOH buffer (pH 9.6) and the assays were started by the addition of 0.5 mM KNO_2 and, where indicated, 0.5 mM NH_4Cl.

Treatment	Additions	Nitrite taken up (nmol/μg chlorophyll per 30 min)
None	NO_2^-	27.9
None	NO_2^-, NH_4^+	7.1
MSX	NO_2^-	45.7
MSX	NO_2^-, NH_4^+	42.9

TABLE IV

EFFECT OF DCCD AND MSX ON NITRITE UPTAKE BY *A. NIDULANS* AT pH 7.2

The assays were started by the addition of KNO_2 to cell suspensions (8 μg chlorophyll/ml 25 mM Mops-NaOH, pH 7.2) that had been preincubated for 15 min (at 40°C in the light) without any addition or in the presence of 20 μM DCCD, 1 mM L-methionine-D,L-sulfoximine (MSX) or 20 μM DCCD and 1 mM MSX. Neither MSX or DCCD interfered with the assay for nitrite determination.

Inhibitor added	Nitrite taken up (nmol/μg chlorophyll per 10 min)
None	10.1
MSX	17.5
DCCD	7.5
DCCD, MSX	7.5

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