JOURNAL OF BACTERIOLOGY, Feb. 1980, p. 959-962
0021-9193/80/02-0959/04$02.00/0

Vol. 141, No. 2

Structure and Composition of Cyanophycin Granules in the Cyanobacterium *Aphanocapsa* 6308

MARY MENNES ALLEN* AND PAMELA J. WEATHERS†

Department of Biological Sciences, Wellesley College, Wellesley, Massachusetts 02181

Cyanophycin granules in the unicellular cyanobacterium *Aphanocapsa* 6308 were examined with the electron microscope in both thin section and by freeze-fracture techniques. Purified granules were examined with the electron microscope, by arginine determinations, by chromatography, and by elemental analysis. They are similar in ultrastructure and composition to those isolated from the nitrogen-fixing cyanobacterium *Anabaena cylindrica*, consisting of equal molar quantities of L-arginine and L-aspartic acid.

Cyanophycin or structured granules in the nitrogen-fixing cyanobacterium *Anabaena cylindrica* were shown by Lang et al. (5) to be composed of multi-L-arginyl poly-(L-aspartic acid). Simon (8) first purified and described the structure of this unique chemical polymer, which consists of various lengths of poly-L-aspartate residues to whose β-carboxyl groups arginine is attached in peptide linkage (10); arginine and aspartate are present in equimolar amounts. No detailed study has been carried out on other cyanobacteria, and therefore the structure and composition of cyanophycin granules were studied in *Aphanocapsa* 6308. *Aphanocapsa* is unicellular, does not fix nitrogen, and produces a large yield of granules.

Aphanocapsa 6308 was routinely grown in liquid medium no. 11 (1) supplemented with 2.4 g of sodium carbonate per liter at 35°C in 10,800 lx of cool white fluorescent light and with 5% CO_2 in air. Radioisotopic incorporation studies were made with uniformly labeled L-[^{14}C]arginine (specific activity, 277 mCi/mmol) or L-[^{14}C]aspartate (specific activity, 204 mCi/mmol; New England Nuclear Corp.) added to medium no. 11. Washed cells were either broken in a French pressure cell at 15,000 lb/in^2 with an MSE 150 W ultrasonic disrupter Mk2 or with a Heat Systems sonifier, and isolation was by the procedure of Simon (8, 9). After this procedure, the final granule pellet was purified in one of two ways. (i) The orange layer was removed with a cotton swab, and the remaining pellet was suspended in distilled water and centrifuged for 30 s at top speed with a table centrifuge. The supernatant was spun for 15 min at 1,000 × *g*, and the white pellet was recentrifuged for 30 s at 1,000 × *g*. The white, cloudy supernatant was decanted with a Pasteur pipette and centrifuged at 27,000 × *g* for 15 min. (ii) The suspended pellet was layered on a 70 to 100% Renografin step gradient containing Triton X-100 and centrifuged for 2 h at 23,000 rpm in an SW25.1 rotor. The highly concentrated granule band at the bottom of the 80% step was collected and washed twice to remove the Renografin. Cyanophycin granule polypeptide (CGP) was analyzed quantitatively by the method of Simon (9), using purified CGP as the standard in the arginine assay. Assay color was proportional to the amount of unhydrolyzed protein assayed, and a correlation factor of 5× was determined between micrograms of arginine hydrochloride per milliliter of sample and micrograms of granules per milliliter of sample. Protein was measured by the Folin method (6), with bovine serum albumin as the standard. Dry weight was determined on washed suspensions by using tared containers in a 90°C oven or using washed Whatman GF/C glass fiber filter papers dried at 50°C. Quantitative microanalysis of purified granules was carried out by Galbraith Laboratories, Inc., Knoxville, Tenn. Isolated granules were hydrolyzed overnight in 6 N constant boiling HCl at 105°C. The hydrolysate was evaporated to dryness under nitrogen or by vacuum evaporation, suspended in distilled water, and evaporated again. The residue was suspended in 20% isopropanol or water and spotted on Whatman no. 1 filter paper, and two-dimensional paper chromatography was carried out by using methanol-water-pyridine (80:20:4) and tertiary butyl alcohol-butanone-water-diethylamine (40:40:20:4). One-dimensional chromatography was carried out in phenol-water-NH_4OH (88:12:0.5).

Cells were prepared for examination of thin sections under conditions previously described (2). For freeze-fracturing, a pellet of cells was suspended and equilibrated for 2 h in phosphate buffer (pH 7.4) containing 20% glycerol. The cell

† Present address: Department of Life Sciences, Worcester Polytechnic Institute, Worcester, MA 01609.

suspension was subsequently centrifuged at 10,000 × *g* for 5 min. Small droplets of cells were transferred by micropipettes to the central well of gold specimen carriers, rapidly frozen in the liquid phase of partially solidified Freon 22 (monochlorodifluoromethane), cooled, and stored in liquid nitrogen. Specimens were fractured and replicated with carbon-platinum in a Balzer's 360 M apparatus (Balzer's High Vacuum Corp., Santa Anna, Calif.) fitted with an electron beam gun for platinum shadowing and a quartz crystal monitor for standardizing replica thickness. Replicas were cleaned for 24 h in methanol and for 2 h in 5% sodium hypochlorite; they were subsequently mounted on 300-mesh copper grids and examined with the electron microscope.

Figure 1 is a light micrograph of logarithmi-

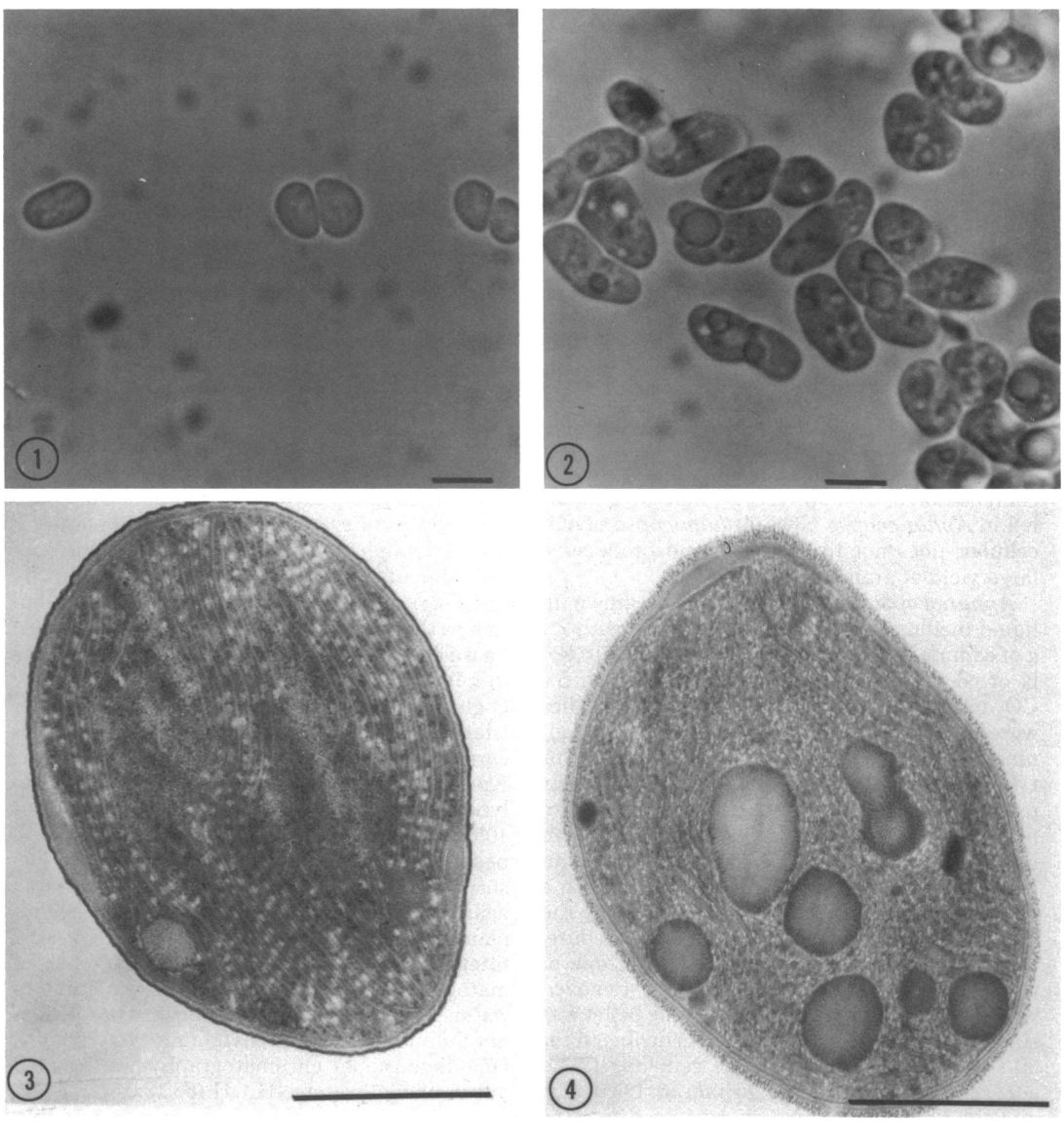

FIG. 1. *Light-limited late stationary-phase cells. Phase micrograph of Aphanocapsa 6308. Bar represents 1 μm.*

FIG. 2. *Logarithmically grown cells in high light. Phase micrograph as in Fig. 1.*

FIG. 3. *Logarithmically grown cell in high light. Thin section through glutaraldehyde-osmium fixed Aphanocapsa 6308. Bar represents 1 μm.*

FIG. 4. *Light-limited stationary-phase cell. Thin section as in Fig. 3.*

cally grown cells. Unstained granulated cells in light-limited late stationary phase are shown in Fig. 2. Structured granules are the most prominent inclusions in the light-limited cells and are present throughout the cell; distortion of coccoid shape occurs in these cells.

Fine structural analysis shows that cells grown logarithmically in medium no. 11 under optimal conditions (3) contained no or one small granule (Fig. 3). Light-limited cells (either cells in stationary phase due to light limitation or those grown at very low light intensity) formed prominent granules (Fig. 4) even, in the latter case, during slow logarithmic growth. Best preservation of the structured nature of granules was obtained after glutaraldehyde-osmium fixation; this was also shown by Lang et al. (5) and Lang and Fisher (4) for *Anabaena* sp. The structure appeared in most cases to radiate out from one or more centers within the granule, and the granules appeared individually or joined together; in some low-light cells, the bulk of cell mass was composed of granular material.

Freeze-fracturing of purified granules revealed defined areas of small particles (Fig. 5) which also were visible in freeze-fractured early stationary-phase cells (Fig. 6). The irregularity in shape and heterogeneity in size of granules is similar to what is observed in thin section. The freeze-fractured granule substructure is punctate in appearance and is interpreted as cross sections through fibers. Such a fracture suggests a possible grouping of the less polar backbone of the CGP with the more polar arginine residues radiating outwards. No membrane is visible in either thin sections or freeze-fractured material, and an irregular granule perimeter is observed in many thin sections (Fig. 4). Figure 6 shows a cross-fractured cell and therefore is comparable to the image seen in fixed thin sections.

Paper chromatographic analysis of acid hydrolysates of purified granules shows the presence of only the amino acids arginine and aspartic acid, in approximately equal molar quantities. The results of microanalysis of purified granules and their correspondence to what is expected for multi-L-arginyl poly-(L-aspartic acid) (10) were as follows (values represent observed and expected percentages, respectively): carbon, 38.89 and 39.2; hydrogen, 6.80 and 6.5; nitrogen, 21.91 and 22.9; oxygen, 31.26 and 31.3; phosphorus, 0 (or trace) and 0. When granules were isolated from cells grown in medium no. 11 with [^{14}C]arginine or [^{14}C]aspartate and then subjected to the fractionation procedure of Roberts et al. (7), 94.8% of the label appeared as ether-insoluble, alcohol-soluble protein; this corresponds to the solubility patterns described by Simon (8). The limited solubility of CGP allows both the purification procedure and the possibility of studying labeling patterns. Table 1 shows the distribution of label in acid-hydro-

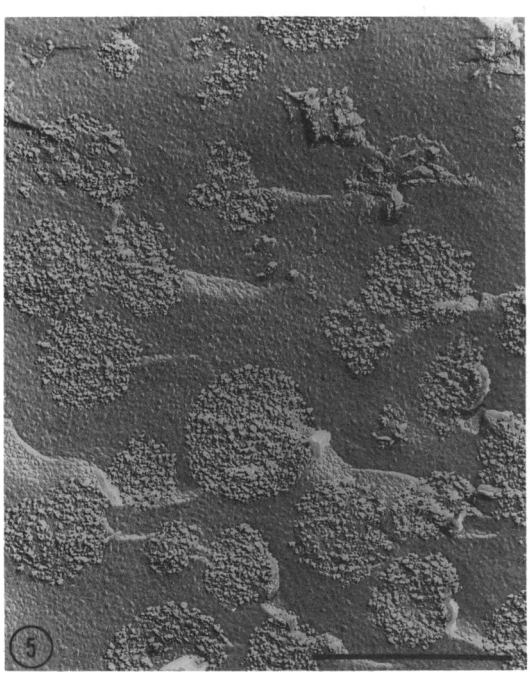

FIG. 5. *Freeze-fractured preparation of purified granules. Bar represents 1 μm.*
FIG. 6. *Freeze-fractured early stationary-phase Aphanocapsa 6308. Bar represents 1 μm.*

TABLE 1. *Distribution of label in acid-hydrolyzed isolated granules*[a]

Granules isolated from cells grown in:	% Label	
	Arginine moiety	Aspartate moiety
[^{14}C]arginine	100	0
[^{14}C]aspartate	60.4	39.6
[^{14}C]aspartate + [^{12}C]arginine	66.0	34.0
[^{14}C]arginine + [^{12}C]aspartate	94.4	5.6

[a] Cells were grown in radioactive medium 14 days before granule isolation.

lyzed granules from cells grown in various isotopically labeled media. When acid-hydrolyzed granules from cells grown in [^{14}C]arginine medium were chromatographically separated into their arginine and aspartate moieties, all the label was found in the arginine moiety. Granules isolated from [^{14}C]arginine + [^{12}C]aspartate-grown cells had 94% of the label in the arginine moiety, whereas 60 to 66% of the label was found in the arginine moiety from granules isolated from either [^{14}C]aspartate or [^{14}C]aspartate + [^{12}C]arginine-grown cells. The rate of incorporation of isotope was equal to the rate of increase in dry weight of cells grown under the conditions described; arginine contributed 3.1 mg (dry weight) of cells per 100 mg, and aspartate contributed 5.0 mg (dry weight) per 100 mg.

The limited incorporation of arginine and aspartate into logarithmically growing cells elucidates the photoautotrophic nature of *Aphanocapsa* 6308. When granules were isolated from cells grown in 5% CO_2 with [^{14}C]arginine, the majority of the ^{14}C from arginine was incorporated into the arginine moiety of CGP and not the aspartate moiety; this suggests little catabolism of arginine. During growth in 0.3% CO_2, arginine has been shown to be catabolized to form CO_2, which can then be photosynthesized (11). Aspartate, on the other hand, was used for arginine synthesis as shown by the ^{14}C aspartate data. Since approximately the same proportion of label appeared in the arginine moiety of CGP from cells grown in [^{14}C]aspartate alone as from cells grown in [^{14}C]aspartate + [^{12}C]arginine, it is suggestive that there is little control over the biosynthesis of arginine used in the formation of CGP.

Aphanocapsa 6308, growing under conditions described here, can form as much as 16% CGP on a dry-weight basis in late light-limited stationary phase, twofold higher than the amount found in *A. cylindrica* (9). The present work suggests a correspondence between cyanophycin granule fine structure and CGP chemical composition in this non-nitrogen-fixing cyanobacterium similar to that in the nitrogen fixer (5). The function of this polymer is being studied under nitrogen-limiting conditions.

This work was supported by National Science Foundation grant DEB 75-17633 and by a Research Corporation Cottrell College Science grant.

Freeze-fracturing was carried out by S. Schaeffer, and we also thank her for critical evaluation and discussion of electron micrographs. The technical assistance of N. Pearson, F. Hutchison, and J. Cameron is gratefully acknowledged. We are indebted to G. F. Gauthier and H. A. Padykula for the use of the facilities of the Laboratory of Electron Microscopy.

LITERATURE CITED

1. **Allen, M. M.** 1968. Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. **4**:1-4.
2. **Allen, M. M.** 1968. Photosynthetic membrane system in *Anacystis nidulans*. J. Bacteriol. **96**:836-841.
3. **Allen, M. M.** 1968. Ultrastructure of the cell wall and cell division of unicellular blue-green algae. J. Bacteriol. **96**: 842-852.
4. **Lang, N. J., and K. A. Fisher.** 1969. Variation in the fixation image of "structured granules" in *Anabaena*. Arch. Mikrobiol. **67**:173-181.
5. **Lang, N. J., R. D. Simon, and C. P. Wolk.** 1972. Correspondence of cyanophycin granules with structured granules in *Anabaena cylindrica*. Arch. Mikrobiol. **83**:313-320.
6. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
7. **Roberts, R. B., P. H. Abelson, D. B. Cowie, E. J. Bolton, and R. J. Britten.** 1963. Studies of biosynthesis in *Escherichia coli*. Carnegie Institution of Washington Publications, Washington, D.C.
8. **Simon, R. D.** 1971. Cyanophycin granules from the blue-green algae *Anabaena cylindrica*: a reserve material consisting of copolymers of aspartic acid and arginine. Proc. Natl. Acad. Sci. U.S.A. **68**:265-267.
9. **Simon, R. D.** 1973. Measurement of the cyanophycin granule polypeptide contained in the blue-green alga *Anabaena cylindrica*. J. Bacteriol. **114**:1213-1216.
10. **Simon, R. D., and P. Weathers.** 1976. Determination of the structure of the novel polypeptide containing aspartic acid and arginine which is found in cyanobacteria. Biochim. Biophys. Acta **420**:165-176.
11. **Weathers, P. J., H. L. Chee, and M. M. Allen.** 1978. Arginine catabolism in *Aphanocapsa* 6308. Arch. Microbiol. **118**:1-6.