Enzyme Activities Related to Cyanophycin Metabolism in Heterocysts and Vegetative Cells of *Anabaena* spp.

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(Received 24 November 1980)

The cyanophycin granule polypeptide (CGP) is known to serve as a nitrogen reserve protein in cyanobacteria and is mobilized during nitrogen starvation. An exopeptidase, provisionally called cyanophycinase, which degraded CGP in vitro has been studied, and its product characterized, in two N₂-fixing *Anabaena* species. The enzyme had a pH optimum of 8.5, had no requirement for monovalent or divalent cations and was inhibited by L-arginine and L-aspartic acid. The product of this enzyme was an aspartic acid-arginine dipeptide. A higher activity of both arg-poly(asp) synthetase and cyanophycinase was observed in extracts of heterocysts of both *Anabaena* species than in vegetative cells. This supports the view that CGP has a dynamic role in nitrogen metabolism of cyanobacteria as well as a storage function.

INTRODUCTION

All cyanobacteria so far examined possess electron-dense bodies, termed cyanophycin granules or structured granules (Lang, 1968; Lang *et al.*, 1972). They consist of a polypeptide, composed only of arginine and aspartic acid, which has been shown to be multi-L-arginyl-poly(L-aspartic acid), abbreviated to arg-poly(asp) and given the trivial name cyanophycin (Simon, 1971; Simon & Weathers, 1976; Allen & Weathers, 1980). The unusually simple composition of this polymer, which has molecular weights between 25 000 and 100 000, is reflected in its resistance to enzymic hydrolysis and to sequential breakdown by Edman degradation (Simon & Weathers, 1976). The fluctuation in cyanophycin granule polypeptide (CGP) content of cyanobacteria reflects the relative availability of nitrogen, and the role of CGP as a nitrogen storage material has been emphasized (Simon, 1973*a*; Allen *et al.*, 1980). Although reduced carbon skeletons which are central to biosynthesis can be derived from arginine and aspartic acid, Stanier & Cohen-Bazire (1977) suggested that CGP may in addition act as an ATP supply by the breakdown of arginine. In contrast to phycocyanin, the other source of reserve nitrogen in cyanobacteria (Allen & Smith, 1969), CGP is not synthesized on ribosomes (Simon, 1973*b*). Simon (1976) has purified and characterized arg-poly(asp) synthetase in extracts of vegetative cells of *Anabaena cylindrica* and shown it to require a 'primer' of pre-formed CGP for its activity.

Many filamentous cyanobacteria contain specialized cells – heterocysts – which are the major or sole site of N₂ fixation and which exist in an interdependent relationship with their vegetative supporting cells (for reviews, see Haselkorn, 1978; Carr, 1979). Heterocysts contain amorphous polar bodies, which stain deeply with Sakaguchi reagent, indicating arginine content (Fogg, 1951). These bodies are now recognized as cyanophycin granules (Lang *et al.*, 1972). We describe in this communication the content of CGP in cell-free extracts of heterocysts and vegetative cells of two *Anabaena* species and characterize its enzymic synthesis and breakdown.

METHODS

Organisms and growth. *Anabaena cylindrica* (strain 1403/2a; Culture Centre of Algae and Protozoa, Cambridge) and *Anabaena* 7120 (PCC 7120; Institut Pasteur, Paris) were maintained and grown at $29 \pm 1^\circ\text{C}$ in the medium of Allen & Arnon (1955) gassed with 5% (v/v) CO_2 in air, with a light intensity of $75 \mu\text{E m}^{-2} \text{s}^{-1}$. Cultures were grown in 500 ml flasks containing 250 ml medium supplemented with 3 mM- NH_4Cl (A^+ medium) when a population without heterocysts was required. To obtain a heterocyst-containing population, 10 l cultures were supplied with only 0.3 mM- NH_4Cl . Nitrogen became limiting after approximately 72 h and heterocysts began to differentiate; after a further 24 h, the heterocyst population was complete (approximately 10% of the cells in *Anabaena* 7120 and 8% in *A. cylindrica*). An intermediate stage of heterocyst differentiation, occurring 14 to 16 h after the limitation of nitrogen, consisted of 6 to 8% proheterocysts and their associated, nitrogen-limited, vegetative cells. Such a culture was termed 'differentiating filaments'.

Isolation of heterocysts. Exponentially growing heterocyst-containing filaments from 20 l of culture were harvested in a continuous-flow centrifuge at room temperature; subsequent steps were carried out at 4°C . For the assay of cyanophycinase activity, organisms were washed three times in 500 ml NP buffer (20 mM-sodium phosphate, pH 8.5) and resuspended (20 l culture in 200 ml) in TEM buffer (50 mM-Tris, 25 mM-EDTA, 25 mM-mannitol, pH 8.5). Lysozyme (1 mg ml^{-1}) was added and the mixture was incubated for 45 min at 30°C on a rotary shaker. Vegetative cells were then ultrasonically disrupted (for about 4 s ml^{-1}) in an MSE 150 W instrument, by a series of 15 s bursts. The intact heterocysts were isolated by centrifugation at 1000 g for 10 min, washed twice with 200 ml TEM buffer, and suspended in 1 to 2 ml NP buffer. Heterocyst preparations contained less than 0.5% vegetative cells.

For the assay of arg-poly(asp) synthetase, the cells were harvested and washed with TM buffer (50 mM-Tris, 5 mM- β -mercaptoethanol, 5 mM-EDTA, pH 8.2). Heterocysts were then isolated (as above) in TEMM buffer (TEM buffer containing 5 mM- β -mercaptoethanol, pH 8.2) and finally suspended in 2 ml TM buffer.

Harvesting of vegetative cells. Vegetative cells from an exponential phase culture grown in A^+ medium were harvested at 4°C , washed and suspended in 2 ml NP or TM buffer for assay of cyanophycinase and arg-poly(asp) synthetase, respectively.

Preparation of cell-free extracts. Vegetative cells in the appropriate buffer were disrupted ultrasonically (15 s ml^{-1}) with continuous cooling. When a heterocyst-containing filament was ultrasonically disrupted, only vegetative cells were broken, and the resultant cell-free extract was separated from debris and intact heterocysts by centrifugation. Heterocysts were broken by passing four or five times through a chilled French press at 110 MPa (which disintegrated 99% of heterocysts) and centrifuged at 124 000 g for 1 h; the supernatant was used for the enzyme assays.

Radioactive labelling of CGP. Radioactively labelled CGP was prepared from vegetative cells of *Anabaena* 7120 and used as substrate to assay the cyanophycinase activity of both heterocysts and vegetative cells of *Anabaena* 7120 and *A. cylindrica*. *Anabaena* 7120 was grown in 500 ml A^+ medium, harvested after 4 to 5 d, when the culture density was approximately 0.2 mg dry wt ml^{-1} , and resuspended in 200 ml A^+ medium. After 4 h growth chloramphenicol (5 $\mu\text{g ml}^{-1}$) was added, followed 2 h later by 20 $\mu\text{Ci L-[5(n)-}^3\text{H]arginine}$ (specific activity 10 Ci mmol^{-1} ; 370 GBq mmol^{-1}). The organism was harvested after a further 60 h growth. For doubly labelled CGP, 15 $\mu\text{Ci L-[U-}^{14}\text{C]aspartic acid}$ (specific activity 10 mCi mmol^{-1} ; 370 MBq mmol^{-1}) was also added.

Isolation of cyanophycin granules. Cyanophycin granules from all types of cells were isolated by a method based on that of Simon (1973a). Labelled or unlabelled cells from 200 ml cultures were disrupted ultrasonically and granules were washed twice in 10 ml 5 mM-sodium phosphate buffer, pH 7.5. The pellet was resuspended in 10 ml 2% Triton X-100 and centrifuged. This step was repeated four times followed by washing four times with 10 ml sodium phosphate buffer. Each centrifugation following washing was carried out at 27 000 g for 15 min at 4°C . Isolated granules were resuspended in 1 to 2 ml sodium phosphate buffer, transferred to 1.5 ml Sarstedt microcentrifuge tubes and purified by centrifugation at 12 000 g for 30 s in a micro-haemocrit centrifuge. The top amorphous layer of the pellet was carefully removed and the lower white compact layer was resuspended in 1 ml buffer and centrifuged at the same speed. Remaining top layer material was again removed and the granules were resuspended in 1 ml buffer. When radioactively labelled granules purified by this procedure were dissolved in 0.1 M-HCl (Simon, 1973a), 99.9% of the isotope was solubilized, indicating a high degree of purity.

CGP to be used as a primer for arg-poly(asp) synthetase was purified as above, except that 5 mM unlabelled L-arginine was added to chloramphenicol-treated cells instead of radioactively labelled arginine. CGP was assayed as described by Simon (1973a).

Assay for cyanophycinase activity. The principle of the assay was the release into the soluble fraction of radioactivity from ^3H arginine-labelled CGP. The standard reaction mixture contained, in a total volume of 240 μl : 50 mM-sodium phosphate buffer, pH 8.5; CGP equivalent to about 70 μg arginine, labelled with ^3H arginine or ^3H arginine and ^{14}C aspartic acid; and cell-free extract containing 150 to 200 μg protein. The reaction was carried out in polycarbonate centrifuge tubes, incubated with agitation at 30°C . The reaction was stopped by transferring the tubes into ice and then to -18°C . The thawed suspension was centrifuged at 27 000 g

for 15 min at 4 °C. Protein was precipitated from the supernatant (to prevent possible quenching during isotope counting) by the addition of 60 µl 20% (w/v) trichloroacetic acid and centrifuging at 5000 g for 10 to 15 min. The supernatant was mixed with Aquasol and radioactivity was counted in an Intertechnique scintillation counter, to an accuracy of at least 1%.

Characterization of product of cyanophycinase. The heterocyst enzyme was employed for the characterization of the cyanophycinase product, with the cyanophycinase assay mixture scaled up threefold. After centrifugation at 27000 g the supernatant was loaded on to a Bio-Gel P-2 column (1 × 22 cm, 17 ml vol.) without prior trichloroacetic acid precipitation. The column was pre-equilibrated with 60 mM-sodium phosphate buffer, pH 8.5; the flow rate was 6 ml h⁻¹ and the fraction volume 0.2 ml. The column was calibrated using the following standards: cyanocobalamin (mol. wt 1355); oxidized glutathione (613); reduced glutathione (307); arginine (174). The molecular weights of the enzyme reaction products in the fractions were calculated on the basis of elution volume to void volume ratio (V_e/V_0) as compared to the standard curve. When a Sephadex G-10 column (1 × 22 cm) was employed the column volume was 13 ml and 0.5 ml fractions were eluted with 50 mM-sodium phosphate buffer, pH 8.5.

Thin-layer chromatographic analysis was carried out after precipitation of protein from the reaction mixture by 8 vol. 90% ethyl alcohol. The alcohol was evaporated, under N₂, at 40 °C and the products were dissolved in 1 ml water at 80 °C. The 5000 g supernatant was extracted twice with diethyl ether. The aqueous phase was lyophilized and then dissolved in 50 ml propan-2-ol/water (20:80, by vol.); 10 µl portions were spotted on MN-300 cellulose plates (20 × 20 cm) which were run in butan-1-ol/acetic acid/water (80:20:20, by vol.) Areas corresponding to arginine and aspartate were removed and their radioactivity was counted in a scintillation counter in toluene plus 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.01% (w/v) 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP).

Assay for arg-poly(asp) synthetase activity. The principle of the assay was the incorporation of radioactively labelled arginine or aspartate into trichloroacetic acid-insoluble material, after normal protein synthesis had been inhibited by chloramphenicol. The assay mixture (Simon, 1976) contained, in a total volume of 250 µl: 50 mM-Tris, pH 8.2; 10 mM-β-mercaptoethanol; 20 mM-KCl; 20 mM-MgCl₂; 4 mM-ATP; 34 µM-chloramphenicol; 0.5 µCi [¹⁴C]aspartic acid; 1 µCi [³H]arginine; purified CGP containing 40 µg arginine; and cell-free extract containing 200 to 300 µg protein. The reaction was carried out for different periods and then stopped by adding 2 ml cold (4 °C) distilled water to the mixture, which was then immediately frozen at -18 °C. After thawing, the reaction mixture was centrifuged at 27000 g for 15 min. The pellet was washed twice with 5 ml 50 mM-Tris buffer, pH 8.0, and then with 5 ml 10% trichloroacetic acid, and recentrifuged. The pellet was dissolved in 0.5 ml 0.1 M-HCl for 30 min at 30 °C and then mixed with 3.3 ml Aquasol, and radioactivity was counted.

Protein estimation. Protein was estimated by the microbiuret method of Itzhaki & Gill (1964).

Heterocyst and vegetative cell counts. Isolated heterocysts and vegetative cells were counted in a haemocytometer. Vegetative filaments were broken into 2- to 4-celled sections with a homogenizer prior to counting.

Chemicals. L-[5(n)-³H]Arginine and L-[U-¹⁴C]aspartic acid were from The Radiochemical Centre, Amersham; Aquasol, PPO and POPOP were from New England Nuclear; amino acids and *p*-aminobenzamidinium.HCl, and glutathione (oxidized and reduced) were from Sigma; Bio-Gel P-2 was from Bio-Rad Laboratories. Cyanocobalamin and all other chemicals were purchased from BDH.

RESULTS.

Properties of the cyanophycinase reaction

The pH optimum of the enzyme reaction was 8.5. Activity increased when the CGP concentration was increased over the range 0 to 80 µg arginine equivalent. Activity also increased linearly with the amount of enzyme protein when up to 100 µg enzyme protein per assay was used with 35 µg arginine equivalent CGP. The release of [³H]arginine was linear with time for up to 40 min with extracts of vegetative cells and for up to 120 min with heterocyst extracts. Under the conditions used, not more than 5% of the labelled CGP substrate was converted into soluble product. The crude enzyme extract lost activity by 7% when frozen for 18 h at -18 °C. The enzyme was inhibited by its end-products: 10 mM-arginine and 10 mM-*p*-aminobenzamidinium.HCl (an analogue of arginine) inhibited the cyanophycinase activity by 62% and 73%, respectively, while 10 mM-aspartic acid caused only 37% inhibition.

When the enzyme extract was passed through a Sephadex G-10 column, the eluted enzyme did not show any drop in specific activity. Activity was not enhanced by the addition of Na⁺,

Fig. 1

Fig. 2

Fig. 1. Distribution of [^3H]arginine in fractions eluted from a Bio-Gel P-2 column after application of the products of incubating ^3H -labelled CGP with cyanophycinase from heterocysts of *Anabaena* 7120 for 30 min (O) and for 2 h (●). The column was calibrated (inset) with cyanocobalamin (mol. wt 1355), oxidized glutathione (613), reduced glutathione (307) and arginine (174).

Fig. 2. Distribution of [^3H]arginine (O) and [^{14}C]aspartic acid (●) in fractions eluted from a Sephadex G-10 column after application of the products of a 2 h incubation of doubly labelled CGP with cyanophycinase from heterocysts of *Anabaena* 7120.

K^+ , Ca^{2+} , Mn^{2+} or Mg^{2+} (each at 5 to 20 mM) to the eluted enzyme; the divalent cations in fact slightly inhibited activity. NH_4Cl (10 mM) had no effect on cyanophycinase activity.

Characterization of the product of cyanophycinase

When the product of a 2 h incubation with enzyme was passed through a Bio-Gel P-2 column, the distribution of labelled arginine in the eluted fractions showed two distinct peaks (Fig. 1), corresponding to molecular weights of about 320 (A) and 180 (B). Since the molecular weight of arginine is 174, peak B was assumed to comprise free arginine, while peak A was taken to be a dimer consisting of a peptide of aspartic acid and arginine.

When doubly labelled CGP was employed in order to examine the degree of release of each amino acid by cyanophycinase, there was an exact equivalence of the two amino acids in the peptide eluted from a Sephadex G-10 column, showing that an arg-asp fraction (rather than an asp-asp fraction) is the product of CGP breakdown (Fig. 2).

Thin-layer chromatography confirmed that only a small proportion of the soluble products of cyanophycinase were free amino acids. The major part of the released material remained at the origin, indicating its peptide nature.

Arg-poly(asp) synthetase

The cell-free extract of heterocysts of *Anabaena* 7120 incorporated both [^3H]arginine and [^{14}C]aspartic acid into CGP. Primer CGP was necessary for the synthetase activity, as reported by Simon (1976). The product of the enzyme reaction was insoluble in 10% trichloroacetic acid and soluble in 0.1 M-HCl, consistent with it being CGP. The specific activity of the heterocyst enzyme from *Anabaena* 7120 increased linearly with the increase in

Fig. 3. Specific activity of arg-poly(asp) synthetase as a function of supplied primer concentration, in an extract of heterocysts from *Anabaena* 7120. The reaction was performed with 200 μg protein per assay for 40 min.

Table 1. Activity of arg-poly(asp) synthetase in cell-free extracts of heterocysts and vegetative cells of *Anabaena* 7120 incubated with radioactively labelled arginine and aspartic acid

Substrate	Specific activity [pmol amino acid incorporated (mg protein) ⁻¹ h ⁻¹]		Activity in vegetative cells relative to activity in heterocysts (%)
	Heterocysts	Vegetative cells	
[³ H]Arginine	146	5	3.4
[¹⁴ C]Aspartic acid	11070	1090	9.8

CGP primer concentration, up to 20 μg arginine equivalent CGP (Fig. 3). Thereafter increase in activity became less pronounced. A CGP concentration equivalent to 40 μg arginine was subsequently used for comparative experiments.

Specific activities of cyanophycinase and arg-poly(asp) synthetase in vegetative cells and heterocysts

The activity of the arg-poly(asp) synthetase was measured in *Anabaena* 7120 by following the incorporation of both [³H]arginine and [¹⁴C]aspartic acid into trichloroacetic acid-insoluble material. Direct comparison of the specific activities obtained with the two amino acids is not possible because each would be diluted to a different degree by the endogenous pools. Furthermore, incorporation of arginine was dependent on the pre-formation of the poly(asp) backbone of CGP (Simon, 1976). The only comparison that can be made is between the activity in vegetative cells and in heterocysts: the latter showed considerably more synthetase activity (Table 1).

Heterocysts of both *Anabaena* species also showed considerably greater cyanophycinase activity than vegetative cells (about 90-fold greater in *Anabaena* 7120 and 20-fold in *A. cylindrica*: Table 2). Differentiating filaments, containing only proheterocysts and vegetative cells, showed much less activity than mature heterocysts. Vegetative cell extracts from N₂-fixing cultures, prepared by ultrasonic treatment that did not disrupt heterocysts, contained cyanophycinase with a specific activity of 0.50 pmol arginine solubilized (mg protein)⁻¹ h⁻¹ in *A. cylindrica* and 0.57 pmol (mg protein)⁻¹ h⁻¹ in *Anabaena* 7120. These activities were close to those found in extracts of cultures grown in the presence of NH₄Cl (Table 2).

Table 2. *Cyanophycinase activity in heterocysts, differentiating filaments and vegetative cells of two Anabaena species*

Results show the mean specific activities, with the range of values and number of determinations in parentheses.

Species	Specific activity [pmol arginine solubilized (mg protein) ⁻¹ h ⁻¹]		
	Heterocysts	Differentiating filaments	Vegetative cells
<i>Anabaena</i> 7120	35.1 (34.9–36.8) (4)	1.2 (1.1–1.5) (3)	0.38 (0.22–0.54) (3)
<i>A. cylindrica</i>	17.0 (15.0–20.0) (3)	4.0 (3.8–4.5) (3)	0.77 (0.51–0.99) (3)

Content of CGP in heterocysts and vegetative cells

Simon (1973*a*) has shown that the actual amount of CGP is fivefold greater than the arginine measured; presumably only a proportion of arginine in CGP is available for the colorimetric estimation. The arginine content as a measure of CGP was higher in vegetative cells than in heterocysts of both *Anabaena* species by about 50%, with significant variation between replicate experiments. *Anabaena* 7120 had average values of 3.9 µg arginine equivalent CGP in 10⁶ heterocysts and 5.6 µg in 10⁶ vegetative cells.

DISCUSSION

The presence of CGP in heterocysts was confirmed by isolation of the polymer from heterocysts that had been freed from vegetative cells; it was presumed that this was the material which stained with the Sakaguchi reaction (Fogg, 1951) and was located in the heterocyst poles. The identification of a CGP-degrading enzyme, apparently an exopeptidase, to which we give the trivial name cyanophycinase, is of interest in view of the resistance of the polymer to other proteases (Simon & Weathers, 1976). The reaction catalysed by cyanophycinase may be written as:

The small amount of free arginine produced is probably the result of cleavage of the arg-asp dipeptide, and experiments to identify an enzyme causing this cleavage are now under way. The synthesis of CGP involves the establishment of a poly(asp) backbone of undetermined length, then the addition of the arginine residue to each aspartic acid (Simon, 1976). The difference between the routes of synthesis and breakdown of CGP is consistent with our knowledge of many other biopolymers.

The considerable difference between the specific activity of arg-poly(asp) synthetase obtained with the two substrates [³H]arginine and [¹⁴C]aspartic acid can be accounted for in two ways. Firstly, there may be a difference in the degree of dilution of radioactive isotope by endogenous pools of arginine and aspartic acid, which may differ. More importantly, the presence of a poly(asp) backbone of CGP is necessary for any arginine incorporation (Simon, 1976). Thus any measure of arg-poly(asp) synthetase that is based on arginine incorporation must assume the prior incorporation of aspartic acid. The point that emerges unequivocally is that arg-poly(asp) synthetase activity is considerably greater in heterocysts than in vegetative cells of *Anabaena* 7120.

Cyanophycinase is 20-fold more active in heterocysts than vegetative cells in *A. cylindrica* and 90-fold greater in the case of *Anabaena* 7120. Other than nitrogenase activity this is probably the greatest enzyme difference yet observed between heterocysts and vegetative cells. Cyanophycinase activity in differentiating filaments was only slightly greater than in

vegetative cells, which had similar activities after growth in the presence or absence of NH_4Cl . We conclude that nitrogen limitation as such does not induce the marked increase in cyanophycinase activity, and that the higher activity is associated only with the functioning of mature heterocysts. The role of CGP as a reserve material (Simon, 1973*a*; Allen *et al.*, 1980) is entirely consistent with its accumulation under varied environmental conditions. However, the relatively similar amounts of the polymer in heterocysts and vegetative cells, and the high activity of CGP-metabolizing enzymes in heterocysts, indicate a greater degree of turnover of CGP in heterocysts and suggest a more direct role in nitrogen assimilation. We suggest that CGP may act as a reservoir between nitrogen fixation and the export of glutamine (Thomas *et al.*, 1977) from heterocysts into vegetative cells, thus separating the constant requirement for fixed nitrogen for biosynthesis from the possibly non-constant rates of nitrogen fixation in the heterocysts.

M.G. is a Commonwealth Fellow and the work was supported by the Science Research Council. We thank Dr R. J. Beynon for advice and discussion.

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