Measurement of the Cyanophycin Granule Polypeptide Contained in the Blue-Green Alga *Anabaena cylindrica*

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Received for publication 26 January 1973

An assay was developed to determine the amount of cyanophycin granules in blue-green algae. The amount of this polypeptide in cells of *Anabaena cylindrica* was measured as a function of culture age and was compared with changes in other proteinaceous cellular components. The data presented support the notion that the cyanophycin granule is a cellular nitrogen reserve.

Although the Cyanophyta lack membrane-bound organelles of the type found in eukaryotes, they possess distinctive subcellular particles: cyanophycin granules, glycogen granules, gas vacuoles, polyphosphate bodies, and polyhedral bodies (8). The composition of certain of these subcellular particles has recently been determined (4, 7, 17). In particular, the cyanophycin granule is composed of high-molecular-weight (25,000 to 100,000) copolymers of L-aspartic acid and L-arginine (17).

Little is known about the function or metabolism of the subcellular particles which are found in the blue-green algae. Light and electron microscopy observations have shown that cyanophycin granules are present in older cultures and are particularly abundant in spores, but are lacking in actively growing cultures (5, 13, 18). These observations, together with the composition of the granules, suggest that this particle serves as a nitrogen reserve.

An assay by which the amount of cyanophycin granule polypeptide (CGP) present in a sample of blue-green algae may be quantified is herein described. The assay is based upon the size and density of the intact cyanophycin granules and upon the unique solubility properties of CGP. The amount of CGP and other proteinaceous components present in cells of the blue-green alga *Anabaena cylindrica* has been determined as a function of culture age. The resulting data are consistent with the idea that cyanophycin granules are a nitrogen reserve.

**MATERIALS AND METHODS**

**Organism and culture.** *Anabaena cylindrica* Lemm. was grown axenically in 13-liter batches by using fermentors (New Brunswick Scientific Co.) as previously reported (19). The fermentors were illuminated with 12 cool-white lamps (ITT F15T81 CW) and were gassed with air at a flow rate of 4 liters/min. The growth medium used throughout the experiments described below consisted of the medium of Allen and Arnon (2) diluted 16-fold and modified by the addition of 1 g of NaNO₃ per liter.

For small-volume cultures, *A. cylindrica* was grown axenically in 1-liter batches of the modified medium of Allen and Arnon described above by using 3-liter diphtheria bottles. Air previously passed through sterile, 0.45-µm membrane filters (Millipore Corp.; HAWP 013) was bubbled through the culture medium by using a coarse Pyrex gas dispersion tube. The air flow rate of approximately 1 liter/min served both to supply CO₂ and to agitate the culture. The culture was illuminated from one side by three 40-W cool-white fluorescent lamps.

**Chemical determinations.** The amino acid composition of cell extracts was qualitatively determined by two-dimensional paper chromatography after hydrolysis with 6 N HCl at 105 C for 24 h. Amino acids were separated on Whatman no. 1 paper by using butanol-acetic acid-water (3:1:1, vol/vol/vol) in one dimension and 80% phenol-water-34% ammonium hydroxide (176:24:1, vol/vol/vol) in the other dimension. Aspartic acid and arginine were quantitatively determined in hydrolysates by separating them by paper chromatography with the latter solvent system. Spots corresponding to aspartic acid and arginine were cut out, eluted with 0.1 N HCl, and assayed for free alpha amino groups (16). Measurement of aspartic acid and arginine standards gave correction factors.
for the losses during hydrolysis and elution.

Arginine was measured by a modified Sakaguchi reaction by using a procedure similar to that reported by Messineo (12). Figure 1A shows a standard curve using arginine-hydrochloride salt. An advantage of the Messineo (12) procedure is that arginine within proteins can be assayed directly without having to first hydrolyze the protein. By using purified CGP as a standard for the arginine assay, it was found that the assay color developed at 520 nm was proportional to the amount of unhydrolyzed protein assayed (Fig. 1B), although the amount of color which developed was about 60% less than would be expected from calculations based upon both the amount of arginine in the protein and the color developed by free arginine (Fig. 1A) (R. D. Simon, unpublished data).

Chlorophyll was measured by the method of Mackinney (11), phycocyanin was measured by the method of Myers and Kratz (14), and dry weight was measured by filtering culture samples through tared 0.45 μm membrane filters (Millipore Corp.; HAWG 013). The filters were dried at 50 C and reweighed. The protein content (10) of the supernatant fluid after centrifugation (27,000 x g, 15 min) of cell-free sonicates was measured and called “soluble protein.” “Soluble protein” includes truly soluble protein as well as a large fraction of the cellular membrane protein.

**Determination of CGP.** The amount of CGP in a culture can be determined quantitatively by taking advantage of the size, density, and unusual solubility properties of cyanophycin granules. Intact cyanophycin granules may be pelleted by centrifugation at 27,000 x g for 15 min, and CGP is soluble in dilute acid but is insoluble in distilled water and the detergent Triton X-100 (9, 17). Portions (25 ml) of a cell suspension (with known dry weight) were sonicated at setting three for 4 min in the continuous flow chamber of a Branson sonifier, model S-125 (20). The exit port of the chamber had been closed with a small serum cap. Almost all of the vegetative cells were broken by this procedure. The resulting suspension was then centrifuged for 15 min at 27,000 x g. The supernatant fluid was used for the assay of “soluble protein” (see above) or was discarded. The pellet was washed with distilled water, with 2% Triton X-100, and then two additional times with distilled water. Next, 1.0 ml of 0.1 N HCl was added to the pellet, and the pellet was resuspended. After 0.5 h at room temperature, the tube containing the resuspended pellet was centrifuged as before. The supernatant fluid was saved, and the pellet was re-extracted with 1.0 ml of 0.1 N HCl. The supernatant fluids from the two extractions were pooled. Control experiments showed a third, dilute-acid extraction of the pellet to be unnecessary (R. D. Simon, unpublished data). The HCl extract was then directly assayed for arginine by the method described above. The amount of CGP in the extract was determined by comparing the arginine value with a standard curve prepared by assaying isolated cyanophycin granules for arginine (17; Fig. 1B).

**RESULTS**

The CGP assay was checked by examining the amino acid composition of the final acid extract. After hydrolysis in 6 N HCl for 24 h, only two amino acids could be identified by paper chromatography: aspartic acid and arginine. Occasionally other faint spots could be seen, but these always accounted for less than 5% of the total amino acids in the extract. Arginine and aspartic acid were found to be present in the acid extracts in a 1:1 molar ratio, a ratio equal to that found for these amino acids in isolated cyanophycin granules (17). Thus, the only significant source of arginine in the acid extract was CGP.

Figure 2 shows the amount of CGP present in cells of *A. cylindrica* at different stages of culture growth and compares this with the dry weight and the amount of chlorophyll in the culture. At day 13 the culture was just leaving the phase of exponential growth and entering the stationary phase. Granule synthesis began at day 14 and proceeded throughout the stationary phase. The amount synthesized reached a maximum at 18 days. When a culture in stationary phase was diluted, and growth recom-
menced, the granules were rapidly broken down from 7.8 to 1.3% of the dry weight in 1 day (Fig. 2).

The level of phycocyanin (Fig. 3A) and "soluble protein" (Fig. 3B) also changed with culture age. The phycocyanin content, as percent of cell dry weight, increased during the exponential phase of culture growth and fell slightly after 14 days, the time of maximum CGP synthesis. Similarly, "soluble protein" increased from 46 to 66% of the cellular dry weight during exponential growth and then fell back to 46% during the stationary phase. Upon dilution of the culture and resumption of growth, the phycocyanin content of cells fell in one day from 6.7 to 2.2% of the dry weight. The phycocyanin content also dropped when measured as percent of "soluble protein," going from 15.8 to 4.8% in one day. However, the "soluble protein," when measured as the percent of cell dry weight, did not change immediately after an exponential culture resumed growth (Fig. 3B), in marked contrast to the change seen in phycocyanin content.

DISCUSSION

The assay for CGP which is described in this paper is a convenient and rapid method to determine quantitatively the content of one of the subcellular particles of the blue-green algae. The observations that the only amino acids present in the 0.1 N HCl extracts of the CGP assay are aspartic acid and arginine and that these two amino acids are present in a molar ratio of 1:1 suggest that the arginine content of the extracts measures CGP only. The values for CGP present are underestimated, because no correction is made for the small percent of unbroken cells and heterocysts or for losses occurring during the assay procedure.

The quantitative data presented agree with earlier microscope observations (5, 6, 18) that cyanophycin granules are found principally in old cultures and only to a much lesser extent in young, growing cultures. The maximal rate of CGP production occurs after the end of the exponential growth phase of the culture, and
the maximal amount of CGP is found in the stationary-phase cells. When an old culture is diluted and growth resumes, CGP largely disappears. This result supports the concept that the granules are a reserve that is built up at the end of the growth phase. The reserve is presumably utilized in building new cellular components when growth recommences.

CGP is not the only protein whose concentration changes with culture age. The phycocyanin content of cells increases during exponential growth. Total "soluble protein" also increases during exponential growth, but falls when the cells enter the stationary phase. The decrease of "soluble protein" during the stationary phase correlates with the production of CGP.

Upon transition of cultures from stationary to exponential phase, the phycocyanin content of the vegetative cells drops extensively in one day, whereas the "soluble cell protein," measured as percent of cell dry weight, is not decreased. Thus the phycocyanin may be specifically broken down. Because phycocyanin content is measured by observing the optical density at 618 nm, the apparent decrease in pigment content may correspond to an effect on the chromatophore of the protein or may represent degradation of the entire protein. Although dilution of a culture also results in an increased light intensity, which conceivably might bleach the phycocyanin chromatophore, some chlorophyll bleaching under the same conditions also would be expected (1), a phenomenon which was not observed (Fig. 2A). The degradation of the phycocyanin protein is a more probable explanation of phycocyanin disappearance. The idea that the auxiliary, photosynthetic pigment phycocyanin serves in part as a reserve protein has been suggested by Allen and Smith (3) and by Neilson et al. (15). These authors observed that the pigment disappears under conditions of nitrogen starvation and that resynthesis occurs when starved cells are furnished with a nitrogen source.

The data presented here show that the relative amounts of different cellular proteins in Anabaena cylindrica change with culture conditions. One interpretation of these changes is that soluble protein serves as a source of amino acids to build up a protein reserve (CGP) during the stationary phase. When growth resumes, the reserve and certain other specific proteins (e.g., phycocyanin) are degraded and utilized.

ACKNOWLEDGMENTS

I thank C. P. Wolk for his extremely helpful comments on the manuscript.

This work was supported by the U.S. Atomic Energy Commission contract AT(11-1)-1538 and by research grant BG 35536 from the National Science Foundation.

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