SHORT COMMUNICATION

Glutamate Synthase Activity in Symbiotic Cyanobacteria

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Glutamate synthase (GOGAT) activities of the symbiotic cyanobacteria of the lichens *Peltigera canina* and *Peltigera aphthosa* and of the water fern *Azolla caroliniana* have been determined and, like glutamine synthetase (GS) activity, found to be substantially reduced compared with the activities found in the free-living cyanobacteria. A similar reduction in GOGAT activity was not noted in the symbiotic green alga (*Coccomyxa* sp.) in *P. aphthosa*. The selective reduction of GS–GOGAT activity in symbiosis may be related to the production of extracellular nitrogen by the symbiotic cyanobacterium.

INTRODUCTION

Various N_2 -fixing cyanobacteria which occur in symbiotic association show different metabolic characteristics in symbiosis and in the free-living state (Rodgers & Stewart, 1977; Stewart & Rodgers, 1977; Peters *et al.*, 1979; Stewart *et al.*, 1980). In relation to nitrogen metabolism, the primary route of NH_4^+ assimilation in free-living cyanobacteria is the glutamine synthetase–glutamate synthase (GS–GOGAT) pathway (Stewart *et al.*, 1975; Wolk *et al.*, 1976). We have shown that in symbiosis, cyanobacterial GS, which may be involved in the regulation of nitrogenase (Shanmugam *et al.*, 1978), shows much reduced activity (Stewart & Rowell, 1977; Peters *et al.*, 1979) concomitant with the transfer of much of the fixed nitrogen from the symbiotic cyanobacterium to its eukaryotic partners (Stewart & Rowell, 1977; Peters *et al.*, 1979; Rai *et al.*, 1980, 1981). Since N_2 -fixing cyanobacteria which occur in symbiosis have negligible glutamate dehydrogenase activity (Stewart *et al.*, 1975; Stewart & Rowell, 1977; Stewart, 1980), GOGAT may be particularly important in these organisms for glutamate production which, in turn, is required for NH_4^+ assimilation via GS and for amino acid biosynthesis via transamination. In this paper we present information on the GOGAT activities of free-living and symbiotic cyanobacteria (*Nostoc* spp.) from the lichens *Peltigera aphthosa* and *P. canina*, and from the water fern *Azolla caroliniana*, where the N_2 -fixing symbiont is *Anabaena azollae*. Our results show that, as with GS activity, the GOGAT activities of the symbiotic cyanobacteria in *Peltigera canina* (a lichen composed of fungus and *Nostoc* only), in *Peltigera aphthosa* (a lichen where the main photosynthetic partner is a green alga – *Coccomyxa* sp. – located only in the main thallus, and the N_2 -fixing *Nostoc*, located only in the cephalodia, is a secondary photosynthetic partner), and in the water fern *Azolla caroliniana* (where the cyanobacterium occurs in ventral cavities in the dorsal lobe of each leaf) are considerably reduced relative to what is found when the cyanobacteria grow in the free-living state.

METHODS

Experimental material. *Peltigera aphthosa* and *P. canina* were collected locally and maintained in a Sherer-Gillet model CEL 255-6 growth cabinet at 22 °C. 3000 lx for 48 h before use. *Azolla caroliniana* was grown as before (Peters & Mayne, 1974).

The *Nostoc* from *P. canina* (*Nostoc* CAN) and *Anabaena azollae* were kindly provided by Dr J. W. Millbank (Imperial College, London) and Dr J. W. Newton (Newton & Herman, 1979), respectively. The free-living *Nostoc* of *P. aphthosa* (*Nostoc* APH) was isolated by us and maintained in axenic culture. All the cyanobacteria were grown in nitrogen-free BG-11 medium (Stanier *et al.*, 1971) at $22 \pm 1^\circ\text{C}$, 3000 lx. The symbiotic *Coccomyxa* was isolated as before (Richardson *et al.*, 1968) and the free-living *Coccomyxa* was isolated by us and maintained in axenic culture in the medium of Kessler *et al.* (1957). The symbiotic *Anabaena azollae* packets were separated from the *Azolla* as before (Peters & Mayne, 1974). The cephalodia and medullary fungus were excised from the main thallus using a scalpel and a pair of fine forceps (Rai *et al.*, 1980).

Glutamate synthase (GOGAT; EC 1.4.7.1) assay. The test material was washed and homogenized in 28 mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) buffer, pH 7.2, passed through a French press at 110 MPa then centrifuged at 10000g for 30 min. The supernatant was used for assay after passage through a Sephadex G-25 column (2 × 5 cm). GOGAT activity was determined by measuring the formation of [^{14}C]glutamate from 2-oxo[^{14}C]glutarate and unlabelled glutamine in the presence of the aminotransferase inhibitor aminooxyacetate (AOA), under anaerobic conditions. The reaction mixture, in a total volume of 0.2 ml, was as described before (Thomas *et al.*, 1977). The reaction was allowed to proceed for 20 min, then the samples were deproteinized by adding 0.2 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 2 M-HCl (Givan *et al.*, 1970). The samples were chromatographed on Whatman no. 1 paper by descending chromatography using *n*-butanol/acetic acid/water (12:3:1, by vol.) as the solvent system. The areas corresponding to the position of the glutamate standard were cut out and counted, in a toluene-based scintillant, on a Packard Tri-carb 2660 scintillation spectrometer.

Nitrogenase activity. This was assayed as before using the acetylene reduction assay (Stewart *et al.*, 1967).

$^{15}\text{N}_2$ fixation rates. Lichen discs, or excised cephalodia, were exposed to a gas phase of $\text{N}_2/\text{O}_2/\text{Ar}/\text{CO}_2$ (30:20:49.96:0.04, by vol.), the N_2 being enriched with 95 atom % ^{15}N . Samples of exposed material were harvested at various intervals and assayed for ^{15}N enrichment using a V.G. Micromass 601 mass spectrometer (Rai *et al.*, 1981).

Protein estimations. Protein was determined according to the method of Lowry, using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

The data on GOGAT activities of the free-living and symbiotic cyanobacteria of *Peltigera canina*, *P. aphthosa* and *Azolla caroliniana* are presented in Table 1. The GOGAT activities found in *P. canina* discs, expressed on a cyanobacterial protein basis, were markedly lower than those of the free-living *Nostoc* (*Nostoc* CAN). No GOGAT activity was detected in fungal hyphae of *P. canina* (or those of the related lichen *P. polydactyla*: results not shown). In the case of *Peltigera aphthosa*, the GOGAT activity of the symbiotic *Nostoc* (in cephalodia) was very low, but the GOGAT activities of the green alga (*Coccomyxa*), separated from the main thallus, or grown in axenic culture, were comparable to each other and very much higher than in the symbiotic *Nostoc*. No GOGAT activity was detected in the fungus of the main thallus. That is, in this symbiosis there was a preferential inhibition of GOGAT activity of the N_2 -fixing cyanobacterium, with the GOGAT activity of the primary photosynthetic partner, the *Coccomyxa*, remaining unimpaired.

In the case of *Anabaena azollae*, the complete symbiosis showed high GOGAT activity, as reported before (Ray *et al.*, 1978), but the isolated cyanobacterial packets showed activities which were only 32% of those found in the free-living *Anabaena azollae*.

Thus, in all the symbiotic cyanobacteria tested, cyanobacterial GOGAT activity was substantially lower than that present when the cyanobacterium was growing in the free-living state. The reductions in GOGAT activity noted here for the symbiotic cyanobacteria (98% for the symbiotic *Nostoc* CAN, 98% for symbiotic *Nostoc* APH and 68% for *Anabaena azollae* packets) correspond well with the reductions in GS activity (95%, 97% and 75%, respectively) of these symbiotic cyanobacteria (Ray *et al.*, 1978; Sampaio *et al.*, 1979), suggesting that both enzymes may be co-regulated in symbiosis.

The interpretation of the quantitative significance of the reductions in GS-GOGAT activity is difficult, because within any one symbiosis cyanobacteria in different stages of symbiosis and of different metabolic activities may be present. It is clear that the overall

Table 1. *Glutamate synthase (GOGAT) activities and N₂ fixation rates in the free-living and symbiotic cyanobacteria from Peltigera canina, P. aphthosa and Azolla caroliniana*

Values in parentheses show activities obtained on the basis of cyanobacterial protein, which is 36% of *Peltigera canina* total protein and 80% of the *P. aphthosa* cephalodial protein (Sampaio *et al.*, 1979).

Organism	GOGAT activity [nmol product formed min ⁻¹ (mg protein) ⁻¹]	N ₂ fixation rate [nmol NH ₄ ⁺ produced min ⁻¹ (mg protein) ⁻¹]
<i>P. canina</i> discs	0.28 (0.84)	0.09 (0.25)
Free-living <i>Nostoc</i> CAN	33.50	—
Excised cephalodia of <i>P. aphthosa</i>	0.58 (0.72)	0.50 (0.63)
Free-living <i>Nostoc</i> APH	31.29	—
Symbiotic <i>Coccomyxa</i> of <i>P. aphthosa</i>	20.94	—
Symbiotic fungus of <i>P. aphthosa</i>	ND	—
Free-living <i>Coccomyxa</i> from <i>P. aphthosa</i>	23.10	—
<i>Anabaena</i> packets separated from <i>Azolla</i> leaf cavities	13.70	0.61
Free-living <i>Anabaena azollae</i>	42.92	—

ND, Not detectable; —, not determined.

activities detected are just sufficient to assimilate, via GS–GOGAT, the total amounts of NH₄⁺ produced during N₂ fixation (Table 1). However, the fact that NH₄⁺ is liberated extracellularly from the prokaryotes in these symbioses (Peters, 1976; Stewart & Rowell, 1977; Rai *et al.*, 1980) suggests that within the symbiotic cyanobacterial population the ratio of GS–GOGAT activity:nitrogenase activity varies substantially. This latter aspect is currently under study.

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REFERENCES

- GIVAN, C. V., GIVAN, A. L. & LEECH, R. M. (1970). Photoreduction of α -ketoglutarate to glutamate by *Vicia faba* chloroplasts. *Plant Physiology* **45**, 624–630.
- KESSLER, E., ARTHUR, W. & BRUGGER, J. E. (1957). The influence of manganese and phosphate on delayed light emission, fluorescence, photoreduction and photosynthesis in algae. *Archives of Biochemistry and Biophysics* **71**, 326–335.
- NEWTON, J. W. & HERMAN, A. I. (1979). Isolation of cyanobacteria from the aquatic fern, *Azolla*. *Archives of Microbiology* **120**, 161–165.
- PETERS, G. A. (1976). Studies on the *Azolla*–*Anabaena azollae* symbiosis. In *Proceedings of the International Symposium on Nitrogen Fixation*, 2, pp. 592–610. Edited by W. E. Newton & C. J. Nyman. Pullman: Washington State University Press.
- PETERS, G. A. & MAYNE, B. C. (1974). The *Azolla*–*Anabaena azollae* relationship. I. Initial characterization of the association. *Plant Physiology* **53**, 813–819.
- PETERS, G. A., MAYNE, B. C., RAY, T. B. & TOIA, R. E., JR (1979). Physiology and biochemistry of the *Azolla*–*Anabaena* symbiosis. In *Nitrogen and Rice*, pp. 325–344. Los Banos, Philippines: International Rice Research Institute.
- RAI, A. N., ROWELL, P. & STEWART, W. D. P. (1980). NH₄⁺ assimilation and nitrogenase regulation in the lichen *Peltigera aphthosa* Willd. *New Phytologist* **85**, 545–555.
- RAI, A. N., ROWELL, P. & STEWART, W. D. P. (1981). ¹⁵N₂ incorporation and metabolism in the lichen *Peltigera aphthosa* Willd. *Planta* (in the Press).
- RAY, T. B., PETERS, G. A., TOIA, R. E., JR. & MAYNE, B. C. (1978). The *Azolla*–*Anabaena azollae* relationship. VII. Distribution of ammonia assimilating enzymes, protein and chlorophyll between host and symbiont. *Plant Physiology* **62**, 463–467.
- RICHARDSON, D. H. S., HILL, D. J. & SMITH, D. C. (1968). The role of the alga in determining the pattern of carbohydrate movement between lichen symbionts. *New Phytologist* **67**, 469–486.
- RODGERS, G. A. & STEWART, W. D. P. (1977). The cyanophyte–hepatic symbiosis. I. Morphology and physiology. *New Phytologist* **78**, 441–458.
- SAMPAIO, M. J. A. M., RAI, A. N., ROWELL, P. & STEWART, W. D. P. (1979). Occurrence, synthesis and activity of glutamine synthetase in N₂-fixing lichens. *FEMS Microbiology Letters* **6**, 107–110.
- SHANMUGAM, K. T., O'GARA, F., ANDERSEN, K. & VALENTINE, R. C. (1978). Biological nitrogen fixation. *Annual Review of Plant Physiology* **29**, 263–276.
- STANIER, R. Y., KUNISAWA, R., MANDEL, M. & COHEN-BAZIRE, G. (1971). Purification and proper-

- ties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews* **35**, 171–205.
- STEWART, W. D. P. (1980). Some aspects of structure and function in N_2 -fixing cyanobacteria. *Annual Review of Microbiology* **34**, 497–536.
- STEWART, W. D. P. & RODGERS, G. A. (1977). The cyanophyte–hepatic symbiosis. II. Nitrogen fixation and interchange of nitrogen and carbon. *New Phytologist* **78**, 459–471.
- STEWART, W. D. P. & ROWELL, P. (1977). Modifications of nitrogen fixing algae in lichen symbioses. *Nature, London* **265**, 371–372.
- STEWART, W. D. P., FITZGERALD, G. P. & BURRIS, R. H. (1967). *In situ* studies on N_2 -fixation using the acetylene reduction technique. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 2071–2078.
- STEWART, W. D. P., HAYSTEAD, A. & DHARMAWARDENE, M. W. N. (1975). Nitrogen assimilation and metabolism in blue-green algae. In *Nitrogen Fixation by Free-living Microorganisms*, pp. 129–158. Edited by W. D. P. Stewart. Cambridge: Cambridge University Press.
- STEWART, W. D. P., ROWELL, P. & RAI, A. N. (1980). Symbiotic nitrogen-fixing cyanobacteria. In *Nitrogen Fixation*, pp. 239–277. Edited by W. D. P. Stewart & J. R. Gallon. London & New York: Academic Press.
- THOMAS, J., MEEKS, J. C., WOLK, C. P., SHAFFER, P. W., AUSTIN, S. M. & CHIEN, W. S. (1977). Formation of glutamine from ^{13}N ammonia, ^{13}N dinitrogen and ^{14}C glutamate by heterocysts isolated from *Anabaena cylindrica*. *Journal of Bacteriology* **129**, 1545–1555.
- WOLK, C. P., THOMAS, J., SHAFFER, P. W., AUSTIN, S. M. & GALONSKY, A. (1976). Pathways of nitrogen metabolism after fixation of ^{13}N -labelled nitrogen gas by the cyanobacterium, *Anabaena cylindrica*. *Journal of Biological Chemistry* **251**, 5027–5034.