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Protistology

Urea transport during gametogenesis of the unicellular green alga *Chlamydomonas reinhardtii*

Zhanneta M. Zalutskaya[1], Tatyana V. Lapina[1], Nicolaus von Wirén[2] and Elena V. Ermilova[1]

[1] *Laboratory of Adaptation in Microorganisms, Biological Research Institute of St. Petersburg State University, Russia*
[2] *Leibniz-Institute for Plant Genetics and Crop Plant Research, Gatersleben, Germany*

Summary

Direct urea transport mechanisms are present in *Chlamydomonas reinhardtii*. Urea uptake system(s) are repressed by ammonium and they can be induced by urea or acetamide in ammonium-starved vegetative cells. Urea transport ability of the alga is altered during gametogenesis. Unlike vegetative cells, mature gametes showed a low urea uptake. Incubation of gametes with urea or acetamide resulted in the increasing of urea uptake ability and the regaining of chemotactic activity. The data suggest a tight coupling between changes in chemotaxis towards ammonium and restoration of transport/metabolism of urea.

Key words: *Chlamydomonas,* urea transport chemotaxis gametogenesis

Abbrevations: TAP - Tris-acetate-phosphate (medium); TAP-N - Nitrogen-free TAP (medium)

Introduction

Ammonium is a preferential nitrogen source for many unicellular organisms including the unicellular green alga *Chlamydomonas reinhardtii*. Besides ammonium *C. reinhardtii* can also utilize other nitrogen forms as urea or acetamide (Grossman and Takahashi, 2001). Higher plants take up urea by different types of transporters (Liu et al., 2003a; Liu et al., 2003b; Kojima et al., 2006; Mérigout et al., 2008). In lower plants the molecular mechanisms of urea transport have not yet been elucidated. The biflagellate green alga *Chlamydomonas reinhardtii* is well suited to molecular-genetic studies of many fundamental processes in photosynthetic organisms (Harris, 2001).

In *C. reinhardtii* urea is incorporated into cells by an active transport mechanism (Watson et al., 1994). Two transport systems have been described to participate in urea uptake by *Chlamydomonas* cells: one with a low affinity and another with a high affinity, and both are negatively regulated by ammonium. In cells, urea is hydrolyzed to ammonia by the sequential action of two enzymes, urea carboxylase and allophanate lyase, which form the enzyme complex ATP:urea amidolyase (Leftley and Syrett, 1973; Hodson et al., 1975). Urea transporter systems should also be present in the chloroplast

membrane, because there urea can accumulate (Dagestad et al., 1981).

The ability of unicellular organisms to differentiate in response to nutrient availabilities is essential for their survival in a changing environment (Lengler et al., 2000). Nitrogen also plays an important role in sexual differentiation since the lack of an utilizable nitrogen source in *C. reinhardtii* is the signal that initiates gamete formation (Beck and Haring, 1996). In gametogenesis, haploid vegetative cells are converted into mating-competent gametes. Gametic differentiation has been shown to be associated with changes not only in cellular biochemistry and subcellular morphology (Beck and Haring, 1996) but also in chemotactic behavior of the cells (Ermilova et al., 2003a; Ermilova et al., 2004). Unlike vegetative cells, gametes lack chemotaxis towards ammonium. Although *Chlamydomonas* cells do not display chemotaxis to urea, this compound interferes with changes in chemotaxis (Ermilova et al., 2004). Very little is known about the molecular basis of this regulation.

The aim of the present study was to investigate urea uptake at different stages of the gametogenesis. For this purpose, uptake capacities of [^{14}C]-urea were determined in vegetative cells, grown under different nutritional conditions, and in gametes. Furthermore, chemotaxis was measured under different nitrogen regimes to determine the interaction of two processes, urea uptake and chemotaxis.

Material and methods

Strains and culture conditions

Chlamydomonas reinhardtii wild-type strain CC-124 (*mt* $^-$) was obtained from the *Chlamydomonas* Culture Collection at Duke University, USA. *Chlamydomonas reinhardtii* wild-type strain CC-124 (*mt* $^+$), obtained from the *Chlamydomonas* Culture Collection at Duke University, USA, was used Another wild-type strain CC-620 (*mt* $^+$), obtained from S.Purton, University College London, GB, was used as the tester strain. Cells were grown at 22° C under a 12-h light/12-h dark regime in Tris acetate phosphate (TAP) medium (Gorman and Levin, 1965) or in urea-containing TAP-N medium. 2 mM urea was added to the medium.

Gametes were obtained by incubation of vegetative cells in nitrogen-free medium with continuous illumination (30 $\mu molm^{-2}s^{-1}$). In the experiments where the effect of cycloheximide (Sigma) on chemotaxis and urea uptake was tested, the inhibitor was added to gametes 15 min prior to start the incubation with urea or acetamide at a final concentration of 1 μg/ml.

Determination of mating competence

The percentage of gametes was assayed by mixing the cells to be tested with a threefold excess of gametes of opposite mating type. Mating was allowed to proceed in the dark for one h and stopped by adding glutaraldehyde (final concentration 0.5%). The number of biflagellate cells and quadriflagellate cells in the mating mixture was recorded microscopically. The percentage of mating competent gametes was calculated as described (Beck and Acker, 1992).

Chemotaxis assay

Chemotactic responses were tested as described (Ermilova et al., 1998). Switch-off of chemotaxis was recorded as 1.0. Data represent means ± SD of triplicate determinations from a representative experiment with at least three replicas.

Uptake assays

Uptake of [^{14}C]-urea was determined as described (Williams and Hodson, 1977). Cells were harvested and subsequently washed and suspended in assay buffer (20 mM HEPES; pH to 7.2 with KOH) to a chlorophyll *a* + *b* content of ~15 μg/ml. Suspended cells were incubated in a shaker at 25° under light and radiolabeled urea (6 μM; specific activity 6 or 50 Ci/mol) was added. At appropriate intervals, 200 μl samples were removed from the culture and filtered through 25-mm-diameter glass fiber disks (Watman GF/C) that had been soaked in 20 mM urea solution in assay buffer. Cells were filtered and washed twice by suction with assay buffer. The damp filters were placed in 2 ml of scintillation cocktail, and the radioactivity was determined by liquid scintillation.

Results

Effects of urea and acetamide on urea uptake

As described earlier, cells grown with ammonium as a nitrogen source did not take up urea (Hodson et al., 1975). Nitrogen deprivation was shown to be required for a derepression of the urea transport ability after removal of ammonium from the medium (Williams and Hodson, 1977). Since high-affinity urea transport in higher plants has been found to be inducible by urea (Kojima et al., 2007), the question

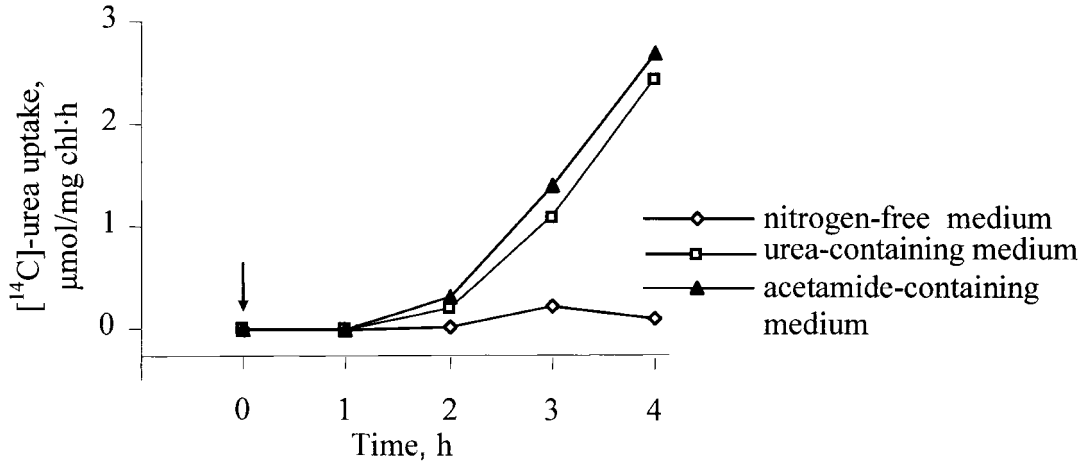

Fig. 1. Effects of urea or acetamide supply on urea uptake in nitrogen-deprived vegetative cells grown in TAP medium. At the time indicated by the arrow, 2 mM urea or 10 mM acetamide was added.

arose whether the addition of urea or acetamide to cells transferred into ammonium-free medium would cause an increase of transport ability to a similar extent as in nitrogen-deprived vegetative cells. If ammonium is depleted and urea or acetamide was immediately added, , the velocity of urea transport into cells increased dramatically after incubation for 2 to 4h in the light and urea transport was higher than in nitrogen-starved cultures (Fig. 1). The stimulating effect of urea and acetamide on urea uptake was similar. Two hours of incubation in nitrogen-free medium were required for a derepression of urea uptake by cells that had been grown in ammonium-containing medium, suggesting that i) an incubation for 2h is the minimal time required before nitrogen-deprived vegetative cells are competent for urea uptake, and ii) the urea and acetamide are acting as inducers for the transport system(s).

Alteration of urea uptake during gametogenesis

Effective formation of mature gametes has been shown to depend on the removal of nitrogen from the medium and on the presence of light (Beck and Haring, 1996). Moreover, it has been shown that urea affects the conversion of vegetative cells into gametes (Matsuda et al., 1992). We therefore wondered whether mature gametes still possess the ability for urea uptake. [^{14}C]-urea uptake was compared between gametes and vegetative cells, which were grown in urea as a sole nitrogen source (Fig. 2). After illumination for 24 h in nitrogen-free medium, *Chlamydomonas* cells differentiated into gametes that exhibited no significant [^{14}C]-urea uptake. Thus, mature gametes were unable to efficiently take up urea, which blocks the program of their differentiation.

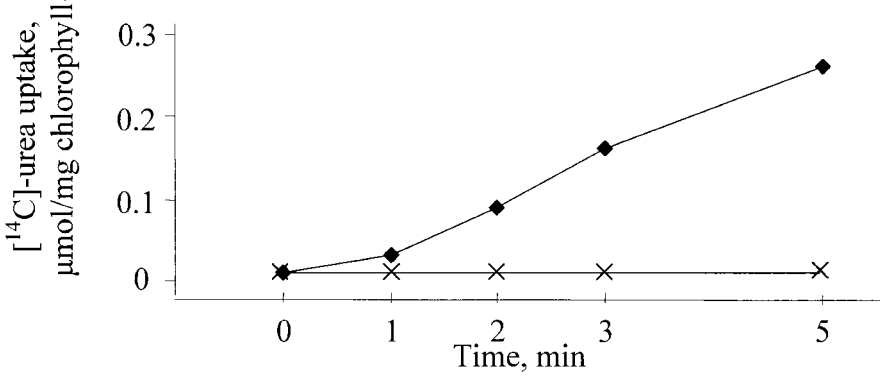

Fig. 2. [^{14}C]-urea uptake in vegetative cells and gametes. Vegetative cells of strain CC-124 were grown for 72 h in urea-containing medium (•). Gametes (×) were generated by incubation of vegetative cells in nitrogen-free medium under continuous light irradiation for 24 h.

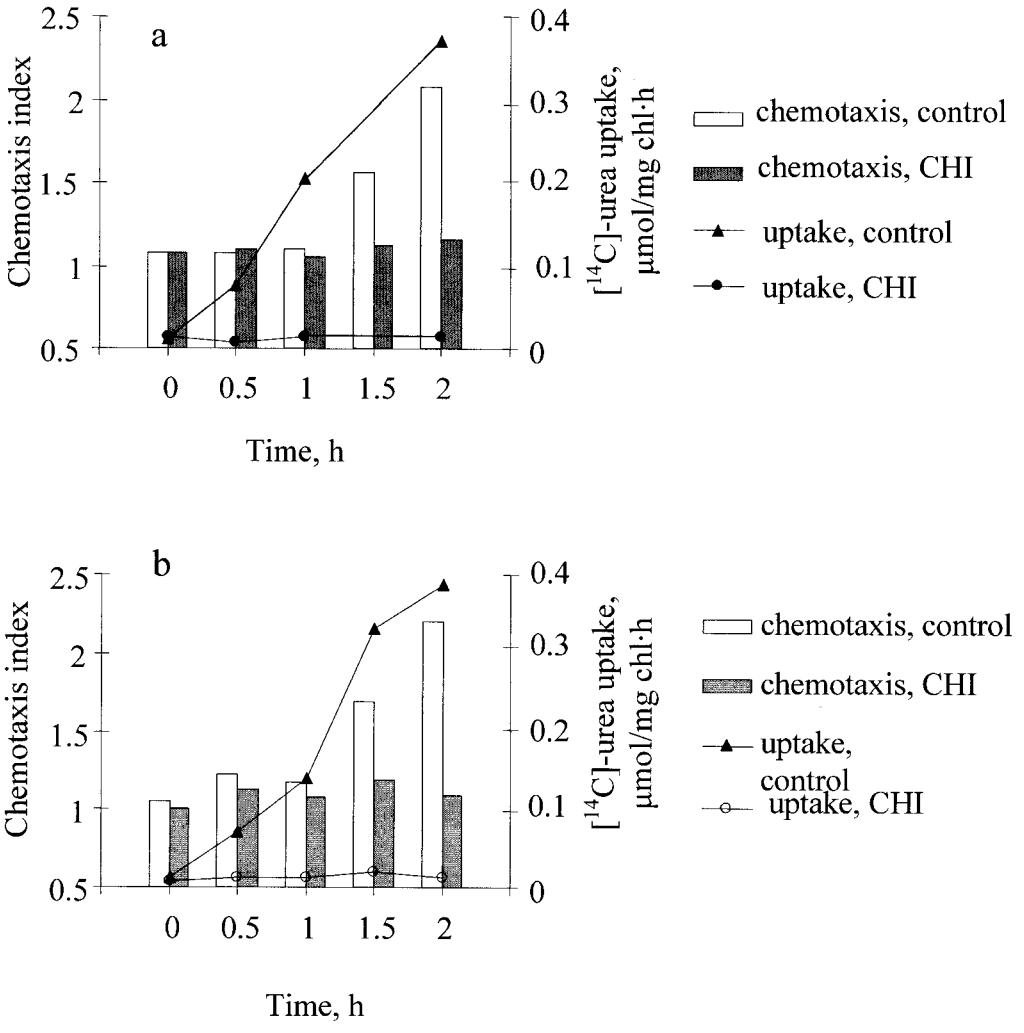

Fig. 3. Effects of urea (a) and acetamide (b) on the restoration of urea uptake and chemotaxis to ammonium. Cycloheximide, CHI, (1μg/ml) was added to gametes 15 min prior to start of incubation with urea (2mM) or acetamide (10 mM).

Urea uptake and chemotaxis to ammonium are restored after addition of urea or acetamide to gametes

After re-supply of urea or acetamide to gametes, urea uptake could be restored (Fig. 3). During the first 10-20 min, urea uptake was not increased and averaged 0,011 μmolmg/chl h. After further incubation in the presence of the urea or acetamide, there was a slight increase in the urea uptake rate at 30 min, and about a dramatic increase after 2 h. We noted that the rate and extent of uptake was much lower compared with nitrogen-deprived vegetative cells incubated with these compounds (Fig. 1). The increase in urea uptake in gametes after incubation with urea or acetamide was dependent on a *de novo* protein synthesis, since it did not occur in the presence of cycloheximide.

Since the loss of chemotaxis to ammonium is a trait that is characteristic for gametes, we investigated whether urea or acetamide treatment resulted also in a recovery of their chemotaxis to ammonium. Indeed, chemotaxis towards ammonium was activated within 2 hours after an incubation of gametes with urea or acetamide (Fig. 3). Thus, chemotaxis restored approximately 1.5 h after urea uptake was increased. The recovery of chemotactic activity may have been caused by a loss of inhibiting factor(s) that is (are) repressed in gametes. An involvement of intracellular urea in the control of chemotaxis was supported by the observation that cycloheximide completely blocked the recovery of chemotaxis. The same concentrations of urea or acetamide also initiated a loss of the mating ability and de-differentiation of gametes. Cells were sexually noncompetent and showed 0% of

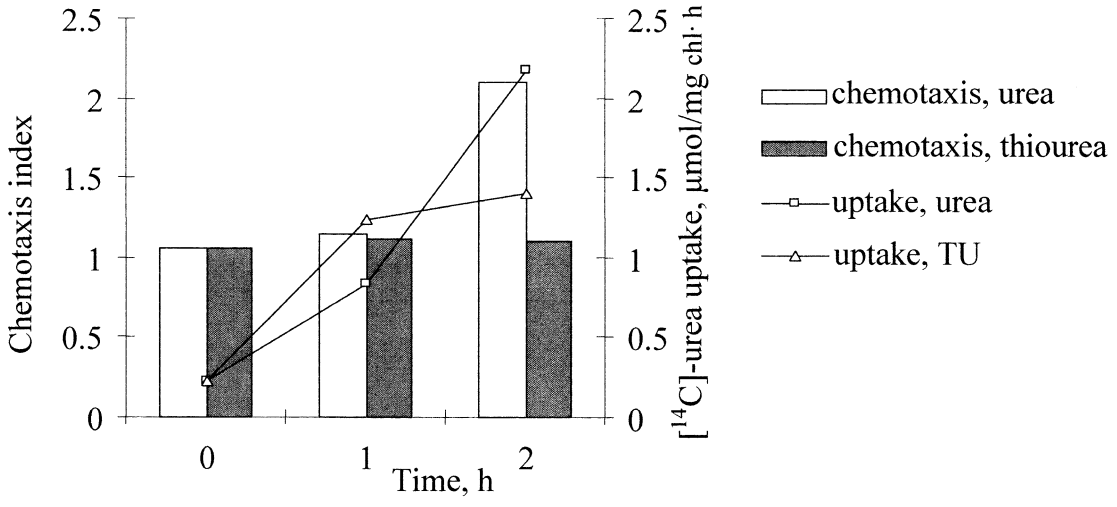

Fig. 4. Effects of thiourea (2 mM) on the restoration of urea uptake and chemotaxis to ammonium.

mating ability. However, addition of urea to gametes resuspended in acetate-free medium did not result in a loss of mating ability or the recovery of chemotaxis to ammonium. Cells were chemotactically inactive and exhibited 80 % of mating competence.

Effects of thiourea on urea uptake and chemotaxis

The block of the chemotaxis recovery in acetate-free medium suggests that urea may serve as a source of ammonium to control the appearance of chemotaxis to ammonium. To test this possibility, thiourea, which not supported the *Chlamydomonas* growth, was used as an inducer. Thiourea was not shown as an inducer of allophanate lyase (Williams and Hodson, 1977). Thiourea did not cause the recovery of chemotaxis to ammonium (Fig. 4). However, after the addition of thiourea to gametes, urea transport increased in 2 h (Fig. 4). The data suggest that (1) thiourea is an inducer of urea uptake, and (2) ammonium is the principal regulator of derepression of chemotaxis activity.

Discussion

The present study shows that the capacity for urea uptake in *Chlamydomonas* cells differs according to the stage of their life cycle. After uptake across the plasma membrane urea can be readily assimilated by *C. reinhardtii* if ammonium is absent (Grossman and Takahashi, 2001), and urea uptake was undetectable in ammonia-fed vegetative cells (Williams and Hodson, 1977). In *Arabidopsis* urea uptake was also reduced by presence of ammonium in the growth medium (Mérigout et al., 2008). In our study, nitrogen-sufficient cells required an incubation period of 2h in the absence of ammonium to restore the uptake of urea – indicating that the nitrogen-mediated derepression is not a rapid process (Fig. 1). Our data further showed that urea uptake is more robust in nitrogen-deprived cells resuspended with urea or acetamide than in only nitrogen-free medium. Allophanate lyase, which mediates the second step in the two-step urea amidolase reaction, can be induced by urea or acetamide in ammonium-starved cells (Semler et al., 1975). Urea uptake system(s) and allophanate lyase appeared to be subject to a coordinate regulation by the same inducers, urea and acetamide. A similar observation has been made in *Arabidopsis thaliana*, where the high-affinity urea transporter AtDUR3 was strongly induced after re-supply of to nitrogen-stared roots (Kojima et al., 2007; Mérigout et al., 2008). The initial step in the sexual life cycle of *C. reinhardtii* is gametogenesis. Gametic differentiation of vegetative cells is induced by the depletion of an utilizable nitrogen source (usually ammonium) (Treier et al., 1989; Beck and Acker, 1992). Nitrogen depletion enhances many nitrogen transport systems in bacteria, yeast and algae including *C. reinhardtii* (Fernandez and Galvan, 2007). However, gametes do not show effective urea uptake (Fig. 2). The loss of the ability to take up urea by gametes, which is known to repress their sexual differentiation (Matsuda et al., 1992; Ermilova et al., 2003b), may be seen as a cellular adaptation to changing environmental conditions.

Addition of urea or acetamide to gametes restored urea uptake in cells (Fig. 3), and the cycloheximide treatment indicated that a *de novo* protein synthesis is required to restore urea uptake. An incubation of gametes with these inducers also resulted in regaining restoration of chemotactic

activity (Fig. 3). These results indicate that a loss of chemotactic activity in gametes is unstable, like their inability to take up urea. Furthermore, these data also indicate a tight coupling between changes in chemotaxis towards ammonium and restoration of transport and metabolism of urea. However, while transport activity appeared within 30 min, the recovery of chemotaxis required 2 hours. Thus, reactivation of chemotaxis in gametes upon urea or acetamide treatment is clearly a slower response and dependent on protein synthesis. Since the reactivation of chemotaxis was not induced by urea in acetate-free medium, urea assimilation may be involved in the control mechanism. This idea is supported by the data with non-metabolizing thiourea, which did not induce the reactivation of chemotaxis (Fig. 4).

We assume that urea and acetamide serve as sources of ammonium to regulate the program of de-differentiation of gametes that involves the alteration in mating ability and chemotactic activity as integral parts.

Acknowledgements

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Address for correspondence: Elena V. Ermilova. Laboratory of Adaptation in Microorganisms, Biological Research Institute of St. Petersburg State University, Oranienbaumskoe schosse 2, Stary Peterhof, St. Petersburg, 198504 Russia, e-mail: ermilova@EE6439.spb.edu