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Absence of the glutamine-synthetase-linked methylammonium (ammonium)-transport system in the cyanobiont of *Cycas*-cyanobacterial symbiosis

A.N. Rai[2], P. Lindblad[1] and B. Bergman[1]

[1] Institute of Physiological Botany, Box 540, S-751 21 Uppsala, Sweden, and
[2] Department of Biochemistry, North-Eastern Hill University, Shillong-793 014, India

Abstract. Using the ammonium analogue $^{14}CH_3NH_3^+$, ammonium transport was studied in the cyanobiont cells freshly isolated from the root nodules of *Cycas revoluta*. An L-methionine-DL-sulphoximine (MSX)-insensitive ammonium-transport system, which was dependent on membrane potential ($\Delta\psi$), was found in the cyanobiont. However, the cyanobiont was incapable of metabolizing exogenous $^{14}CH_3NH_3^+$ or NH_4^+ because of the absence of another ammonium-transport system responsible for the uptake of ammonium for assimilation via glutamine synthetase (EC 6.3.1.2). Such a modification seems to be the result of symbiosis because the free-living cultured isolate, *Anabaena cycadeae*, has been shown to possess both the ammonium-transport systems.

Key words: Ammonium transport – *Anabaena* – Cycad-cyanobacterium symbiosis – Cyanobacterium – *Cycas* – Methylammonium transport – Symbiosis.

Introduction

Some N_2-fixing cyanobacteria form associations with eukaryotes where the ammonia produced during N_2 fixation is liberated by the cyanobiont (see Stewart et al. 1983). In cases so far studied (lichens, bryophytes, *Azolla*) a reduction in the level of the primary ammonia-assimilating enzyme, glutamine synthetase (GS), has been shown to be the cause of such ammonia liberation (Stewart et al. 1983). However, we find that unlike other cyanobacterial symbioses, the cyanobiont of *Cycas revoluta* shows normal levels of GS activity. This prompted us to check whether the ammonium-transport system (ATS) was modified in the cyanobiont, as speculated earlier (Kleiner et al. 1981). Our studies reported here show that the cycad cyanobiont, unlike its free-living cultured isolate *Anabaena cycadeae* (Singh et al. 1985), lacks the ATS responsible for import of ammonium for assimilation via GS.

Abbreviations and symbol: ATS/ATSs = ammonium transport system/systems; Chl = chlorophyll; GS = glutamine synthetase; MSX = L-methionine-DL-sulphoximine; $\Delta\psi$ = membrane potential

Material and methods

Nitrogen-fixing cyanobacterial cells were collected from the root nodules of *Cycas revoluta* Thunb. collected from the Uppsala University green house. Thin slices of root nodules, cleaned and washed with distilled water, were cut and incubated in 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes)-NaOH buffer, pH 7, for 1 h. During this time, cyanobiont filaments were found to have come out into the buffer. Such cells were taken up in fresh buffer, washed twice and resuspended in the same buffer to a density of 25 μg chlorophyll (Chl) $a \cdot cm^{-3}$. Methylammonium uptake, as an indicator of ammonium transport, was assayed at 25 °C and at a photon fluence rate of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ as described by Rai et al. (1984). Cells were separated from the medium using silicon-oil microcentrifugation (Scott and Nicholls 1980). The enzymes GS (EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.7.1) were assayed according to Sampaio et al. (1979) and Rai et al. (1982), respectively. Ammonia estimation was done according to Solorzano (1969), Chl according to Harborne (1973), and amino-acid analysis according to Rai et al. (1984).

Results and discussion

The ammonium analogue $^{14}CH_3NH_3^+$ has been used to study ATS in cyanobacteria, since both NH_4^+ and $CH_3NH_3^+$ share a common transport system in these organisms as evidenced by a competitive inhibition of $CH_3NH_3^+$ uptake by NH_4^+ and by an efflux of the preaccumulated free $CH_3NH_3^+$ pool (see Kleiner 1985). In general, $^{14}CH_3NH_3^+$ uptake has been found to be biphasic: the first phase representing an ATS responsible for

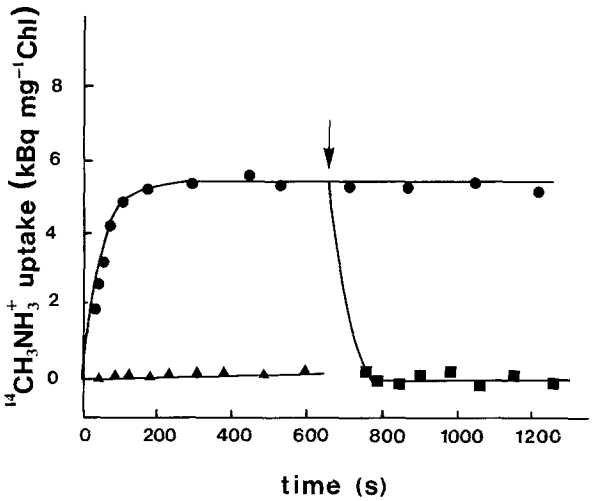

Fig. 1. Uptake of $^{14}CH_3NH_3^+$ by cyanobiont cells of *Cycas revoluta* root nodules. Cells were suspended in 10 mM Hepes-NaOH buffer, pH 7.0, and $^{14}CH_3NH_3^+$ added to a final concentration of 50 μm (specific activity 180 kBq·μmol^{-1}). At time intervals indicated cells were separated from their bathing medium and ^{14}C label determined. NH_4Cl was added to a final concentration of 200 μM at times arrowed. Values are plotted after substracting ^{14}C label in toluene-treated cells and are means of three replicates which did not vary by more than 5%. ●, control; ▲, +NH_4Cl (added at zero time); ■, +NH_4Cl (added after 650 s incubation with $^{14}CH_3NH_3^+$)

Table 1. Effect of NaCl, NH_4Cl, toluene, triphenylmethylphosphonium ($TPMP^+$) and MSX on $^{14}CH_3NH_3^+$ accumulation by cyanobiont cells of *Cycas revoluta* root nodules. $^{14}CH_3NH_3^+$ was added at zero time to the cyanobiont cell suspension in 10 mM Hepes-NaOH buffer, pH 7.0, to a final concentration of 50 μM (specific activity 180 kBq·μmol^{-1}). After 120 s, ^{14}C label in the cells was determined (control). The choice of a 120-s time period for these experiments was based on the fact that no cellular increase in the ^{14}C label was found beyond this time (see Fig. 1). NH_4Cl and NaCl, when used, were added just before the addition of $^{14}CH_3NH_3^+$; $TPMP^+$ and MSX were added 30 and 60 min, respectively, prior to the addition of $^{14}CH_3NH_3^+$. To measure nonspecific binding, cyanobiont cells were treated with toluene for 15 min, after which the toluene layer was removed and $^{14}CH_3NH_3^+$ added. Values are means ±SEM ($n=10$)

Treatments	$^{14}CH_3NH_3^+$ Accumulation		
	Bq·mg^{-1}Chl	Bq·mg^{-1}Chl (after substracting toluene values)	% Control
Control (no additions)	13375±590	5042	100.0
+NaCl (200 μM)	13183±566	4850	96.2
+NH_4Cl (200 μM)	8372±236	39	0.8
+Toluene (1%, v/v)	8333±330	0	0.0
+$TPMP^+$ (100 μM)	8283±500	−50	−1.0
+MSX (100 μM)	13258±560	4925	97.7

intracellular accumulation of free $^{14}CH_3NH_3^+$, which can be totally displaced by addition of NH_4^+, and the second phase representing conversion by GS of the internal methylammonium pool into γ-methylglutamine, which accumulates in the cell and cannot be displaced by NH_4^+ (Rai et al. 1984; Kleiner 1985).

In the present investigation we have studied ATS in the cyanobiont of *C. revoluta* and compared it with the free-living isolate studied earlier (Singh et al. 1985). The cyanobiont cells showed that the initial rapid uptake of $^{14}CH_3NH_3^+$ became saturated within 100 s, the label in the cells remaining constant thereafter (Fig. 1). This contrasts with the pattern of $^{14}CH_3NH_3^+$ uptake in the free-living isolate *A. cycadeae* and in other free-living cyanobacteria where, in addition to the rapid phase observed here, a slower second phase representing $CH_3NH_3^+$ assimilation has been observed (Singh et al. 1985; Rai et al. 1984; Kerby et al. 1986).

Since $^{14}CH_3NH_3^+$ uptake in the cyanobiont was similar to the first phase of $^{14}CH_3NH_3^+$ uptake in the free-living isolate *A. cycadeae* (Singh et al. 1985) and since the second phase of uptake, linked to $CH_3NH_3^+$ assimilation via GS and detectable in free-living cyanobacteria (Rai et al. 1984; Singh et al. 1985), was not apparent in the cyanobiont, we thought it possible that the cyanobiont does not metabolize $CH_3NH_3^+$ and that ^{14}C label in the cyanobiont cells remains as a free $CH_3NH_3^+$ pool. Since NH_4^+ is known to cause a total displacement of the intracellular $CH_3NH_3^+$ pool we investigated the effect of NH_4^+ addition on ^{14}C release from cells to which $^{14}CH_3NH_3^+$ had been added previously. As seen in Fig. 1, addition of NH_4^+ caused a total efflux of the ^{14}C label from the cells, indicating that indeed all the ^{14}C label in the cells represented a free $CH_3NH_3^+$ pool and that $CH_3NH_3^+$ assimilation did not occur. Sodium chloride did not produce the above-observed effect shown by NH_4^+, indicating that this effect was specific for NH_4^+ (Table 1).

The observation that addition of NH_4^+ inhibited $^{14}CH_3NH_3^+$ uptake (Fig. 1) indicated that both NH_4^+ and $CH_3NH_3^+$ are transported through the same system and that NH_4^+ is the preferred substrate. Similar interpretations have been made for other systems (see Kleiner 1985). Thus, the $^{14}CH_3NH_3^+$-transport data can be interpreted in terms of ATS. This together with the fact that the uptake was sensitive to triphenylmethylphosphonium ($TPMP^+$, which collapses $\Delta\psi$; membrane potential) and insensitive to L-methionine-DL-sulphoximine (MSX) indicates that the cyanobiont

possessed a $\Delta\psi$-dependent MSX-insensitive ATS as reported in other cyanobacteria, based on similar criteria (Kleiner 1985). However, despite the presence of this ATS the cyanobiont was unable to metabolize exogenously provided $CH_3NH_3^+$, as discussed above. We suggest that this is because the cyanobiont lacks the second, MSX-sensitive, ATS which has been reported in the free-living isolate and has been shown to be linked to $CH_3NH_3^+$ assimilation via GS. The absence of a second phase of $^{14}CH_3NH_3^+$ uptake in the cyanobiont supports this. However, for this suggestion to be valid it was necessary to preclude several other possible explanations. First, the lack of $CH_3NH_3^+$ uptake could have been caused by a lack of either GS activity or GS affinity for $CH_3NH_3^+$. Both these possibilities were discounted because the cyanobiont possessed GS and glutamate-synthase activities of 113 and 86 nmol product formed $\cdot min^{-1} \cdot mg^{-1}$ protein, respectively, and cell-free preparations of the cyanobiont showed a GS biosynthetic activity of 145 nmol product formed $\cdot min^{-1} \cdot mg^{-1}$ protein using $CH_3NH_3^+$ as substrate.

Second, the product of $CH_3NH_3^+$ assimilation, γ-methylglutamine, may have been liberated by the cells into the medium. However, no methylglutamine was detectable in the medium, or in the cells, when these were analysed over a period of upto 3 h using an amino-acid analyser.

Third, the second ATS may not have been detectable with $CH_3NH_3^+$, as has been suggested for *Anacystis nidulans* R-2 (Boussiba et al. 1984). However, the cyanobiont was equally unable to take up NH_4^+ when we measured the disappearance of exogenously provided NH_4^+ over a 3-h period.

It is not clear at present how the second ATS is repressed in the cyanobiont. However, the observations of Pargent and Kleiner (1985) that microaerobiosis may cause ATS repression in *Rhizobium meliloti* may be relevant here. Since the cyanobiont is located in roots and therefore functionally non-photosynthetic, existence of microaerobiosis in such a system is a distinct possibility.

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