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Nitrite uptake system in photosynthetic bacterium *Rhodopseudomonas capsulata* E1F1

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Nitrite was taken up by *Rhodopseudomonas capsulata* E1F1 cells by means of an inducible energy-dependent system distinguishable from the enzymatic reduction process. Bacterial cells grown on ammonia or yeast extract were unable to consume nitrite, although there was nitrite reductase activity. The nitrite uptake system required either nitrate or nitrite in the light to be induced, depended on protein synthesis de novo, and was repressed by ammonia. Nitrite uptake showed saturation kinetics lacking diffusion component with an apparent K_T lower than 1 μM and an activation energy of 1.75 kJ · mol^{-1}. 50 μM pHMB inhibited nitrite uptake, whereas nitrite reductase resulted unaffected. Uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone and dinitrophenol and the inhibitor of electron flow 2-thenoyltrifluoroacetone inhibited nitrite uptake without affecting enzymatic nitrite reduction. Nitrate completely suppressed nitrite uptake, whereas ammonia or L-methionine-DL-sulfoximine, which immediately stopped nitrate entrance, only inhibited partially nitrite consumption. On the basis of these and other published differences we conclude that in *Rps. capsulata* E1F1 nitrite is taken up by an inducible energy-dependent transport process different from the nitrate-transport system and distinguishable from enzymatic nitrite reduction.

Introduction

In Rhodospirillaceae, some few strains have been shown to assimilate nitrate and nitrite as sole nitrogen source [1–4], and the mechanisms by which these oxidized forms of nitrogen are translocated inside the cells, as a prerequisite to their reduction, are poorly understood.

In non-vacuolate microorganisms, like photosynthetic bacteria, nitrate or nitrite transport and reduction are tightly coupled processes not easily separable, although a distinction can be established between these two processes involved in the uptake system of both anions.

Rhodopseudomonas capsulata E1F1 assimilates nitrate and nitrite as sole nitrogen sources [3–5] and consumes nitrate by means of an uptake system energy-dependent and distinguishable from nitrate reductase [6–8], but nothing is known as how nitrite enters these bacterial cells.

In the present work, we describe, for the first time in an assimilatory purple bacteria, the nitrite uptake system as consisting of an energy-dependent transport process distinguishable from the following intracellular enzymatic nitrite reduction, and different from the bacterial nitrate active transport.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; pHMB, *p*-hydroxymercuribenzoate; TTFA, 2-thenoyltrifluoroacetone; RCV, see Ref. 9 and references therein.

Materials and Methods

Organism and culture conditions

Rps. capsulata E1F1 cells (kindly supplied by Prof. W.G. Zumft, Karlsruhe, F.R.G.) were grown phototrophically in the RCV medium [9] with DL-malate and KNO_3 (1 g/l) or KNO_2 (1 mM) as nitrogen source. In the experiments of transport-system induction, NH_4Cl or yeast extract both at concentrations of 1 g/l were used. Cultures were illuminated continuously with actinic light (4 $W \cdot m^{-2}$) at 30°C. Anaerobic conditions were achieved by filling up with culture medium 125 ml screw-capped bottles.

Experimental procedures

Cells were harvested after 24–30 h (A_{680} 0.8–1.2) by centrifugation at 20000 × *g*, 15 min, in a Beckman J2-21 refrigerated centrifuge, washed once with 50 mM Tris-HCl buffer (pH 7.5) and resuspended in RCV nitrogen-free medium. Nitrite, nitrate or ammonium disappearance was determined by estimating the concentration of the corresponding ion in the medium after removal of the cells by centrifugation at 11600 × *g*, 1 min, in a Beckman Microfuge 11.

Enzyme assay and analytical methods

Nitrite reductase (EC 1.7.99.3) activity was determined in whole cells as previously described [10].

Nitrate was measured spectrophotometrically at 210 nm [11], nitrite at 540 nm as described by Snell and Snell [12], and ammonium at 410 nm by the Conway microdiffusion technique [13]. Protein was determined by the Lowry procedure [14], after digesting cells with 2 M NaOH [15], using bovine serum albumin as standard.

Kinetic determinations

K_T was measured following analysis of progress curves of nitrite uptake from plots of integrated Michaelis-Menten equation by representing $t/\ln(S_0/S)$ vs. $(S_0 - S)/\ln(S_0/S)$, where S_0 is initial nitrite concentration and S nitrite concentration at time t. Aliquots were taken every 20 s after addition of variable amounts of nitrite to cells previously induced with nitrite. Lowest allowed concentration was 10 μM which permitted to calculate a reliable limit value of 1 μM for K_T.

Chemicals

Antimycin A, CCCP, DCCD, and TTFA were prepared as ethanolic solutions and used at final ethanol concentrations lower than 0.2%.

Antimycin A, bovine serum albumin, CCCP, chloramphenicol, dinitrophenol, pHMB, and TTFA were purchased from Sigma Chemical Co., St. Louis (U.S.A.), and DCCD was from Aldrich, Steinheim (F.R.G.). Other chemicals used were of the highest quality available.

Results

Induction of the nitrite uptake system

Nitrite uptake was detectable in bacterial cells grown on ammonia and transferred to nitrite-containing media after a lag period of 40 min, reaching maximal rates at about 2 h (Fig. 1). Likewise, the nitrite uptake system was induced after a similar period when cells were transferred to media with nitrate (not shown). When ammonia-grown cells were transferred to media containing nitrite and ammonia, nitrite was only consumed once external ammonia was exhausted, thus requiring a longer time for the nitrite uptake system to be induced. This induction was light-dependent, since in darkness consumption of nitrite was not observed. Likewise, induction of nitrite uptake was dependent on de novo protein synthesis, since nitrite was not taken up in the light when chloramphenicol was present. Under all the studied conditions and treatments nitrite reductase of whole cells was active over at least an 8 h period (not shown). The nitrate uptake system was similarly induced in the light after a lag period of 80 min incubation in media containing nitrate and devoid of ammonia (not shown).

Characterization of nitrite uptake system

The rate of nitrite uptake by *Rps. capsulata* E1F1 cells induced with nitrate or nitrite was higher in the light than in the dark (13.9 nmol nitrite consumed/min per mg protein under light-aerobic and anaerobic conditions vs. 9.4 under dark-aerobic conditions), and was negligible under dark-anaerobic conditions (0.7), which indicates

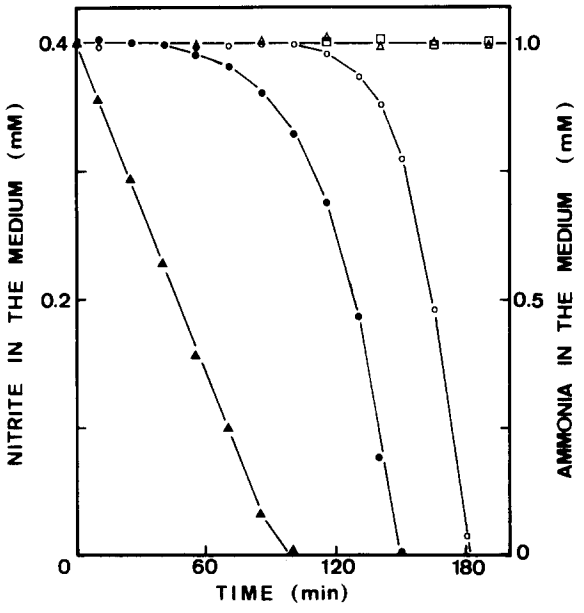

Fig. 1. Induction of the nitrite uptake system in *Rps. capsulata* E1F1. Cells (0.68 mg protein/ml) grown phototrophically on ammonia as nitrogen source were harvested, resuspended in RCV media with 0.4 mM nitrite and subjected to the treatments indicated in the figure. □, Dark conditions; △, chloramphenicol, 20 μg/ml; ○, ammonia, 1 mM; ●, control. Cells were allowed to stand in the light at 30°C, and at the indicated times nitrite was determined in aliquots from the different cultures. When ammonia was present, disappearance of ammonia was also estimated (▲).

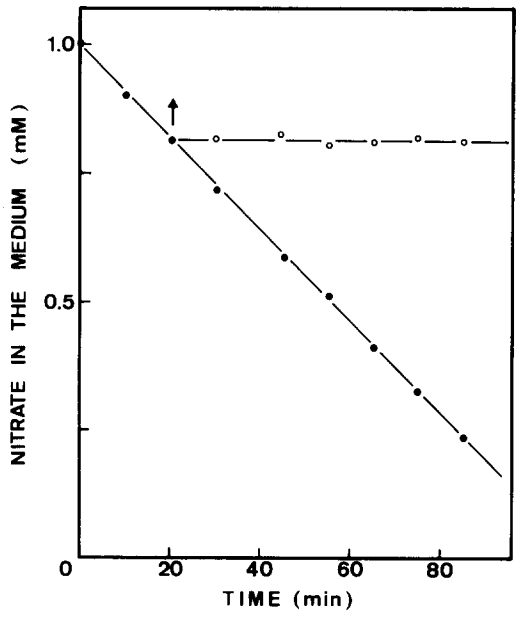

Fig. 2. Nitrate uptake dependence on anaerobic-light conditions in *Rps. capsulata* E1F1. Cells induced with nitrate were placed in RCV media containing 1 mM nitrate under light-anaerobic conditions. Where indicated by the arrow light was switched off and nitrate uptake was followed at different time intervals. ●, nitrate uptake in the light; ○, nitrate uptake in the dark.

an energy-dependent uptake system.

Nitrate uptake also was strictly light-dependent and, like nitrite uptake system, it was not operative under dark-anaerobic conditions (Fig. 2).

The nitrite uptake system displayed saturation kinetics, exhibiting a V_{max} of 13.9 nmol nitrite/min per mg protein and an apparent $K_T < 1$ μM, and lacking diffusion component (not shown).

The optimum pH for nitrite uptake was 6.5 (Fig. 3). The uptake rate was temperature-dependent, reaching a maximum at 40°C with an activation energy of 1.75 kJ · mol^{-1} and a Q_{10} value (20–30°C) of 1.02 (Fig. 4).

Various metabolic inhibitors which interrupt active transport were studied to determine whether or not nitrite uptake is an energy-requiring process in *Rps. capsulata* E1F1 under phototrophic conditions and the extent to which these metabolic poisons affected nitrite reductase activity (Table

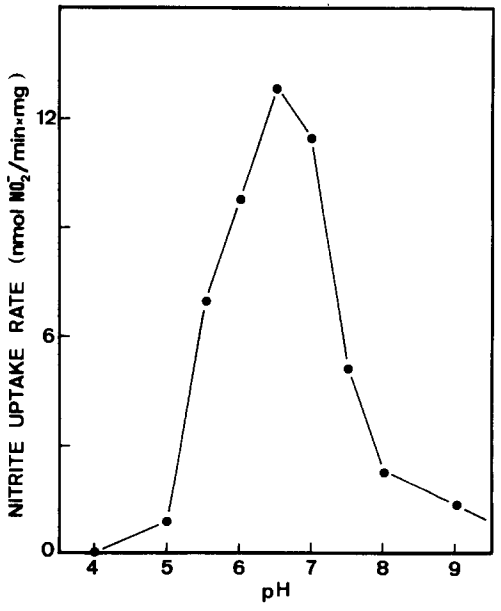

Fig. 3. Influence of pH on nitrite uptake rate in *Rps. capsulata* E1F1. Uptake rates were measured in cells resuspended in RCV media containing 0.4 mM nitrite at the pH values indicated in the figure under conditions described in Materials and Methods.

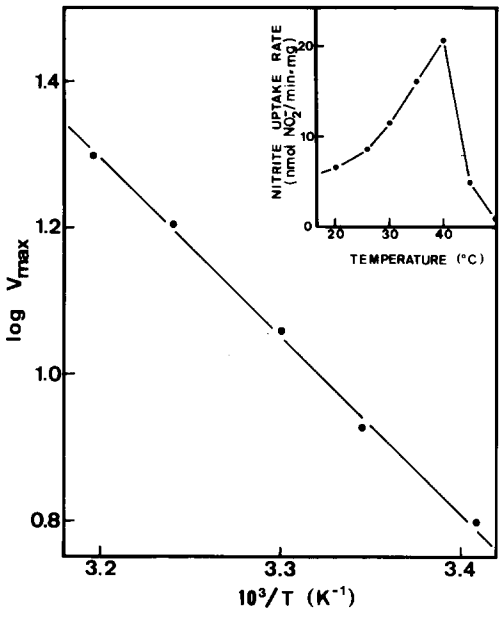

Fig. 4. Arrhenius' plot for nitrite uptake in *Rps. capsulata* E1F1. Experiments were performed with induced cells in RCV media with 0.4 mM nitrite at the temperatures indicates in the upper figure (inset). Activation energy was calculated from the slope of Arrhenius' representation.

TABLE I

EFFECT OF METABOLIC INHIBITORS ON NITRITE UPTAKE AND REDUCTION IN *RPS. CAPSULATA* E1F1

Inhibitors were added at the indicated concentrations to cells actively consuming nitrite in the light at a rate of 13.9 nmol/min per mg protein (100% nitrite uptake rate) and exhibiting a nitrite reductase activity of 16 mU/mg protein (100% nitrite reductase activity). Experiments were protracted during 45 min.

Inhibitor	Concentration (mM)	Nitrite uptake rate (%)	Nitrite reductase activity (%)
None	–	100	100
pHMB	0.05	0	95
CCCP	0.05	8	85
Dinitrophenol	0.25	100	90
	1	0	67
TTFA	0.1	0 [a]	90
Antimycin A	0.1	76	42
DCCD	0.1	85	84
NaCN	0.1	0	0

[a] Nitrite uptake decreased steadily, becoming 0 after 45 min.

I). The SH-groups reagent pHMB abolished nitrite consumption without affecting the enzymatic nitrite reduction activity. Similar effects were observed with the photophosphorylation uncouplers CCCP or dinitrophenol, although a higher concentration of the latter was needed to suppress the nitrite uptake. The inhibitor of electron flow TTFA also interrupted, though at a lower rate, nitrite uptake without changing nitrite reductase activity. In contrast, cyanide, DCCD, and antimycin A inhibited to a similar extent both nitrite uptake and reduction.

Effect of nitrate on nitrite uptake

Rps. capsulata E1F1 cells exhibited preferential assimilation of nitrate. In cells actively consuming nitrite nitrate addition prevented nitrite uptake without affecting at all nitrite reductase activity. Nitrite uptake recommenced only when most of the nitrate had been consumed (Fig. 5).

Effect of ammonia on nitrite and nitrate uptake

In contrast, ammonia was incapable of halting

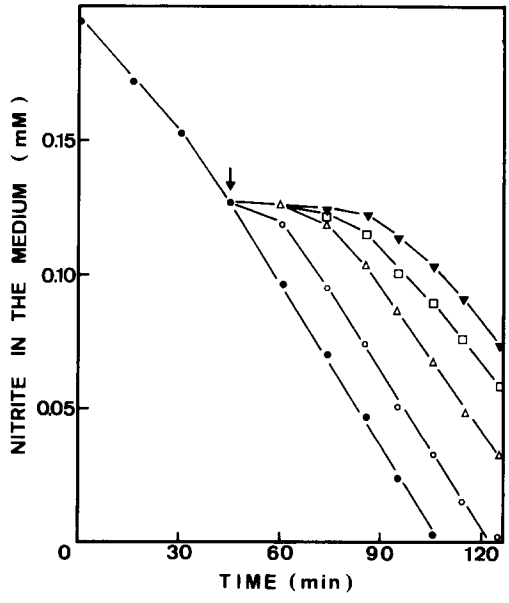

Fig. 5. Nitrate inhibition of nitrite uptake in *Rps. capsulata* E1F1. Different concentrations of nitrate were added to cells actively consuming nitrite (arrow) and, at the indicated times, nitrite disappearance was followed as described in Materials and Methods. ●, control; ○, +50 μM KNO_3; △, +150 μM KNO_3; □, +300 μM KNO_3; ▼, +500 μM KNO_3.

nitrite uptake. When ammonia was added at 1 mM concentration, the rate of nitrite uptake was only inhibited by 57% and both nitrite and ammonia were simultaneously consumed (Fig. 6). On the contrary, nitrate uptake was quickly abolished by addition of 1 mM ammonia (Fig. 7). Under these conditions both nitrate and nitrite reductases were full active along the experiment.

Effect of L-*methionine-*DL-*sulfoximine on nitrite and nitrate uptake*

In the presence of L-methionine-D,L-sulfoximine, a known inhibitor of the glutamine synthetase reaction, a decrease in the rate of nitrite uptake with a concomitant stoichiometric excretion of ammonia to the medium was observed (Fig. 8).

Addition of the inhibitor to *Rps. capsulata* E1F1 cells growing on nitrate blocked almost completely the nitrate uptake. Both nitrate and nitrite reductase activities resulted unaffected by the

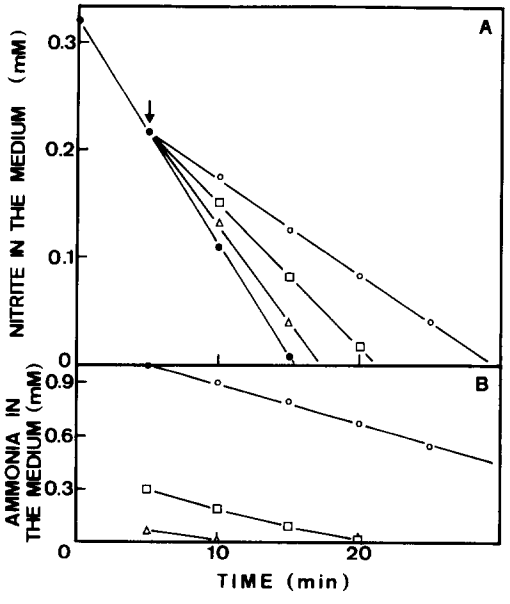

Fig. 6. Simultaneous nitrite and ammonia uptake by *Rps. capsulata* E1F1 cells. (A) Cells induced with nitrite were placed in RCV media containing 0.33 mM KNO_2 and at the time indicated by the arrow cultures were supplemented with ammonia 1 mM (○), 300 μM (□) and 50 μM (△); ●, control medium with nitrite alone. Nitrite was determined during 30 min at 5 min intervals. (B) When indicated, ammonia was determined. Symbols are as in (A).

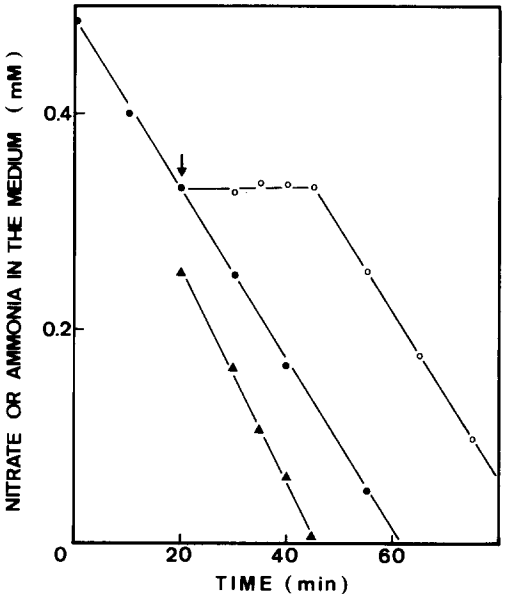

Fig. 7. Effect of ammonia on nitrate uptake in *Rps. capsulata* E1F1. Where indicated by the arrow ammonia 0.25 mM was added to cells actively consuming nitrate under light-anaerobic conditions. ●, nitrate uptake in the absence of ammonia; ○, nitrate uptake in the presence of ammonia; ▲, ammonia uptake.

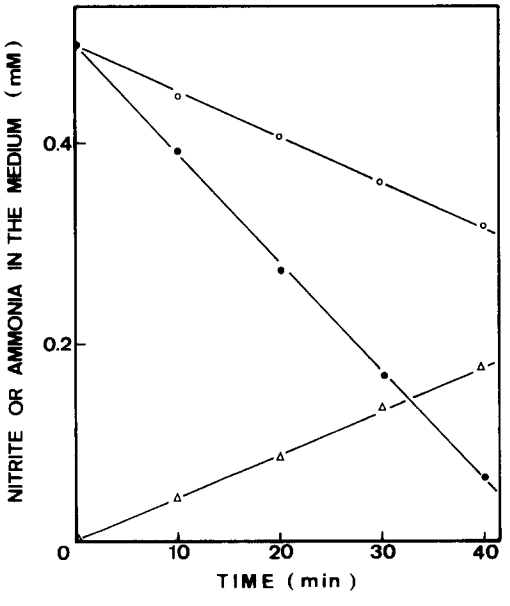

Fig. 8. Effect of L-methionine-DL-sulfoximine on nitrite uptake in *Rps. capsulata* E1F1. 1 mM L-methionine-DL-sulfoximine was added to cells induced with nitrite under light-anaerobic conditions. ●, nitrite uptake in the absence of L-methionine-DL-sulfoximine; ○, nitrite uptake in the presence of L-methionine-DL-sulfoximine; △, ammonia excreted to the medium in the presence of L-methionine-DL-sulfoximine.

L-methionine-DL-sulfoximine treatment (not shown).

Discussion

The presented results demonstrate that in *Rps. capsulata* E1F1 exists an inducible energy-dependent uptake system for nitrite with a transport component.

The nitrite uptake system showed an absolute dependence on nitrate or nitrite to be induced and in the absence of these nitrogen sources nitrite translocation into the cells did not occur even under conditions in which significant levels of nitrite reductase were detected. The development of the nitrite uptake system is dependent upon de novo protein synthesis with carbon coming from photosynthetic CO_2 fixation as revealed by the effect of chloramphenicol and dark conditions on the uptake induction (Fig. 1). A similar dependence on protein synthesis for nitrite and nitrate uptake has been found in *Neurospora crassa* [16,17] and algae [18–21].

Nitrite uptake is energy-dependent as indicated by its absolute requirement of light for induction (Fig. 1) and by the negligible rate of nitrite uptake under dark-anaerobic conditions in cells with the uptake system induced. Similar links between photosynthetic apparatus and the nitrite uptake have been described in cyanobacteria [22,23] and algae [21,24]. The high rate of nitrate uptake under dark-aerobic conditions corroborates the energy dependence of the process. This conclusion is strengthened by the inhibition exerted on nitrite uptake by various energy poisons. Uncouplers CCCP and dinitrophenol as well as the electron flow inhibitor TTFA prevented nitrite entrance without appreciably affecting nitrite reductase in whole cells, which stresses a requirement of metabolic energy for the uptake to proceed.

The nitrite uptake system of *Rps. capsulata* E1F1 lacks passive diffusion component as reported for the same system in *Phaseolus vulgaris* L. [25]. However, in *Anacystis nidulans* [23] and *Phaeodactylum tricornutum* [26] a passive component for diffusion of HNO_2 into the cells has been postulated.

Kinetic and thermodynamic characteristics of nitrite uptake and its enzymatic reduction by *Rps. capsulata* E1F1 cells allow us to establish a difference between both processes. K_T for nitrite ($< 1\ \mu M$) is of the same order of magnitude as those described for the uptake of nitrate in bacteria ($< 20\ \mu M$ in *Rps. capsulata* N22, Ref. 27; $7\ \mu M$ in *Pseudomonas fluorescens*, Ref. 28) and cyanobacteria (1–10 μM in *A. nidulans*, Ref. 29), and much lower than K_m for nitrite of nitrite reductase of *Rps. capsulata* E1F1 cells (80 μM, Ref. 5). Both uptake and reduction processes differ also in optimum pH and activation energy (6.5 and 1.75 $kJ \cdot mol^{-1}$, and 7.0 and 26.7 $kJ \cdot mol^{-1}$ for the uptake and enzymatic nitrite reduction, respectively). The Q_{10} value of 1.02 for nitrite uptake is characteristic of an enzyme energy-dependent process and differs from that of the nitrite reductase (1.43, Ref. 5).

Another distinction between both nitrite uptake and reduction can be deduced from their different sensitivity towards various energy poisons. pHMB blocked nitrite uptake at concentrations that did not affect nitrite reduction, which indicates that SH-groups can be involved in nitrite uptake. The same proposal has been made for nitrite and nitrate uptake in diatoms [20,30]. Uncouplers such as CCCP and dinitrophenol inhibited preferentially nitrite transport leaving practically unaffected nitrite reductase. Similar findings have been reported in cyanobacteria [23] and algae [20,21]. A sudden cessation of nitrate uptake in the presence of FCCP has been observed in *Rps. capsulata* N22 [27]. In *Rps. capsulata* kb1 an inhibition of photosynthetic electron transport by TTFA has been found in a process related to succinate reduction [31]. TTFA also inhibited nitrite uptake in *Rps. capsulata* E1F1 without diminishing nitrite reductase activity in whole cells, which links nitrite transport and photosynthesis in this bacterium. The effect of DCCD and antimycin A on nitrite uptake may reflect the inhibition exerted by these antibiotics on nitrite reductase and points to the possible participation in vivo of a *b*-type cytochrome in nitrite reduction [32]. Similar effects of DCCD on both nitrate and nitrite uptake in *A. nidulans* have been recently reported [23] and antimycin A has been found to inhibit nitrate uptake in *N. crassa* [17].

The presented results indicate also that nitrite uptake system is endowed with a transport compo-

nent. Throughout this work we use transport to denote the transfer of unmodified nitrite across the bacterial membrane as mediated by a saturable carrier whereas uptake has been referred to as two processes operating in tandem: nitrite transport followed by its intracellular metabolism [33]. In photosynthetic bacteria, transport and reduction of nitrite are two processes hard to separate, but still distinguishable. When bacterial cells grown on ammonia were transferred to nitrate or nitrite-containing media, nitrite uptake was induced de novo in the light after a 40 min period. Nitrite uptake was not immediately observed even though in whole cells nitrite reductase, glutamine synthetase and glutamate synthase were active [8], which indicates the existence of a transport process that needed to be induced for the uptake to take place.

The results of Fig. 5 clearly indicate also the existence of a transport process. Nitrate addition prevented nitrite uptake under conditions in which nitrite reductase, glutamine synthetase and glutamate synthase were active [8], which suggests that the nitrite transport component is the only process of the uptake affected by nitrate addition.

The entrance of nitrite in cells subjected to dark-aerobic conditions or in the presenceo f L-methionine-DL-sulfoximine (Fig. 8), clearly links nitrite uptake with nitrite reduction, since under these circumstances bacterial cells lack active glutamine synthetase. Thus, the pointed differences between uptake and nitrite reduction, namely light or oxygen dependence, the fact that some energy poisons block preferentially nitrite uptake, differences in optimum pH, activation energy and K_T, become strong support in favour of a energy-dependent transport process. Similar reasoning is applicable to nitrate uptake experiments presented here.

A last point to be discussed is whether or not nitrate and nitrite are translocated by the same transport system into *Rps. capsulata* E1F1 cells. Although the uptake of both nitrogen forms is energy dependent, several dissimilarities can be emphasized. Nitrate uptake occurred only in the light (Fig. 2) and in the absence of ammonia (Fig. 7), whereas nitrite was also consumed in the dark-aerobic conditions or in the presence of ammonia (Fig. 6). *Anabaena cylindrica* behaved much alike *Rps. capsulata* E1F1 [22]. However, in *Chlamydomonas* and *Phaeodactylum* ammonia inhibited both nitrate and nitrite uptake [18,20,21,24,26,34].

Other differences are the reciprocal behavior in respect to each other translocation and the effect of L-methionine-D,L-sulfoximine on uptake. Low nitrate concentrations reversibly suppressed nitrite uptake in *Rps. capsulata* E1F1 cells (Fig. 5), which seems to rule out an inhibition of the competitive type. In addition, L-methionine-D,L-sulfoximine blocked nitrate uptake whereas it only decreased the rate of nitrite consumption. Similarly, in *Chlamydomonas* L-methionine-D,L-sulfoximine has been reported to inhibit the uptake of nitrate, but not of nitrite [24], whereas in *A. nidulans* L-methionine-D,L-sulfoximine stimulated nitrate utilization [29].

On the basis of these dissimilarities we conclude that in *Rps. capsulata* E1F1 nitrite and nitrate are taken up by two different energy-dependent systems. The existence of separate transport systems for nitrate and nitrite has been indicated in *N. crassa* [16] and in *Phaseolus vulgaris* L. [25]. In *Phaeodactylum*, however, the uptakes of nitrate and nitrite have been cautiously described as showing many similarities and possibly occurring by the same mechanism [21].

Acknowledgements

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