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Short-term regulation of nitrate uptake by a 'pump and leak' mechanism in the acidophilic nonvacuolated alga, *Cyanidium caldarium*

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In the nonvacuolated acidophilic thermophilic red alga, *Cyanidium caldarium*, nitrate uptake and reduction can be separated by measuring disappearance of nitrate from the suspension medium using, in in vivo experiments, cyanate, a competitive inhibitor of algal nitrate reductase. Cyanate selectively inhibited nitrate reduction at concentrations that did not significantly affect nitrate uptake, photosynthesis or respiration. Its use proved that short-term control of intracellular nitrate through the increase of a carrier-mediated nitrate efflux took place when nitrate reduction was inhibited. The occurrence of the high- and low-affinity nitrate uptake systems in cells grown in nitrogen-limited conditions, as previously reported, suggests a 'pump and leak' mechanism operating at the plasmalemma level to regulate nitrate uptake and intracellular nitrate: the high-affinity nitrate transport system mediated by proton cotransport (irreversible) operates the influx, while the low-affinity transport (reversible) operates influx or efflux according to cell requirements. Kinetic analysis of cyanate inhibition in cells taken from low-nitrate medium supports this hypothesis and reveals that, in *Cyanidium*, intracellular nitrate is probably compartmented in the cytosol.

Introduction

Nitrate uptake can be easily studied in vacuolated algae, which accumulate NO_3^- largely in the vacuoles [1–8]. In these organisms it is possible to distinguish between nitrate uptake and nitrate reduction by measuring the intracellular accumulation of nitrate.

Disappearance of nitrate from suspension medium and integration of time-dependent curves of nitrate uptake have also been used to evaluate intracellular nitrate in barley plants and in *Chara corallina*, taking account of the fact that, in these organisms, reduction is negligible compared with uptake [9,10].

Similar studies are not possible in nonvacuolated microorganisms, because uptake and reduction can never be easily separated. The intracellular nitrate content generally cannot be assayed in algae that assimilate it [8,11–13]. In any case, uptake and reduction have been considered distinct processes because of their different regulation in long-term and short-term experiments [12,14–16]. The failure to measure intracellular nitrate has been attributed to the activity of nitrate reductase greatly exceeding that of nitrate permease(s), as supported by comparing in vitro and in vivo findings on nitrate reductase activity and nitrate assimilation rate, respectively [6]. The hypothesis that the same system operates uptake and reduction of nitrate [17] has not received significant support from studies on these organisms, even though *Chlamydomonas* mutants lacking nitrate reductase activity were unable to accumulate nitrate [18]. In this respect, short-term control of nitrate assimilation remained questionable, because, depending on the organisms, uptake and/or reduction have been suggested as regulating points to sense multiple environmental signals [7,8,11,19,20].

To study nitrate uptake specifically by measuring intracellular nitrate accumulation, mutants lacking nitrate reductase [18,21,22] and cells in which nitrate reductase had been suppressed by tungstate treatment [3,23] have been used.

In the nonvacuolated acidophilic and thermophilic alga, *Cyanidium caldarium* [24], it was possible by measuring the disappearance of nitrate from the cell suspension to distinguish between nitrate uptake and reduction, treating *Cyanidium* cells in vivo with cyanate, a competitive inhibitor of nitrate reductase in algae. This

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inhibitor affected nitrate reduction at concentrations not significantly affecting nitrate uptake, photosynthesis or respiration [25].

In *Cyanidium*, a short-term control of nitrate uptake was evidenced. This kept internal nitrate at relatively low concentration, even when nitrate reductase was severely inhibited by cyanate [26].

In this paper a 'pump and leak' mechanism for setting the intracellular concentration of NO_3^- is proposed and discussed. Maintenance of the intracellular solute levels by controlling the influx and/or efflux across cell membranes has been reported for inorganic and organic substances [27]. In addition, short-term control of nitrate uptake through efflux rather than influx has been reported in higher plants and algae [9,28–30].

Materials and Methods

Cyanidium caldarium strain 0206, supplied by T.D. Brock, Wisconsin University, was grown autotrophically in continuous light, in a modified Allen medium adjusted to pH 1.9 and supplied with nitrate as nitrogen source. Continuous cultures were carried out in a chemostat maintained at 42° C and flushed with air enriched with 5% CO_2 under conditions of nitrate limitation, as previously described [31].

The experiments were performed collecting cells by low-speed centrifugation (2000 × *g* for 5 min), washing, and resuspending them in a fresh nitrogen-free medium. The reaction vessel, maintained at 37° C, was equipped with side-arms through which pH and nitrate electrodes (Orion Research) were inserted into the cell suspension.

Nitrate assimilation was also determined by simultaneously monitoring the photosynthetic O_2 evolution and pH changes due to nitrate assimilation in a cell suspension in which CO_2 was kept at the compensation point. The oxygraph cuvette (5 ml) was equipped with a Clark electrode (Radiometer) and a pH electrode. To measure O_2 exchange, N_2 was flushed through the suspension to adjust the oxygen concentration in the cell suspension to 20–30% of O_2 saturation. Respiration was determined by evaluating the O_2 consumption in the dark. Full photosynthetic rates were determined upon adding up to 1 mM bicarbonate to the suspension in the light. For comparison, the photosynthesis related to the fixation of the CO_2 previously produced in a dark period was determined. Addition of test solution was made by a microsyringe.

Experiments were carried out in the light using an incandescent lamp (Philips Comptalux E44, 100 W) at 60 $W \cdot m^{-2}$ PAR light fluence. Lamp light was filtered through a 2% $CuSO_4$ solution. Cell density was determined by centrifuging at low speed a defined aliquot of cell suspension in a hematocrit test-tube.

Nitrate reductase in cell-free extracts was determined as previously described [31].

The values in the text are mean ± S.E. (standard error).

Results

Effect of cyanate on nitrogen-limited cell suspensions of C. caldarium

Cyanate has been reported as a strong inhibitor of nitrate reductase in vitro in algae [32,33] and as an inducer of nitrate uptake and assimilation in vivo in some bacteria [34]. In the acidophilic microalga, *C. caldarium*, it was also a strong competitive inhibitor of nitrate reductase at the nitrate site, the inhibition constant being 3 ± 0.3 μM when assayed at pH 7. The Michaelis constant, K_m, determined under the same assay conditions was 80 ± 5 μM (unpublished results). Since HCNO, as small uncharged molecule, freely crosses the cellular membranes, cyanate has been also used in vivo to study its effect on nitrate assimilation in cell suspensions of *Cyanidium*.

As reported in a previous paper [16], cells of *Cyanidium* grown in nitrogen-limited conditions possess two nitrate-uptake systems: a high-affinity and a low-affinity system. The high-affinity system involves the cotransport of two protons per nitrate ion [35], and some simple thermodynamic considerations on the proton gradient between medium and cell sap suggested that it would be able to concentrate nitrate with great efficiency within the cell if nitrate reduction were strongly inhibited. Hence, the following experiments were done using nitrogen-limited cells and low concentrations of nitrate (5–10 μM) in order to have only the high-affinity system operative for nitrate influx.

Cyanate at very low concentration added to a cell suspension of *Cyanidium* affected nitrate assimilation in a peculiar way (Fig. 1): before cyanate treatment, cells took up nitrate at a constant rate to depletion, as indicated by the nitrate electrode trace, and continued with the same rate even upon subsequent nitrate additions. Upon cyanate treatment, the same cells took up nitrate at the previous rate only immediately after the addition. Thereafter, the rate declined to a lower steady-state value that remained unchanged until depletion of nitrate in the medium. Further nitrate additions before depletion caused no change in the steady rate, but if the new additions were delayed by more than 1 min after nitrate depletion, the assimilation pattern turned out to be similar to that upon the first addition. Fig. 1 also shows that also the proton uptake varied accordingly, cyanate-treated cells which had a reduced nitrate assimilation rate also had a reduced steady proton uptake rate [36].

Fig. 2 shows the quantitative evaluation of the traces in Fig. 1. The initial nitrate uptake rate upon addition

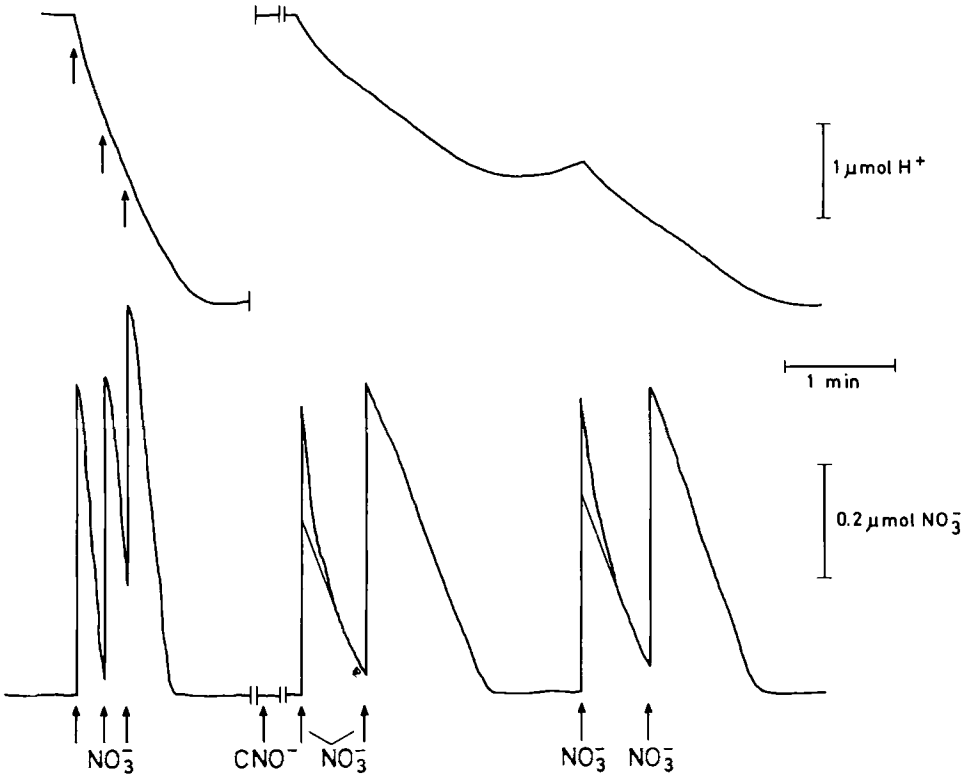

Fig. 1. Effect of addition of cyanate to cell suspensions of *C. caldarium* grown in nitrogen-limited conditions. Disappearance of nitrate and the corresponding pH variations were monitored. The arrows indicate solute additions: CNO^- was 5 nmol; nitrate as indicated. Nitrate addition was delayed 2 min from cyanate addition in order to allow the distribution of CNO^- into the cell. The suspension volume was 100 ml with 15 µl packed cells/ml. Experiments were carried out at 37 °C in a medium adjusted to pH 3.68.

of nitrate to a nitrate-free cell suspension was independent of the concentration of the added cyanate and was similar to the uptake rate shown before cyanate treatment. The steady uptake rate of cyanate-treated cells, on the contrary, was lower than that in untreated cells and was dependent on additions of cyanate to the suspension.

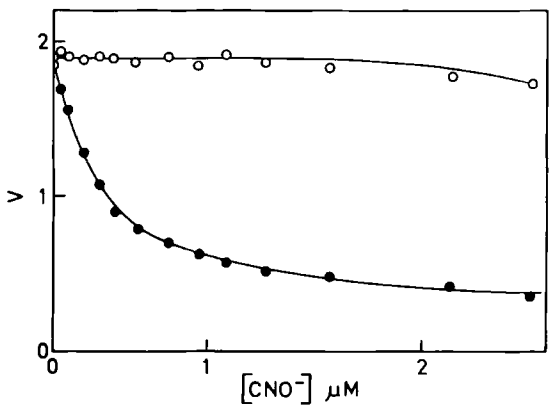

Fig. 2. Comparative effect of cyanate on the initial (○) and on the steady-state (●) rates of nitrate uptake by a cell suspension treated with various concentrations of cyanate. Experiments as in Fig. 1. The uptake rate is measured in µmol/(min per ml packed cells). Cyanate concentration is referred to the suspension volume (100 ml) containing 1 ml packed cells.

Cyanate is an unstable molecule. *Cyanidium* cells were reversibly inhibited by it under the conditions of the previous experiments. After a certain time, cyanate-treated cells took up nitrate as did the untreated ones. The half-time for recovery of the activity observed before cyanate treatment was about 120 min.

Effect of cyanate on proton exchange and photosynthetic O_2 evolution related to nitrate uptake and assimilation

In *Cyanidium*, nitrate uptake through the high-affinity system was energetically linked to proton cotransport [35]. At the same time, in the light, nitrate assimilation was coupled to photosynthetic O_2 evolution and it could be determined by keeping the CO_2 concentration of the suspension at the compensation point [37]. Fig. 3 shows the pH and the O_2 electrode traces during nitrate uptake and assimilation before and after cyanate addition.

Cyanate affected nitrate-dependent proton uptake and oxygen evolution differently: in cyanate-treated cells, nitrate-dependent O_2 evolution was strongly inhibited just upon addition of nitrate. The initial proton uptake rate, on the contrary, was not affected. The inhibition of proton uptake to a rate corresponding stoichiometrically to the reduced O_2 evolution was delayed. Cyanate complexes metals and, as a weak acid, is also reported as uncoupler of oxidative and photosyn-

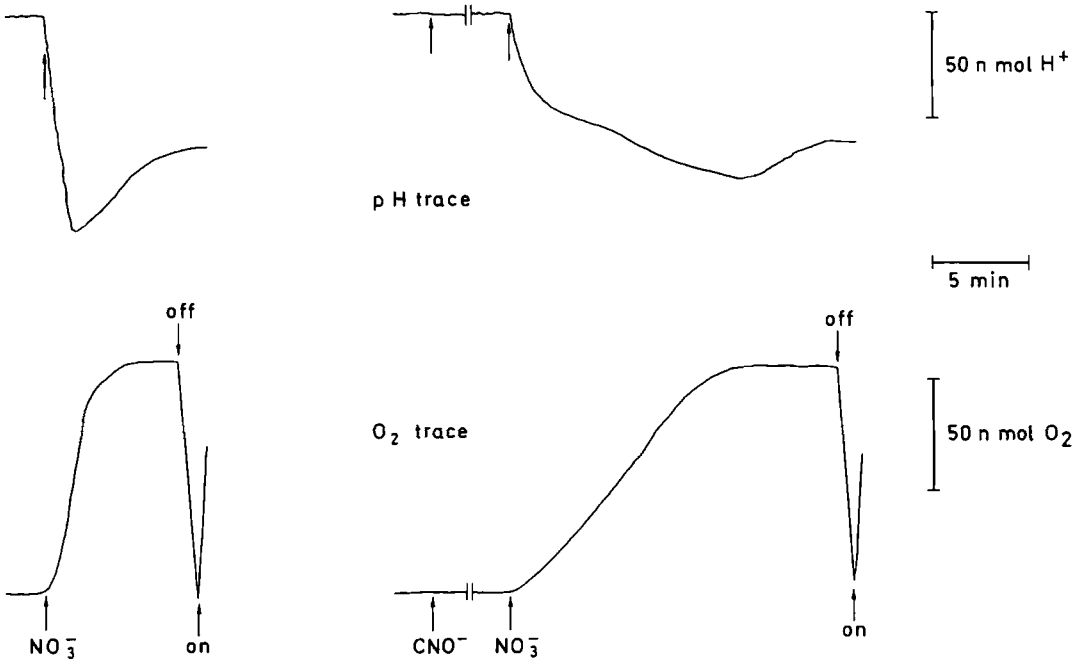

Fig. 3. Simultaneous monitoring of pH changes and photosynthetic O_2 evolution related to nitrate assimilation before and after treatment with cyanate in a cell suspension adjusted to pH 3.7. The arrows indicate nitrate addition (50 nmol) and cyanate addition (2 nmol); 'off' and 'on' in the plot indicate 'light off' and 'light on', respectively. The 5 ml of suspension in the oxygraph cell contained 30 μl packed cells. Details in Materials and Methods.

thetic phosphorylation; hence, it could affect also other metabolic processes. Fig. 3 shows that both the O_2 consumption rate in the dark and the O_2 evolution rate in the light with the excess CO_2 produced during respiration remained unaffected following a cyanate addition which inhibited nitrate assimilation more than 50%. Under the same conditions O_2-dependent nitrite assimilation was not significantly affected, either. To inhibit photosynthesis, respiration and nitrite assimilation rates at to the same extent as nitrate assimilation, the cyanate concentration had to be about 10-times higher.

Kinetics of cyanate inhibition of the steady nitrate assimilation rate

A 2 min interval was chosen between cyanate and nitrate additions to allow the cells to take up the bulk quantity of cyanate added. In acidic media that are physiological for *Cyanidium*, due to permeation through the cellular membranes and due to the neutral pH of the cell sap, cyanate should be distributed and, this means, concentrated into the cell. In agreement with this assumption, a given amount of cyanate caused an inhibition which decreased when the cell content and the medium pH were increased: in acidic media, a large fraction of the added cyanate was trapped in the cell sap. Thus, a better evaluation of the inhibition has been made relating the amount of added cyanate to the total cell content of the suspension instead of the total volume of the medium. Fig. 4 shows the Dixon-like plot of the steady nitrate uptake rate versus cyanate 'concentration' evaluated on a cell volume basis. As can be seen, the plot is not linear, indicating that at increased cyanate concentrations the apparent inhibition constant of cyanate was also increased. At higher concentrations cyanate also significantly inhibited photosynthesis and respiration.

Nitrate efflux in cyanate-treated cells

As previous shown, cyanate in vitro strongly inhibited *Cyanidium* nitrate reductase; on the other hand,

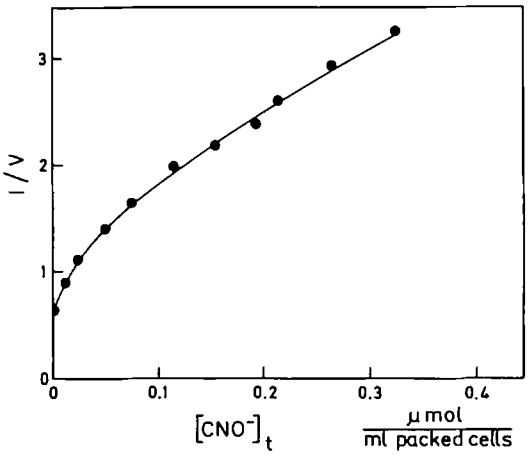

Fig. 4. Dixon-like plot of nitrate uptake rate under steady-state conditions versus cyanate concentration evaluated on a per cell volume basis. Data were derived from experiments as in Fig. 1, except with pH adjusted at 4.1. The nitrate assimilation rate is expressed in μmol/min per ml packed cells.

in vivo it affected significantly only the steady nitrate uptake, not the initial one (Fig. 1). The absence of effect on photosynthesis and respiration at the given cyanate concentrations suggests that only the nitrate assimilation pathway is affected. Since nitrite assimilation was not affected, either, the inhibition should be located at the level of nitrate reductase, in vivo as well as in vitro.

The uptake rate (net uptake rate) is the difference between influx and efflux rates. On condition that the initial uptake, i.e., influx, is not affected (Fig. 1), and as the high-affinity nitrate uptake system operates far from equilibrium [35], the decline of the net uptake rate in cyanate-treated cells can be due to an increase in the efflux rate. If only the influx operated, no competitive inhibition could be observed because the intracellular concentration of nitrate would rise to the extent required to sustain a reduction corresponding to the uptake rate.

Direct measurements of nitrate efflux by desorption experiments cannot be made in *Cyanidium*: the release of the intracellular nitrate should occur in few seconds. In this respect, the nitrate efflux rate under steady-state conditions was calculated from experiments as in Fig. 1, by subtracting the steady uptake rate from the initial one determined upon addition of nitrate to the nitrate-free suspension. The decline of the net nitrate uptake rate upon nitrate addition in cyanate-treated cells was exponential (Fig. 1) and was consistent with the filling of a cellular compartment. The pattern of Fig. 1 allowed the calculation of the NO_3^- accumulated within the cell by time integration of the difference between the net nitrate uptake rate, upon nitrate addition, and the steady-state uptake rate (reduction rate). Similar calculations have been reported for barley plants [9], in which, as in cyanate-treated cells, the reduction rate of nitrate was lower that the uptake rate. Such a value leads to the steady-state nitrate concentration at the nitrate reductase. Table I shows that on a per cell volume basis the concentration of nitrate, $[NO_3^-]_i^d$, determined in such a way, was function of cyanate concentration: it was higher at higher cyanate.

TABLE I

Effect of cyanate on intracellular nitrate concentrations

$[NO_3^-]_i^d$ was determined according experiments as in Fig. 1 at pH 4.1 (details in the text). Data are mean ± S.E. of four experiments. $[NO_3^-]_i^c$ was calculated by Eqn. 4. S.E. < 10%.

$[CNO^-]$ (μM)	$[NO_3^-]_i^d$ (μM)	$[NO_3^-]_i^c$ (μM)
0	-±	11
14.5	44±10	40
35.0	75±10	66
50.0	90±15	82
75.0	115±10	101
100.0	125±15	115
130.0	130±10	124
150.0	140±15	134
185.0	150±20	144

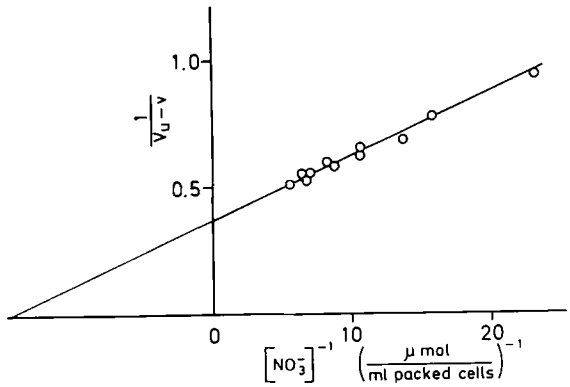

Fig. 5. Double-reciprocal plot of differences between the initial nitrate uptake rate and the steady-state uptake rate versus the intracellular concentration of nitrate extrapolated from experiments as in Fig. 1. Details in the text. The uptake rate was expressed in μmol/(min per ml packed cells). Intracellular nitrate concentration is expressed in μmol/ml packed cells.

Fig. 5 shows a Lineweaver-Burk plot of the nitrate efflux rate of cells treated with various amount of cyanate versus the corresponding intracellular nitrate concentration $[NO_3^+]_i^d$. A straight line could be drawn through the data points, suggesting that a saturable type of efflux (leakage) system was involved.

Mechanism for controlling nitrate uptake in C. caldarium

The data presented above could be interpreted by the model shown in Fig. 6. It suggested that three major processes were involved in determining the intracellular concentration of nitrate: (1) an active nitrate-uptake system that operates irreversibly; (2) a reversible carrier-mediated nitrate-transport system (both of these operating at the level of the plasmalemma). (3) a nitrate-reducing system independent of nitrate uptake. These assumptions could be justified by the following considerations. (1) The active nitrate influx was identical to the high-affinity uptake system, energetically

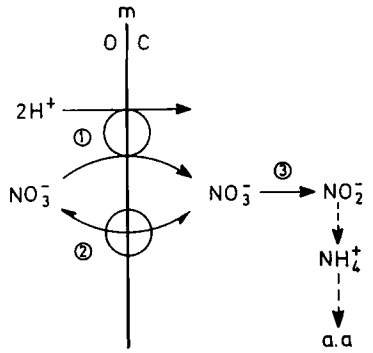

Fig. 6. 'Pump and leak' mechanism for regulating intracellular nitrate in *C. caldarium*. (1) Proton-linked high-affinity nitrate-uptake system; (2) reversible nitrate-uptake system; (3) nitrate reductase. Details in the text.

linked to proton cotransport. Under the experimental conditions (pH 4.1 or lower) it was able to sustain maximal rate almost up to nitrate depletion. The close coupling of two proton per nitrate ion and the pH gradient across the plasmalemma (the cell sap was neutral) made it irreversible [35]. (2) The reversible carrier-mediated nitrate transport was inferred from Fig. 5 and from the low-affinity nitrate uptake system [16]. At low extracellular nitrate (Fig. 1) it could operate essentially as a leak system. A nitrate reductase independent of uptake was suggested by the data as in Fig. 2 [24].

For a quantitative analysis of these assumptions at low nitrate in the medium, an expression could be derived as follows: V_u is the rate of nitrate uptake system saturated at even less than 1 μM of NO_3^- and, hence, independent of the extracellular nitrate concentration in the range used in experiments as in Fig. 1 [35]:

$$V_l = \frac{V_l \cdot [NO_3^-]_i}{K_l + [NO_3^-]_i}$$

is the leakage rate under steady-state conditions (Fig. 3) ($[NO_3^-]_i$ is the intracellular nitrate concentration; V_l and K_l are the maximal leak rate and the apparent Michaelis constant for the leakage, respectively). The uptake through this system is neglected in a low-nitrate medium.

$$V_r = \frac{V_r \cdot [NO_3^-]_i}{K_r + [NO_3^-]_i}$$

is the rate of nitrate reduction by nitrate reductase (V_r and K_r are the maximal nitrate reduction rate and the apparent Michaelis constant for nitrate reductase).

The intracellular concentration of nitrate can then be expressed as:

$$\frac{d[NO_3^-]_i}{dt} = V_u - \frac{V_l \cdot [NO_3^-]_i}{K_l + [NO_3^-]_i} - \frac{V_r \cdot [NO_3^-]_i}{K_r + [NO_3^-]_i}$$

and, under steady-state conditions ($d[NO_3^-]_i/dt = 0$):

$$0 = V_u - \frac{V_l \cdot [NO_3^-]_i}{K_l + [NO_3^-]_i} - \frac{V_r \cdot [NO_3^-]_i}{K_r + [NO_3^-]_i}$$

In this equation, the net uptake rate of nitrate, v, is the algebraic sum of the influx and efflux rates:

$$v = V_u - \frac{V_l \cdot [NO_3^-]_i}{K_l + [NO_3^-]_i} \quad (1)$$

and, under steady-state conditions, is also equal to the reduction rate:

$$v = \frac{V_r \cdot [NO_3^-]_i}{K_r + [NO_3^-]_i} \quad (2)$$

Eqns. 1 and 2, by substituting $x = 1/v$ and rearranging, are combined and written:

$$\frac{K_r}{K_l} = \frac{(V_u \cdot x - 1) \cdot (V_r \cdot x - 1)}{(V_l - V_u) \cdot x - 1} \quad (3)$$

According to the assumption that cyanate is a competitive inhibitor of nitrate reductase in vivo as in vitro, Eqn. 3 is modified as:

$$\frac{K_r(1 + [CNO^-]/K_{in})}{K_l} = \frac{(V_u \cdot x - 1) \cdot (V_r \cdot x - 1)}{(V_l - V_u) \cdot x - 1} \quad (4)$$

where $[CNO^-]$ and K_{in} are respectively the concentration of cyanate and its inhibition constant for nitrate reductase within the cell.

Because the intracellular cyanate concentration is a function of cyanate added, Eqn. 4 can describe the nonlinear Dixon-like plot of cyanate inhibition (Fig. 4).

Eqn. 4 was evaluated knowing that: (1) the initial uptake rate, the influx rate, V_u, was measured from experiments as in Fig. 1; (2) the maximal reduction rate, V_r, was measured in experiments as in Fig. 3, using nitrate at high concentration (60–100 mM) at which reduction is the limiting step [38]. The values of V_l and K_l on a per cell volume basis were determined from Fig. 5. The ratio K_r/K_l was calculated from the steady uptake rate in nontreated cells using Eqn. 3. Furthermore, measurements of steady nitrate assimilation rates in cells treated with different amounts of cyanate, using the parameters shown in Table II, permitted calculation of the ratio $[CNO^-]/K_{in}$ in Eqn. 4 and comparison of them with the applied cyanate concentration $[CNO^-]_t$.

Fig. 7 shows the relationship between the measured $[CNO^-]_t$ and the calculated ratio, $[CNO^-]/K_{in}$. A straight line interpolates the experimental data over the whole range considered, supporting the previous assumptions. In agreement with this, Table I shows the fit of the measured intracellular nitrate concentration $[NO_3^-]_i^d$ and that calculated through Eqns. 4 and 1 $[NO_3^-]_i^c$, when $[CNO^-]_t$ was given.

K_{int}, the apparent cyanate inhibition constant in vivo at pH 4.1, referred to the total cyanate on a per cell

TABLE II

Parameters determined in vivo in experiments at pH 4.1 and used to calculate the ratio $[CNO^-]/K_{in}$ in Eqn. 4

Parameter	Value
	μmol NO_3^-/min per ml packed cells
V_u	2.3 ±0.1
V_i	2.64 ±0.14
V_r	3.44 ±0.2
	μmol NO_3^-/ml packed cells
K_l	0.068 ±0.05
K_r/K_l	0.12 ±0.01
K_r	0.0084 ±0.001

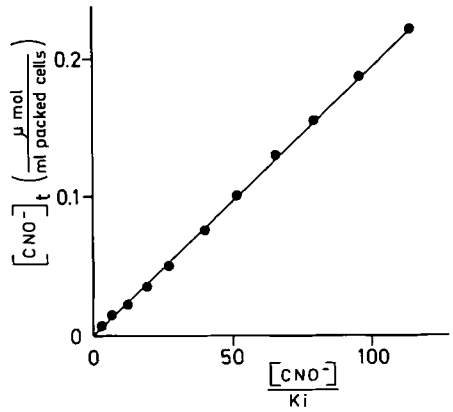

Fig. 7. Fitting of the experimental $[CNO_3^-]_t$ and the computed $[CNO^-]/K_{in}$ using Eqn. 4. Details in the text. The computation was based on experiments performed at pH 4.1.

volume basis $[CNO^-]_t$, could be calculated from Fig. 7: $K_{int} = 2.0 \pm 0.1$ nmol CNO^-/(ml packed cells).

In Table II is also reported the value K_r in vivo, calculated from K_1 and the ratio K_r/K_1.

Discussion

Discrimination of nitrate uptake and nitrate reduction is obscured in nonvacuolated algae by the lack of storage compartments, thus implying that the nitrate taken up is rapidly reduced and assimilated [7,8,13]. This complicates the analysis of the uptake systems and their role in the regulation of the nitrate transport.

The data presented above show that in the nonvacuolated alga, *C. caldarium* cyanate allows us to discriminate between the two processes, affecting reduction selectively in contrast to uptake (Figs. 1 and 2). When the high-affinity nitrate uptake system [35] is operating alone, the initial proton uptake rate immediately upon nitrate addition is not affected by the inhibitor and, in addition, the initial proton uptake occurs when no nitrate reduction has taken place (Fig. 3), giving further support to the concept of proton-linked nitrate cotransport.

The O_2 evolution depending on nitrate reduction and its further assimilation was strongly affected by cyanate throughout the assay. In agreement with this, in steady-state conditions the proton uptake, which is stoichiometrically coupled to nitrate assimilation [36], was also inhibited (Figs. 1, 3).

The steady nitrate uptake rate in cyanate-treated cells was unchanged up to the point of nitrate depletion, suggesting that about 2 min after nitrate addition a homeostatic internal nitrate concentration at the location of nitrate reductase was reached, independent of nitrate concentration in the medium (Fig. 1), but dependent on the added cyanate (Table I).

Since nitrate influx is not affected by CNO^-, the net nitrate uptake has to be regulated through changes in nitrate efflux. Control of net solute uptake and accumulation into the cell has been reported for sugars, amino acids and inorganic ions [39–41]. When active transport systems, which generally function far from thermodynamic equilibrium, operate, two types of transport mechanism have been suggested to control the intracellular solute concentration, as discussed by Eddy [27]: (1) a 'slip' mechanism, in which the same carrier can transport the solute through two channels, one of which is coupled to the driving ion; (2) a 'pump and leak' mechanism, in which two independent carriers operate transport, one of which is the active type. Examples of both have been shown in *Chlorella* species: a slip mechanism for hexose uptake has been extensively studied [42]; A 'pump and leak' mechanism for controlling phosphate uptake has been suggested to explain why cells grown under phosphate limitation, having high- and low-affinity phosphate uptake systems, showed transitory high tracer fluxes of phosphate when transferred to high-phosphate medium [43].

A 'pump and leak' nitrate uptake mechanism has been reported in barley plants and in algae [9,27,28].

In *Cyanidium*, the occurrence of the high- and low-affinity nitrate uptake systems, differently expressed when cells were grown in high- or low-nitrate medium [16], supports the 'pump and leak' mechanism of Fig. 6. It can explain the pattern of Fig. 1: the influx mediated by the high-affinity system is saturated in the range of concentrations used; under the same conditions, the low-affinity system can modulate the efflux to the given net nitrate uptake rate, thus maintaining the intracellular nitrate at a relatively low concentration (Fig. 5; Table I).

The analysis shows that this mechanism can describe the Dixon-like plot reported in Fig. 4 (Eqn. 4; Fig. 7; Table I), as shown previously, and permit further consideration of the apparent nitrate reduction constant in vivo K_r. Assuming that the in vitro value of K_m for NO_3^- of nitrate reductase is equal to that in vivo (the internal pH being neutral), the ratio $K_r/K_m = 0.10 \pm 0.01$ indicates that only 10% of the packed cell volume has to pool the intracellular nitrate. On comparing this volume to the cell water volume, i.e., 37% of the packed cell volume (unpublished data), it is suggested that the nitrate is concentrated in a cellular compartment, probably the cytosol. *C. caldarium* has a large cup-shaped chloroplast that accounts for about 70% of the total cell volume [24]. It is generally reported that nitrate reduction occurs in the cytosol, even in green algae; but recent results obtained by immunolocalization favour the occurrence of nitrate reductase in the chloroplast, also [44]. Similar data for comparison have not been available for *Cyanidium*, a red alga.

The proposed mechanism explains the impossibility of determining nitrate in strongly inhibited cells (unpublished observations) on account of the high efflux

that can rapidly cause leakage of the accumulated nitrate of collected cell samples. It could also explain the low levels of nitrate determined in *Chlamydomonas* mutants lacking nitrate reductase [12,18]. Hence, regulation of nitrate assimilation at the level of nitrate reductase does not necessarily lead to an increase in the intracellular nitrate concentration, due to the control of its pool size, even if specific effectors of the efflux are not considered [28].

This mechanism causes a cycling of nitrate, wasting the energy of the proton gradient, under conditions in which the efflux rate is high, i.e., when cells are transferred to high-nitrate medium. However, this is a transitory response to changes of nutritional conditions, because in a low-nitrate medium the internal nitrate does not sustain significant leakage, whereas in a high-nitrate medium the high-affinity system does not operate [16].

The existence of 'futile' cycles can be useful to obtain a fine modulation of the intracellular homeostasis when transitory pertubations occur in the environment [45]. As pointed out by Deane-Drummond [28], the long-term and short-term control of solute uptake can provide a coarse and a fine regulation, respectively, to optimize the intracellular solute concentrations to the actual cell requirements.

Acknowledgements

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