Differentiation in *Nostoc muscorum*: Nitrogenase Is Synthesized in Heterocysts

(nitrogenase induction/cellular differentiation/polyacrylamide-gel electrophoresis)

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**ABSTRACT** Cellular differentiation can be observed in certain filamentous blue-green algae after transfer of the cells from medium containing NH₄⁺ or NO₃⁻ to nitrogen-free medium. The appearance of differentiated cells (heterocysts) is accompanied by an increase in the activity of nitrogenase, an enzyme complex that reduces N₂ to NH₃. We have separated vegetative cells from heterocysts in differentiated filaments of *Nostoc muscorum*, and analyzed their proteins by polyacrylamide-gel electrophoresis. The pattern of incorporation of ³²SO₄²⁻ into proteins of the two cell types during differentiation indicates that the increase in nitrogenase activity is due to the *de novo* synthesis of nitrogenase proteins predominantly, if not exclusively, in heterocysts.

Cellular differentiation, usually considered to be a property of eukaryotic organisms, does appear to occur in certain prokaryotic species such as the filamentous blue-green algae *Anabaena variabilis* and *Nostoc muscorum*. The differentiation of 5–10% of the cells is observed when the filaments are transferred from a medium containing NH₄Cl or Ca(NO₃)₂ to a nitrogen-free medium. The differentiated cells, called heterocysts, can be observed at regular intervals along the length of the filament 24 hr after the transfer.

Heterocysts are morphologically distinguishable from vegetative cells by an envelope external to the cell wall and specialized intracellular membranes (1). A pore connects the heterocyst to adjacent vegetative cells in the filament (2). Heterocysts contain much less phycocyanin than vegetative cells (3, 4), and their cell walls and lipid composition differ from those of vegetative cells (5, 6). The respiratory activity of heterocysts is apparently higher than that of the vegetative cells (7). Heterocysts also appear to have a reduced ability to fix CO₂ (8).

The appearance of heterocysts after transfer of *Nostoc* from nitrogen-containing to nitrogen-free medium is accompanied by induction of nitrogenase, an enzyme complex that reduces N₂ to NH₃. Most active interest in heterocyst function has centered on the suggestion (7, 9, 10) that differentiation of these cells is necessary for nitrogenase activity. While it has been shown that reducing activity is concentrated in the heterocysts (11) and nitrogenase activity has been measured in isolated heterocysts (7, 11, 12), it has not been shown that nitrogenase is exclusively found in heterocysts.

We have analyzed the proteins contained in heterocysts and vegetative cells of *Nostoc muscorum* by electrophoresis on polyacrylamide gels. It is possible to distinguish three new proteins in the differentiated filaments which can be tentatively identified as nitrogenase components and which are found only in the heterocysts. These proteins appear to be the major products of protein synthesis in differentiating cells; the synthesis of most other proteins, characteristic of vegetative cells, is shut off.

**MATERIALS AND METHODS**

*Algal Growth.* The culture of *Nostoc muscorum* used was originally obtained from the Botany Department of Iowa State University. Complete medium was Chu 10 (13) with 2.8 mM Ca(NO₃)₂ and 1 mM NH₄Cl and 1 mM Na₂CO₃. For differentiation-induction the cultures were transferred to Chu 10 from which combined nitrogen was omitted. The time required for one cell division in aerated nitrogen-free medium was about 30 hr at 22°C with 1610 lux of illumination from GE Mainlighter fluorescent lamps.

*Nitrogenase.* An assay by reduction of acetylene to ethylene (14, 15). The assay was done in stoppered 25-ml serum bottles containing 10 ml of culture, at a cell density of about 10⁶ cells per ml. To start the incubation, 1 ml of acetylene was injected into the air above the culture. The incubation period was generally 1 hr under about 2370 lux of illumination. The amount of acetylene converted to ethylene was measured by chromatography of the gases on a Varian Aerograph 1600 with a Porapak R column. A unit of nitrogenase is defined as that amount of enzyme that converts 1 μmol of acetylene to ethylene in 1 min.

*Preparation of Cell Extracts and Gel Electrophoresis.* *Nostoc* cultures (100 ml containing about 10⁹ cells) were labeled for 36 hr with ³²SO₄²⁻, 1.0 μCi/ml, with a specific activity of 100 Ci/mol. The labeled cells were harvested, washed twice with fresh medium, and suspended in a small volume of MET buffer [0.5 M mannitol–20 mM EDTA–1 mM Tris·HCl (pH 7.6)] (1). Lysozyme was added to a concentration of 1 mg/ml, and the cells were incubated for 2–3 hr at 37°C.

The lysozyme treatment does not appear to affect intracellular proteins, because samples withdrawn before and at half-hour intervals during the lysozyme incubation show identical stained protein patterns when examined by acrylamide-gel electrophoresis. The undifferentiated filaments are completely lysed by this treatment. Such lysates are centrifuged at 10,000 rpm for 10 min (12100 × g) to yield a soluble fraction (Vₗ⁺) and a pellet fraction containing membrane-associated proteins (Vₘ⁺). About 50% of the total protein in undifferentiated filaments is recovered in each fraction.

When differentiated filaments are treated with lysozyme in the same way, a sticky green pellet forms. It is dispersed by drawing through a Pasteur pipette several times, and the suspension is then centrifuged at 1000 rpm for 3–5 min to yield a pellet containing heterocysts and some membrane
fragments. This pellet is washed by centrifugation several times with buffer to remove membrane fragments. The supernatants from these spins are combined and centrifuged at 10,000 rpm for 10 min. This supernatant and pellet furnish the two protein fractions ($V_s^-$ and $V_m^-$) from vegetative cells of differentiated filaments. Each of these fractions contains 45% of the total protein in differentiated filaments. The washed heterocysts from the 1000 rpm pellet are disrupted by sonication and centrifuged at 10,000 rpm for 10 min to yield two comparable protein fractions ($H_1$ and $H_m$), containing 7.5 and 3.5% of the protein in differentiated filaments, respectively.

Proteins in the supernatant fractions were concentrated by precipitation with 10% trichloroacetic acid. These precipitates, as well as the pellet fractions, were washed twice with 80% ethanol and once with ether. After they were dried they were solubilized in a mixture of 2% sodium dodecyl sulfate, 0.25 M Tris·HCl (pH 7.0), 1% mercaptoethanol, and a trace of bromthymol blue by immersion in boiling water for 3 min. The samples were subjected to electrophoresis on 20% polyacrylamide slab gels (16, M. Anderson, personal communication), stained for 2 hr at 37° with 0.15% Coomassie brilliant blue, destained in 7.5% acetic acid–5% methanol, and/or dried onto filter paper and autoradiographed.

**RESULTS**

Heterocysts appear within 18–24 hr after transfer from nitrogen-containing medium to nitrogen-free medium. Differentiation of heterocysts coincides with induction of nitrogenase (Fig. 1). This event is the culmination of a sequence of changes that are first apparent 4–6 hr after the transfer. During this period the grainy appearance characteristic of cells grown in nitrogen-rich medium is lost. This change probably results from depletion of storage materials, particularly the cyanophycin granules which contain a copolymer of arginine and aspartic acid (17). Within another 3–4 hr, it becomes possible to identify cells whose pigmentation and structure differ from those of the vegetative cells. These have been called proheterocysts (18). They are smaller than mature heterocysts and do not have polar thickenings. Their distribution is characteristic of heterocysts, appearing at regular intervals every 5–10 cells along the length of the filament (19). During the next 10–12 hr the proheterocysts become mature heterocysts, and the increase of nitrogenase activity parallels this further differentiation. The micrographs in Fig. 2.1–C illustrate these stages of development. Extensive ultrastructural changes accompany a similar cellular differentiation in *Anabaena* (20).

Although the period required for development of heterocysts from proheterocysts is rather constant, the time from transfer to complete induction–differentiation varies under...
FIG. 3. NH₄⁺ delays induction of nitrogenase in Nostoc. A culture was transferred to medium containing 1 mM (●), 3 mM (○), or 5 mM (□) NH₄Cl, and samples were subsequently assayed for nitrogenase activity.

different conditions. Increased aeration, increased light intensity, or addition of NaHCO₃ to the medium each results in more rapid induction-differentiation (data not shown). These differences appear to be related to exhaustion of combined nitrogen. Fig. 3 demonstrates that the length of the period from transfer to differentiation-induction can be directly related to the amount of NH₄Cl present at the time of transfer. The decrease in nitrogenase activity after maximum differentiation-induction is probably due to the NH₄⁺-dependent inactivation of nitrogenase observed by others (21).

The increase in nitrogenase activity, induced by removal of NH₄⁺, requires continuous de novo protein synthesis since the enzyme activity no longer increases when chloramphenicol is added to the culture (data not shown). Microscopic observation indicates that the frequency of heterocysts in the popu-

FIG. 4. Comparison of proteins synthesized in vegetative cells and heterocysts of differentiated Nostoc. Cultures labeled with ³⁵SO₄⁻ were separated into two cell types by low-speed centrifugation (1000 rpm for 3 min). The crude heterocyst fraction, vegetative cell fraction, and washed heterocysts were analyzed on polyacrylamide gels; electrophoresis was from left to right on the densitometer tracings of the autoradiograms shown. On the lowest tracing the horizontal scale is expanded 2-fold. The gels contained, top to bottom, 400 µg of protein (100,000 cpm); 300 µg of protein (75,000 cpm); and 350 µg of protein (100,000 cpm).

lation also remains constant in the presence of chloramphenicol.

We wished to compare the proteins synthesized in the filaments grown in medium containing combined nitrogen with

FIG. 5. Comparison of “soluble” and “membrane-associated” proteins synthesized in vegetative cells and heterocysts of Nostoc. Cultures labeled with ³⁵SO₄⁻ were fractionated and analyzed on polyacrylamide gels. Gels a-c contain fractions H m, V m−, and V m⁺, respectively; gels d-f contain fractions H α, V α−, and V α⁺, respectively. Numbers in the left-hand column provide a rough molecular weight calibration (x10⁻⁵) based on external standards. H₁–H₄ are the major heterocyst-specific proteins described in the text. P is the phycocyanin region (22). Gel samples contained the following amounts of total protein: (a) 100 µg, 38,000 cpm; (b) 150 µg, 38,000 cpm; (c) 150 µg, 34,000 cpm; (d) 65 µg, 26,000 cpm; (e) 150 µg, 36,000 cpm; (f) 150 µg, 34,000 cpm.
those synthesized in the two cell types of differentiated filaments. The various methods used in the past to separate heterocysts from vegetative cells rely on the greater resistance of the heterocysts to destruction by French press, sonication, or other physical means. These techniques do extensive damage to the heterocysts (1). However, heterocysts separated by a scheme that relies on lysozyme incubation for a short period do not appear to be damaged when examined in an electron microscope (2). Fig. 2D shows a phase-contrast micrograph of the heterocysts isolated in this manner, used for the following experiments.

Ultrastructural studies of undifferentiated filaments of Anabaena indicate that they contain a single cell type (20). Filaments of Nostoc from nitrogen-containing medium (Fig. 2A) appear to be composed of a single cell type; the treatment we use to separate heterocysts from vegetative cells yields only a vegetative cell fraction from such filaments.

We studied the program of protein synthesis in fully differentiated filaments by labeling most cellular proteins with $^{35}$S for a 36-hr period starting 35 hr after transfer to nitrogen-free medium, and analyzing the proteins of the two cell types by electrophoresis on polyacrylamide gels. In the two upper traces of Fig. 4, we compare the labeled proteins contained in a crude pellet of heterocysts with the proteins in the vegetative cells of the same filaments. Four proteins ($H_1-H_4$) found in the heterocysts are missing from the vegetative cells. When heterocysts are washed free of contaminating membranes from vegetative cells, the proteins $H_1-H_4$ are found to be the major components synthesized in differentiated heterocysts. The lowest trace in Fig. 4 shows, on an expanded scale, the central portion of an electropherogram of the proteins from washed heterocysts.

Comparison of the protein synthesis patterns is more clearly made when the proteins are separated into classes, termed “soluble” and “membrane-associated,” based on their ability to be pelleted at 10,000 rpm in 10 min. These two classes of protein from three cell types (heterocysts, vegetative cells from differentiated filaments, and vegetative cells from undifferentiated filaments) are shown in Fig. 5. Gels a–c of Fig. 5 contain the soluble proteins from the three cell types; gels d–f contain membrane-associated proteins. Together these constitute all the proteins significantly labeled with $^{35}$S. We assume that the cells contain many other proteins not seen because they contain little sulfur or are present in minute quantities.

The pattern of synthesis of soluble proteins in the undifferentiated vegetative cell is very similar to that of the differentiated vegetative cell. There are, however, at least four protein bands that are more prominent in $V_r$ than in $V_r^*$. The differences between membrane-associated proteins of these two cell types are less pronounced.

There are very striking differences between the vegetative cell proteins and those of the heterocyst. The new program of protein synthesis after differentiation involves the loss of ability to synthesize at least 20 vegetative cell proteins, two of which can be identified as components of phycocyanin (22). Six protein bands dominate the heterocyst pattern. Two of these appear to be similar to proteins associated with vegetative cell membranes, but the other four ($H_1-H_4$) are unique to heterocysts.

We have tentatively identified the three bands $H_1$, $H_2$, and $H_4$ as the components of nitrogenase. In making this identification, we have assumed that the nitrogenase of Nostoc resembles this enzyme in aerobic bacteria (23) and anaerobic bacteria (24). The enzyme in these organisms is similar: it consists of two proteins, one containing molybdenum and iron, the other containing iron. The Mo–Fe protein found in Clostridium (25) consists of subunits of molecular weight 60,000 and 50,000. A sample of crystalline Mo–Fe protein from Azotobacter (26) (the kind gift of John Stansby) was subjected to electrophoresis and compared to the heterocyst proteins. The stained bands in Fig. 6B have mobilities identical to the radioactive proteins $H_1$ and $H_2$ shown in Fig. 6A. Their molecular weights are about 60,000 and 54,000.

The iron protein from Clostridium (27) is composed of subunits of molecular weight 28,000. This is the molecular weight of band $H_4$. We do not know the significance of band $H_5$. It could be a precursor of $H_4$, another protein involved in fixation or in processing of NH$_3$, or a protein involved in some other heterocyst function such as glycolipid synthesis.

Further evidence that supports the designation of $H_1$, $H_2$, and $H_4$ as nitrogenase proteins is the finding in this experiment, and in others, that the proteins in these bands are regulated appropriately. They are not synthesized in a culture containing NH$_3$ or Ca(NO$_3$)$_2$, and, when induced, the extent of their synthesis parallels the increase in nitrogenase activity.

The last two sets of gels in Fig. 5 provide a comparison of supernatant and membrane proteins from heterocysts and vegetative cells. Although some of the nitrogenase protein is released into the supernatant by sonication, much appears to be associated with membranes, in agreement with previous reports (2, 28).

The evidence that bands $H_1$, $H_2$, and $H_4$ are components of nitrogenase is indirect. More direct proof of their identity may be provided either by purification of the Nostoc enzyme, or by their specific precipitation with antibody prepared against one of the bacterial nitrogenases.

**DISCUSSION**

Isolated heterocysts are responsible for a disproportionately large percentage of the total nitrogenase activity in disrupted filaments of Anabaena (11, 12). Furthermore, several experiments have shown that reducing activity, as measured by reduction of triphenyltetrazolium chloride, is greater in heterocysts than in vegetative cells (11). This evidence has
been used to support the theory that the morphologically
differentiated heterocysts are functionally specialized for
nitrogen reduction (7, 9, 10). Our results demonstrate a
basis for such functional specialization in the differences
between the patterns of protein synthesis in the two cell types.
We have shown that induction of nitrogenase activity in
aerobically grown *Nostoc* is accompanied by de novo syn-
thesis of the protein components of nitrogenase in dif-
ferentiated heterocysts, but not in vegetative cells. Earlier
reports of nitrogenase in vegetative cells (29) are probably
the result of leakage of the enzyme from heterocysts produced
by sonication (12).

Our results are consistent with the hypothesis that hetero-
cyst development begins when the concentration of NH$_4^+$, or
a derivative, falls below some threshold. A corollary of this
model is that the products of nitrogen fixation in heterocysts
repress development of, and nitrogenase synthesis in, neigh-
bouring vegetative cells (30). However, Wolk (31) and Wilcox
(19) have pointed out that the ability to inhibit heterocyst
development is acquired by proheterocysts before they have
the capacity to fix nitrogen. Thus, fixation of nitrogen in
heterocysts alone cannot account for the early arrest of
development in other cells of the filament. We can only specu-
late on the nature of this early event: one possibility is that
the proheterocyst acquires an active system for export of
NH$_4^+$ to neighboring cells.

If the products of nitrogen fixation repress the genes for
nitrogenase structural proteins, the original cell in which
nitrogenase was derepressed must become refractory to
repression, in order to continue synthesis of those proteins.
Indeed, during differentiation-induction, cells destined to be
heterocysts appear to pass a critical point beyond which
their morphological development, and their ability to syn-
thesize nitrogenase components, cannot be reversed by ex-
ternally added NH$_4^+$, although the NH$_4^+$ does reduce the
activity of nitrogenase substantially (unpublished results).

Refractoriness to repression may be only one manifestation of
a very extensive set of related phenomena. The synthesis of
most vegetative cell proteins is shut off in heterocysts (Fig.
5). We would like to think that irreversible commitment of
heterocyst development, shut-off of vegetative-cell protein
synthesis, and constitutive synthesis of nitrogenase proteins
have a common basis. Moreover, it seems possible that the
event triggering this developmental sequence could occur
(infrequently) in cultures grown in nitrogen-containing
medium, and might account for those few heterocysts ob-
served under such conditions.

Our discussion thus far has centered on a single model for
regulation in which NH$_4^+$, or a derivative, is the corepressor
of nitrogenase component structural genes. The data are also
compatible with more complex schemes. For example, NH$_4^+$
might regulate an element that in turn is the regulator of
nitrogenase gene activity. Alternatively, a metabolite nor-
manly kept in low concentration by NH$_4^+$ might accumulate
in its absence, and serve as a positive inducer of development.

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