Protein Synthesis during Encystment of *Azotobacter vinelandii*†

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Proteins synthesized during the encystment of *Azotobacter vinelandii* were radiolabeled with [³⁵S]methionine and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Pulse labeling was used to demonstrate that early encystment-specific proteins were beginning to be synthesized at 2 h and reached peak levels about 12 h after initiation of encystment. One such protein was identified as a β -ketoacyl acyl-carrier protein synthase. The concentration of early proteins began to decrease at 16 h, when intermediate proteins specific to the differentiation process began to be synthesized. The cessation of synthesis of intermediate proteins began at 20 h postinitiation, and the labeling pattern of proteins then remained constant throughout the remaining 4 days of encystment.

Azotobacter vinelandii are free-living, nitrogen-fixing bacteria which differentiate to form cysts under adverse environmental conditions (7). Encystment can also be induced when exponentially growing vegetative cells are shifted from growth on glucose to β -hydroxybutyrate (BHB) as the sole carbon source (7). Like the sporulation of *Bacillus* species, encystment is a process which must involve the sequential turning on and off of specific genes during development. Furthermore, encystment-specific proteins appear to be synthesized at the expense of nonessential proteins, which are degraded to provide amino acids for the synthesis of the new proteins (14, 15).

The genetic analysis of sporulation in *Bacillus* species and the identification of sporulation genes have been approached by the use of mutants which are blocked at unique developmental stages (8, 10, 23). Unfortunately, this kind of approach has not been possible with *Azotobacter* species because of the difficulty of inducing mutations other than in nitrogen fixation (16). As an alternative, we have studied the synthesis of proteins at different times during the encystment of *A. vinelandii* in an effort to identify those genes which are inactive during vegetative growth but become activated to produce encystment-specific proteins.

Our initial interest has been in the enzymes of lipid synthesis, because membrane lipid shifts are among the first discernible differentiation-specific events occurring after induction of encystment (12, 13, 19). The membranes of vegetative cells of *A. vinelandii* contain phospholipids (P-lipids) whose type and acyl group composition are similar to those of *Escherichia coli* phospholipids (18). The chain-lengths of the P-lipid acyl groups are C₁₆ (palmitate), C_{16:1} (palmitoleate), and C_{18:1} (*cis*-vaccenate). Cyst membranes, however, are devoid of P-lipids, having bilayers consisting of alkylresorcinols and alkylpyran-2-ones (12). These are newly synthesized during encystment from saturated C₂₈ and C₃₀ keto fatty acids (17). The transition from cell to cyst membrane is achieved early in encystment by cessation of

synthesis and rapid turnover of P-lipids concomitant with resorcinol and pyran synthesis (13).

The fatty acid-synthesizing complex of enzymes operative during encystment must be different from that functional during vegetative growth of *A. vinelandii*. The minimal differences between the two enzyme sets producing such different products must be (i) a means for achieving fatty acid chain lengths 10 to 12 carbons greater during encystment than during vegetative growth and (ii) the loss of the specific β -hydroxydecanoyl thioester dehydrase which functions in the formation of *cis*-double-bonded acyl chains (5). During the course of this research we have identified a protein produced very early during encystment as a β -ketoacyl-acyl-carrier protein (ACP) synthetase which can be differentiated from a similar protein in vegetative cells by its catalytic properties.

MATERIALS AND METHODS

Preparation of vegetative cells and cysts. *A. vinelandii* ATCC 12837 was used throughout this study. Vegetative cells were grown in Burk nitrogen-free medium (22) with 1% glucose as the sole carbon source. The induction of encystment consisted of the following procedure. A culture of vegetative cells was grown in 1% glucose to mid-exponential phase (OD₆₀₀, 0.7), cooled to 0°C quickly, and then harvested by centrifugation. The pellet was washed once with Burk buffer, the cells were suspended in their original volume of Burk buffer containing 0.2% BHB, and the suspension was incubated with aeration at 30°C.

Labeling of cell proteins. High-specific-activity L-[³⁵S]methionine (>600 Ci/mmol) in aqueous potassium acetate solution was purchased from Amersham Corp., Arlington Heights, Ill., and used for labeling studies. The [³⁵S]methionine was transferred into 25-ml capped, sterile Corex centrifuge tubes (100 μ Ci per tube), stored frozen at -70°C, and used within 2 weeks of delivery.

Before pulse labeling, tubes containing frozen [³⁵S]methionine were thawed and equilibrated at 30°C for 10 min. Six-milliliter samples of a culture either growing exponentially or induced to encyst were then taken at appropriate times. These were pipetted into centrifuge tubes containing [³⁵S]methionine, mixed immediately, and incubated at 30°C for 10 min with shaking (pulse) to allow [³⁵S]methionine to be incorporated into the cell proteins. After incubation, the tubes were cooled in ice and the incorporation of radioac-

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FIG. 1. SDS-PAGE analysis of proteins during encystment of *A. vinelandii*. The proteins were extracted directly from vegetative cells and cysts by boiling in SDS sample buffer for 5 min to avoid any proteolytic modification. The undissolved cell debris was removed after boiling. Protein samples equivalent to 1 ml of culture were loaded in each well. Because nitrogen fixation stopped shortly after the induction of encystment, the total protein content per milliliter of sample was relatively constant. Shown are photographs of an 8% polyacrylamide gel (top) and a 15% polyacrylamide gel (bottom). Samples of vegetatively growing cells were obtained 0, 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, 48, 60, 72, 96, and 120 h after induction of encystment (lanes A through P, respectively). Protein sizes (in kilodaltons) are indicated.

tivity was determined in cells collected from 1 ml of sample on passage through a double layer of glass fiber filters. The retained cells were washed twice with 10 ml of Burk buffer, dried, and counted in a scintillation spectrometer. The remaining 5 ml of labeled cells was collected by centrifugation and washed twice with 10 ml of Burk buffer, and the cell pellets were frozen at -70°C until used for electrophoretic studies.

Protein extraction. We attempted a number of cell-free treatments prior to the extraction of soluble and membrane-bound proteins of vegetative cells and cysts. These treatments had no effect on the extraction procedure, which was carried out by adding an equal volume of $2\times$ sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris hydrochloride, pH 6.8, 4% SDS, 20% glycerol, 10% β -mercaptoethanol) to a cell or cyst suspension and heating to 100°C for 5 min. The samples were cooled in an ice bath, and insoluble material was removed by centrifugation. The supernatant solution was used for protein assay or SDS-polyacrylamide gel analysis.

Enzyme extraction. Cells were concentrated 100-fold in buffer A (1 mM Tris hydrochloride, pH 7.8, 1 mM MgCl_2) and broken by passing through a French pressure cell three times at $12,000\text{ lb/in}^2$. The resulting suspension was then

centrifuged at $6,000 \times g$ for 20 min to remove the unbroken cells and large particulate material. The supernatant fluid from the first centrifugation was respun at $41,000 \times g$ for 30 min and used for enzyme purification.

β -Ketoacyl-ACP synthase. The enzyme was assayed by measuring the oxidation of reduced NADH by the condensation product of acetyl acylcarrier protein (acetyl ACP) and malonyl ACP in the presence of pig heart β -hydroxyacyl coenzyme A dehydrogenase (1). One unit of activity is defined as 1 μmol of NAD formed per min.

Protein determination. Soluble proteins were determined directly by a dye absorbance protein assay (Bio-Rad Laboratories, Richmond, Calif.). Insoluble proteins were solubilized either by boiling in SDS sample buffer or with 0.5 N NaOH. The solution was then diluted or neutralized before protein determination.

SDS-PAGE of proteins. The proteins in all cell or cyst extracts were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (6). Eight percent polyacrylamide gels were used to separate high-molecular-weight proteins ($>40,000$), and 15% polyacrylamide gels were used to separate low-molecular-weight proteins. The gels were stained with Coomassie blue and destained in methanol-acetic acid- H_2O (50:75:875) until the backgrounds were clear. They

were then photographed. Gels with [35 S]methionine-labeled proteins were further processed for autoradiography with En 3 Hance (New England Nuclear Corp., Boston, Mass.) and overlaid with X-ray films.

The molecular weight standards were purchased from Bio-Rad Laboratories. The radioactive standards used in gels subjected to autoradiography were 14 C-labeled low-molecular-weight and high-molecular-weight proteins from Bethesda Research Laboratories, Gaithersburg, Md.

RESULTS

The effect of incubation temperature on the SDS-polyacrylamide gel pattern during the protein extraction was examined because the boiling of *E. coli* envelope proteins in SDS solutions for 10 min results in an apparent molecular weight change of three proteins compared with the values obtained after incubation at 70°C for 30 min (4). Identical SDS-polyacrylamide gel patterns of *A. vinelandii* proteins were obtained with either heating regimen, but higher protein recoveries were obtained at 100°C.

The SDS-polyacrylamide gel analysis of proteins from exponentially growing vegetative cells as well as from cysts at different times during encystment are shown in Fig. 1. Approximately 50 protein bands could be resolved in gels of extracts of vegetative cells. The largest protein had an estimated molecular size of 180 kilodaltons (P-180). P-59.5 and P-63.5 were two major protein species; other major protein bands included P-155, P-153, P-110, P-82, P-73, P-54, P-44, P-43, and P-30.5. Although there were many similar protein bands present in vegetative cells and cysts, there were also many new proteins which were synthesized only during encystment. P-210 and P-170 were such proteins, which appeared 16 h after the initiation of encystment, reached maximal concentrations at 24 h, and then started to decrease until they disappeared at 60 h postinitiation. P-167 and P-145 appeared at 24 h after initiation of encystment and lasted for 5 days. P-120 appeared 12 h after initiation of encystment.

Some protein bands increased in staining intensity during the encystment while others decreased. This increase could be due to the synthesis of a new protein of a size similar to one already present or to the increased synthesis and accumulation of a particular protein. P-41.5 and P-40.5 started to increase in intensity 8 h after encystment. Many proteins, including P-73, P-63.5, P-28.5, P-25.3, P-24.5, and P-21, started to increase in intensity between 8 and 12 h after induction of encystment. The protein band P-76 stained more intensively 16 h after encystment began, whereas P-18.4 increased in apparent concentration 24 h after encystment and became a major cyst protein. On the other hand, P-30.5 decreased in staining intensity within 2 h after encystment began.

The intensity of stained protein bands on SDS-polyacrylamide gels reflects the total amount of a particular species of protein at the sampling time. This is the summation of past synthesis minus protein turnover and does not reflect the synthesis rate of an individual protein at the time of sampling. To examine this parameter, samples were taken at various times during the encystment and pulse-labeled with [35 S]methionine. Figure 2 shows the uptake of [35 S]methionine per milliliter of cells after 5 min of incubation at 30°C. Assuming that [35 S]methionine uptake is not the rate-limiting factor, this figure should reflect total protein synthesis activity at various stages during encystment. Immedi-

FIG. 2. Uptake of [35 S]methionine during encystment of *A. vinelandii*. *A. vinelandii* cells were induced to encyst by shifting the exponentially growing cells from 1% glucose to 0.2% BHB as the sole carbon source. A 6-ml sample of culture was taken at different time points during encystment. The samples were incubated with 85 μ Ci of [35 S]methionine for 5 min at 30°C. After incubation, the samples were cooled immediately at 4°C, and 1 ml of cells was taken and collected on double layers of glass fiber filters. The filters were washed extensively with Burk buffer and dried, and the radioactivity was determined by scintillation counting.

ately after the cells were transferred from growth on glucose to the BHB encystment medium, protein synthesis decreased sharply, as might be expected in a metabolic shutdown. After 2 h of encystment, protein synthesis increased and reached a peak at about 12 to 18 h postinduction. It then decreased gradually over a 4-day period.

The protein species which were being synthesized during the encystment were studied by pulse-labeling the encysting cells at various times during encystment and then extracting and separating proteins by SDS-PAGE. The [35 S]methionine-labeled proteins were detected by autoradiography (Fig. 3). In vegetatively growing cells of *A. vinelandii* at the late exponential phase, the most active synthesis involved P-110, P-88, P-82, P-70, P-63.5, P-58, P-54, P-44, and P-30.5. Following an almost complete cessation of synthesis, a completely new set of proteins were being made, indicating the reprogramming of genes. At 2 h into encystment, P-56, P-40.5, and P-25.3 were the proteins that were most strongly labeled. As encystment proceeded, the level of protein synthesis increased but the protein labeling pattern did not change appreciably. At 4 h into encystment, several new protein bands were visible on autoradiograms of gels. Long exposure of the X-ray film revealed that those proteins were already being synthesized at 2 h after induction, but were not detectable with short exposures. P-85, P-73, P-63.5, P-58, P-44, P-26.5, P-24.5, and P-14.5 were all being labeled significantly at 4 h after initiation of encystment. The same protein synthesis pattern continued at a higher rate for 4 h more, at which time two more cyst-specific bands, P-82 and P-116, were detected.

At 12 h postinduction, [35 S]methionine uptake reached a peak as reflected in the protein-labeling pattern. Newly detected proteins P-155, P-153, P-91, P-59.5, P-54, P-46, P-30.5, and P-28.5 were all labeled very strongly. Among these proteins, P-91, P-59.5, and P-30.5 were only actively synthesized at this point in the encystment process. At 16 h

FIG. 3. Autoradiography of proteins pulse-labeled with [35 S]methionine at various times during encystment of *A. vinelandii*. Cells (5 ml) were pulse-labeled with [35 S]methionine (from the previous experiment, Fig. 2), harvested by centrifugation, and washed twice with Burk buffer to remove unincorporated methionine. The proteins were extracted and separated on SDS-polyacrylamide gels. [35 S]methionine-labeled protein equivalent to 1 ml of original culture was loaded on each lane. After electrophoresis, the gels were processed with En 3 Hance, dried under vacuum, and exposed to X-ray films. Shown are an 8% polyacrylamide gel (top) and a 15% polyacrylamide gel (bottom). Samples of vegetatively growing cells were taken 0, 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, 48, 60, 72, and 96 h after induction of encystment (lanes A through O, respectively). Protein sizes (in kilodaltons) are indicated.

into encystment, the protein synthesis pattern went through another transition period in which some previously rapidly synthesized proteins underwent a sharp decrease while the rate of synthesis of other proteins increased. Synthesis of P-73, P-63.5, P-61.5, and P-14.3 decreased substantially, but synthesis of P-210, P-170, P-76, P-41.5, P-40.5, and P-18.4 increased dramatically. At 20 h into encystment, protein synthesis activity decreased further. P-210, P-170, P-76, P-58, P-41.5, P-40.5, P-25.3, and P-24 were the major protein species being synthesized at that time. Protein synthesis then gradually decreased, but the labeling pattern remained constant throughout the rest of the 4-day encystment.

Chloramphenicol (100 μ g/ml of culture) blocked the synthesis of early cyst proteins and the overall encystment process when it was introduced 0 to 4 h after the addition of BHB to vegetative cells. There was no shift from P-lipid to resorcinol and pyran-2-one type of membrane in such treated cells, nor was there significant synthesis of P-63.5. Chloramphenicol had no effect on encystment when it was added 6 h or more after initiation by BHB.

From these observations we assumed that P-63.5 could be involved in new lipid synthesis and that it could be a

β -ketoacyl-ACP synthase (21). The following tests were conducted to validate our assumptions.

A survey was made of β -ketoacyl-ACP synthase activity in extracts of encysting cells (Fig. 4). Within an hour of initiation of encystment, the specific activity of the enzyme dropped from its vegetative cell level to near zero. The enzyme specific concentration then increased rapidly to a peak value at 5 h postinduction, from which it diminished sharply to 15% of its peak value. The synthase concentration remained at about that level for the initial 24 h of the encystment process and decreased to zero by 65 h postinitiation.

The identity of P-63.5 with the β -ketoacyl-ACP synthase was established as follows. The putative enzyme protein was cut from gels and electrophoretically eluted, and 500 μ g was injected intraperitoneally into BALB/c mice weekly for 3 weeks to elicit antibody formation. After week 4, serum was prepared from the blood of the immunized mice and bound to cyanogen bromide-activated Sepharose 4B beads, and the beads were used for affinity chromatography.

β -Ketoacyl-ACP synthases from vegetative and 5-h encysting cells were prepared by published methods (1). These

two enzyme preparations reacted in an identity pattern with the immune mouse serum in double-diffusion reactions, suggesting that they had common antigenic sites. The vegetative and encystment enzymes were then further purified by affinity chromatography to homogeneity when tested by SDS-PAGE (A. da Cunha, manuscript in preparation). The enzymes were identical in size on gel filtration through Superose 6 (Pharmacia), 66,000 kilodaltons, but they differed remarkably in their pH activity profiles and isoelectric points. The synthase from vegetative cells reacted optimally at pH 7.5, while the encystment enzyme reacted optimally at pH 6.5 (Table 1), and when subjected to isoelectric focusing, the two proteins had pIs of 5.7 and 4.1, respectively.

DISCUSSION

Encystment of *A. vinelandii* is a differentiation process that has been studied extensively in this laboratory at the morphological and biochemical levels (3, 7, 14–16, 20). In this research we used a simple, reliable protein separation procedure because we were looking for major differences between the proteins of cells and cysts. The limited resolution of one-dimensional SDS-PAGE imposes a difficulty in determining whether a single band detected in a gel represents a single protein or contains more than one protein species of similar size. Two-dimensional gel electrophoresis (2, 9) would have provided higher resolution, but the procedures employed were adequate to demonstrate that a programmed sequence of unique genes functions during encystment of *A. vinelandii*. Furthermore, the studies revealed that some unique gene expression is transitory and occurs only during the cell-cyst transition, while other genes code for proteins that are only found in cysts.

The results of pulse-labeling and subsequent electrophoresis were in agreement with those obtained by electropho-

FIG. 4. β -Ketoacyl-ACP synthase of encysting *A. vinelandii* cells. The culture was sampled at intervals from 0 to 65 h after induction, cell-free preparations were made, and the enzyme was assayed in microcuvettes as follows (1). The reaction mixture contained 15 μ l of 1 M potassium phosphate, pH 7.0, 20 μ l of 0.4 M mercaptoethanol, 10 μ l of 10 mM EDTA, 15 μ l of 1 mM acetyl ACP, 15 μ l of 1 mM malonyl ACP, 20 μ l of 1 mM NADH, and 0.1 U of β -hydroxyacyl coenzyme A dehydrogenase in a 150- μ l volume. The reaction was started by the addition of approximately 0.001 U of β -ketoacyl-ACP synthase. Controls to assess NADH oxidase activity of the crude extracts were run by omitting malonyl ACP from the reaction mixture. The plotted values are corrected for endogenous oxidation of NADH.

TABLE 1. Specific activities of fatty acid synthases versus pH^a

pH	Sp act (mU/mg of protein)	
	Vegetative cells	Encysting cells
5.7	0	0
6.0	0	0
6.5	0	158.5
7.0	111.7	132.7
7.5	116.5	62.1
8.0	23.1	0
8.5	0	0
9.0	0	0

^a The assays were conducted as described in the legend to Fig. 4. The buffer over the pH range 5.7 to 8.0 was 0.1 M phosphate and from 8.5 to 9.0 was 0.1 M Tris.

retic analysis of extracts of encysting cells. Protein bands which increased in staining intensity during encystment were also being labeled heavily. The only exception we observed was P-21 (Fig. 1), which started to increase in staining intensity at 12 h after induction of encystment but was not ³⁵S-labeled sufficiently to be detected in Fig. 3. This protein could be a proteolysis product of an extant higher-molecular-weight protein (14). Alternatively, P-21 may not contain a significant amount of methionine to become labeled. P-167 and P-145 also stained strongly at 36 h after induction of encystment but were only slightly labeled. These two proteins could be proteolytic products of higher-molecular-weight proteins; alternatively, they were being synthesized at low rate and were not being actively turned over.

After induction of encystment with BHB, protein synthesis decreased sharply but resumed within 4 h, producing a completely different set of proteins from those of vegetative cells. One of the newly synthesized proteins was identified as a β -ketoacyl-ACP synthase, which had immunological properties similar to those of the vegetative enzyme but differed from it in catalytic properties and net charge. This enzyme may function in the biosynthesis of the unique lipids during encystment, since there was a temporal relationship between the appearances of the enzyme and the lipids. Other observed proteins may not have been encystment specific but required for the cells to switch from glucose to acetate metabolism (viz., BHB dehydrogenase and enzymes of the gluconeogenesis pathway [3]).

It should be noted that even proteins which were not being actively synthesized during encystment may have been required for that process. Such proteins would be common to cells and cysts and probably constitute the majority of proteins in either cell type. The finding of similar protein patterns in vegetative cells and cysts supports this view.

Pulse-labeling studies of protein synthesis indicate that (i) certain proteins are synthesized throughout encystment (P-116, P-59.5, P-30.5, and P-28.5) and (ii) the encystment process in *A. vinelandii* occurs in three discernible stages. During early encystment, 0 to 8 h postinduction, the major protein species being synthesized were P-85, P-82, P-73, P-63.5, P-44, P-40.5, P-25.3, and P-14.3. [³⁵S]methionine uptake reached a peak at 12 h, approximately half-way through the middle stage. The synthesis of late-stage proteins began at 20 h into encystment, producing P-91, P-76, P-44, P-41.5, P-40.5, P-25.3, P-24, and P-18.4. The rate of protein synthesis then decreased with time and with little change in the protein species being synthesized over a 4-day period.

Other biochemical studies also indicate that encystment reaches an important transition point at 8 to 12 h postinduc-

tion. DNA synthesis stops at about 4 h postinduction, but net RNA synthesis continues until 12 h of encystment (3).

The synthesis of unique membrane lipids also started between 6 and 12 h after induction (11, 19). Studies of several enzymes of *A. vinelandii*, including BHB dehydrogenase, isocitrate dehydrogenase, isocitrate lyase, and malate synthetase, reveal that their specific concentrations vary in the course of encystment in a programmed fashion (3).

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