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Mechanism of proton-linked nitrate uptake in *Cyanidium caldarium*, an acidophilic non-vacuolated alga

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The unicellular non-vacuolated alga *Cyanidium caldarium*, grown under conditions of nitrogen limitation, possesses two permease systems for nitrate uptake, one of which, the so-called 'high-affinity nitrate uptake system', enables the alga to take up nitrate through a mechanism involving cotransport of protons. Measurements of nitrate and proton stoichiometry, and determination of the kinetic parameters of uptake in cells resuspended in medium adjusted at different pH values, are consistent with a mechanism of uptake in which two protons for each nitrate ion are transported across the plasmalemma. Furthermore, kinetic data suggest that the carrier first binds nitrate and, subsequently, protons. Permutations of this binding sequence do not agree with the experimental results.

Introduction

It is generally recognized that the hydrogen ion is the major driving species for cotransport processes in bacteria, fungi and higher plants [1–3]. In algae H^+-dependent cotransport systems have been described for sugars and amino acids, as well as for mineral ions [4]. A proton-dependent hexose transport system has been extensively studied in the green alga *Chlorella vulgaris* by Komor et al. [5–8]. On the basis of the kinetic and stoichiometric data they have proposed a mechanism for hexose uptake whereby a carrier-sugar complex could cross the plasma membrane as protonated and non-protonated species, the protonated one being involved in the high-affinity transport and the non-protonated being involved in the low-affinity transport. Depolarization of plasma membrane potential, when the high-affinity hexose transport operates, was interpreted as evidence of an electrogenic transfer, in support of the previously proposed proton-linked uptake mechanism [9].

No such extensive studies have yet been performed for nitrate. It has been suggested that nitrate uptake in the giant celled alga *Hydrodictyon africanum* occurs through a proton cotransport of nitrate with more than one proton for each nitrate ion [10,11]. On the other hand, depolarization of transmembrane potential associated with proton-linked nitrate uptake in *Lemna gibba* [12,13] suggests a cotransport of two protons per nitrate ion. In the unicellular non-vacuolated algae similar results have so far not been produced.

It was previously reported [14] that in the acidophilic non-vacuolated alga *Cyanidium caldarium* there are two transport systems for nitrate uptake: a high-affinity system which is able to take up nitrate from a low-nitrate medium, and a low-affinity system which takes up nitrate from a relatively high-nitrate medium. This study on the high-affinity transport system of nitrate uptake strongly suggested that it is a secondary active transport system involving cotransport of protons. This alga is particularly suitable for studying H^+-dependent cotransport. It grows in acidic media

where pH changes related to proton-cotransport phenomena are not complicated by CO_2 exchange [15]. *Cyanidium* grows in the pH range 0.5–6.0, and can respire even at pH values above 8. Hence different and large proton gradients across the plasmalemma can be established by changing external pH, the cytosol remaining at neutrality. Thanks to these features it has been possible to study the effect of the proton gradient on the nitrate uptake mediated by the high-affinity transport system, and also to establish the stoichiometry of the nitrate and proton cotransport.

Materials and Methods

Cyanidium caldarium, strain 0206, was supplied by Professor T.D. Brock, Wisconsin University. It was grown autotrophically at pH 1.9, at 42°C, in continuous light. Continuous cultures were carried out in a chemostat under conditions of nitrate limitation as previously described [16]. The fresh medium containing 2 mM nitrate was added to the chemostat culture at a dilution rate of 0.15 day^{-1}.

Cells for experiments were collected by low-speed centrifugation, washed and resuspended in fresh medium without any nitrogen source at 40°C. Experiments were carried out in the light (60 $W \cdot m^{-2}$; Philips comptalux E44 100W incandescent lamp; lamp light was filtered through a 2% $CuSO_4$ solution). The disappearance of nitrate and the related pH-changes were measured by putting the cell suspension in a reaction chamber with side-arms through which nitrate and pH electrodes were inserted in the suspension [15]. The nitrate and pH electrodes were supplied by Orion Research. Cell density was estimated by low-speed centrifugation of a definite aliquot of cell suspension in a hematocrit test tube.

Results

Stoichiometry of NO_3^- and H^+ uptake

It has been demonstrated that *C. caldarium* cells assimilating nitrate in steady-state conditions displayed a proton uptake related to the overall proton requirement for nitrate reduction to ammonium and for further ammonium assimilation [15]. However, cells grown under nitrogen-limiting conditions exhibited a complex exchange of protons with the medium when they were analyzed for nitrate uptake in short-term experiments. As shown in Fig. 1, from nitrate addition to its disappearance from the medium, as ascertained by the nitrate electrode trace, there was a proton uptake by the cell. After the nitrate had disappeared, there was a proton extrusion. This second step was followed by a further proton uptake and, finally, after some time, by a further proton extrusion.

Fig. 2 involves short-term experiments in which only the first step of proton uptake, after nitrate addition, was considered. Changes in H^+ concentration resulted from the addition of 0.05 μmol KNO_3 and then later of 0.05 μmol HNO_3 to 100 ml of cell suspension at pH 3.5. The addition of KNO_3 produced an intermediate rise in the nitrate concentration with no change in H^+ concentra-

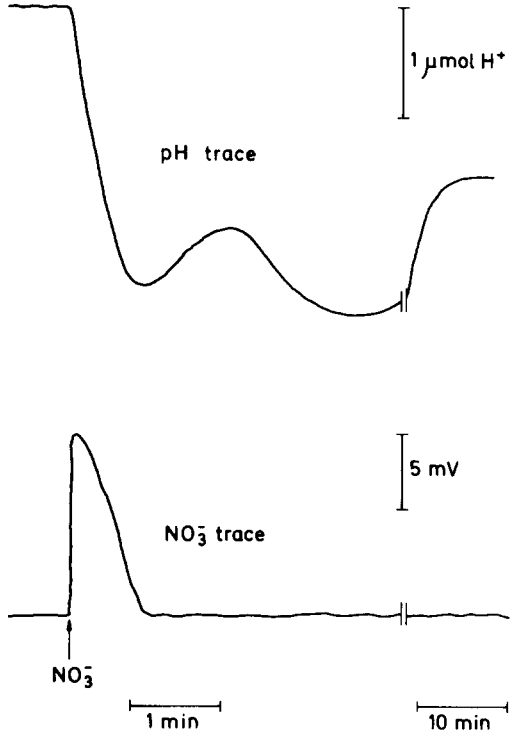


Fig. 1. Nitrate depletion and related pH changes in cell suspension of *Cyanidium caldarium* monitored by nitrate and pH electrodes; pH trace upward indicates pH decrease. The working volume was 100 ml. The suspension contained 20 μl packed cells/ml. The experiments were carried out at pH 3.5 with cells grown under conditions of nitrate limitation. The arrow indicates addition of KNO_3 (1.3 μmol).

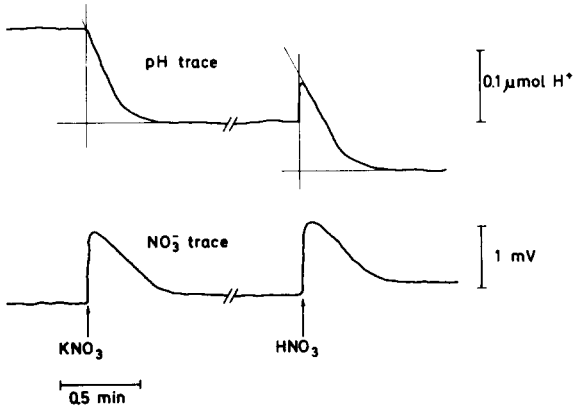


Fig. 2. Short-term experiments of nitrate and proton uptake by nitrogen-limited cell suspension of *C. caldarium*; pH trace downward shows pH increase. Arrows indicate nitrate addition respectively as KNO_3 and HNO_3. The working volume of the suspension was 100 ml. The suspension contained 20 μl packed cells/ml.

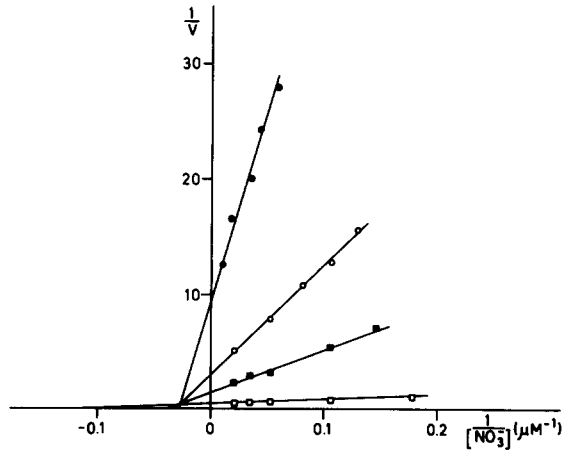


Fig. 3. Lineweaver-Burk plots of nitrate uptake rates versus NO_3^- concentrations at various pHs: ●———●, pH 6.47; ○———○, pH 6.18; ■———■, pH 5.98; □———□, pH 5.49. The suspensions were buffered with 1 mM citrate and contained 20 μl packed cells/ml.

tion. Uptake of the added nitrate by the cells commenced at once and was completed in about 0.2 min. (The nitrate electrode has a longer delay time than the pH electrode.) NO_3^- uptake was accompained by an uptake of H^+; the quantity of H^+ taken up when nitrate absorption ceased was 0.10 μmol. Upon the addition of HNO_3 there was an immediate increase in both NO_3^- and H^+ concentration, and, again, uptake of 0.05 μmol of NO_3^- occurred with 0.10 μmol of H^+. During such short-term periods (0.2 min) it may be assumed that the only process affecting the external pH is nitrate uptake (unpublished observations). The value of the ratio of H^+ to NO_3^-, average from several experiments, was 2.0 ∓ 0.1 (S.E.). Consequently it appeared that nitrate uptake was linked to H^+ uptake with a stoichiometry of 2 hydrogen ions per nitrate ion.

Saturation kinetics of nitrate uptake at different pH values

C. caldarium is a non-vacuolated alga that, as demonstrated in a previous paper [14] allows nitrate uptake to be assessed by measuring nitrate disappearance from the medium. In fact, nitrate uptake was the limiting step throughout the whole process of nitrate assimilation in physiological conditions. In addition, the experiments were done by choosing a range of nitrate concentration in which only the high-affinity system contributed to the uptake, the low-affinity system being inoperative.

Fig. 3 shows the double reciprocal plots of nitrate uptake rate against nitrate concentration in cell suspension taken at various pH values. All the plots gave straight lines, that is, the uptake process follows Michaelis-Menten-type saturation kinetics. However, the apparent K_m values (K'_m) and the apparent V_{max} values (V'_m), obtained from these plots vary with pH. Table I lists K'_m and V'_m

TABLE I

EXERIMENTAL VALUE OF THE APPARENT K_m (K'_m) AND THE APPARENT V_{max} (V'_m) FOR THE HIGH-AFFINITY SYSTEM OF NITRATE UPTAKE DERIVED FROM DOUBLE RECIPROCAL PLOTS AS SHOWN IN Fig. 3.

Data are mean ± S.E.

pH	K'_m (μM)	V'_m (μmol/min)
4.00	–	2.20±0.01
5.00	–	2.00±0.01
5.24	2.0±0.2	1.90±0.02
5.32	5.1±0.2	1.80±0.02
5.49	7.0±0.2	1.70±0.03
5.64	12.2±0.2	1.30±0.01
5.98	28.0±0.3	0.68±0.02
6.18	33.0±0.3	0.37±0.01
6.47	37.5±0.3	0.09±0.01

determined at the given pH values and shows that an increase in pH caused a decrease in V'_m and an increase in K'_m.

When the reciprocal of V'_m is plotted against $1/[H^+]$ (Fig. 4A) and the reciprocal of K'_m is plotted against $[H^+]$ (Fig. 5A) parabolic relationships are obtained. That the curves are truly parabolic is shown by Figs. 4B and 5B), respectively, when the data are plotted in such a way as to produce straight lines if the relationships are parabolic. (The general equation of the parabola: $y = a + bx + cx^2$, is written as:

$$(y - a)/x = b + cx.)$$

Consequently, the phenomenological equations describing the graphs in Fig. 4A and Fig. 5A must have, at most, second-order terms with respect to $1/[H^+]$ and $[H^+]$ respectively.

Kinetic mechanism of nitrate uptake

In order to discuss Figs. 3, 4 and 5 it is necessary to postulate a mechanism for nitrate uptake. This allows rate equations to be obtained which

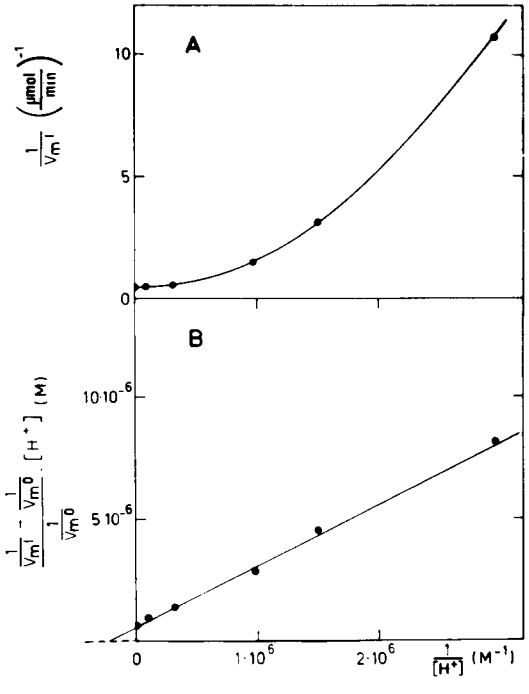


Fig. 4. (A) Double reciprocal plot of the apparent maximal uptake rate values V'_m obtained at given pH values (Table I) versus H^+ concentrations. (B) Transformation of the plot in A that linearizes it with respect to $1/[H^+]$. V^0_m indicates the value of V'_m extrapolated from the plot A at $1/[H^+] = 0$.

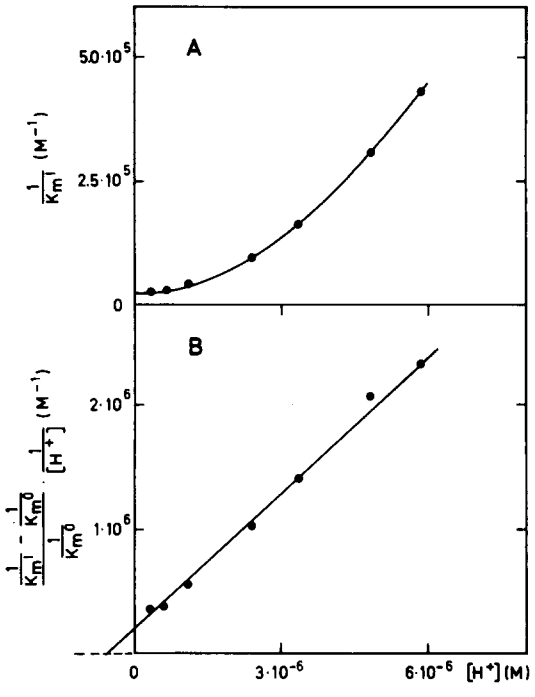


Fig. 5. (A) Plot of the reciprocal apparent K_m values (K'_m) obtained at given pH values (Table I) versus $[H^+]$. (B) Transformation of the plot in A that linearizes it with respect to $[H^+]$. K^0_m indicates the value of K'_m extrapolated at $[H^+] = 0$.

can be compared with the experimental results. The analysis of cotransport systems is complex [17,18] and generally needs some simplification. In the equilibrium-binding assumption (the transfer

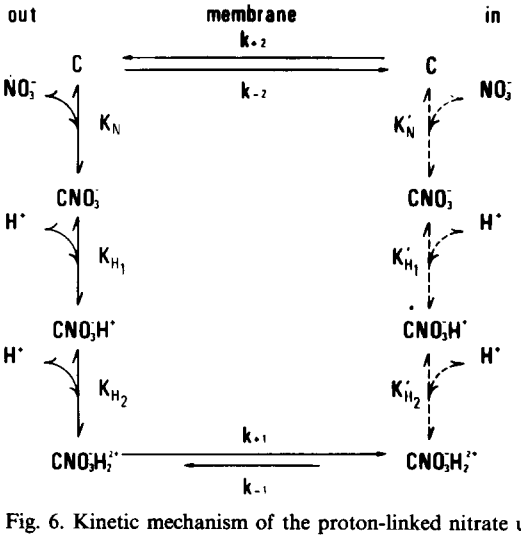


Fig. 6. Kinetic mechanism of the proton-linked nitrate uptake of the high-affinity system in *C. caldarium*.

of ligands across a membrane is limiting in the overall rate of transport) the mechanism reported in Fig. 6 fits in with the results given above. In fact, each partial reaction being close to equilibrium, the following equations can be written (in them C represents a macromolecular carrier with which NO_3^- and H^+ combine, movement of NO_3^- across membrane taking place in the form of $C \cdot NO_3^- H_2^{2+}$):

$$\frac{[C][NO_3^-]}{[C \cdot NO_3^-]} = K_N \qquad \frac{[C]'[NO_3^-]'}{[C \cdot NO_3^-]'} = K'_N$$

$$\frac{[C \cdot NO_3^-][H^+]}{[C \cdot NO_3^- H^+]} = K_{H1} \qquad \frac{[C \cdot NO_3^-]'[H^+]'}{[C \cdot NO_3^- H^+]'} = K'_{H1}$$

$$\frac{[C \cdot NO_3^- H^+]}{[C \cdot NO_3^- H_2^{2+}]} = K_{H2} \qquad \frac{[C \cdot NO_3^- H^+]'[H^+]'}{[C \cdot NO_3^- H_2^{2+}]'} = K'_{H2}$$

$$\frac{[C]'}{[C]} = \frac{k_{-2}}{k_{+2}} = K_c$$

The mass balance equation for the carrier is:

$$C_t = [C] + [C \cdot NO_3^-] + [C \cdot NO_3^- H^+] + [C \cdot NO_3^- H_2^{2+}] + [C]' + [C \cdot NO_3^-]' + [C \cdot NO_3^- H^+]' + [C \cdot NO_3^- H_2^{2+}]'$$

where C_t indicates the total amount of carrier species; [ ]′ and K' refer to the cytoplasmic side of the plasma membrane; K_C is the ratio of the unloaded carrier concentrations.

In *Cyanidium*, as in other non-vacuolated algae [4,19], internal nitrate concentration is very low because of the further nitrate metabolism [14] and, moreover, the pH of the cytoplasm is around neutrality (unpublished observations). The inside concentrations of the loaded carrier species are expected to be negligible. So the reverse reaction characterized by k_{-1} in Fig. 6 can be disregarded, and the rate of the overall process can be written:

$$v = K_{+1}[C \cdot NO_3^- H_2^{2+}]$$

All the carrier species defined for the internal side of membrane can be collected:

$$C^* = [C]' + [C \cdot NO_3^-]' + [C \cdot NO_3^- H^+]' + [C \cdot NO_3^- H_2^{2+}]'$$

with $C^* = \alpha' \cdot [C]' = \alpha' \cdot K_C \cdot [C] = \alpha \cdot [C]$. On the previous assumption α should be constant.

Substituting the equivalent terms as function of v and of substrate concentrations in the mass balance equation, putting $V_m = k_{+1} \cdot C_t$ and taking the reciprocals, the following kinetic equation is obtained:

$$\frac{1}{v} = \frac{1}{V_m}\left(1 + \frac{K_{H2}}{[H^+]} + \frac{K_{H1}K_{H2}}{[H^+]^2} + \frac{K_{H1}K_{H2}}{[H^+]^2}\frac{K_N}{[NO_3^-]}(1+\alpha)\right)$$

which can be rearranged as:

$$\frac{1}{v} = \frac{1}{V_m}\left(1 + \frac{K_{H2}}{[H^+]} + \frac{K_{H1}K_{H2}}{[H^+]^2}\right) \times \left(1 + \frac{1}{[NO_3^-]} \cdot \frac{K_N(1+\alpha)}{1 + \frac{[H^+]}{K_{H1}} + \frac{[H^+]^2}{K_{H1}K_{H2}}}\right) \qquad (1)$$

This kinetic equation is consistent with the results of Fig. 2, because at a constant pH the double reciprocal plot $1/v$ versus $1/[NO_3^-]$ will be linear.

The apparent V_m values (V'_m) and the apparent K_m value (K'_m) derived from Eqn. 1 are respectively:

$$\frac{1}{V'_m} = \frac{1}{V_m}\left(1 + \frac{K_{H2}}{[H^+]} + \frac{K_{H1}K_{H2}}{[H^+]^2}\right) \qquad (2A)$$

which can be written in a linear form against $1/[H^+]$ as:

$$\frac{\frac{1}{V'_m} - \frac{1}{V_m}}{\frac{1}{V_m}}[H^+] = K_{H2} + K_{H1}K_{H2}\frac{1}{[H^+]} \qquad (2B)$$

and

$$\frac{1}{K'_m} = \frac{1}{K_N(1+\alpha)}\left(1 + \frac{[H^+]}{K_{H1}} + \frac{[H^+]^2}{K_{H1}K_{H2}}\right) \qquad (3A)$$

which can be written in a linear form against $[H^+]$ as:

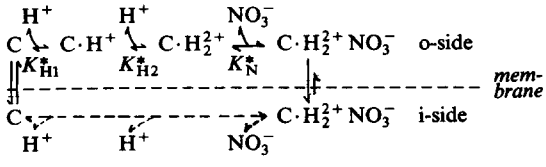
$$\frac{\frac{1}{K'_m} - \frac{1}{K_N(1+\alpha)}}{\frac{1}{K_N(1+\alpha)}}\frac{1}{[H^+]} = \frac{1}{K_{H1}} + \frac{1}{K_{H1}K_{H2}}[H^+] \qquad (3B)$$

Eqns. 2A and 2B are consistent with Fig. 4A and B, respectively. The plot of $1/V'_m$ against $1/[H^+]$ will be non-linear and parabolic, as Eqn. 2A contains terms of second order with respect to $1/[H^+]$. The plot of Fig. 4B is equivalent to Eqn. 2B with $V_m^0 = V_m$. Eqns. 3A and 3B are consistent with Fig. 5A and B, respectively. The plot $1/K'_m$ against $[H^+]$ will be non-linear and parabolic, as the second member of Eqn. 3A contains terms of second order with respect to $[H^+]$. The plot of Fig. 5B is equivalent to Eqn. 3B with $K_m^0 = K_N(1+\alpha)$.

Thus the postulated mechanism yields kinetic equations in agreement with the experimental data regarding the dependence of the uptake rate from NO_3^- and H^+ concentrations, i.e., the permease system transfers nitrate across the plasmalemma with a symport of two protons per nitrate ion.

According to the scheme proposed in Fig. 6, the binding of nitrate to the carrier molecule precedes the binding of the two protons. This is the only sequence that is consistent with the experimental results; other mechanisms which introduce permutations of the postulated sequence are not. Thus:

(1) if it is assumed that nitrate binds to the carrier after protons, i.e.:

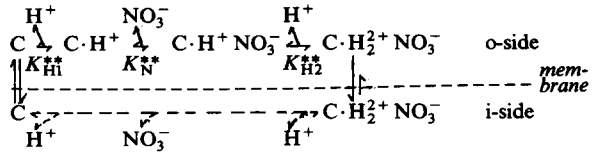


the kinetic equation corresponding to Eqn. 1 would be ($\overline{K^*_N}$ contains terms constant or quite constant):

$$\frac{1}{v} = \frac{1}{V_m}\left(1 + \frac{\overline{K^*_N}}{[NO_3^-]}\left(1 + \frac{K^*_{H2}}{[H^+]} + \frac{K^*_{H1}K^*_{H2}}{[H^+]^2}\right)\right) \qquad (4)$$

According to this equation the apparent V_{max} (V'_m) is equal to V_m, $V'_m = V_m$, that is, V'_m is independent of $[H^+]$. This result does not agree with the results of Fig. 3A. Hence, this mechanism can be rejected.

(2) If binding of nitrate follows the binding of the first proton and precedes the binding of the second proton, i.e.:



then, the kinetic equation corresponding to Eqn. 1 would be ($\overline{K^{**}_N}$ contains terms constant or quite constant):

$$\frac{1}{v} = \frac{1}{V_m}\left(1 + \frac{K^{**}_{H2}}{[H^+]}\right)\left(1 + \frac{\overline{K^{**}_N}}{[NO_3^-]}\frac{K^{**}_{H2}}{[H^+]}\frac{K^{**}_{H1} + [H^+]}{K^{**}_{H2} + [H^+]}\right) \qquad (5)$$

According to this equation the apparent V_{max} (V'_m) is:

$$V'_m = V_m\left(1 + \frac{K^{**}_{H2}}{[H^+]}\right)$$

and the double reciprocal plot $1/V'_m$ versus $1/[H^+]$ would be linear. This result does not agree with the plot in Fig. 3A, where the relationship is parabolic. Hence, this mechanism also can be rejected.

Discussion

The stoichiometrical data, obtained in short-term experiments (Fig. 2), and the kinetic analysis of initial uptake rate (Figs. 3, 4 and 5) strongly support the idea that, in *Cyanidium*, nitrate transport by the high-affinity uptake system involves the symport of two protons per nitrate ion.

The mechanism, shown in Fig. 6, that fits in with the experimental results, suggests some further consideration. (1) Nitrate transport by this system requires the formation of the complex $C \cdot NO_3^- H_2^{2+}$. This is the only complex, together with the unloaded carrier, that can cross the membrane. The mechanism differs from that proposed for the carrier of hexoses in *Chlorella*, where protonated and non-protonated carrier-sugar complexes were traslocated [7]. Further study is necessary to establish whether charge-transit step are involved in the process. In this respect many cotransport and countertransport systems operate electrophoretically, transporting net positive charges inward across cell membranes [18]. Net positive charge-transfer dependent on nitrate uptake has been reported in *Lemna* [12,13]. (2) The binding sequence supports the view that the carrier first binds nitrate and subsequently protons. (No binding sequence can be derived for the inside from the results; in Fig. 6 only a probable sequence is indicated, considering that H^+ is the driver ion.)

From the data of Table I and Figs. 5 and 6 the constants occurring in Eqn. 1 can be determined (the values are expressed as means ± S.E.): $K_N(1+\alpha) = (4.5 \pm 0.2) \cdot 10^{-5}$ M; $K_{H1} = (4.8 \pm 0.3) \cdot 10^{-6}$ M; $K_{H2} = (5.5 \pm 0.3) \cdot 10^{-7}$ M. $K_N(1+\alpha) > K_{H1} > K_{H2}$, ($\alpha$ can be assumed not to be large), indicates that the binding of nitrate promotes the binding of the first proton and this promotes the binding of the second proton. This result strengthens the ordered binding sequence proposed above on the basis of the kinetic analysis. In particular, $K_{H1} > K_{H2}$ suggests cooperative interactions and, according to Sanders et al. [18], could favour a sterically ordered to a statistically ordered binding. (3) The quite large value of $K_N(1+\alpha)$ suggests that at very low nitrate concentration the limiting step in the overall transport could be the formation of the complex $C \cdot NO_3^-$, and the equilibrium binding assumption could be of no use in deriving the kinetic equation. This consideration is particularly important at pH below 4, where the expected K'_m would be much less than 1 μM. In this respect short-term experiments such as those shown in Fig. 2 and analysis of initial uptake rate are complementary methods: the former is useful at pH below 4 where the nitrate uptake rate is high even at low nitrate concentration. Thus, determination of protons linked to the uptake process can be made by using a very small amount of nitrate in pre-steady state conditions [4,10,20]. The latter is useful at pH above 4 where the nitrate concentrations for determining the kinetic parameters can be relatively large.

Finally, even if some of the conclusions derived from the kinetic data may be model-dependent [17,18], they are a basis for any further improvement.

Acknowledgements

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