KINETICS AND REGULATION OF UREA UPTAKE IN *ANABAENA DOLIOLUM* AND *ANACYSTIS NIDULANS*

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The apparent K_m and V_{max} values for urea uptake in *Anabaena doliolum* Bharadwaja were 250 μM and 568 nmol of urea (mg protein)⁻¹ hr⁻¹. In comparison, *Anacystis nidulans* IU 625 showed lower affinity, K_m being 400 μM and V_{max} 500 nmol of urea (mg protein)⁻¹ hr⁻¹. Urea uptake was optimal at pH 7.5, 15 W m⁻² illumination and 30°C. Maximum uptake was evident during the exponential growth phase. Cells pregrown on N₂, urea or nitrate transported urea equally well, but there was essentially no uptake of urea in cells preincubated with ammonia for more than 24 hr. Nevertheless, ammonia did not inhibit urea uptake by urea grown cells. But the uptake of ammonia was reduced in the presence of urea. N-deficiency invariably stimulated uptake of urea. Experiments under different metabolic conditions revealed that uptake is an active process driven mainly by cyclic photophosphorylation.

Nitrate and ammonia are probably the main nitrogen sources for the growth of algae under natural conditions and have received more attention than organic nitrogen. Urea can support excellent growth of some green algae and cyanobacteria (1–3), but little is known about the mechanism(s) responsible for its transport. Only recently, there have been some studies on the kinetics of urea transport in eukaryotic algae (4–8). These investigations have provided evidence for carrier-mediated active transport of urea at the plasmalemma of the algal cells and the existence of an active transport mechanism with high affinity for urea.

Active uptake in plant cells may be driven by either respiration or photosynthesis through the generation of ATP. In green cells transport is limited by photosynthesis rather than by respiration. Since there is no study on how urea is taken up in cyanobacterial cells (2), we describe here some characteristics of urea

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transport in the cyanobacteria *Anabaena doliolum* and *Anacystis nidulans*.

MATERIALS AND METHODS

Organism and culture conditions. *Anabaena doliolum* Bharadwaja (axenic isolate from a local population) and *Anacystis nidulans* IU 625 (ATCC 27144) were used in this study. Axenic cultures were grown in Chu No. 10 medium (9). Nitrogen-free medium (N_2 -medium) was obtained by replacing calcium nitrate with calcium chloride (0.007 mM). Combined nitrogen was provided in the medium as 0.5 mM NH_4Cl , 0.25 mM $Ca(NO_3)_2$ and 1 mM urea for *A. doliolum* and 0.5 mM NH_4Cl , 1 mM $Ca(NO_3)_2$ and 5 mM urea for *A. nidulans*. The cultures were incubated in a culture room at $24 \pm 1^\circ C$ and illuminated with day light fluorescent tubes (intensity $15 W m^{-2}$) for 14 hr per day.

Analytical methods. Urea was determined by the diacetylmonoxime method (10). Ammonia was estimated by the method of CHANEY and MARBACH (11). The concentration of phenate and hypochlorite reagent was modified as described by KAPLAN (12). Cell concentration was estimated from the absorbance values at 430 nm of the cell suspension, after a correlation between these two parameters had been established. Protein was estimated by the method of LOWRY et al. (13), standardized with bovine serum albumin.

Uptake assays. Exponentially growing cells were collected by centrifugation at $5,000 \times g$ for 10 min, washed twice with sterile distilled water and suspended in sterile growth medium (N_2 -medium). Uptake was initiated by addition of urea (50–2,000 μM). Unless otherwise stated, the uptake experiments were conducted at a light intensity (from daylight fluorescent tubes) of $15 W m^{-2}$ at $24 \pm 1^\circ C$. From samples withdrawn at regular intervals, the cells were removed by centrifugation and cell-free supernatants were analyzed for residual urea. Uptake was assayed measuring the depletion of urea from the external medium.

The linear portions of the curve (uptake during 0–30 min) were used to calculate the initial rate of urea uptake ($nmol (mg \text{ protein})^{-1} hr^{-1}$). Values of K_m and V_{max} were calculated by using S/V against S plots. For the rest of the uptake experiments, an initial concentration of 2 mM urea was taken. For anaerobic uptake assays the air of the vessels was replaced by N_2 .

Urease assay. Cells were collected by centrifugation, resuspended in 5 mM phosphate buffer, pH 7.3 and ground in a pre-chilled glass pestle and mortar at $4^\circ C$ with an equal volume of acid-washed sand. The resulting extract was centrifuged at $10,000 \times g$ for 20 min. Supernatants were made 2 mM in urea and 5 mM phosphate buffer, pH 7.3 and incubated at $37^\circ C$. The reaction was terminated by addition of 4 ml of the mixed reagent used for colorimetric assay of urea (10).

pH profile. The uptake medium was buffered with either sodium citrate-HCl (pH 2.0–5.0), $NaHPO_4-KH_2PO_4$ (pH 6.0–8.5) or $NaHPO_4-NaOH$ (pH 9.0–12.0) with buffer concentrations adjusted to 5 mM in each case.

Inhibitors. Acetohydroxamic acid, carbonylcyanide-*m*-chlorophenyl hy-

drazone (CCCP), dicyclohexyl carbodiimide (DCCD), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), hydroxyurea and imidazole were obtained from Sigma Chemical Company (U.S.A.). Stock solutions of DCMU, DCCD, CCCP were prepared in 95% ethanol before diluting 100 times in the basal medium to achieve the desired concentrations. Incubation medium devoid of inhibitors but with an equal amount of ethanol served as the control. Algal cells were incubated for 1 hr with the inhibitors prior to uptake experiments.

RESULTS AND DISCUSSION

Acetohydroxamic acid and hydroxyurea inhibited urease in vitro (Table 1) as well as in vivo, which is apparent from the level of urea in the cells in the presence of these inhibitors (Table 2), but had little or no effect on the disappearance of urea from the medium. This indicates that urease does not contribute to the short-term uptake results. A single kinetic system mediated urea uptake in both algal species with an apparent K_m of $250\ \mu\text{M}$ and a $V_{\max}$ of $568\ \text{nmol urea (mg protein)}^{-1}\ \text{hr}^{-1}$ for *A. doliolum* and a K_m of $400\ \mu\text{M}$ and a $V_{\max}$ of $500\ \text{nmol (mg protein)}^{-1}\ \text{hr}^{-1}$ for *A. nidulans* (Fig. 1). Uptake of urea by both algal species was optimum during their exponential growth phase (results not shown) at 30°C , pH 7.5 and light intensity $15\ \text{W m}^{-2}$ (Fig. 2). The apparent high affinity for urea possibly accounts for utilization of this nitrogen source by algae even at relatively lower levels prevalent in nature. The K_m values also indicated that under N-rich conditions, *A. doliolum* ($K_m = 250\ \mu\text{M}$) which has the additional ability to fix N_2 under N-depleted conditions may compete with the unicellular, non- N_2 -fixing (14) *A. nidulans* ($K_m = 400\ \mu\text{M}$) and thereby establish a significant population.

The urea uptake system of *A. doliolum* and *A. nidulans* showed two phenotypes: a repressed state (preincubation or growth with ammonia) and a constitutive state

Table 1. Effect of acetohydroxamic acid and hydroxyurea on the urea uptake rate and urease activity of *Anabaena doliolum* and *Anacystis nidulans*.

Inhibitor, μM	<i>A. doliolum</i>		<i>A. nidulans</i>	
	Urea uptake	Urease	Urea uptake	Urease
None	100	100	100	100
Acetohydroxamic acid, 2.5	97	50	100	58
10	99	30	97	26
50	99	21	97	10
Hydroxyurea, 20	96	3	100	4

Rates represent the percentage of the control. Urease activity in the control was $498\ \text{nmol (mg protein)}^{-1}\ \text{hr}^{-1}$ for *A. doliolum* and $447\ \text{nmol (mg protein)}^{-1}\ \text{hr}^{-1}$ for *A. nidulans*. The urea uptake was 550 and $453\ \text{nmol (mg protein)}^{-1}\ \text{hr}^{-1}$ for *A. doliolum* and *A. nidulans*, respectively. For details see MATERIALS AND METHODS.

Table 2. Effect of acetohydroxamic acid and hydroxyurea on the uptake (disappearance of urea from the medium) and intracellular level of urea in *Anabaena doliolum* and *Anacystis nidulans*.

Inhibitor, μM	nmol urea (mg protein) $^{-1}$ hr $^{-1}$			
	<i>A. doliolum</i>		<i>A. nidulans</i>	
	Urea uptake	Intracellular urea	Urea uptake	Intracellular urea
None	558	37	442	33
Acetohydroxamic acid, 2.5	541	231	456	133
10	553	362	443	295
50	551	410	452	385
Hydroxyurea, 20	553	489	456	415

Acetohydroxamic acid and hydroxyurea were added to the urea-grown cells 1 hr before the uptake experiments.

Fig. 1. Effect of different urea levels on uptake rate by urea-grown *Anabaena doliolum* ($\square$, $\blacksquare$) and *Anacystis nidulans* ($\circ$, $\bullet$). Open symbols ($\square$, $\circ$) refer to V (the left vertical line) and closed symbols ($\blacksquare$, $\bullet$) refer to S/V (the right vertical line). Uptake was followed as described in MATERIALS AND METHODS (final urea concentration 50–2,000 μM , pH 7.3).

Fig. 2. Effect of pH (A), light intensity (B) and temperature (C) on urea uptake by *Anabaena doliolum* (○) and *Anacystis nidulans* cells (●). (A) uptake was performed at various pH values in 5 mM of Na citrate-HCl (pH 2.0–5.0); NaHPO₄-KH₂PO₄ (pH 6.0–8.5) and NaHPO₄-NaOH (pH 9.0–12.0). Algal cells (with 2 mM urea) were incubated at various light intensities (B) and temperature (C) indicated 10 min before the uptake assay as described in MATERIALS AND METHODS.

Fig. 3. Uptake of urea by N-depleted *Anabaena doliolum* (○) and *Anacystis nidulans* cells (●). Urea grown cells were washed and resuspended in N₂-medium in light. Samples were withdrawn at times indicated and allowed to take up urea (final urea concentration 2 mM).

(growth on dinitrogen (N₂) and nitrate) (Tables 3 and 5, Fig. 4). In urea-grown cells depleted of nitrogen by preincubation in N₂-medium, urea uptake increased proportionally to the N-depletion period (Fig. 3) as observed with *Phaeodactylum*

Table 3. Urea uptake by *Anabaena doliolum* and *Anacystis nidulans* grown on different nitrogen sources.

Nitrogen source for growth	Urea uptake rate (%)	
	<i>A. doliolum</i>	<i>A. nidulans</i>
N ₂	106	—
Urea	100	100
KNO ₃	99	96
NH ₄ Cl	44	29

Table 4. Light and dark uptake of urea by N-depleted and N-replete cells of *Anabaena doliolum* and *Anacystis nidulans*.

Experiment	Urea uptake nmol (mg protein) ⁻¹ hr ⁻¹			
	<i>A. doliolum</i>		<i>A. nidulans</i>	
	Light	Dark	Light	Dark
N-depleted	1,250 (100)	750 (60)	1,102 (100)	503 (46)
N-replete	562 (45)	208 (17)	550 (50)	116 (11)

Urea-grown cells were washed and resuspended in sterile N₂/urea medium in light. After 24 hr the cells were collected, washed and resuspended in sterile medium supplemented with 2 mM urea to follow urea uptake in light and dark. Values in parentheses indicate the percentage of urea uptake rate.

Table 5. Urea uptake by *Anabaena doliolum* and *Anacystis nidulans* cells (urea-grown) preincubated with ammonia (5 mM) and urea (5 mM).

Experiments	Urea uptake nmol (mg protein) ⁻¹ hr ⁻¹	
	<i>A. doliolum</i>	<i>A. nidulans</i>
Urea-grown cells	550 (100)	453 (100)
Cells incubated in ammonia (5 mM) for 24 hr	48 (9)	20 (4)
Cells preincubated in ammonia (5 mM) for 24 hr, washed and incubated in urea (5 mM) for 24 hr	354 (64)	275 (61)

Values in parentheses indicate the percentage of urea uptake rate.

cells (8). During the first 8 hr of N-depletion, increase in the activity of urea uptake was not so marked in *A. nidulans* as in *A. doliolum*, so the increase was almost linear. To examine the effect of light and N-deficiency on urea uptake, the N-deprived (for 24 hr) and N-replete cells were tested for urea uptake in light and dark (Table 4).

Light and N-deprivation both stimulated urea uptake. In *A. doliolum*, N-depletion dominates over illumination in stimulating urea uptake, but in *A. nidulans* light had an edge over N-depletion. The apparent reason for such a discrepancy may be the degree of N-depletion. The N-deficient *A. doliolum* differentiates heterocysts and the first heterocyst appeared 30 hr following transfer to N_2 -medium. It may be that during the 24 hr of N-depletion the available cellular nitrogen was rapidly utilized to synthesize nucleic acids and proteins prior to heterocyst differentiation. Thus, on comparative grounds, *A. doliolum* cells would have become more N-deplete than *A. nidulans* and would have responded maximally to urea uptake.

Cells pregrown in urea when exposed to ammonia (5 mM) lost their urea uptake capacity (Fig. 4). The ability of these cells (preincubated with ammonia for 24 hr) to

Fig. 4.

Fig. 4. Uptake of urea by urea-grown algal cells preincubated for different periods with urea (5 mM) or ammonium chloride (5 mM). Samples from light-grown cells were taken at intervals to compare their ability to take up urea. Uptake conditions were the same as in Fig. 3; *Anabaena doliolum* preincubated in urea (○) and ammonia (△), *Anacystis nidulans* preincubated in urea (●) and ammonia (▲).

Fig. 5. Simultaneous uptake of urea (A) and ammonia (B) by urea-grown *Anabaena doliolum* (○, ●) and *Anacystis nidulans* (▲, △). Uptake was followed under illumination in the presence (●, ▲) and absence (○, △) of ammonia/urea. In the assay medium ammonium chloride was 1 mM and urea 2 mM.

Fig. 5.

Table 6. Effect of different metabolic situations and inhibitors on urea uptake by *Anabaena doliolum* and *Anacystis nidulans*.

Addition or incubation conditions	Concentration (M)	Urea uptake nmol (mg protein) ⁻¹ hr ⁻¹	
		<i>A. doliolum</i>	<i>A. nidulans</i>
None (light-aerobic)		550	460
Light-anaerobic		503	414
Dark-aerobic		52	46
Dark-anaerobic		25	23
3-(3,4-dichlorophenyl)-1,1-dimethylurea	10 ⁻⁵	398	310
Carbonyl cyanide- <i>m</i> -chlorophenyl hydrazone	2.5 × 10 ⁻⁵	0	0
Dicyclohexylcarbodiimide	10 ⁻⁴	0	0
2,4-dinitrophenol	10 ⁻³	0	0
Imidazole	10 ⁻³	332	254

The inhibitors were added to the concentrations as indicated 1 hr before the start of uptake assay. Anaerobic conditions were achieved by replacing air of the vessels by N₂.

take up urea was regained when transferred to urea medium (for 24 hr) (Table 5). Urea uptake not affected in cells exposed to a combination of urea (2 mM) and ammonia (1 mM) (Fig. 5A) led to the conclusion that the repression of the urea uptake system depends on ammonia assimilation products (feed-back system). The simultaneous presence of urea greatly reduced ammonia uptake (Fig. 5B). Since intracellular ammonia inhibits its own uptake, it seems logical to assume that once urea is inside the cells, it is hydrolyzed to ammonia, which in turn inhibits its own uptake.

The ability of algae to take up urea in light-anaerobic condition (CO₂-free N₂-atmosphere) comparable to the rates observed for light-aerobic cells (Table 6) indicates the possible linkage of uptake system with cyclic photophosphorylation, as CO₂ exclusion from the atmosphere allows only cyclic electron flow. Urea uptake was almost abolished in cells dependent on oxidative phosphorylation (dark-aerobic condition). The metabolic inhibitors 2,4-dinitrophenol, an uncoupler of photophosphorylation, and dicyclohexyl carbodiimide, which can inhibit both the ATP generating and consuming process, inhibited urea uptake completely in both algae. This indicates the involvement of ATP derived through photophosphorylation. In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea or imidazole (15) when the algae are capable of carrying out cyclic photophosphorylation, the inhibition of urea uptake was incomplete. Carbonyl cyanide-*m*-chlorophenyl hydrazone, a cyclic electron flow uncoupler abolished the uptake of urea completely. These results affirm the vital role of cyclic photophosphorylation in urea uptake process.

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