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Kinetic Characterization of Nitrite Uptake and Reduction by *Chlamydomonas reinhardtii*[1]

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ABSTRACT

Kinetics of nitrite uptake and reduction by *Chlamydomonas reinhardtii* cells growing phototrophically has been studied by means of progress curves and the Michaelis-Menten integrated equation. Both uptake and reduction processes exhibited hyperbolic saturation kinetics, the nitrite uptake system lacking a diffusion component. Nitrite uptake and reduction showed significant differences in K_s for nitrite at pH 7.5 (1.6 *versus* 20 micromolar, respectively), optimal pH, activation energy values, and sensitivity toward reagents of sulfhydryl groups. K_s values for nitrite uptake were halved in cells subjected to darkness or to nitrogen-starvation. Nitrate inhibited nitrite uptake by a partially competitive mechanism. The same inhibition pattern was found for nitrite uptake by *C. reinhardtii* mutant 305 cells incapable of nitrate assimilation. The results demonstrate that *C. reinhardtii* cells take up nitrite via a highly specific carrier, probably energy-dependent, kinetically responsive to environmental changes, distinguishable from the enzymic nitrite reduction and endowed with an active site for nitrite not usable for nitrate transport.

Nitrate uptake and reduction by higher plants, fungi, algae, and microorganisms are processes well established and characterized (4, 16, 36). However, nitrite assimilation has been poorly investigated, most of the studies being centered on the enzymic nitrite reduction (36, 37). In nonvacuolated microorganisms, uptake and reduction of nitrite are tightly coupled processes hard to study separately (36).

In microalgae, the scanty existing data on nitrite uptake indicate that this process, like that of nitrate uptake, is highly sensitive to ammonium, requires light and CO_2, and is inhibited by uncoupling agents (8, 10, 14, 31, 33, 34). Besides, in diatoms nitrite uptake exhibits hyperbolic kinetics with a diffusion component (5), and it is still unclear whether or not nitrite is taken up by the same carrier system as nitrate (5, 10, 36).

In the present work, uptake and reduction of nitrite by *Chlamydomonas reinhardtii* cells are characterized as two distinguishable processes on the basis of their differential kinetic properties. In addition, inhibition experiments demonstrate the existence of two separate sites for the uptake of nitrate and nitrite.

MATERIALS AND METHODS

Organisms and Enzyme Preparation. *Chlamydomonas reinhardtii* wild type 6145*c* was obtained from Dr. R. Sager (Sidney Farber Cancer Center, New York) and mutant 305 was isolated by Sosa *et al.* (29). The mutant is incapable of nitrate assimilation and carries a single mutation in the structural gene *nit-1a* (12, 13). Wild and mutant cells were grown with 8 mM NH_4Cl and derepressed for 5 to 6 h with 3 mM KNO_2 or N-free media, pH 7.5, as previously described (12). Cells were harvested at the midlogarithmic phase of growth by centrifugation at 15,000*g* for 10 min, and disrupted by freezing-thawing treatment with liquid N_2 (12). Nitrite reductase was extracted with 0.5 M phosphate buffer (pH 7.5), the suspension was centrifuged at 30,000*g* for 5 min, and the resulting supernatant used as source of enzyme.

Kinetics of Nitrite Uptake. Kinetics of nitrite uptake was studied by using the two following procedures:

Initial Rate Method. Initial rate of nitrite uptake at different nitrite concentrations in the medium was estimated at pH 7.5 in diluted cultures (less than 10 mg Chl/L) as the mean velocity over a short period (1–2 min), which is assumed to be the initial velocity corresponding to the mean substrate concentration in the same period. Kinetic parameters were calculated from the Hanes-Woolf plot (S/v *versus* S).

Progress Curve Method. Nitrite uptake rates were determined at pH 7.5 by measuring nitrite disappearance from the medium at different times till its complete exhaustion. Duration for each experiment depended on initial nitrite and Chl concentrations used. Progress curves obtained were analyzed by using the integrated form of the Michaelis-Menten equation, rearranged to yield linear plots (9, 23):

$$\frac{t}{\ln (S_o/S)} = \frac{1}{V_{max}} \cdot \frac{(S_o - S)}{\ln (S_o/S)} + \frac{K_s}{V_{max}}$$

where t is time in min, S_o is the initial concentration of nitrite, S is the concentration of nitrite at time t, K_s is the apparent half-saturation constant and V_{max} is the maximum uptake rate.

Nitrite Reductase Assay. Nitrite reductase activity was determined *in vitro* according to Ramírez *et al.* (25) with modifications. The reaction mixture contained, in a final volume of 1 ml, 0.4 mM KNO_2, 0.2 M K-phosphate (pH 7.5), 46 mM sodium dithionite, 0.8 mM methyl viologen, and the adequate amount of enzyme extract. Reaction was started by addition of dithionite. One unit of enzymic activity is defined as the amount of enzyme which catalyzes the reduction of 1 μmol of nitrite per min.

Kinetics of Nitrite Reduction. Kinetics of nitrite reduction by cell-free extracts was performed according to the progress curve method described above but using the reaction mixture for nitrite reductase assay. The incubation time is defined as the time elapsed from the moment of nitrite addition (0 time) till its complete exhaustion, which depended on initial nitrite concentration and nitrite reductase activity used. When indicated, dithionite concentration was modified.

Analytical Determinations. Nitrite was estimated colorimetrically by the diazotization method of Snell and Snell (27). Protein was measured colorimetrically according to Bailey (2), using BSA as standard. Chl was determined spectrophotometrically at 652 nm after Arnon (1).

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RESULTS

Kinetic Characterization of Nitrite Uptake and Reduction. From the progress curves of nitrite uptake (Fig. 1A) and reduction (Fig. 2A), K_s values for nitrite of 1.6 ± 0.1 μM and 25 ± 2 μM, respectively, were calculated. Both processes fit Michaelis-Menten kinetics (Figs. 1B and 2B). A diffusion component for nitrite uptake was not detected. The same kinetic parameters for nitrite uptake were found in mutant 305 (results not shown).

Nitrite uptake was highly responsive to the physiological state of the cells. Nitrogen starvation significantly decreased K_s for nitrite without affecting V_{max} of uptake, whereas under dark conditions both K_s and V_{max} were halved (Table I). In these circumstances nitrite uptake displayed hyperbolic kinetics, and intracellular levels of nitrite reductase activity were as high as

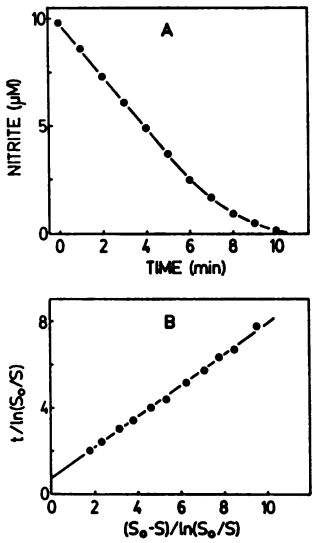

FIG. 1. Kinetic analysis of nitrite uptake by *C. reinhardtii*. Nitrite grown cells were transferred to media containing 25 μM nitrite. A, Nitrite concentration was determined in aliquots from the media taken every minute. B, Data in A are analyzed by the integrated Michaelis-Menten equation. S_o and S are expressed in μM and t in min.

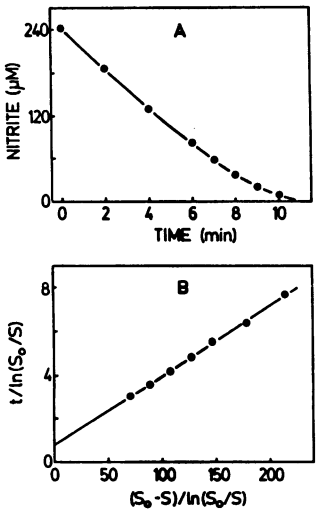

FIG. 2. Kinetic analysis of *in vitro* enzymic nitrite reduction by *C. reinhardtii* extracts. A, Nitrite (240 μM) was added to reaction mixtures containing adequate amounts of crude extract from *C. reinhardtii* and 23 mM sodium dithionite. Depletion of nitrite was followed for 10 min. B, Data in A are analyzed by the integrated Michaelis-Menten equation. S_o, S, and t are expressed as in Figure 1.

Table I. *Kinetic Parameters for Nitrite Uptake by C. reinhardtii Cells Subjected to Different Treatments*

Cells grown on nitrite were centrifuged, washed and transferred to nitrite media 0.1 mM (control), 0.1 mM nitrite in darkness or nitrogen free media (−N). After 8 h treatment, 0.1 mM nitrite was added and when nitrite was 25 μM, kinetic studies were carried out as indicated in "Materials and Methods." Data are expressed as mean ± SD. Number of experiments is indicated between parentheses.

Treatments	K_s		V_{max}
	Initial rate method	Progress curve method	
	μM		μmol(mg Chl·h)⁻¹
Control	2.43 ± 0.24 (5)	1.57 ± 0.11 (5)	10.05 ± 0.61 (5)
Darkness	1.06 ± 0.14 (3)	0.64 ± 0.08 (3)	4.60 ± 0.59 (3)
−N	1.11 ± 0.12 (3)	0.49 ± 0.06 (3)	10.88 ± 0.81 (3)

Table II. *Effect of Dithionite on K_s for Nitrite Reductase from C. reinhardtii*

Dithionite concentration corresponded to a final volume of 1 ml of assay mixture.

Dithionite	Incubation Time	K_s
mM	min	μM
23	10	27
23	30	48
23	120	178
46	30	57
69	30	79

those of control cells in nitrite media in the light (results not shown). K_s values for nitrite uptake calculated by using the initial rate method were higher than those obtained from the kinetic analysis of the progress curves of the integrated Michaelis-Menten equation (Table I).

Kinetic characterization of enzymic nitrite reduction was performed *in vitro* by using methyl viologen chemically reduced with dithionite as artificial electron donor.

First, the influence of variable amounts of dithionite used for nitrite reduction on the kinetic parameters was studied. Analysis of progress curves indicated that the K_s values for nitrite reduction increased with increasing incubation time. The direct correlation between incubation time and K_s is presumably due to an increase in the oxidation products of dithionite. When increasing initial concentrations of dithionite were used, increasing K_s values for nitrite were also observed, although smaller than when incubation time was modified (Table II). However, although K_s values found depended on incubation time and on initial concentration of dithionite, maximum rates of nitrite reduction remained constant in all tested conditions (results not shown). When data of Table II were extrapolated to zero concentration of oxidized dithionite, K_s values of nitrite reductase for nitrite of 15 to 20 μM were obtained.

Effect of pH and Temperature on Nitrite Uptake and Reduction. The curve of pH dependence of nitrite uptake showed a sharp maximum at pH 6.0 (Fig. 3A). Photosynthetic oxygen evolution was practically unaffected between pH 5.0 and 8.0 (results not shown). In contrast, the pH optimum for nitrite reduction was 8.0 (Fig. 3B).

Nitrite uptake and nitrite reduction exhibited different apparent optimum temperatures. Q_{10}(20–30°C) and activation energies were also different for both processes, the activation energy for nitrite uptake being 65% higher than for nitrite reduction (Table III).

Effects of Reagents of Sulfhydryl Groups on Nitrite Uptake

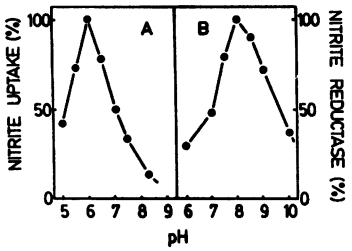

FIG. 3. Effect of pH on nitrite uptake and nitrite reductase activity in *C. reinhardtii*. A 50 mM citrate-MOPS-glycine buffer was used in the overall pH interval investigated. The uptake rates (A) and enzyme activity (B) were measured after 30 min of incubation at different pH. 100% of nitrate uptake rate and nitrite reductase activity corresponded to 13.7 μmol (mg Chl·h)$^{-1}$ and 94 milliunits/mg protein, respectively.

Table III. *Temperature Dependence of Nitrite Uptake and Reduction by C. reinhardtii*

Cells or crude extracts were incubated at different temperatures for 30 min, and uptake rate of nitrite or nitrite reductase activity determined after 10 min assay as indicated in "Materials and Methods."

	Nitrite Uptake	Nitrite Reduction
Apparent optimum temperature (°C)	30	45
Q_{10} (20–30°C)	2.5	1.9
Activation energy (kJ·mol^{-1})	72.0	43.5

Table IV. *Effect of Reagents of Sulfhydryl Groups on Nitrite Uptake and Reduction by C. reinhardtii*

NEM or *p*HMB was added to cells grown on nitrite at the indicated concentrations. After 1 h treatment, uptake rate of nitrite and nitrite reductase activity were determined as indicated in "Material and Methods."

Treatments	Concentration	Nitrite Uptake Rate	Nitrite Reductase Activity
	μM	%	
None		100[a]	100[b]
NEM	1	106	98
	10	7	91
	100	6	76
*p*HMB	10	109	102
	20	91	92
	100	41	94

[a] 9.0 μmol(mg Chl·h)$^{-1}$. [b] 71.3 milliunits/mg protein.

and Reduction. The nitrite uptake system was highly sensitive to the membrane-penetrating reagent NEM.[2] However, the non-penetrating reagent *p*HMB was significantly less effective as inhibitor. In both cases, nitrite reduction was practically unaffected by the above compounds even at concentrations as high as 100 μM (Table IV).

Nitrate Inhibition of Nitrite Uptake. Nitrite uptake was inhibited by low concentrations of nitrate (Fig. 4). This inhibition was studied by analyzing the effect of varying nitrate concentrations on nitrite uptake. The Hanes-Woolf plots indicate that nitrate inhibition only affected K_s values, which suggests a competitive inhibition of nitrate on nitrite uptake (Fig. 5). However, secondary replots of $1/v$ versus (nitrate) (Fig. 6A) and K_s' *versus* (nitrate) (Fig. 6B) produced curves which responded to equations of a partially competitive inhibition by nitrate (11):

[2] Abbreviations: NEM, *N*-ethylmaleimide; MOPS, *N*-morpholino-3-propane sulfonic acid; *p*HMB, *p*-hydroxymercuribenzoate.

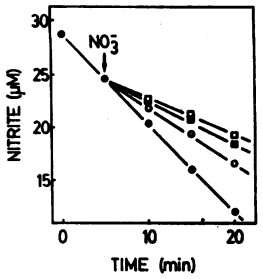

FIG. 4. Nitrate inhibition of nitrite uptake by *C. reinhardtii*. Nitrite depletion was followed in nitrite grown cells (●). When indicated by the arrow, 10 (○), 100 (■) or 1000 (□) μM nitrate was added.

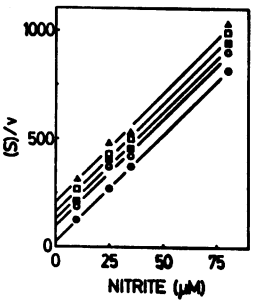

FIG. 5. Hanes-Woolf representation of nitrate inhibition of nitrite uptake by *C. reinhardtii*. Cells were grown in nitrite media. When the concentration of nitrite reached the indicated values, nitrate 12.5 (○), 25 (■), 50 (□), or 100 μM (▲), or none (●), was added and the initial rate of nitrite uptake determined. S is expressed in μM and v in μmol NO_2^-·(mg Chl·h)$^{-1}$.

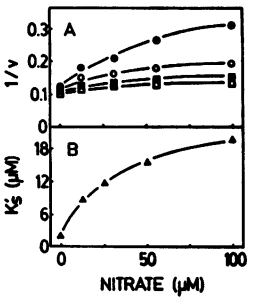

FIG. 6. Secondary plots of nitrate inhibition of nitrite uptake in *C. reinhardtii*. Data of Figure 5 were plotted by using the Dixon representation ($1/v$ *versus* | nitrate |) (A), and the secondary plot of K_s' *versus* | nitrate | (B). A, nitrite 10 (●), 25 (○), 35 (■), and 80 μM (□). v is expressed in μmol NO_2^-·(mg Chl · h)$^{-1}$.

$$v = \frac{V_{max}(S)}{K_s' + (S)},$$

where

$$K_s' = \frac{1 + (I)/K_i}{1 + \frac{(I)}{K_i} \cdot \frac{K_s}{K_{is}}} \quad \text{and} \quad \frac{K_i}{K_{si}} = \frac{K_s}{K_{is}}$$

according to the following scheme:

$$\begin{array}{ccc} & \overset{K_s}{\rightleftarrows} & \overset{k_p}{\rightarrow} \\ E + S & ES & \rightarrow E + P \\ + & + & \\ I & I & \\ K_i \updownarrow & \updownarrow & K_{si} \\ EI + S & \underset{K_{is}}{\rightleftarrows} ESI & \underset{k_p}{\rightarrow} E + P + I \end{array}$$

Data fit into the proposed mechanism with an error less than 6%. Since in partially competitive inhibitions it is not possible to determine kinetic parameters by simple graphical methods (11), we have calculated them from the above equations and experimental data (Table V).

When the same inhibition experiments were performed with the mutant strain 305 of *C. reinhardtii*, incapable of nitrate assimilation but able to grow with nitrite (12, 29), no significant differences in the kinetic constants were found in comparison with those of the parental wild strain 6145*c* (Table V).

DISCUSSION

Integrated rate equations are appropriately used in enzyme kinetic studies when product accumulation and substrate depletion are taken into account in the reaction rate calculation. This approach allows one to follow a reaction over an extended period and to obtain kinetic data by a simplified integrated equation in enzyme reactions with high K'_{eq} and low affinity of enzyme for the products (11, 23). Besides, this method yields more accurate kinetic parameter values than the usually preferred initial rate method which involves high errors in the initial rate determinations at low substrate concentrations. These errors are specially significant when the reaction cannot be followed continuously (15).

Nitrite uptake exhibited Michaelis-Menten kinetics with a K_s of 1.6 μM, which suggests the involvement in the process of a protein carrier. Unlike other algae and higher plants (5, 17, 19), nitrite uptake by *C. reinhardtii* lacked a diffusion component, which is consistent with the very low proportion of nitrous acid/nitrite (1/10,000) existing under our experimental conditions at pH 7.5. Darkness or nitrogen starvation changed values of kinetic parameters of nitrite uptake. The observed decrease in V_{max} for nitrite uptake by cells under dark conditions can be interpreted in terms of a diminution of nitrite reductase activity *in vivo* closely linked to photosynthetic mechanisms (21, 28, 32), although it can be also explained by a decrease in available energy required for nitrite transport, since *in vitro* nitrite reductase levels of darkened cells were the same as those of the cells grown in the light, under our experimental conditions. The significantly lower K_s values found in nitrogen-starved cells or subjected to dark conditions clearly indicate that nitrite uptake in *C. reinhardtii* is regulated by some intracellular effector (*s*). Physiologically, in darkness, a lower K_s may compensate the effect of a lower V_{max} on the uptake rate at the low nitrite concentrations existing in natural environments, and in nitrogen starvation conditions lower K_s would allow the uptake of nitrite present at low concentrations, at a higher rate. K_s value of nitrate for nitrate uptake in *Chlorella sorokiniana* decreased in nitrate grown cells subjected to nitrogen deprivation (33). In *C. reinhardtii*, K_s values of nitrite obtained by the initial rate method were always higher than those found by means of the progress curve method, although they changed similarly in cells subjected to the different treatments. This reflects errors in the uptake rate estimation by the former method as previously discussed.

Enzymic nitrite reduction to ammonium with reduced methyl viologen has a calculated K'_{eq} of about 4.5×10^{77} and is not inhibited by ammonium (15, 22, 24). Thus, the kinetic parameters of nitrite reductase can be satisfactorily calculated from progress curves of integrated Michaelis-Menten equations. Dithionite, the reducing chemical of methyl viologen in the electron donor system of nitrite reductase, inhibits nitrite reduction activity probably by formation of sulfite or other oxidation products (18, 26). In *C. reinhardtii*, K_s for nitrite increased linearly with incubation time during the nitrite reductase reaction, which probably reflects a competitive inhibition of oxidation products of dithionite on nitrite binding to the enzyme. Similarly, increasing concentrations of dithionite in the assay also raised K_s values but to a lesser extent. V_{max} of the reaction remained unchanged in both situations, which shows that activity in routine assay of nitrite reductase is unaffected by oxidation products of dithionite. The dithionite effect can also explain the wide variation (1 μM to 40 mM) in the reported figures of K_s (or K_m) of nitrite for nitrite reductase in green algae and higher plants (18, 36, 37).

Nitrite uptake exhibited an optimum pH of 6.0. Since in whole cells photosynthetic O_2 evolution was not affected by changing pH from 5.0 to 8.0, a distinction can be made between a nitrite transport system and the photosynthetic reducing power required to reduce nitrite. The distinction is confirmed by the optimal pH of 8.0 for the nitrite reductase assay *in vitro*. This value coincides with that of the chloroplast stroma (6), the suggested intracellular location of nitrite reductase in higher plants (37). Throughout this work we have performed kinetic studies of nitrite uptake and reduction at pH 7.5 instead of at optimal pH in order to establish similar experimental conditions between both processes so that the differences on K_s values for nitrite uptake and reduction could not be attributed to pH effects. On the other hand, the selected pH value is the usual pH of *C. reinhardtii* culture media.

Nitrite uptake has a Q_{10} of 3.1 and an activation energy of 72 $kJ \cdot mol^{-1}$, similar to those found for nitrate uptake in *C. sorokiniana* (34). These results, together with the aforementioned effect of darkness on kinetic parameters and the reported inhibition by several uncouplers (8, 10, 14), strongly suggest that nitrite uptake is energy-dependent. The lower values of Q_{10} and activation energy (1.9 and 43.5 $kJ \cdot mol^{-1}$, respectively) for nitrite reduction indicate that nitrite uptake is the kinetic rate-limiting step in nitrite assimilation. This is consistent with the proposed role for inorganic nitrogen permeases as the first control point in its assimilatory pathway (16, 30).

Nitrite uptake is strongly inhibited in the presence of NEM, and to a lesser extent when *p*HMB is present. Since these reagents possess different capabilities to penetrate the cells (20), our results suggest that HS-groups, nonaccessible from the outer face of the plasmalemma are involved in the nitrite transport system of *C. reinhardtii*. Unlike *in vitro* (3), we have not observed *in vivo* any effect of *p*HMB or NEM on nitrite reductase activity. However,

Table V. *Kinetic parameters for the Nitrate Inhibition of Nitrite Uptake in 6145c Wild Type and Mutant 305 Strains of C. reinhardtii*

Kinetic constant values corresponding to wild strain were obtained from data of Figures 5 and 6. Kinetic parameters corresponding to mutant strain were caculated from an identical experiment to that carried out with wild strain and described in the legends of Figures 5 and 6. Data are means of four independent experiments ± SD.

Strain	Kinetic Constants			
	K_s	K_i	K_{is}	K_{si}
	μM			
6145*c*	2.0 ± 0.2	2.7 ± 0.8	25.8 ± 5.6	33.8 ± 4.3
305	1.9 ± 0.2	2.9 ± 0.5	23.1 ± 4.8	35.3 ± 4.8

unspecific effects of these reagents on metabolic processes linked to nitrite uptake can not be ruled out.

Up to now, whether or not there is one or two different specific permeases for nitrate and nitrite in photosynthetic organisms is still unresolved. Kinetic and regulatory similarities in the uptake of nitrate and nitrite (10, 14) as well as nitrate competitive inhibition of nitrite uptake (5) suggest that both anions might be taken up by the same transport system, although Ullrich (36) has proposed two different systems on the basis of a different pH dependence of nitrate and nitrite uptakes (35) and different effects of monochromatic light on both processes (7). Our results clearly reveal that nitrate is a partially competitive inhibitor of the nitrite uptake and that this inhibition is independent of the existence of a functional nitrate reductase, since the same inhibition pattern was observed in mutant 305 incapable of assimilating nitrate. According to this kinetic mechanism nitrate does not bind to the site for nitrite in the nitrite permease. The observed inhibition by nitrate suggests that nitrate and nitrite uptake systems are closely connected, presumably by the "nitrogen charge." Since data do not fit into a pure competitive inhibition mechanism of nitrite uptake by nitrate (11), the existence of a single permease with a common active site responsible for the transport of both nitrate and nitrite is ruled out.

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