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Nitrate Uptake in the Halotolerant Cyanobacterium *Aphanothece halophytica* is energy-dependent driven by ΔpH

Aran Incharoensakdi* and Surasak Laloknam

Department of Biochemistry and Program of Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

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The energetics of nitrate uptake by intact cells of the halotolerant cyanobacterium *Aphanothece halophytica* were investigated. Nitrate uptake was inhibited by various protonophores suggesting the coupling of nitrate uptake to the proton motive force. An artificially-generated pH gradient across the membrane (ΔpH) caused an increase of nitrate uptake. In contrast, the suppression of ΔpH resulted in a decrease of nitrate uptake. The increase of external pH also resulted in an enhancement of nitrate uptake. The generation of the electrical potential across the membrane (Δψ) resulted in no elevation of the rate of nitrate uptake. On the other hand, the valinomycin-mediated dissipation of Δψ caused no depression of the rate of nitrate uptake. Thus, it is unlikely that Δψ participated in the energization of the uptake of nitrate. However, Na^+-gradient across the membrane was suggested to play a role in nitrate uptake since monensin which collapses Na^+-gradient strongly inhibited nitrate uptake. Exogenously added glucose and lactate stimulated nitrate uptake in the starved cells. *N, N'*-dicyclohexylcarbodiimide, an inhibitor of ATPase, could also inhibit nitrate uptake suggesting that ATP hydrolysis was required for nitrate uptake. All these results indicate that nitrate uptake in *A. halophytica* is ATP-dependent, driven by ΔpH and Na^+-gradient.

Keywords: *Aphanothece halophytica*, Cyanobacteria, Nitrate uptake, ΔpH, Proton motive force

Introduction

Cyanobacteria are photoautotrophic organisms that perform oxygen-evolving photosynthesis typical of higher plant systems using carbon dioxide as carbon source. All cyanobacteria are capable of utilizing nitrate as their sole nitrogen source. However, cyanobacteria usually utilize reduced nitrogen sources such as ammonia and urea in preference to nitrate (Flores and Herrero, 1994). The active transport of nitrate is thought to be the rate-limiting step for nitrate assimilation. After entry into the cell, nitrate is reduced to ammonium by the sequential action of nitrate reductase and nitrite reductase before being fixed into amide nitrogen of glutamine.

Aphanothece halophytica is the halotolerant cyanobacterium able to grow under a wide range of NaCl concentrations up to 3 M (Takabe *et al*., 1988). The successful strategies used by *A. halophytica* to cope with high external salinity include the accumulation of glycine betaine (Incharoensakdi and Wutipraditkul, 1999), and the extrusion of Na^+ mediated by an Na^+/H^+ antiporter (Waditee *et al*., 2001). Carbon assimilation in *A. halophytica* also increased in response to salt stress (Takabe *et al*., 1988). On the other hand, nitrate assimilation with respect to the uptake of nitrate was recently shown to decrease in *A. halophytica* grown under salt-stress condition (Incharoensakdi and Wangsupa, 2003). In addition, it was also shown that nitrate uptake is Na^+-dependent in cells grown under both non-stress and salt-stress conditions.

Nitrate uptake has been studied extensively in plants (Crawford and Glass, 1998). Three kinetically distinct nitrate transport systems are found in plant roots, i. e. two high affinity transport systems (HATS), one is constitutive and the other is inducible, and the third is constitutive low affinity transport system (LATS). The energy for nitrate uptake in plants is provided by the proton gradient or the proton motive force (Crawford and Glass, 1998) and nitrate uptake is mediated by $2H^+/1NO_3^-$ symport mechanism (Meharg and Blatt, 1995; Miller and Smith, 1996). Only recently, nitrate uptake in the marine higher plant *Zostera marina* L. was shown to be mediated by a high affinity Na^+-symport system (Garcia-Sanchez *et al*., 2000). Until now the study on the nitrate uptake in cyanobacteria with regard to energy requirement has been very scarce. Most studies in cyanobacteria have dealt mainly with the characterization and regulation of genes for nitrate assimilation (Flores and Herrero, 1994).

In the present study we were able to demonstrate that

*To whom correspondence should be addressed.
Tel: 662-2185419; Fax: 662-2185418
E-mail: iaran@sc.chula.ac.th

nitrate uptake in *A. halophytica* was driven by ΔpH term of the proton motive force and that sodium motive force might also be involved in nitrate uptake.

Materials and Methods

Organism and culture *Aphanothece halophytica* was grown photoautotrophically in BG 11 medium supplemented with 18 mM $NaNO_3$ as described previously (Incharoensakdi and Waditee, 2000). A slight modification was made in which molybdenum in the medium was replaced with tungsten to induce the cells to synthesize nitrate reductase in an inactive form (Lara *et al*., 1987). Cells were grown in a 250-mL flask containing 100 mL medium on a rotary shaker at 30°C without CO_2 supplementation. Continuous illumination was provided by cool white fluorescent lamps at an irradiance of 60 $\mu Em^{-2}s^{-1}$. The concentration of NaCl in the medium was adjusted to 0.5 M.

Assay of nitrate uptake Log-phase cells were washed twice in 25 mM Hepes-KOH buffer, pH 8.2, containing 10 mM $NaHCO_3$ and 0.5 M sorbitol, and were suspended in the same buffer at a chlorophyll (Chl) concentration of 25 μg/mL determined as described by Mackinney (1941). The reaction was started by the addition of 100 μM $NaNO_3$ to the suspension and kept at 30°C in the light with an irradiance of 60 $\mu Em^{-2}s^{-1}$. Samples were removed at various time intervals and rapidly filtered through a 0.45 μm membrane filter. The nitrate content remaining in the filtrate was determined by anion-exchange high performance liquid chromatography (Hypersil-10 Sax column, 250 mm × 4.6 mm) (Incharoensakdi and Wangsupa, 2003).

EDTA-treated cells Prior to incubation with monensin or valinomycin, the cells were incubated 15 min at 30°C in 100 mM Tris-HCl pH 7.0, supplemented with 1 mM EDTA, and washed twice with the assay buffer in absence of EDTA. This treatment suppresses impermeability of cell membrane for these ionophores (Joshi *et al*., 1989).

Uptake assays in absence of potassium were performed using 100 mM Tris-maleate buffer for pH 5.5 and 100 mM Tris-HCl buffer for pH 7.0 or 8.2. Assays having potassium in the uptake medium were performed with 100 mM potassium phosphate buffer for the three pH values.

Artificial generation and suppression of pH gradient (ΔpH) and membrane potential (Δψ) The ΔpH was generated in acetate loaded cells (80 mM potassium acetate buffer at pH 5.5 or 7.0) (Allende *et al*., 2000), the supernatant was then removed and cells were diluted in 100 mM Tris-maleate buffer (pH 5.5) or Tris-HCl buffer (pH 7.0). ΔpH was generated at pH 8.2 in ethanolamine-HCl loaded cells instead of acetate loaded cells. The ΔpH was suppressed using 80 mM potassium acetate buffer at pH 5.5 and pH 7.0, 80 mM ethanolamine-HCl buffer at pH 8.2 (Booth, 1985; Allende *et al*., 2000).

The generation and suppression of Δψ were done according to Allende *et al*., (2000). The artificial Δψ was imposed using 20 mM KSCN or 3 μM valinomycin with EDTA-treated cells in absence of K^+ using 100 mM Tris-maleate buffer (pH 5.5) or 100 mM Tris-HCl buffer (pH 7.0 or 8.2). The Δψ term was suppressed using 3 μM valinomycin and EDTA-treated cells with 100 mM potassium phosphate buffer for the three pH values.

Results

Energy-dependent nitrate uptake To determine whether nitrate uptake was energy-dependent, the effects of some inhibitors on nitrate uptake were investigated. As shown in Table1 transport uncouplers such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2, 4-dinitrophenol (DNP), and gramicidin D, which dissipate proton motive force (Kroll and Booth, 1981), could effectively inhibit nitrate uptake to a similar extent by about 70-75%. Sodium azide, an inhibitor of cytochrome oxidase, which also dissipates proton motive force strongly inhibited the uptake. All these results indicate that nitrate uptake by *A. halophytica* was energized by proton motive force. Interestingly, monensin which collapses Na^+-electrochemical gradient also caused drastic inhibition of nitrate uptake. *N,N'*-dicyclohexylcarbodiimide (DCCD), an ATPase inhibitor, also inhibited nitrate uptake with strong inhibition being observed at 100 μM DCCD. This suggests the involvement of ATP hydrolysis in the uptake of nitrate.

Effect of different energy sources on nitrate uptake To assess the roles of ATP and proton motive force in energizing the uptake of nitrate, *A. halophytica* cells were starved to deplete endogenous energy sources. Nitrate uptake was then monitored after reenergization with glucose or lactate (Table 2). Both glucose and lactate could energize the uptake of nitrate in the starved cells. These results indicate that nitrate uptake is an energy-dependent process and depends on the

Table 1. Effect of metabolic inhibitors on nitrate uptake[a]

Inhibitor	Concentration	Nitrate uptake (%)
None		100
CCCP	20 μM	50
	40 μM	26
DNP	1 mM	60
	2 mM	28
Gramicidin D	10 μg/ml	34
	20 μg/ml	24
NaN_3	10 mM	50
	30 mM	16
Monensin	20 μM	46
	30 μM	25
DCCD	40 μM	60
	100 μM	35
KF	15 mM	101
	30 mM	100

[a]Cells were preincubated with inhibitors in the dark for 30 min before the addition of 100 μM $NaNO_3$ to initiate the uptake as described in Materials and Methods.

Table 2. Effect of energy sources on nitrate uptake[a]

Addition	Nitrate uptake (%)
None	100
Glucose (20 mM)	128
Lactate (10 mM)	116
KCN (20 mM)	24
Glucose + KCN	28
Lactate + KCN	33

[a]Cells were starved by suspending cells in the growth medium lacking carbon and nitrogen source in the dark for 24 h. The starved cells were then assayed for nitrate uptake in the presence of different energy sources or respiratory inhibitor. Starved cells were preincubated with the tested compound(s) in the dark for 30 min before the addition of 100 μM $NaNO_3$ to initiate the uptake as described in Materials and Methods.

proton motive force. The respiratory inhibitor, KCN, strongly inhibited nitrate uptake, either alone or together with glucose or lactate.

Role of ΔpH on nitrate uptake The proton motive force, which is known to energize a number of active transport processes, has two components namely the electrical potential (Δψ) and the chemical hydrogen ion concentration gradient (ΔpH) across the membrane. To test whether ΔpH played an energetic role in the uptake of nitrate, we artificially generated pH as well as suppressed ΔpH and followed the extent of nitrate uptake at three different pHs. At pH 5.5, ΔpH was generated by diluting acetate-loaded cells (pH 5.5) into a solution containing Tris-maleate as a less permeable anion (pH 5.5). This would cause the diffusion of acetate across the membrane in its protonated form. As shown in Fig. 1A, an increase in nitrate uptake by such treatment was clearly evident. In contrast, the suppression of ΔpH imposed with 80 mM potassium acetate buffer resulted in a reduction of nitrate uptake. Similar results were obtained when the experiments were done at pH 7.0 (Fig. 1B) and pH 8.2 (Fig. 1C).

Role of Δψ on nitrate uptake at different pHs To investigate whether another component of the proton motive force, Δψ, contributed the driving force for nitrate uptake, we artificially generated Δψ either by supplementation of KSCN or by valinomycin in the absence of K^+ and then monitored the uptake of nitrate. Table 3 shows that at the three pHs tested, the rates of nitrate uptake did not increase under the influence of Δψ by the addition of 20 mM KSCN to the medium (cell inside is more negative than the control). Indeed, a slight decrease of nitrate uptake was observed. Likewise, the generation of Δψ by an imposition of an outwardly-directed, valinomycin-mediated (in the absence of K^+) potassium diffusion gradient caused no increase of the rates of nitrate uptake at the three pHs. On the other hand, the dissipation of Δψ by the addition of valinomycin in the presence of K^+ resulted in a modest stimulation of the rates of nitrate uptake at the three pHs assayed (Table 3). Taken together, these results indicated that Δψ constituted little or no contribution for the energization of nitrate uptake in *A. halophytica*.

Effect of external pH on nitrate uptake The data presented above indicate that the main driving force for nitrate uptake was contributed by ΔpH. We therefore further tested the influence of external pH on nitrate uptake. Figure 2 shows the uptake of nitrate as a function of time under different pHs of the assay medium. The uptake of nitrate apparently increased with the increase of the external pH. Within 5 min nitrate uptake appeared to reach maximum for all pHs tested. The initial rates of nitrate uptake at pHs 5.5, 7.0, and 8.2 were calculated to be 0.63 ± 0.04, 0.84 ± 0.06, and 1.16 ± 0.08 $mmol \cdot min^{-1} \cdot mg^{-1}Chl$, respectively.

Discussion

The results shown in this study clearly indicated that there is energy-dependent nitrate uptake in *A. halophytica*. The energetic component for nitrate uptake was ascribed to the

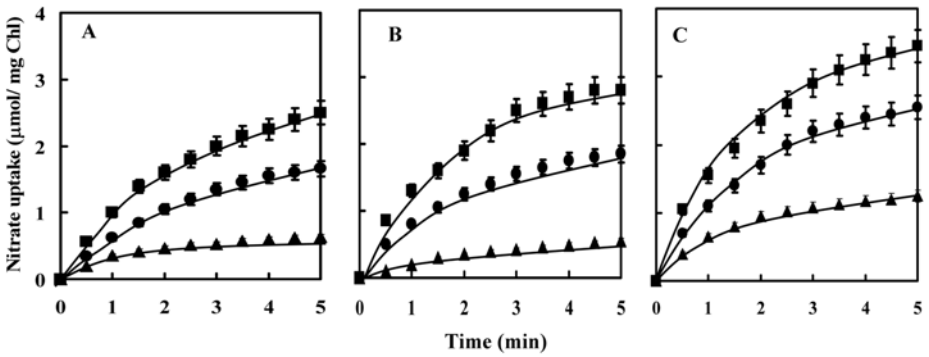

Fig. 1. Effect of ΔpH on nitrate uptake at three pH values of 5.5 (A), 7.0 (B) and 8.2 (C). Artificial generation and suppression of ΔpH were done as described in Materials and Methods. Symbols are ●: control, ■: ΔpH generation, ▲: ΔpH suppression. Data are means from three independent experiments with vertical bars representing standard errors of the means.

Table 3. Effect of Δψ on nitrate uptake at different pHs[a]

pH	Nitrate uptake (μmol · min^{-1} · mg^{-1} Chl)			Nitrate uptake (μmol · min^{-1} · mg^{-1} Chl)	
	Control	Δψ generated by		Control	Δψ dissipated by
		KSCN	Valinomycin (-K$^+$)		Valinomycin (+K$^+$)
5.5	0.79 ± 0.06	0.71 ± 0.05	0.78 ± 0.05	0.84 ± 0.06	0.92 ± 0.06
7.0	0.94 ± 0.07	0.83 ± 0.06	0.86 ± 0.06	0.90 ± 0.06	0.99 ± 0.07
8.2	1.12 ± 0.08	0.97 ± 0.07	0.99 ± 0.07	1.03 ± 0.07	1.14 ± 0.08

[a]For KSCN and valinomycin (-K$^+$) experiments, 100 mM Tris-maleate buffer was used at pH 5.5 and 100 mM Tris-HCl buffer was used at pH 7.0 and 8.2. For valinomycin (+K$^+$) experiments, 100 mM potassium phosphate buffer was used for the three pHs. Data are means ± standard errors of the means (n = 3).

contribution by the proton motive force (Δp) which consists of two components, i. e. the pH gradient (ΔpH) and the membrane potential (Δψ) with the relationship, $\Delta p = \Delta\psi - 59\Delta pH$, where ΔpH equals the pH_{out} minus pH_{in} (The value 59 is a combination of constants for expression of ΔpH in millivolts at 25°C). This conclusion is based on the results showing (i) inhibition of nitrate uptake by various protonophores (Table 1), (ii) an increased nitrate uptake when glucose or lactate was added as an energy source (Table 2).

The data from Fig. 1 show an increase of nitrate uptake upon artificially generating ΔpH. This increase was observed independent of external pH. On the other hand, the suppression of ΔpH always resulted in a significant decrease of nitrate uptake, and again was independent of external pH. This behavior is expected when the ΔpH term of the proton motive force is the driving force for nitrate uptake. In contrast to the complete abolition of 4-hydroxybenzoate uptake in *Klebsiella planticola* strain DSZ1 when ΔpH was suppressed (Allende *et al*., 2002), our results showed some nitrate uptake activity remaining when ΔpH was suppressed (Fig. 1). However, this nitrate uptake activity disappeared when ΔpH was suppressed in the presence of monensin (data not shown). Thus, it was suggested that Na^+-electrochemical gradient dissipated by monensin (Pressman, 1976) was involved in nitrate uptake. The dependence of nitrate uptake on Na^+ was previously reported in *A. halophytica* (Incharoensakdi and Wangsupa, 2003). The fact that the protonophores, CCCP, 2, 4-DNP and gramicidin D could not completely inhibit the uptake of nitrate (Table 1) also supports the notion that there exists another energetic component in addition to proton motive force, i. e. the so called sodium motive force which was previously suggested to play a role in nitrate uptake in *Anacystis nidulans* R2 (Rodriguez *et al*., 1992; Rodriguez *et al*., 1994). The contribution by either a proton or a sodium gradient to amino acid and glycine betaine transport has previously been shown in some bacteria (Ekiel *et al*., 1985; Proctor *et al*., 1997).

The overall results in Fig. 1 and Table 3 suggest that the uptake of nitrate by *A. halophytica* is driven by ΔpH. The contribution by Δψ was rather unlikely due to the findings that Δψ generated by either KSCN or valinomycin in the absence

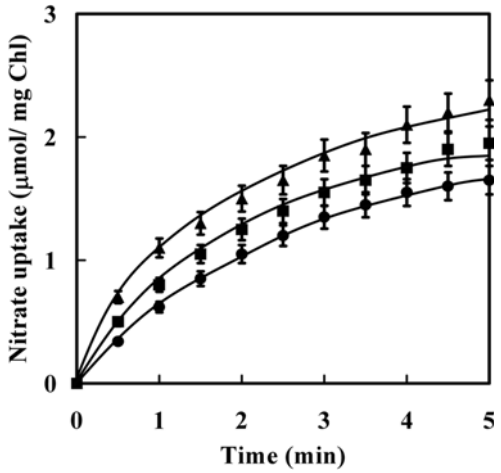

Fig. 2. Dependence of nitrate uptake on external pH. Nitrate uptake assay was done as described in Materials and Methods with the modification where 100 mM Tris-maleate buffer was used at pH 5.5 (●) and 100 mM Tris-HCl pH 7.0 (■) or pH 8.2 (▲). Data are means from three independent experiments with vertical bars representing standard errors of the means.

of K^+ had no stimulatory effect on the uptake of nitrate in acidic, neutral, and alkaline conditions (Table 3). Moreover, when Δψ was dissipated by valinomycin in the presence of K^+, no depression of nitrate uptake was observed (Table 3). The absence of an inhibitory effect by valinomycin suggests the absence of the contribution of Δψ to nitrate uptake. On the contrary, a slight stimulation of nitrate uptake occurred under the condition depleted of Δψ by valinomycin (+K$^+$) treatment (Table 3). This stimulation could occur due to (i) the action of Na^+-motive force (Na^+-gradient driven) since valinomycin specifically abolishes Δψ contributed by K^+ transport (Reed, 1979) and/or (ii) an increase of ΔpH term of the proton motive force is generated by a compensatory mechanism, i. e. an increase in ΔpH simultaneous to a decrease in Δψ (Reed, 1979; Kroll and Booth, 1981). Similar observations were reported for 4-hydroxybenzoate uptake in *Klebsiella planticola* whereby ΔpH was also implicated as the driving force (Allende *et al*., 2002). Previously, it was also shown in the cyanobacterium *Plectonema boryanum* that ΔpH is the

major energetic component with no involvement of $\Delta\psi$ for energy coupling in photosynthesis (Padan and Schuldiner, 1978).

Extracellular pH also affected the uptake of nitrate in *A. halophytica*. It was shown that raising the extracellular pH from 5.5 to 8.2 resulted in the enhancement of the rate of nitrate uptake (Fig. 2). The dependence of betaine uptake on the extracellular pH was previously reported in *Lactococcus lactis* (Molenaar *et al*., 1993). However, the increase of the extracellular pH resulted in the decrease of betaine uptake. This inverse relationship between extracellular pH and betaine uptake was proposed to be mediated by the intracellular K^+ concentration. It is intriguing to speculate that the intracellular Na^+ might mediate nitrate uptake in response to extracellular pH in *A. halophytica*. Previous work has shown that *A. halophytica* contains an Na^+/H^+ antiporter which can confer salt tolerance on the cells (Waditee et al., 2001; Waditee *et al*., 2002). Apart from protecting cells against salt stress, Na^+/H^+ antiporter can also regulate intracellular H^+ levels (Padan and Schuldiner, 1996). During the generation of a pH gradient across the membrane, an increase in the extracellular pH would lead to a decrease in the pH gradient and consequently the intracellular Na^+ might also decrease. The observed increase of nitrate uptake at increasing extracellular pH (Fig. 2) might be accounted for by an increase of Na^+-gradient mediated by Na^+/H^+ antiporter. Indeed, the activity of Na^+/H^+ antiporter in *A. halophytica* was shown to increase with increasing pH (Waditee *et al*., 2001). Moreover, we also observed a reduction of nitrate uptake *in A. halophytica* in the presence of amiloride, an inhibitor of Na^+/H^+ antiporter (Mochizuki-Oda and Oosawa, 1985) (data not shown). Thus, the involvement of Na^+/H^+ antiporter in the uptake of nitrate cannot be precluded. That the role of Na^+/H^+ antiporter in the generation of sodium motive force to power Na^+/ solute symport has previously been proposed for the transport of anions across the membranes (Krulwich and Guffanti, 1989; Espie and Kandasamy, 1994).

The fact that DCCD, the ATPase inhibitor, and the ionophores, CCCP, 2, 4-DNP, and gramicidin D strongly inhibited the uptake of nitrate (Table 1) highly suggests that the energy needed is provided by electron transport in the cytoplasmic membrane through a proton motive force, with the participation of ATP hydrolysis. Previous studies have shown that an ATP-binding cassette (ABC)-type system with the two ATP-binding proteins is involved in nitrate uptake in the cyanobacterium *Synechococcus* sp. PCC7942 (Omata, 1995). Further work on the characterization of proteins responsible for nitrate uptake in *A. halophytica* is needed for a better understanding of nitrate uptake in cyanobacteria.

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