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Cooperative regulation by ammonium and ammonium derivatives of nitrite uptake in *Chlamydomonas reinhardtii*

Francisco Córdoba, Jacobo Cárdenas and Emilio Fernández

Departamento de Bioquímica, Facultad de Ciencias, Universidad de Cordoba, Córdoba (Spain)

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The role of ammonium in the regulation of nitrite uptake in *Chlamydomonas reinhardtii* has been investigated under conditions that prevented ammonium assimilation. Prolonged carbon-starvation or inhibition of glutamine synthesis with L-methionine-DL-sulfoximine partially relieved ammonium inhibition of nitrite uptake. However, nitrite uptake was inhibited in both methionine sulfoximine-treated and carbon-starved cells preincubated with ammonium, the inhibition extent in the two cases being directly dependent on the ammonium concentration in the preincubation media. Methionine sulfoximine treatment caused an increase of intracellular ammonium levels. When methionine sulfoximine-treated cells were transferred to ammonium media there existed a linear correlation between intracellular and extracellular ammonium concentration. Addition of methionine sulfoximine to cells with their nitrite uptake system inhibited by ammonium counteracted the effect of ammonium and restored nitrite uptake rate. These results strongly suggest that ammonium itself and a (some) product(s) of its metabolism must act together to block completely nitrite uptake by *C. reinhardtii* cells. Partial inhibition of nitrite uptake by methylammonium, a structural analogue of ammonium incapable of being used for cell nutrition, supports the above conclusion.

Introduction

Nitrite assimilation by the unicellular green alga *Chlamydomonas reinhardtii* includes two consecutive steps ion transport across the cytoplasmic membrane via a highly specific permease kinetically distinguishable from the nitrate permease [1] and subsequent reduction of internal nitrite to ammonium catalyzed by nitrate reductase [2–4] Both nitrate and nitrite uptake rates are very sensitive to ammonium regulation and, like nitrate reductase, nitrite reductase synthesis is repressed in the presence of ammonium [5–9] However, nitrite reductase is not inactivated when ammonium is added to culture media, whereas nitrate reductase activity decreased immediately after addition of ammonium [4,5,10,11]

At present it is still unresolved whether ammonium per se or a product of its metabolism is the inhibitor of the nitrate uptake Most data suggesting this latter possibility have been obtained from experiments carried out with methionine sulfoximine, an inhibitor of glutamine synthetase [12,13] or in carbon-deficient cells [14,15], whereas Florencio and Vega [5,6] have proposed that nitrate uptake is regulated by ammonium per se and not by any of its assimilation products

The effect of ammonium on nitrite uptake has been poorly investigated in algae, although it has been suggested that some metabolic ammonium

Correspondence J Cárdenas, Departamento de Bioquímica, Facultad de Ciencias, Universidad de Córdoba, Avenida San Alberto Magno s/n, 14071 Córdoba, Spain

derivative is the true inhibitor of nitrite consumption [6]

In the present work, we have studied the effect of ammonium on nitrite uptake rate in *C reinhardtii* cells by using experimental conditions under which ammonium assimilation is prevented Our data indicate that ammonium itself inhibits nitrite uptake rate but that this inhibition is total only under conditions that allow ammonium assimilation Thus, ammonium and one (or several) metabolite(s) derived from it must act together in the inhibition process

Materials and Methods

C reinhardtii wild-type strain 6145*c* was grown with 8 mM NH_4Cl and derepressed for 5–6 h with 3 mM KNO_2, as previously described [16] When indicated, CO_2 was eliminated by bubbling air through saturated KOH and $BaCl_2$ solutions In the in vivo experiments media were buffered at pH 7 0 with 50 mM potassium phosphate solutions For the in vitro enzyme determinations, cells were harvested by centrifugation at 15 000 × *g* for 10 min, and disrupted by freezing-thawing treatment [16] Nitrite reductase was extracted with 0 5 M phosphate buffer (pH 7 5) and glutamine synthetase with 0 5 M Mops-KOH buffer (pH 7 0) Suspensions of disrupted cells were centrifuged at 30 000 × *g* for 5 min, and the resulting supernatant was used as source of enzymes

Nitrite reductase was determined colorimetrically according to the procedure of Ramírez et al [17], with modifications The reaction mixture contained, in a final volume of 1 ml 0 4 mM KNO_2, 0 2 M phosphate buffer (pH 7 5), 0 8 mM methyl viologen and the adequate amount of enzyme extract Reaction was carried out at 30 °C for 15 min and was started by adding 46 mM sodium dithionite At 5-min intervals, 0 1-ml aliquots were taken out, diluted to 1 ml with distilled water and nitrite determined as described below The transferase activity of glutamine synthetase was determined by measuring colorimetrically the γ-glutamylhydroxamate formed according to the method of Shapiro and Stadtman [18] 1 unit of enzyme activity is defined as the amount of enzyme which catalyzes the transformation or production of 1 μmol substrate or product per min

Nitrite was estimated colorimetrically by the diazotization method of Snell and Snell [19] Ammonium in the media was determined after nesslerization according to the method of Folin and Denis [20] For the intracellular ammonium determination, cells were three times washed with ice-cold water, disrupted by freezing-thawing in 0 25 M triethanolamine buffer (pH 8 6) and centrifuged at 20 000 × *g* for 5 min to eliminate cell debris The resulting cell-free extracts were heated in a boiling water bath for 2 min to prevent any uncontrolled alteration of intracellular ammonium pools Ammonium was estimated by measuring at 340 nm NADH oxidation dependent on ammonium and 2-oxoglutarate in the presence of bovine liver glutamate dehydrogenase [21] Protein was measured colorimetrically according to the method of Bailey [22], using bovine serum albumin as standard Chlorophyll was determined spectrophotometrically by the method of Arnon [23] Cell volume was calculated by measuring the packed cell volume as described by Gfeller and Gibbs [24]

Results

Addition of ammonium to *Chlamydomonas* cells growing on nitrite, under saturating light and CO_2 conditions, blocked suddenly the uptake of nitrite (Fig 1A) However, when ammonium-grown cells were kept for 12 h in the absence of carbon source or in the presence of methionine sulfoximine, nitrite uptake rate was moderately inhibited by ammonium (20–40%) (Fig 1B, C) In both CO_2-depleted and methionine sulfoximine-treated cells preincubated with increasing concentrations of ammonium, an increasing inhibition of nitrite uptake was observed (Fig 2) Under these conditions, the maximal inhibition found never exceeded 80%, even at high ammonium concentrations

The intracellular content of ammonium in nitrite-grown cells was more than doubled when 1 mM ammonium was added to culture media (Table I) However, in the presence of methionine sulfoximine the intracellular ammonium concentration was raised ten times over control value whereas nitrite uptake rate decreased by only 18% and ammonium was excreted into the medium (Table I) Addition of ammonium to methionine

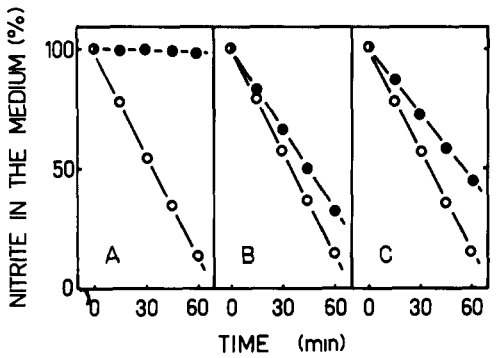

Fig 1 Effect of methionine sulfoximine-treatment and CO_2 depletion on nitrite uptake inhibition by ammonium in *C reinhardtii* Ammonium grown cells were washed, transferred to and kept for 12 h in media containing 3 mM KNO_2 (A), 3 mM $KNO_2 - CO_2$ (B) or 3 mM $KNO_2 + 1$ mM methionine sulfoximine (C) At zero time, 1 mM ammonium was added (●) to control cultures without ammonium (○), and nitrite was measured in the media at the indicated times 100% corresponded to 0 19 (A), 0 14 (B) and 0 16 (C) mM nitrite The chlorophyll content of cells was 17 5 (A), 27 1 (B), and 16 7 (C) mg/l

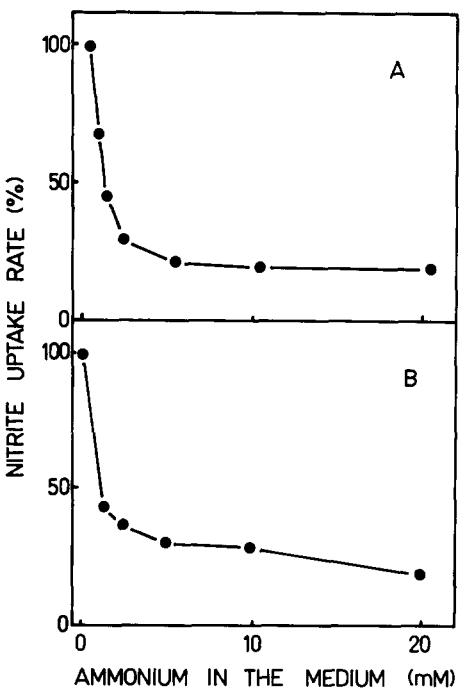

Fig 2 Relationship between nitrite uptake rate and extracellular ammonium concentration in methionine sulfoximine-treated (A) and carbon-starved (B) cells of *C reinhardtii* Cells were subjected to methionine sulfoximine treatment (A) or kept in CO_2-depleted media (B) in the presence of 3 mM KNO_2 After 12 h, cells were harvested and transferred to media containing 0 5–20 mM ammonium and, after 2 h treatment, the nitrite uptake rate was determined by measuring periodically nitrite content in the media 100% of nitrite uptake rate corresponded to 8 6 (A) and 4 3 (B) μmol/mg Chl per h

TABLE I

AMMONIUM AND/OR METHIONINE SULFOXIMINE EFFECTS ON THE NITRITE UPTAKE BY *C REINHARDTII*

Nitrite-grown cells were harvested in the mid-phase of growth and transferred to media containing 1 mM KNO_2, 1 mM NH_4Cl or 5 mM methionine sulfoximine (MSX), as indicated After 1 h treatment nitrite and ammonium uptake (↓) or excretion (↑) rates were determined by measuring periodically their concentrations in the media, and intracellular ammonium concentration was measured enzymatically as described in Materials and Methods 100% of uptake or excretion rate corresponded to 9 2 μmol/mg Chl per h and 100% of intracellular ammonium concentration corresponded to 0 08 mM

Treatment	Uptake or excretion rate (%)			Intracellular $[NH_4^+]$ (%)
	NO_2^- (↓)	NH_4^+ (↓)	NH_4^+ (↑)	
KNO_2	100		0	100
$NH_4Cl + KNO_2$	3	182		267
methionine sulfoximine $+ KNO_2$	82		112	990
NH_4Cl		132		362
methionine sulfoximine $+ NH_4Cl$			26	1116
methionine sulfoximine $+ NH_4Cl + KNO_2$	51		79	1140

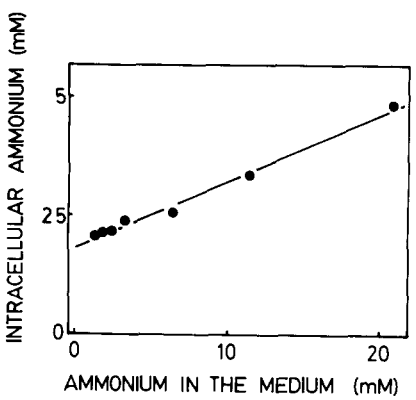

Fig 3 Linear correlation between extra- and intracellular ammonium concentration in methionine sulfoximine-treated cells of *C reinhardtii* 0 5–20 mM ammonium was added to cells treated with methionine sulfoximine for 12 h, as in the legend to Fig 2 After 2 h in the presence of ammonium, its intracellular and extracellular concentrations were determined as described in Materials and Methods

sulfoximine-treated cells did not alter intracellular ammonium levels, but caused a 50% inhibition of nitrite uptake rate (Methionine sulfoximine + NH_4Cl + KNO_2, Table I) Besides, ammonium concentration in the media was linearly correlated with intracellular ammonium concentration in methionine sulfoximine-treated cells (Fig 3)

Methionine sulfoximine partially relieved nitrite uptake inhibition by ammonium (Fig 4A) The relief was accompanied by ammonium excretion to the media (Fig 4B) and a significant increase of intracellular ammonium levels (Fig 4C) About 75% of the ammonium excreted was due to enzymatic reduction of nitrite and the remaining 25% probably came from catabolic or photorespiratory reactions (Table I and Fig 4)

In methionine sulfoximine-treated cells glutamine synthetase activity was absent In contrast, nitrite reductase was only partially inhibited (10–20%) in methionine sulfoximine-treated and CO_2-starved cells (results not shown)

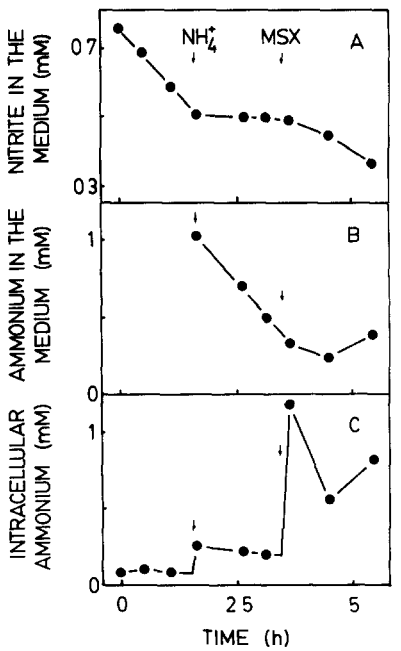

Fig 4 Reversion by methionine sulfoximine (MSX) of the ammonium inhibitory effect on the nitrite uptake in *C reinhardtii* cells 1 mM ammonium or 5 mM methionine sulfoximine was added to nitrite-grown cells when indicated by arrows, and nitrite in the media (A), ammonium in the media (B) and intracellular ammonium (C) concentrations determined at the indicated times

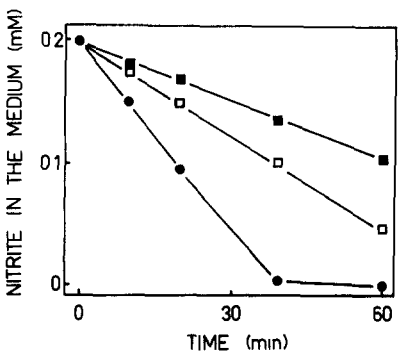

Fig 5 Nitrite uptake inhibition by methylammonium in *C reinhardtii* 2.5 (□) or 5 mM (■) methylammonium was added to nitrite-grown cells, and nitrite in the media determined at the indicated times Control without methylammonium is expressed by (●)

Methylammonium partially inhibited nitrite uptake by nitrite growing cells (2.5 and 5 mM methylammonium inhibited nitrite uptake by 50 and 70%, respectively) (Fig 5) However, nitrite reductase was practically unaffected by methylammonium addition (results not shown)

Discussion

Our results show that nitrite uptake inhibition by ammonium in *Chlamydomonas* is complete when ammonium is assimilated without any restrictions, which might lead up to conclude that a product derived from ammonium metabolism is the true inhibitor of nitrite uptake in this alga This conclusion has been previously reached for nitrate uptake inhibition by ammonium in *Chlamydomonas* and other photosynthetic organisms [13–15, 25–27] On the other hand, when ammonium assimilation is blocked, by inhibiting glutamine synthetase activity with methionine sulfoximine or by subjecting the cells to carbon starvation conditions, an enhancement of the intracellular ammonium accompanied by nitrite uptake inhibition takes place The conclusion now would be that ammonium per se is the true inhibitor of nitrite uptake, as proposed by Florencio and Vega [5,6] for nitrate uptake in *C reinhardtii* Therefore, a mixed interpretation of our results can be elaborated nitrite uptake in *Chlamydomonas* is probably regulated via a double mechanism that involves ammonium per se

and a metabolite derived from its assimilation In this respect, Bagchi et al [28] have recently reported, by using glutamine auxotrophs of *Anabaena cycadeae,* that in addition to ammonium itself a product of ammonium assimilation via glutamine synthetase may also be involved in the regulation of nitrate uptake

Intracellular ammonium determinations were performed in order to ascertain whether or not use of this parameter is valid to predict the inhibition degree of nitrite uptake Actually, it has been reported that an intracellular threshold level of ammonium exists above which this compound behaves as a co-repressor of nitrate reductase in *C reinhardtii* [8] Besides, nitrite reductase synthesis in the same organism is inversely related to intracellular ammonium levels in cells transferred from ammonium to nitrite media [9] Our data show that the nitrite uptake system is little sensitive to ammonium alone since in methionine sulfoximine-treated cells without external ammonium added and displaying high intracellular ammonium levels of ammonium, nitrite was taken up at rates similar to those in control cells. However, a strong inhibition of nitrite uptake in methionine sulfoximine-treated cells resulting from increasing extracellular ammonium levels was observed and directly correlated with the intracellular levels of this compound Thus, we conclude that an increase of intracellular ammonium concentration is a necessary but not sufficient condition to explain the nitrite uptake inhibition by ammonium, and that product(s) of ammonium assimilation can act together with ammonium (probably synergistically) in the regulation of nitrite uptake Possibly, the nitrogen status of cells, which depends on experimental conditions, and the particular ratio external/internal concentrations of ammonium and nitrite are decesive factors to account for the particular contribution of ammonium itself and the product of its metabolism to the inhibition of nitrite uptake In this connection, Deane-Drummond [29,30] has proposed a model for the nitrate uptake system in which, basically, the metabolic state of cells is responsible for the ratio influx/efflux of nitrate Other interpretations based on ammonium assimilation via pathways alternative to glutamine synthetase (e g , via glutamate dehydrogenase) do not seem probable since ammonium from photorespiratory or catabolic origin is excreted to media in methionine sulfoximine-treated cells

The ammonium analogue methylammonium mimics all the effects of ammonium on nitrate assimilation [31–33] In *C reinhardtii*, ammonium and methylammonium utilize the same transport system [34] Besides, methylammonium cannot be used as nitrogen source for growth, and acts as a substrate of glutamine synthetase, rendering as a unique product γ-*N*-methylglutamine [8] We have shown that, depending on its concentration, methylammonium inhibits nitrite uptake by *C reinhardtii* Methylammonium itself must be the inhibitor of the nitrite uptake If γ-*N*-methylglutamine were the true inhibitor of the nitrite uptake, it an inhibition higher than that observed would be expected, in keeping with the rate at which that compound accumulates inside the cells Our results show that methylammonium inhibition pattern is linear during 1 h, which rules out the preceding interpretation This linear pattern can be the result of the rapid accumulation of methylammonium inside the cells and of its slow transformation rate by glutamine synthetase [8] In *Phaeodactylum tricornutum,* a partial inhibition of nitrate and nitrite uptake by methylammonium has been also found, but took a longer time to develop as result of a slow rate of methylammonium accumulation inside the cells [35] The lower sensitivity of nitrite uptake to methylammonium with respect to that exhibited toward ammonium under the same conditions can be due to the absence of the metabolite(s) derived from ammonium assimilation, which further supports our conclusion that both ammonium itself and a product of its metabolism act synergistically to inhibit nitrite uptake Besides, methylammonium may be not recognized as efficiently as ammonium by the regulatory sites or proteins in the membrane

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