Protein Synthesis and Degradation in *Anabaena* During Nitrogen Starvation

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Nitrogen starvation and heterocyst development were induced in the cyanobacterium *Anabaena* 7120 by growth in nitrogen-free medium or by treatment with the amino acid analogue methionine sulphoximine. During the first 6 h of nitrogen deprivation, amino acid levels and rates of protein synthesis, as measured by the incorporation of [^3H]leucine, decreased to 50 to 70% of those in ammonia-grown organisms; after this time there was no difference between the rates of protein synthesis in ammonia-grown and nitrogen-starved cultures. The period 4 to 12 h after the onset of starvation was marked by the release of [^3H]leucine from previously labelled proteins at a rate 6 to 7.5 times that of ammonia-grown organisms. These results are consistent with the idea that nitrogen starvation in cyanobacteria causes a reduction in protein synthesis and leads to the rapid degradation of storage proteins. In rapidly growing *Anabaena* 7120, the doubling time for total cell protein was estimated to be 14.9 ± 1.0 h and the half-life was 139 ± 88 h.

INTRODUCTION

Heterocysts are the primary site of nitrogen fixation in *Anabaena* and many other filamentous blue-green algae (cyanobacteria) under aerobic conditions. These cell types are generally absent from cultures of cyanobacteria grown in a medium containing ammonia, but develop and become functional within 24 to 36 h after transfer to nitrogen-free medium (Talpasayi & Kale, 1967). Since lack of nitrogen in some way triggers heterocyst development, we have investigated the effects of nitrogen deprivation on protein metabolism to determine how protein synthesis and degradation are altered during the period coincident with heterocyst formation.

Protein turnover has been studied extensively in a variety of organisms (for reviews, see Huffaker & Peterson, 1974; Goldberg & St John, 1976). In bacteria, the degradation of total cell protein is very low in growing cells, $0.6\% \text{ h}^{-1}$ (Willetts, 1967), but may increase to 4 to $5\% \text{ h}^{-1}$ in nutrient-starved cells (Mandelstam, 1958) and to 8 to $10\% \text{ h}^{-1}$ in sporulating cells (Mandelstam & Waites, 1968). The enhanced rate of protein breakdown apparently results from the activity of proteolytic enzymes synthesized during starvation (Doi, 1972). A similar system seems to exist in cyanobacteria. Lau *et al.* (1977) have reported increased degradation of phycocyanin, which may function as a storage protein, in nitrate-starved *Anacystis nidulans*, while proteases which hydrolyse phycocyanin have recently been described in species of *Anabaena* (Wood & Haselkorn, 1976; Foulds & Carr, 1977).

We have induced nitrogen starvation and heterocyst formation in ammonia-grown *Anabaena* 7120 by transferring the organism to nitrogen-free medium and by treatment with the amino acid analogue L-methionine-DL-sulphoximine (MSO). MSO has previously been shown to initiate heterocyst development in several species of *Anabaena* (Stewart &

Rowell, 1975; Ownby, 1977). It apparently acts by irreversible inactivation of glutamine synthetase, the first enzyme in the pathway of assimilation of ammonia in cyanobacteria (Thomas *et al.*, 1975). Since the use of MSO permits rapid induction of functional heterocysts without physical manipulation (e.g., centrifugation or filtration), it may be useful in fermenter or chemostat studies of heterocyst development.

METHODS

Growth conditions. The origin and growth of *Anabaena* 7120 have been described previously (Ownby, 1977). Briefly, cultures essentially free of heterocysts were obtained by growing *Anabaena* 7120 on a reciprocating shaker without aeration in the medium of Allen & Arnon (1955) supplemented with 2 mM-NH₄Cl and 1 mM-NaNO₃. Nitrogen starvation and heterocyst development were initiated by adding MSO to a final concentration of 1 μ M or by transferring the cells to Allen & Arnon's nitrogen-free medium after centrifuging at 480 g and rinsing once in nitrogen-free medium.

Measurement of protein synthesis. Samples (18 ml) of *Anabaena* 7120 grown as above were transferred to 50 ml flasks and allowed to grow for 24 h. Half the flasks were then treated with 1 μ M-MSO. After 0, 2, 5, 11 or 23 h of treatment, selected flasks were pulse-labelled for 1 h with [4,5-³H]leucine (New England Nuclear, 40 to 60 Ci mmol⁻¹) at 0.027 or 0.135 μ Ci ml⁻¹ in 0.1 mM carrier leucine. At the end of the labelling period, the filaments in each flask were collected by centrifugation, resuspended in cold 6% (w/v) trichloroacetic acid (TCA), sonicated for 20 s (Branson sonifier model 185; Branson Sonic Power Co., Danbury, Conn., U.S.A.) and chilled for 30 min. The TCA-insoluble fraction was collected by centrifuging, rinsed in cold TCA and dissolved in warm 0.5 M-NaOH. The radioactivity in the medium, TCA-soluble fraction and TCA-insoluble, alkali-soluble fraction was measured by liquid scintillation spectrometry by placing 0.2 ml of each fraction directly into a dioxan-based counting medium. The ³H counting efficiency was approximately 40% in 6% TCA and 43% in 0.5 M-NaOH. In this and all subsequent experiments, measurements of total protein in the TCA-insoluble, alkali-soluble fraction were made using the method of Lowry *et al.* (1951).

Measurement of the release of ³H from previously labelled protein. *Anabaena* 7120 was grown in the presence of fixed nitrogen in 1 l flasks for 3 to 4 d, then labelled for 24 h with 60 μ Ci of [³H]leucine in 0.1 mM carrier leucine. The filaments were collected by centrifuging and resuspended in unlabelled medium for a 14 to 15 h chase period, during which time much of the remaining soluble [³H]leucine was incorporated into protein (Halvorson, 1958). The cells were again collected by centrifuging, rinsed in the appropriate medium and resuspended in medium containing NH₃, medium lacking nitrogen or medium containing MSO. All media contained 0.1 mM carrier leucine during the experimental period. Samples (4 ml) were taken 0, 2, 4, 8, 12 and 24 h after the initiation of nitrogen starvation, and ³H in the medium, TCA-soluble and TCA-insoluble, alkali-soluble fractions was determined as described above.

Chromatography of ³H-labelled compounds. Samples (0.2 ml) from the TCA-soluble fractions collected after 12 h of MSO-induced nitrogen starvation were spotted on MN300 cellulose plates and chromatographed in butan-1-ol/acetic acid/water (90:10:29, by vol.) for 4 h. Sections of the chromatogram were analysed for ³H by placing them directly into 10 ml scintillation fluid containing 4 g 2,5-diphenyloxazole l⁻¹ and 50 mg 1,4-di-2-(5-phenyloxazolyl)benzene l⁻¹. The ³H counting efficiency was 18 to 22% under these conditions.

Amino acid analysis. *Anabaena* 7120 was grown to exponential phase in 1 l flasks and treated with MSO at 1 μ M. After 0, 2, 4, 6, 8 and 12 h, 25 ml samples were taken and the filaments were collected by centrifugation. Free amino acids were released from the cyanobacteria by placing the cells in boiling water for 5 min. After removal of insoluble debris by centrifugation, the hot water-extractable fraction was filtered and lyophilized. Amino acids were redissolved in 0.3 ml of pH 2.2 diluting buffer (Moore & Stein, 1954) and assayed using an amino acid analyser modified for narrow bore columns (Liao *et al.*, 1973).

RESULTS

All experiments were conducted with axenic cultures in the middle to late phase of exponential growth, during which the doubling time for total cell protein varied from 15 to 21 h. Cyanobacteria treated with MSO or transferred to nitrogen-free medium ceased to accumulate protein after approximately 2 to 4 h (Fig. 1). After a lag period of 16 to 20 h, cells in nitrogen-free medium once more began to accumulate protein coincident with the reported onset of nitrogen-fixing activity in *Anabaena cylindrica* whose doubling time is

Fig. 1. Effect of nitrogen deprivation on accumulation of cell protein in *Anabaena* 7120. At zero time, filaments were transferred to medium containing 1 μ M-MSO (■), medium lacking nitrogen (●) or medium containing 2 mM- NH_4Cl and 1 mM- NaNO_3 (○). Each point is the average of at least three experiments.

Fig. 2. Incorporation of $[^3\text{H}]$ leucine into protein in control (ammonia-grown) cultures (○) and MSO-treated cultures (■) of *Anabaena* 7120. Cultures were labelled with $[^3\text{H}]$ leucine at 0.027 $\mu\text{Ci ml}^{-1}$ (a) or 0.135 $\mu\text{Ci ml}^{-1}$ (b) for 1 h at various times after treatment with MSO (zero time).

similar to that of *Anabaena* 7120 used in these experiments (Bradley & Carr, 1976). Because of their apparent inability to assimilate NH_3 , MSO-treated cyanobacteria showed no accumulation of protein even after heterocysts had developed (see also Stewart & Rowell, 1975).

The slow rate of uptake of radioactive precursors hinders metabolic studies in cyanobacteria. In the presence of carrier leucine at 0.1 mM which apparently facilitates uptake by active transport systems (R. Haselkorn, personal communication), the initial uptake of $[^3\text{H}]$ leucine by *Anabaena* 7120 was 8 to 12 % h^{-1} . We pulse-labelled cells for 1 h at intervals during the first 24 h of MSO-induced nitrogen starvation and expressed protein synthesis as a percentage of the total ^3H incorporated into protein (Fig. 2). To minimize the possibility that uptake rather than synthesis might limit the appearance of $[^3\text{H}]$ leucine in protein, we labelled cultures at 0.027 $\mu\text{Ci ml}^{-1}$ and 0.135 $\mu\text{Ci ml}^{-1}$ and expressed the results separately (Fig. 2a, b). Although MSO had no effect on $[^3\text{H}]$ leucine uptake, the incorporation of ^3H

Fig. 3. Effect of MSO-induced nitrogen starvation on (a) total hot water-extractable amino acids and (b) relative abundance of major amino acids in the hot water-extractable pool of nitrogen-starved cells. In control (ammonia-grown) cultures, the level of free amino acids was 393 nmol (mg protein)⁻¹ initially, and 288 nmol (mg protein)⁻¹ after 12 h.

Fig. 4. Release of ³H from protein in cultures of *Anabaena* 7120 previously labelled with [³H]leucine for 24 h and then transferred to medium containing 1 µM-MSO (■), nitrogen-free medium (●) or medium containing 2 mM-NH₄Cl and 1 mM-NaNO₃ (○). Since much of the ³H was released into the culture medium, all values have been expressed as c.p.m. (ml culture)⁻¹. At zero time, the ³H in the TCA-insoluble fraction was 10364 c.p.m. (ml culture)⁻¹.

into TCA-insoluble material was only 50 to 70 % of that in control cultures during the first 6 h, but subsequently the rate returned to that of ammonia-grown cultures after 12 and 24 h.

The initial decline in protein synthesis in MSO-treated cultures was in general matched by a decrease in the levels of hot water-extractable amino acids during the first 6 h of treatment (Fig. 3a). MSO-treated cultures also showed changes in amino acid composition, most notably a decline in aspartic acid matched by an increase in alanine (Fig. 3b). Threonine and tyrosine, which were not detectable at zero time, increased to 2.3 % and 1.4 %, respectively, of the total amino acid pool after 12 h.

We measured protein degradation by the release of ³H from proteins previously labelled with [³H]leucine, since Halvorson (1958) has shown that this approach provides a clearer index of protein degradation than when the decline of ³H in protein is measured directly.

Fig. 5. (a) Change in specific activity of ^3H -labelled protein from ammonia-grown *Anabaena* 7120 after transfer to unlabelled medium. The cells were labelled for 24 h with [^3H]leucine [$60 \mu\text{Ci}$ ($200 \text{ ml medium}^{-1}$)], and sampled after a subsequent 16 h chase period. ●, Culture flasks contained 0.1 mM unlabelled leucine during the experimental period. ○, No unlabelled leucine was added to the culture flasks for the experimental period. (b) Accumulation of total cell protein during the experimental period.

Table 1. Rate constants for synthesis and degradation of cyanobacterial protein during rapid growth

Rate constants (d^{-1}) were obtained by multiplying the slopes obtained as in Fig. 5 by $\log_e 10 \times 24$. Doubling time and half-life were determined by dividing $\log_e 2$ by the rate constants for synthesis and degradation, respectively.

Rate constant for synthesis $\pm$ s.d.	$1.12 \pm 0.07 \text{ d}^{-1}$
Rate constant for accumulation $\pm$ s.d.	$1.00 \pm 0.09 \text{ d}^{-1}$
Rate constant for degradation $\pm$ s.d.	$0.12 \pm 0.08 \text{ d}^{-1}$
Doubling time	$14.9 \pm 1.0 \text{ h}$
Half-life	$139 \pm 88 \text{ h}$

During the initial 24 h labelling period, the specific activity of cell protein reached $384 \pm 19 \text{ c.p.m. } (\mu\text{g protein}^{-1})$. In the subsequent 16 h chase period, the percentage of ^3H in the TCA-insoluble fraction increased from 64 to 88% of the total cellular [^3H]leucine. At the end of the chase period, the specific activity of total cell protein was $348 \pm 40 \text{ c.p.m. } (\mu\text{g protein}^{-1})$. The release of ^3H into the medium and TCA-soluble fraction over the following 12 h was measured in cultures transferred to medium containing ammonia, medium lacking nitrogen or medium containing MSO (Fig. 4). Although the TCA-soluble fraction (including free amino acids) underwent a transient increase 4 to 8 h after treatment, the major effect of nitrogen starvation was to stimulate release of ^3H into the medium, particularly in the period 4 to 12 h after treatment began. During this time, the rate of protein breakdown, as measured by release of ^3H , was 6 to 7.5 times greater in nitrogen-starved than in control (ammonia-grown) cultures.

To establish whether the ^3H released from labelled proteins was still in the form of leucine, we chromatographed $200 \mu\text{l}$ samples from the medium and the TCA-soluble fraction as described in Methods. With cyanobacteria grown in fixed-nitrogen medium, 67% of the TCA-soluble ^3H and 57% of the ^3H released to the medium after 12 h co-chromatographed with authentic leucine. By contrast, only 27% of the TCA-soluble ^3H and 9% of the ^3H released into the medium by nitrogen-starved cyanobacteria appeared as leucine.

Since synthesis and degradation of protein in exponentially growing cultures should be linear with time, we attempted to determine rate constants for these processes under conditions of rapid growth. The general approach used, and the assumptions on which it is based, have been described by Trewavas (1972). The method involved obtaining rate constants for protein accumulation from the slope of a graph of $\log_{10}$ total protein plotted against time and the rate constants for protein synthesis from the slope of a graph of $\log_{10}$ specific activity of protein plotted against time, using cultures in which the proteins had been previously labelled with [^3H]leucine. The rate constants for degradation were calculated as

the difference between rate constants for synthesis and rate constants for accumulation. The accumulation of protein and decline in specific activity of proteins previously labelled with [^3H]leucine are shown in Fig. 5. Growth constants calculated from these results are shown in Table 1. Although Scherer & Boylen (1977) showed that 0.1 mM excess unlabelled leucine was effective in preventing the recycling of [^3H]leucine into new protein in *Arthrobacter*, we observed no difference in the decline in specific activity when 0.1 mM-leucine was added to cultures during the experimental period. The growth constants shown in Table 1 have thus been calculated using both cultures without carrier leucine and those to which 0.1 mM unlabelled leucine had been added.

DISCUSSION

Two factors which may contribute to erroneous estimates of protein turnover rates are release of proteins through cell death and recycling of amino acids from hydrolysed proteins into new proteins. The contribution of cell death to protein turnover has not been determined, although microscopic examination of rapidly growing cultures showed very few dead cells or cell fragments. Since the addition of excess unlabelled leucine had no detectable effect on the decline in specific activity of previously labelled proteins, the rate of recycling of amino acids into new proteins also appears to be small. In the absence of data on the rate of re-utilization of [^3H]leucine, however, our estimates are best taken as the *minimum* rates of synthesis and degradation. Another possibility which might explain the relatively rapid rate of protein catabolism in *Anabaena* 7120 compared with eubacteria is that *Anabaena* 7120 secretes proteins into the medium, which would result in an over-estimated rate of degradation. We did observe, however, that over half (57%) of the ^3H in the medium chromatographed as the free amino acid leucine.

The data on amino acid levels and incorporation of [^3H]leucine into protein indicate that the first 6 h of nitrogen starvation are marked by a reduced capacity for protein synthesis. It should be noted, however, that at no time did protein synthesis fall below 50 to 70% of that in control (ammonia-grown) cultures. This suggests that neither depletion of amino acid pools nor total cessation of protein synthesis are characteristic of the period when heterocysts probably start to develop. Fleming & Haselkorn (1976), also working with *Anabaena* 7120, reported that the incorporation of ^{35}S into protein decreased continuously to 10% of the initial rate during the first 27 to 36 h of heterocyst development.

The data showing active release of ^3H from previously labelled proteins and increased incorporation of pulse-labelled [^3H]leucine into new proteins suggest that cyanobacteria rapidly degrade proteins and utilize the amino acids in the synthesis of new proteins 6 to 24 h after the onset of nitrogen starvation. The major form of protein hydrolysed is probably phycocyanin. Bradley & Carr (1976) have shown that phycocyanin declines to 10% of its initial level in *Anabaena cylindrica* during the first 18 h of nitrogen starvation, and cell-free extracts from *Anabaena cylindrica* contain an active, specific phycocyaninase (Foulds & Carr, 1977).

Very little of the ^3H released from protein in nitrogen-starved cultures remains in leucine. It appears that amino acids thus generated are extensively metabolized, including perhaps deamination and release of carbon skeletons into the medium. Newman *et al.* (1976) observed that *Escherichia coli* grown on leucine as the sole nitrogen source caused the release of nitrogen from leucine and excretion of α -ketoisocaproic acid into the medium.

Do the changes in protein turnover reported here bear any direct relation to heterocyst development? Much of the protein degraded and later resynthesized is probably confined to vegetative cells and plays no role in the formation and regulation of heterocysts [i.e. proteins in classes g, h, and i in the model of Fleming & Haselkorn (1974)]. A means by which protein catabolism could regulate heterocyst development, however, would be one in which proteinaceous inhibitors that repress heterocyst development are degraded and not resynthesized during the first 6 h, permitting the expression of genes involved in hetero-

cyst formation. Later resynthesis of the inhibitors, using amino acids from degraded storage proteins, would prevent further commitment of cells to heterocysts. Mandelstam & Waites (1968) proposed a role for proteinaceous inhibitors in *Bacillus subtilis* spore development. Another possibility is that the products of proteolysis inhibit the commitment of cells to heterocysts, as suggested by Fleming & Haselkorn (1974).

Our results show that, in addition to co-ordinated changes in morphological development, *Anabaena* 7120 undergoes marked changes in protein metabolism during nitrogen starvation. The possible interactions between these two processes need to be investigated further.

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