The *Cycas revoluta*–*Nostoc* Symbiosis: Enzyme Activities of Nitrogen and Carbon Metabolism in the Cyanobiont

By PETER LINDBLAD,¹* AMAR N. RAI² AND BIRGITTA BERGMAN¹

¹Department of Physiological Botany, University of Uppsala, Box 540, S-751 21 Uppsala, Sweden

²Department of Biochemistry, North-Eastern Hill University, Shillong 793014, India

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A comparative study was made of enzymes involved in nitrogen and carbon metabolism in the cyanobiont directly isolated from *Cycas revoluta* coralloid roots, and in the cultured isolate *Nostoc* 7422. The symbiotic *Nostoc* showed high activity of glutamine synthetase and glutamate synthase, the primary ammonia-assimilating enzyme system in cyanobacteria. Ammonia-assimilating glutamate dehydrogenase (GDH) activity was undetectable, although the catabolic GDH activity was high. Both glutamate–oxaloacetic acid transaminase and malate dehydrogenase showed higher activities in the symbiotic *Nostoc* than in the cultured *Nostoc* strain. The symbiotic *Nostoc* did not fix CO₂ *in vivo* although in cell-free extracts both ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase activities, similar to those in the cultured strain, were present.

INTRODUCTION

Among gymnosperms, cycads are the only group that form a symbiosis with a nitrogen-fixing organism. Filamentous heterocystous cyanobacteria of the genus *Nostoc* inhabit a cylindrical zone, intercellularly, in the cortex of cycad coralloid roots (Sprenst, 1979; Lindblad *et al.*, 1985). The fixed nitrogen is rapidly transported from the cyanobiont to the cycad (Bergersen *et al.*, 1965; Renaut *et al.*, 1975; Halliday & Pate, 1976), although the form in which the fixed nitrogen is released and transported is unknown. Recently we obtained evidence (Lindblad & Bergman, 1986) that, in contrast to the situation in most other symbiotic cyanobacterial symbioses, e.g. *Anthoceros*, *Azolla* and *Peltigera* (Stewart & Rowell, 1977; Ray *et al.*, 1978; Rai *et al.*, 1981c, 1983; Orr & Haselkorn, 1982; Stewart *et al.*, 1983; Meeks *et al.*, 1985; Rowell *et al.*, 1985), a product of ammonium assimilation, in addition to or instead of ammonia, is transferred from the cyanobacterial cells to the cycad.

The aim of the present work was to extend our studies of the enzymes involved in nitrogen metabolism and to obtain information regarding carbon metabolism in cyanobionts of the cycad *Cycas revoluta*.

METHODS

Organisms and growth. Coralloid roots from the cycad *Cycas revoluta* Thunb. were collected from plants growing in the Botanical Garden, University of Uppsala, Sweden. Cyanobacteria were carefully removed from the coralloid roots as described earlier (Lindblad & Bergman, 1986; Rai *et al.*, 1986). The washed cyanobacteria were resuspended in sterile BG-11₀ medium (Stanier *et al.*, 1971) and used for the experiments within 1 h.

Nostoc 7422 (ATCC 29132), originally isolated from *Cycas*, was obtained from the Pasteur Culture Collection, Paris, France. Axenic cultures were grown in BG-11₀ medium (Stanier *et al.*, 1971) under continuous light at 90

Abbreviations: APT, aspartate–pyruvate aminotransferase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GOT, glutamate–oxalacetate aminotransferase; GPT, glutamate–pyruvate aminotransferase; GS, glutamine synthetase; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate; PRK, phosphoribulokinase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

$\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ [Philips 40 W, TL 33 and 55 (400–700 nm)] at 25 °C and flushed with sterile air. The cultures were harvested after 6–8 d, washed with sterile BG-11₀ medium and resuspended in the same medium.

Preparation of cell-free extracts. The cyanobacterial cells were sedimented by centrifugation and resuspended in the following extraction buffers: for GS, 50 mM-Tris/HCl, pH 7.5, with additions of 10 mM-glutamate, 5 mM-2-mercaptoethanol, 5 mM-MgCl₂ and 1 mM-Na₂EDTA (Lindblad & Bergman, 1986); for GOGAT, 2 mM-TES, pH 7.2 (Rai *et al.*, 1981a); for GDH, 50 mM-Tris/HCl, pH 7.5, containing 10 mM-2-mercaptoethanol and 5 mM-Na₂EDTA (Stewart & Rowell, 1977); for GPT, 100 mM-Tris/HCl, pH 7.5, with additions of 10 mM-2-mercaptoethanol and 1 mM-Na₂EDTA; for APT, GOT and PEP carboxylase, pyruvate carboxylase and MDH, 50 mM-Tris/HCl, pH 7.5; and for RuBisCO and PRK, 10 mM-Tris, pH 8.0, with additions of 25 mM-NaHCO₃, 10 mM-MgCl₂ and 1 mM-Na₂EDTA. After two or three passages through a French press at 110 MPa, the material was centrifuged (35000 g at 4 °C for 30 min unless otherwise stated).

Enzyme assays. *Glutamine synthetase* (GS) was assayed as both Mn²⁺-dependent γ -glutamyltransferase activity (EC 2.3.2.2) and Mg²⁺-dependent biosynthetic activity (EC 6.3.1.2), as described by Sampaio *et al.* (1979). *Glutamate synthase* (GOGAT; EC 1.4.7.1) was assayed by measuring the formation of [¹⁴C]glutamate from [¹⁴C]glutamine in the presence of the aminotransferase inhibitor aminooxyacetate (AOA). Preparation of cell-free extracts was as described by Rai *et al.* (1981a) except that the supernatants were dialysed overnight against the extraction buffer, instead of being passed through Sephadex G25. The reaction mixture, in a total volume of 0.7 ml, was as described by Thomas *et al.* (1977). The reaction was stopped after 20 min by adding 0.7 ml 100% acetone. The samples were chromatographed on TLC plates coated with 0.1 mm cellulose (Merck, Art. 5716) using iso-butanol/formic acid/water (6:1:2, by vol.) as the solvent system. The areas corresponding to the position of the glutamate standard were scraped off and counted in UniSolve 1 Scintillant (Zinsser Analytic), in an LKB Wallac 1217 Rackbeta scintillation spectrometer. For *glutamate dehydrogenase* (GDH), ammonia-assimilating activity (NADPH-dependent, EC 1.4.1.4) was measured as described by Stewart & Rowell (1977) and catabolic activity (NADH-dependent, EC 1.4.1.2) by the same protocol except for the change of cofactor. *Glutamate-pyruvate aminotransferase* (GPT; EC 2.6.1.2) was assayed as described by Jäger & Weigel (1978), *aspartate-pyruvate aminotransferase* (APT; EC 2.6.1.12) by the method of Herbert *et al.* (1978), and *glutamate-oxalacetate aminotransferase* (GOT; EC 2.6.1.1) as described by Rowell & Stewart (1976). For *ribulose-1,5-bisphosphate carboxylase/oxygenase* (RuBisCO; EC 4.1.1.39), carboxylase activity was measured as described by McFadden *et al.* (1975); *phosphoribulokinase* (PRK; EC 2.7.1.19) was assayed as described by Lanaras & Codd (1981); for both assays, ¹⁴C counting was done as described above. *Phosphoenolpyruvate (PEP) carboxylase* (EC 4.1.1.31), *PEP carboxykinase* (EC 4.1.1.49) and *PEP carboxytransphosphorylase* (EC 4.1.1.38) were all assayed as described by Rai *et al.* (1981b) except that the supernatants were dialysed overnight against the extraction buffer, instead of being passed through Sephadex G25. *Pyruvate carboxylase* (EC 4.1.1.1) and NADH-dependent *malate dehydrogenase* (MDH; EC 1.1.1.37) were assayed as described by Codd & Stewart (1973).

In vivo CO₂ fixation. The uptake of CO₂ was followed using a gas chromatograph as described by Pettersson *et al.* (1985). The incorporation of ¹⁴C was measured by incubating 2.0 ml cyanobacterial cells in the presence of NaH¹⁴CO₃ [2.0 $\mu\text{Ci ml}^{-1}$; specific activity 56 mCi mmol⁻¹ (2.07 GBq mmol⁻¹)] at 28 °C in the light (70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or in darkness (preincubated in the dark for 30 min). The incubations were terminated by adding 3.0 ml acidified methanol and the incubation vials, containing cells, were left overnight without caps at room temperature in a fume cupboard to drive off unfixed ¹⁴CO₂. ¹⁴C-counting was then done as described above.

Chlorophyll *a* and protein. Chlorophyll *a* was determined according to Harborne (1973) and protein by the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS

Cyanobacteria freshly isolated from coralloid roots of *C. revoluta* showed high *in vitro* activity of the ammonia-incorporating GS-GOGAT system, but NADPH-dependent (ammonia-assimilating) GDH activity was undetectable. However, catabolic GDH activity was present (Table 1). Among the transaminases tested, GPT, APT and GOT were all present (Table 1). GOT showed the highest activity, followed by APT and GPT. There were certain distinct differences in the enzyme activities of freshly isolated symbiotic *Nostoc* and the cultured *Nostoc* 7422: the symbiotic *Nostoc* showed a 75% higher GS activity, more than double catabolic GDH activity, over threefold higher GOT activity, and the presence of GPT (Table 1).

Net *in vivo* CO₂-fixation, as assayed by gas chromatography or ¹⁴C incorporation, was undetectable in the freshly isolated symbiotic *Nostoc*, in both light and dark conditions. In contrast, *Nostoc* 7422 showed high rates of CO₂ fixation in light [106.5 nmol CO₂ fixed ($\mu\text{g chlorophyll } a$)⁻¹ min⁻¹ or 257 nmol H¹⁴CO₃⁻ incorporated ($\mu\text{g chlorophyll } a$)⁻¹ min⁻¹; means of duplicate experiments]. In spite of the absence of *in vivo* CO₂-fixing ability, the symbiotic

Table 1. *In vitro* activities of various nitrogen-assimilating enzymes in symbiotic Nostoc and Nostoc 7422

Enzyme	Activity*	
	Symbiotic <i>Nostoc</i>	<i>Nostoc</i> 7422
GS		
Transferase	1818.2	1054.4
Coupled biosynthetic	135.4	83.0
GOGAT	86.0	81.8
GDH		
NADH-dependent	17.6	7.4
NADPH-dependent	ND	ND
GPT	3.7	ND
APT	27.0	22.7
GOT	70.2	20.6

ND, Not detectable.

* The results are expressed as nmol product formed min⁻¹ (mg protein)⁻¹ and are means of duplicate experiments.Table 2. *In vitro* activities of RuBisCO, PRK, other CO₂-fixing enzymes and MDH in symbiotic Nostoc and Nostoc 7422

Enzyme	Activity*	
	Symbiotic <i>Nostoc</i>	<i>Nostoc</i> 7422
RuBisCO (carboxylase)	279.4	321.7
PRK	153.2	163.6
PEP carboxylase	< 1.1	3.7
PEP carboxykinase	ND	< 1.9
PEP carboxytransphosphorylase	< 1.8	ND
Pyruvate carboxylase	3.5	4.2
MDH (NADH-dependent)	22.0	2.8

ND, Not detectable.

* The results are expressed as nmol product formed min⁻¹ (mg protein)⁻¹ and are means of duplicate experiments.

Nostoc, in cell-free preparations, showed RuBisCO carboxylase and PRK activities (Table 2). None of the other CO₂-fixing enzymes tested showed any appreciable *in vitro* activity (Table 2), either in the symbiotic *Nostoc* or in *Nostoc* 7422. Interestingly, the symbiotic *Nostoc* showed at least seven times higher MDH (NADH-dependent) activity than *Nostoc* 7422 (Table 2).

DISCUSSION

Apparently there are at least two categories of nitrogen-fixing symbiotic cyanobacteria as regards ammonia-assimilating enzymes. First, there are those with a decreased GS activity, e.g. the cyanobacteria in *Anthoceros*, *Azolla* and *Peltigera* (Stewart & Rowell, 1977; Ray *et al.*, 1978; Haselkorn *et al.*, 1980; Orr & Haselkorn, 1982; Stewart *et al.*, 1983; Meeks *et al.*, 1985; Rowell *et al.*, 1985). In *Azolla* and *Peltigera* the decrease in GS activity has been shown to be due to a decreased level of GS protein (Haselkorn *et al.*, 1980; Orr & Haselkorn, 1982; Stewart *et al.*, 1983) resulting, at least in *Azolla*, from a reduction in the mRNA levels for GS protein (Nierzwicki-Bauer & Haselkorn, 1986). In *Peltigera*, GS protein levels are particularly low in the heterocysts (Hällbom *et al.*, 1986), the site of nitrogen fixation (Bergman *et al.*, 1986). Furthermore, decreased GOGAT activities have also been noted in *Azolla* and *Peltigera* (Rai *et al.*, 1981a). In these symbioses the cyanobiont releases fixed nitrogen as ammonia because the

levels of GS and GOGAT are insufficient to assimilate all the ammonia produced during nitrogen fixation. The ammonia thus released is assimilated by the eukaryotic partner (Ray *et al.*, 1978; Rai *et al.*, 1981*c*, 1983; Stewart *et al.*, 1983; Meeks *et al.*, 1983, 1985; Rowell *et al.*, 1985).

The second category is exemplified by the cycad symbiosis studied here. In *C. revoluta* the cyanobiont shows no decrease in *in vitro* GS activity (Lindblad & Bergman, 1986; Rai *et al.*, 1986; Table 1) or *in vitro* GOGAT activity (Table 1), nor is there any difference in the cellular/subcellular distribution of GS protein between the cyanobiont and the free-living nitrogen-fixing cyanobacterium (Lindblad & Bergman, 1986). Full GS activity was also found in the cyanobiont of the cycad *Zamia skinneri* (Lindblad & Bergman, 1986). Thus, the cyanobiont seems to be fully capable of assimilating all the ammonia produced by nitrogen fixation. However, the cyanobiont in cycad symbiosis does provide fixed nitrogen to the cycad plant (Bergersen *et al.*, 1965; Renaut *et al.*, 1975; Halliday & Pate, 1976); the form in which the fixed nitrogen is released is not known, but in view of the high GS and GOGAT activities in the cyanobiont, it may be a product of ammonia assimilation.

Among the transaminases tested, GOT shows an increased activity in the symbiotic cyanobacteria of cycads. In contrast, no such increase is found in symbiotic cyanobacteria of the lichen *Peltigera* (Stewart & Rowell, 1977; Rai *et al.*, 1980, 1981*c*). GOT requires oxaloacetic acid as one of the substrates. This may be provided by the action of MDH, as we observed higher MDH activity in the cyanobiont of *C. revoluta* than in the cultured *Nostoc* strain. Thus, the possibility exists that these two enzymes might be involved in a malate-aspartate shuttle, transporting reducing equivalents from the cycad cells to the cyanobacterium as a substitute for those generated by light-requiring processes in free-living cyanobacteria.

The cyanobacteria in cycads are exceptional among symbiotic cyanobacteria in not being reached by light. This is manifested by the absence of *in vivo* CO₂-fixation. Thus, the cyanobiont has to depend on heterotrophic carbon metabolism, the fixed carbon being provided by the cycad. A similar situation is found in symbiotic cyanobacteria of *Anthoceros* and *Gunnera*, where ¹⁴C uptake and fixation are virtually absent in both light and dark (Rodgers & Stewart, 1977; Silvester, 1976). In contrast, cyanobacteria in *Azolla* fix atmospheric CO₂ (Ray *et al.*, 1979; Peters *et al.*, 1982; Peters & Calvert, 1983) though the mRNA levels for RuBisCO are decreased (Nierzwicki-Bauer & Haselkorn, 1986). Cyanobacteria in *Peltigera aphthosa* are also capable of fixing CO₂ in the light and to a lower extent in the dark (Englund, 1977; Rai *et al.*, 1983). In the latter case, carbon skeletons are provided by PEP carboxylase (Rai *et al.*, 1981*b*, 1983). It is not evident why CO₂-fixation is lacking in the cyanobiont of cycads even when light is provided, since both RuBisCO (carboxylase) and the ribulose-1,5-bisphosphate-generating PRK were found in cell-free extracts. However, the absence of other enzymes of the Calvin cycle cannot be ruled out. Alternatively, the photosystems of the cyanobiont may be non-functional.

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