Nitrate Uptake in the Halotolerant Cyanobacterium
Aphanthece halophytica is energy-dependent driven by ∆pH

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The energetics of nitrate uptake by intact cells of the halotolerant cyanobacterium Aphanthece 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 (∆pH) caused an increase of nitrate uptake. In contrast, the suppression of ∆pH 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 (∆ψ) resulted in no elevation of the rate of nitrate uptake. On the other hand, the valinomycin-mediated dissipation of ∆ψ caused no depression of the rate of nitrate uptake. 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 ∆pH and Na⁺-gradient.

Keywords: Aphanthece halophytica, Cyanobacteria, Nitrate uptake, ApH, Proton motive force

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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.

Aphanthece 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⁺/1NO₃⁻ 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...
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 photobiotrophically in BG 11 medium supplemented with 18 mM NaNO₃ 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 (Lana et al., 1987). Cells were grown in a 2.90-mL flask containing 100 mL medium on a rotary shaker at 30°C with an irradiance of 60 µmol quanta m⁻² s⁻¹. Cells were preincubated with inhibitors in the dark for 30 min before the addition of 100 µM NaNO₃ to initiate the uptake as described in Materials and Methods.

**Assay of nitrate uptake** Log-phase cells were washed twice in 25 mM Hepes-KOH buffer, pH 8.2, containing 10 mM NaHCO₃ and 0.5 mM 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 NaNO₃ to the suspension and kept at 30°C in the light with an irradiance of 60 µE m⁻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 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 in *A. halophytica* was energized by proton motive force. Interestingly, monensin which collapses Na⁺ electrochemical gradient also caused drastic inhibition of nitrate uptake. *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.

**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 Table 1, 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*-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>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Nitrate uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>CCCP</td>
<td>20 µM</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>40 µM</td>
<td>26</td>
</tr>
<tr>
<td>DNP</td>
<td>1 mM</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>28</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>10 µg/ml</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>20 µg/ml</td>
<td>24</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>10 mM</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>30 mM</td>
<td>16</td>
</tr>
<tr>
<td>Monensin</td>
<td>20 µM</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>30 µM</td>
<td>25</td>
</tr>
<tr>
<td>DCCD</td>
<td>40 µM</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>35</td>
</tr>
<tr>
<td>KF</td>
<td>15 mM</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>30 mM</td>
<td>100</td>
</tr>
</tbody>
</table>

*Cells were preincubated with inhibitors in the dark for 30 min before the addition of 100 µM NaNO₃ to initiate the uptake as described in Materials and Methods.*
Role of \( \Delta pH \) 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 (\( \Delta \psi \)) and the chemical hydrogen ion concentration gradient (\( \Delta pH \)) across the membrane. To test whether \( \Delta pH \) played an energetic role in the uptake of nitrate, we artificially generated \( \Delta pH \) at three different pHs. At pH 5.5, \( \Delta pH \) 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 \( \Delta pH \) 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 \( \Delta \psi \) 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 \( \Delta \psi \) 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 \( \Delta \psi \) 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 \( \Delta pH \). 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·min\(^{-1}\)·mg Chl\(^{-1}\), 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

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**Table 2. Effect of energy sources on nitrate uptake**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nitrate uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Glucose (20 mM)</td>
<td>128</td>
</tr>
<tr>
<td>Lactate (10 mM)</td>
<td>116</td>
</tr>
<tr>
<td>KCN (20 mM)</td>
<td>24</td>
</tr>
<tr>
<td>Glucose + KCN</td>
<td>28</td>
</tr>
<tr>
<td>Lactate + KCN</td>
<td>33</td>
</tr>
</tbody>
</table>

*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 \( \mu \)M NaNO\(_3\) to initiate the uptake as described in Materials and Methods.*

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

\[ \text{Na}^+ \]

was suppressed in the presence of monensin (data not shown). Activity remaining when

\[ \text{Na}^+ \]

was previously shown in some bacteria (Ekiel et al., 1994). The contribution by either a proton or a sodium motive force, i.e. the so-called sodium motive force which exists another energetic component in addition to proton motive force, i.e. the pH gradient (\(\Delta \text{pH}\)) and the electrochemical gradient (\(\Delta \Psi\)) with the relationship,

\[ \Delta \Psi = \Delta \text{pH} - 59 \mu \text{V} \]

where \(\Delta \text{pH}\) equals the pH

\[ L \]

minus pH

\[ H \]

(The value 59 is a combination of constants for expression of \(\Delta \text{pH}\) 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 \(\Delta \text{pH}\). This increase was observed independent of external pH. On the other hand, the suppression of \(\Delta \text{pH}\) always resulted in a significant decrease of nitrate uptake, and again was independent of external pH. This behavior is expected when the \(\Delta \text{pH}\) term of the proton motive force is the driving force for nitrate uptake. In contrast to the complete abolition of 4-hydroxybenzoate uptake in

\[ \text{Anacystis nidulans} \]

strain DSZ1 when \(\Delta \text{pH}\) was suppressed (Allende et al., 2002), our results showed some nitrate uptake activity remaining when \(\Delta \text{pH}\) was suppressed (Fig. 1). However, this nitrate uptake activity disappeared when \(\Delta \text{pH}\) 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

\[ \text{A. halophytica} \]

(Incharoensakdi and Wangsapa, 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

\[ \text{Anacystis nidulans} \]

R2 (Rodríguez et al., 1992; Rodríguez 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

\[ \text{A. halophytica} \]

is driven by \(\Delta \text{pH}\). The contribution by \(\Delta \Psi\) was rather unlikely due to the findings that \(\Delta \Psi\) generated by either KSCN or valinomycin in the absence

\[ \text{K}^+ \]

had no stimulatory effect on the uptake of nitrate in acidic, neutral, and alkaline conditions (Table 3). Moreover, when \(\Delta \Psi\) was dissipated by valinomycin in the presence of \(\text{K}^+\), no depression of nitrate uptake was observed (Table 3). The absence of an inhibitory effect by valinomycin suggests the absence of the contribution of \(\Delta \Psi\) to nitrate uptake. On the contrary, a slight stimulation of nitrate uptake occurred under the condition depleted of \(\Delta \Psi\) 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 \(\Delta \Psi\) contributed by K' transport (Reed, 1979) and/or (ii) an increase of \(\Delta \text{pH}\) term of the proton motive force is generated by a compensatory mechanism, i.e. an increase in \(\Delta \text{pH}\) simultaneous to a decrease in \(\Delta \Psi\) (Reed, 1979; Kroll and Booth, 1981). Similar observations were reported for 4-hydroxybenzoate uptake in

\[ \text{A. halophytica} \]

whereby \(\Delta \text{pH}\) was also implicated as the driving force (Allende et al., 2002). Previously, it was also shown in the cyanobacterium

\[ \text{Plectonema boryanum} \]

that \(\Delta \text{pH}\) is the

\[ \text{K}^+ \]

and \(\Delta \text{pH}\).

**Table 3.** Effect of \(\Delta \Psi\) on nitrate uptake at different pHs

<table>
<thead>
<tr>
<th>pH</th>
<th>Nitrate uptake ((\mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{Chl})) Control</th>
<th>(\Delta \Psi) generated by</th>
<th>Nitrate uptake ((\mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{Chl})) Control</th>
<th>(\Delta \Psi) dissipated by</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>(0.79 \pm 0.06)</td>
<td>(0.71 \pm 0.05)</td>
<td>(0.78 \pm 0.05)</td>
<td>(0.84 \pm 0.06)</td>
</tr>
<tr>
<td>7.0</td>
<td>(0.94 \pm 0.07)</td>
<td>(0.83 \pm 0.06)</td>
<td>(0.86 \pm 0.06)</td>
<td>(0.90 \pm 0.06)</td>
</tr>
<tr>
<td>8.2</td>
<td>(1.12 \pm 0.08)</td>
<td>(0.97 \pm 0.07)</td>
<td>(0.99 \pm 0.07)</td>
<td>(1.03 \pm 0.07)</td>
</tr>
</tbody>
</table>

\(\Delta \Psi\) was generated by either KSCN or valinomycin in the absence of the contribution of \(\text{K}^+\). Data are means ± standard errors of the means (n = 3).

**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.
major energetic component with no involvement of ∆ψ 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* (Molenar 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 (Wadii et al., 2001; Wadii et al., 2002). Apart from protecting cells against salt stress, Na+/H+ antiporter can also regulate intracellular H+ (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+/H+ gradient mediated by Na+/H+ antiporter. Indeed, the activity of Na+/H+ antiporter in *A. halophytica* was shown to increase with increasing pH (Wadii 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 (Krudwich and Gufant, 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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References


