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Protein Degradation and Synthesis of Cyanophycin Granule Polypeptide in *Aphanocapsa* sp.

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Cyanophycin granule polypeptide content increased by 2- to 3-fold, soluble protein content decreased by 1.5-fold, and carbohydrate content increased by 2-fold within 6 h of chloramphenicol addition to exponentially growing cells of *Aphanocapsa* sp. strain 6308. Analysis of ^{14}C- and ^{3}H-labeled cells transferred to unlabeled medium and analysis of pulse-labeled cells both suggested cyanophycin granule synthesis from preformed protein breakdown.

Cyanophycin granule polypeptide (CGP), or multi-L-arginyl-poly(L-aspartic acid), is a unique cellular nitrogen reserve of cyanobacteria (2, 8). That synthesis of CGP is not dependent on new ribosomal protein synthesis was first shown by Simon in nitrogen-fixing *Anabaena cylindrica* (9) and later by Allen et al. in non-nitrogen-fixing *Aphanocapsa* sp. strain 6308 (3). Simon and Weathers (10) reported the isolation of an enzyme that elongates the polypeptide by incorporation of arginine and aspartate, and Simon also showed (8) that a decrease in soluble protein during the stationary phase correlates with the production of CGP. He suggested that soluble protein serves as a source of amino acids to build up the nitrogen reserve during the stationary phase. Allen et al. (3) showed that 48 h of chloramphenicol (CAP) treatment also causes a decrease in soluble protein concentration which correlates with an increase in CGP concentration, but no information was available on changes taking place during the early hours of CAP treatment when its effect might be observed by changes in cellular pools. The present study was therefore designed to determine whether degradation of protein was the source of amino acids for CGP synthesis in the unicellular cyanobacterium *Aphanocapsa* sp. strain 6308 (ATCC 27150). In one set of experiments, cells were isotopically labeled before they were transferred to unlabeled medium with CAP for 6 h. In another set of experiments, exponentially growing cells were pulse-labeled for 1 h with [^{14}C]arginine at various times within 6 h of CAP treatment. Amounts of macromolecules and labeling patterns gave support to the hypothesis that CGP is formed from products of protein degradation.

Cells were grown as described previously (3), and exponentially growing cells were axenically harvested by centrifugation at 27,000 × *g* for 10 min before suspension to an absorbance of 0.200 at 750 nm in fresh culture medium before each experiment. Radioisotopes were added immediately after inoculation in experiments to label cells which were allowed to grow for 45 h (experiments 1 and 2) or 69 h (experiments 3 and 4). Cells were then harvested and washed to remove any unincorporated label. Half of the culture was resuspended in a bubbler flask containing 5 μg of CAP per ml and allowed to grow for 6 h before the cells were harvested and analyzed; these cells were considered to be 6-h cells. The other half of the culture was analyzed and considered to be 0-h cells. In pulse experiments, cells were grown for 45 h, at which time samples of cells were transferred to small bubbler flasks to be used as control cells. The remainder of the culture was treated with 5 μg of CAP per ml, and then samples were transferred to small bubbler flasks. All samples were treated for 1-h periods with radioisotope before analysis. At least two experiments of each type were carried out.

Growth was routinely determined through measurement of cell densities by absorbance at 750 nm with a Gilford 240 spectrophotometer. Radioisotopes used were [^{14}C]aspartic acid (specific activity, 233 mCi/mmol) and [^{3}H]arginine (specific activity, 38 Ci/mmol) in two experiments (experiments 1 and 2) and [^{14}C]arginine (specific activity, 300 mCi/mmol) in five experiments (experiments 3 and 4 as well as three pulse experiments); unlabeled amino acids to a final concentration of 2 mM were added in experiments 1 through 4.

Dry weight determinations and cell breakage were carried out as described previously (3). Soluble protein was measured by the Coomassie blue method as refined by Spector (11) on the 27,000 × *g* supernatant. CGP was isolated and analyzed by the method of Simon (8). Extraction

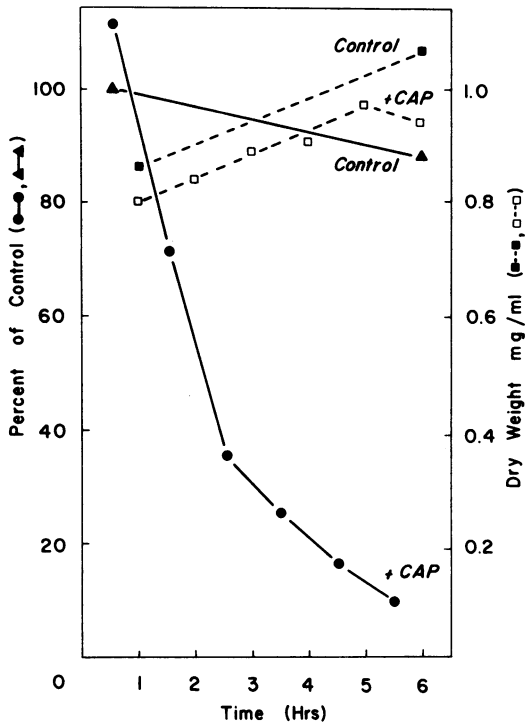

FIG. 1. Comparison of dry weight changes (---) with a decrease in protein synthesis (——) in exponentially growing cells with (●, □) and without (▲, ■) CAP treatment which was measured by [^{14}C]leucine incorporation into cold TCA-precipitable material. The 100% control was 17.8 kdpm/mg of cells (dry weight) per h.

by the method of Roberts et al. (7) was carried out on samples of 0- and 6-h cells in experiments 1 and 2. Triplicate samples were taken from all fractions from which it was desired to determine the amount of radioactivity present, dissolved in Aquasol II, and measured using a Packard Tri-Carb 560C automatic liquid scintillation counter. Total incorporation and cold trichloracetic acid (TCA)-precipitable incorporation were measured by liquid scintillation after filtering samples on Whatman QFA filters, washing, drying, and dissolving in Aquasol II. Glucose was measured by the Glucostat reagent from Worthington Diagnostics.

The decrease in protein synthesis after CAP treatment was measured by [^{14}C]leucine (specific activity, 353 mCi/mmol) incorporation into cold TCA-precipitable protein in logarithmically growing cells. Control cells and six samples of cells treated with CAP at zero time were labeled with leucine for 1-h periods during 6-h experiments.

The decrease in protein synthesis during 6 h of CAP treatment of exponentially growing *Aphanocapsa* sp. strain 6308 is shown in Fig. 1. Protein synthesis increased 111% over the non-CAP-treated control in h 1 of CAP treatment, but decreased to 9.7% of the control by 6 h. This contrasts with *A. cylindrica*, in which leucine incorporation into protein was totally inhibited by CAP within 1 h (9). Cellular dry weight continued to increase for most of this time period (Fig. 1). During this time the glucose concentration increased by an average of 209% of the dry weight in two separate experiments, and the percent dry weight which was soluble protein decreased by an average of 147% in four separate experiments.

In experiments in which cellular components were labeled for 45 or 69 h before the 6-h CAP treatment in unlabeled medium, CGP formed

TABLE 1. Characteristics of CGP isolated before and after CAP treatment

Expt	Sample (h)	CGP (mg)	Cellular dry wt (mg)	% CGP (of dry wt)	kdpm			
					^{14}C in isolated CGP	^{14}C per mg of CGP	^{3}H in isolated CGP	^{3}H per mg of CGP
1[a]	0	1.22	265	0.5	19	15	12	10
	6	6.93	428	1.6	84	12	40	6
2[a]	0	4.88	217	2.2	88	18	18	26
	6	10.3	257	4.0	270	37	25	24
3[b]	0	7.65	326	2.3	24	9.5		
	6	8.43	333	2.5	26	9.1		
4[b]	0	7.03	293	2.4	17	6.2		
	6	11.05	290	3.8	24	5.5		

[a] Cells grown exponentially for 45 h with [^{14}C]aspartic acid and [^{3}H]arginine (0-h sample) before washing and suspension in unlabeled medium with 5 μg of CAP per ml for 6 h (6-h sample).

[b] Cells grown exponentially for 69 h with [^{14}C]arginine (0-h sample) before washing and suspension in unlabeled medium with 5 μg of CAP per ml for 6 h (6-h sample).

TABLE 2. Percent radioactivity recovered in macromolecular fractions[a]

Isotope	Expt	Sample (h)	% Radioactivity recovered by:						
			Roberts extraction					Sonication and differential centrifugation	
			Cold TCA soluble	Alcohol soluble-ether soluble	Alcohol soluble-ether insoluble	Hot TCA soluble	Hot TCA insoluble	Soluble protein	CGP
^{14}C	1	0	11.5	12.1	5.8	22.2	41.3	96.7	3.3
		6	9.5	2.3	12.9	36.2	39.7	84.3	15.7
	2	0	13.3	11.3	5.9	37.0	32.7	91.3	8.8
		6	5.9	7.1	14.3	35.1	37.6	78.3	21.7
$^{3}H^{b}$	2	0	18.6	7.6	7.9	7.4	58.5	78.8	21.2
		6	13.2	3.3	14.8	9.0	59.5	73.0	27.0

[a] Cells grown exponentially for 45 h with [^{14}C]aspartic acid and [^{3}H]arginine (0-h sample) before washing and suspension in unlabeled medium with 5 μg/ml of CAP per ml for 6 h (6-h sample).
[b] Counts too low in experiment 1 to allow differentiation from ^{14}C.

only from the products of protein breakdown or from labeled cell pools of arginine and aspartate was expected to have the same specific activity at 0 and 6 h. Since protein synthesis was inhibited by CAP (Fig. 1) and CGP synthesis is independent of CAP (9), few unlabeled amino acids should have been incorporated into protein during the 6 h. Table 1 gives the characteristics of CGP isolated from 0- and 6-h cells. CGP increased in amount, in percent dry weight of cells, and in radioactivity. A decrease in specific activity (disintegrations per minute per milligram of CGP) was seen in three of four cases in experiments 1 and 2 in which cells were labeled with [^{14}C]aspartic acid and [^{3}H]arginine; this decrease in specific activity could indicate some synthesis of CAP from unlabeled photosynthetic products formed after CAP was added or some interconversion between aspartate and arginine in the 6-h CAP treatment or both. Short-term $^{14}CO_2$ fixation experiments (12), using light-limited stationary-phase cells, suggested that the label is incorporated into cells but that very little is incorporated into CGP, even though the polypeptide is rapidly synthesized during this time. Therefore, newly synthesized photosynthetic products did not appear to be used in CGP synthesis. Hoare and Hoare (5) showed that 2 μM arginine exhibits allosteric control over its own synthesis from glutamate in three of four cyanobacteria tested. Although Hood and Carr (6) found no repression of enzymes involved in arginine biosynthesis, the 2 mM arginine concentration used in these experiments should have prevented any new synthesis of arginine, at least in the 0-h sample. No arginine was present in the culture medium from 0 to 6 h, so that feedback inhibition would cease. Therefore, despite CAP inhibition of protein synthesis, unlabeled arginine and aspartate might be synthesized by enzymes already present in the cells, causing a dilution of the labeled amino acids already present in cellular pools and protein. A decrease in specific activities would result. Since some protein synthesis occurred in the first 6 h of CAP treatment (Fig. 1), some unlabeled amino acids may have been incorporated into protein and then degraded, also explaining a

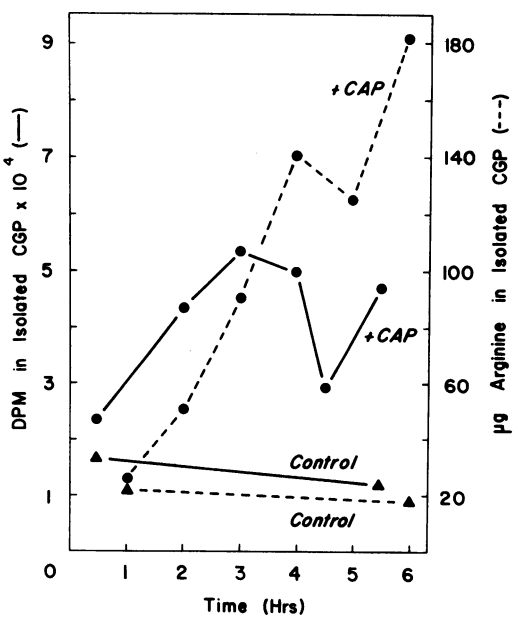

FIG. 2. Comparison of changes in the amount of label (——) and in the arginine concentration (---) of isolated CGP. Each sample of cells treated with (●) and without (▲) CAP was labeled with [^{14}C]arginine for a pulse of 1 h during the 6-h period.

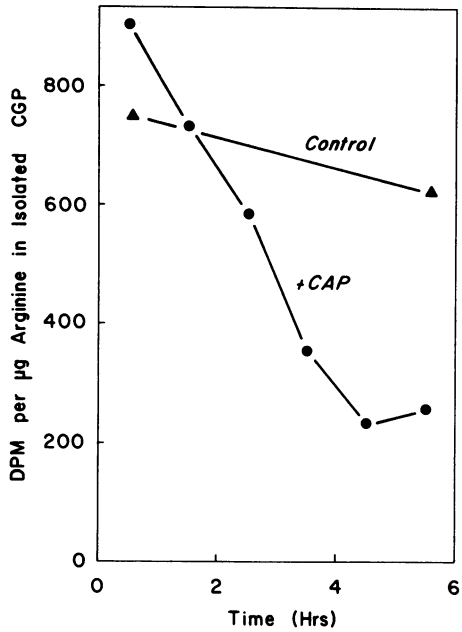

FIG. 3. Changes in the specific activity of CGP isolated from control cells (▲) and from six samples of cells treated with CAP at zero time (●). Each sample was treated with [^{14}C]arginine for a pulse of 1 h.

lower specific activity in CGP. It is more likely, however, that during the 6-h period any protein synthesis took place by using labeled amino acids in cellular pools. The percentage of label in each macromolecular fraction of these cells (Table 2) showed that the percentage of label in the transient intermediates (cold TCA soluble), such as free amino acids and small peptides, and in soluble protein decreased over the 6-h period during the same time that there was an increase in isolated CGP and in the alcohol-soluble–ether-insoluble fraction into which the majority of CGP is extracted (4).

Experiments 3 and 4 (Table 1) were carried out by using [^{14}C]arginine for 69 h to label exponentially growing cells. The specific activity of isolated CGP was nearly similar, suggesting that the majority of CGP synthesis occurred from the breakdown of labeled protein.

Data from 1-h [^{14}C]arginine pulse experiments also suggested the synthesis of CGP from products of protein degradation. In these experiments, cellular protein and pools were unlabeled at the time of the pulse. Figure 2 compares the amount of label and the arginine concentration in isolated CGP from cells treated with and without CAP at zero time and with [^{14}C]arginine for each 1-h period thereafter. The amount of arginine in isolated CGP increased since the CGP concentration increased, and the amount of label in the isolated CGP increased for 3 h. The specific activity of the CGP, however, decreased throughout this time period (Fig. 3) when changes in the specific activity of CGP from CAP-treated cells were compared with changes in the specific activity of CGP isolated from non-CAP-treated control cells. This decrease indicates that the synthesis of CGP occurred from the breakdown of unlabeled protein and not from labeled arginine entering the cells from the medium. The small amount of CGP in control cells was more highly labeled per microgram of arginine than in CAP-treated cells which were producing a large quantity of CGP (Fig. 2). This specific activity decreased with time after h 1 of CAP treatment. Protein synthesis in which labeled arginine was utilized and degradation of that protein were probable in the non-CAP-treated controls, leading to a higher average specific activity in CGP isolated from these cells which were labeled with [^{14}C]arginine for the entire 6-h period. CGP purified from CAP-treated cells labeled with [^{14}C]arginine for the entire 6-h period in this experiment had an average specific activity of 254 dpm/µg of arginine, whereas CGP from non-CAP-treated cells labeled for the 6-h period had a specific activity of 750 dpm/µg of arginine. Arginine was incorporated into the cells at the same rate (average for control cells, 1.87 kdpm/mg [dry weight] per h; average for CAP-treated cells, 2.36 kdpm/mg [dry weight] per h) during the entire 6 h, as a comparison of the total incorporation of [^{14}C]arginine into cells each hour showed.

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