Protein Turnover in *Azobacter vinelandii* During Encystment and Germination†

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Protein turnover occurs during differentiation of *Azobacter vinelandii* 12837 to the extent of 50% during encystment and 7% during germination. The addition of rifampin at the initiation of encystment prevents encystment and inhibits turnover. In germinating cysts, protein turnover is essential owing to an apparent lack of certain amino acid biosynthetic enzymes. The capacity to synthesize sulfur-containing amino acids from inorganic precursors is regained about halfway through the germination process.

*Azobacter vinelandii* are N$_2$-fixing bacteria which can be induced to form metabolically dormant cysts (4). Under the proper nutritional conditions, cysts can germinate, outgrow, and initiate a new round of vegetative growth. Previous studies have shown that there is a striking similarity in the pattern of events that occurs in the life cycles of *A. vinelandii* and the bacilli (11). During sporulation in *Bacillus* species, extensive protein turnover as well as amino acid catabolism occurs as new spore-specific proteins are synthesized (1). Protein turnover also occurs during germination of dormant spores, since they lack many of the enzymes involved in amino acid synthesis. N$_2$ fixation does not occur in *A. vinelandii* during encystment and the initial stages of cyst germination (11, 12), yet protein synthesis takes place. We have assumed that the amino acids necessary for this synthesis were provided by protein turnover.

In this report we show that extensive protein turnover does occur during encystment of *A. vinelandii* and that initial deficiencies in cysts of enzymes for sulfur-containing amino acid synthesis can be overcome by relatively little protein turnover.

**MATERIALS AND METHODS**

**Organisms and growth conditions.** *A. vinelandii* ATCC 12837 was grown in M medium, a modified Burk N-free medium. It contained (per liter) 0.82 mM MgCl$_2$, 0.58 mM CaCl$_2$, 18 μM FeCl$_3$, 1 μM NaMoO$_4$, 2 mM NaCl, 5 mM potassium phosphate (pH 7.4), and 1 mM K$_2$SO$_4$. The carbon source was 30 mM glucose. Cultures were inoculated with exponentially growing cells (5% volume) and incubated with shaking at 30°C, and their growth was monitored by turbidity (optical density) at 540 nm (OD$_{540}$) in a Spectronic 20 colorimeter. When bacterial culture densities reached an OD$_{540}$ of 0.3, the induction of encystment with 0.2% β-hydroxybutyrate (BHB) was carried out as previously described (2). Such cultures at the end of 5 days were comprised of 95% cysts; Cysts were harvested, washed with M medium, and stored as a pellet at 4°C for no more than 2 days before use. Germination was carried out at 30°C in 30 mM glucose-M medium and was greater than 90% complete in 8 to 9 h as judged by phase-contrast microscopy.

**Labeling of cells for protein turnover studies.** The most common method of demonstrating protein turnover is the pulse-chase method of Spudich and Kornberg (15). This technique involves growing the cells in the presence of a labeled amino acid for a certain amount of time, followed by the addition of a large excess of unlabeled amino acid. Turnover is measured by the loss of label from the trichloroacetic acid (TCA)-insoluble protein fraction.

Cells for use in the determination of protein turnover during growth were labeled by adding 0.1 μCi of [U-14C]phenylalanine (specific activity, 412 mCi/mmol) per ml of culture at a growth level corresponding to 0.1 OD$_{540}$. After one doubling, unlabeled phenylalanine was added to a final concentration of 5 mM constituting a 21,000-fold reduction in specific activity of the label and effectively stopping further incorporation. At this time, and hourly thereafter, duplicate 1-ml samples were taken and pipetted into 4 ml of cold 6.7% TCA. After at least 30 min on ice, the samples were collected on Whatman GF/C glass fiber filters, washed with TCA, dried at 80°C, and counted in ACS scintillation fluid (Amersham-Searle). Turnover is expressed as the percentage of loss of TCA-insoluble radioactive label relative to that initially present in the sampling interval.

For the determination of turnover during encystment, exponentially growing cells were labeled with 0.1 μCi of [U-14C]leucine (specific activity, 300 mCi/mmol) per ml of culture in essentially the same manner as described above, except that the cells were exposed to [14C]leucine throughout the entire exponential growth period. The cells were harvested,
washed, and shifted to a medium containing 0.2% BHB and 4 mM unlabeled leucine. Duplicate 1-ml samples were aseptically withdrawn every 10 to 12 h and treated as described above. Cultures were agitated vigorously on a Vortex mixer before sampling.

Labeled cysts were prepared by carrying out encystment in M medium with 0.2% BHB replacing glucose and by employing either 0.1 μCi of [U-14C]leucine or 0.1 μCi of [U-14C]tyrosine (specific activity, 450 mCi/mmole) per ml of culture. After 5 days the cysts were harvested, washed, and stored at 4°C until use. Cyst suspensions, 2.4 × 10⁷ per ml, were germinated in M medium containing 30 mM glucose and 4 mM unlabeled leucine or tyrosine. At hourly intervals, duplicate 1-ml samples were withdrawn into 0.1 ml of 55% TCA. After 30 min at 4°C, the samples were filtered through 0.45-μm Millipore filters, and a portion of the filtrate was counted. The extent of turnover was expressed as the percentage of TCA-soluble counts relative to the total radioactivity in the dormant cysts. Germination was assessed by the loss of cyst refractility over an 8-h period and the subsequent shedding of exines (11).

Nitrogen fixation. The rate of nitrogen fixation was determined by the acetylene reduction technique (16). At regular intervals triplicate 2-ml samples of encysting cultures were added to 5-ml-capacity glass serum bottles. The samples were flushed with a 22% O₂-0.04% CO₂-78% argon gas mixture before fitting the bottles with rubber serum bottle stoppers. Acetylene (0.5 ml) was then injected into each bottle, and they were incubated for 1 h at 25°C. A control received 0.3 ml of 2% HgCl₂ immediately on capping and served to establish the background level of ethylene in acetylene. Nitrogenase was inactivated by the addition of HgCl₂ to the cell suspensions. Ethylene formation in duplicate 0.5-ml gas samples was detected by gas chromatography in a Varian-Aerograph 1440 equipped with a column (3.2 mm by 1 m) of Porapak N at 45°C and a flame detector. The carrier gas was N₂ at a flow rate of 25 ml/min. The standard was a gas mixture of 47 μl of ethylene per liter of N₂. The relative rate of N fixation was expressed as nanomoles of ethylene produced per microgram of cell protein per hour. The protein content of cells was determined by partially solubilizing TCA-precipitated cells with 0.2 N NaOH, removing insoluble debris by centrifugation, and assaying the supernatant solution by the method of Lowry et al. (6) with bovine serum albumin as a standard.

The time course for initiation of nitrogenase activity during cyst germination was a modification of the above procedure. A 10-ml amount of a cyst suspension (2.4 × 10⁷ cysts per ml) in M medium with glucose was placed in a 100-ml serum bottle fitted with a septum; air was replaced by flushing with the O₂-CO₂-Ar mixture, and 5 ml of acetylene was added with a syringe. The cyst suspension was incubated in a rotary shaking water bath at 30°C, and at various times, 50 μl of gas in equilibrium with the suspension was assayed for ethylene by gas chromatography.

RESULTS

Exponentially growing, N-fixing cells of A. vinelandii and those utilizing NH₄⁺ as an N source lost 1.6 and 1.4%, respectively, of radioactive label previously incorporated into their protein per h (Table 1). The generation times (t₉₀) of the two cultures were 200 and 100 min, respectively. After exponential growth, the turnover rates of the cultures decreased, and they approached 0.7 to 0.8% per h (Table 1).

We found that extensive protein turnover occurred during the encystment process, albeit at a slightly lower rate (ca. 1% per h) than in exponentially growing cells. Cells which were labeled with radioactive amino acids during exponential growth and then shifted to BHB to induce encystment lost ca. 50% of their labeled protein within 50 h (Fig. 1). Little additional turnover occurred after this time. To show that the observed loss of label was due to turnover and not to a loss of total protein from the cells, whole cell protein was measured throughout encystment. We found that the amount of protein per milliliter of culture remained constant throughout the 120-h encystment process. These data are in agreement with previous observations (12).

During the course of the induction of encystment in A. vinelandii, some of the inducing agent, BHB, is metabolized to acetate which is then used for synthesis and as an energy source. The organism fixes nitrogen and grows vegetatively on acetate, but the molecule will not induce encystment as does BHB. To test the effect of acetate metabolism on protein turnover, prelabeled glucose-grown cells were shifted into an N-free medium containing acetate (rather than BHB) as the carbon and energy source and incubated at 30°C with aeration. A protein turnover of 10% occurred in the first 50 h of incubation. An additional 20% loss of label had taken place by 80 h, but no further loss occurred after that time. The extent of encystment after 120 h ranged from 0.5 to 1%.

In a different type of experiment, encysting 14C-labeled cultures from which the unlabeled amino acid had been omitted underwent no loss of radioactive label. This result suggests that the loss of label is truly a loss of amino acid that can be chased with unlabeled amino acid. The addition of 20 μg of rifampin per ml of culture at the time of induction of encystment with BHB sharply inhibited protein turnover and blocked encystment. The total loss of radioactivity from prelabeled cells in these cultures was 12% over an 80-h incubation period.

Cysts were examined for their capacity to synthesize sulfur-containing amino acids from 35SO₄²⁻ during germination. Cysts which were germinating in M medium—30 mM glucose containing 0.1 μCi of [methyl-³H]methionine (specific activity, 100 mCi/mmole) per ml began to incorporate that amino acid immediately. Cysts
TABLE 1. Loss of radioactivity from two exponentially growing cultures of A. vinelandii labeled for one generation with [14C]phenylalanine

<table>
<thead>
<tr>
<th>Time</th>
<th>Experimental conditions*</th>
<th>Loss when t_e is 200 min</th>
<th>Loss when t_e is 100 min</th>
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<tr>
<td></td>
<td></td>
<td>OD_w</td>
<td>cpm in cells</td>
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<tr>
<td>0</td>
<td></td>
<td>0.094</td>
<td>0.11</td>
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<tr>
<td>1</td>
<td>Pulse</td>
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<td>13,600</td>
</tr>
<tr>
<td>2</td>
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<td>0.30</td>
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<tr>
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<td>22</td>
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<td>0.86</td>
<td>10,670</td>
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</table>

* Average of duplicate samples rounded off to four significant figures.

germinating in M medium–30 mM glucose containing 0.1 µCi of carrier free 35SO4²⁻ (specific activity, 4,300 Ci/mmol) per ml incorporated significant amounts of label in cold TCA-insoluble material beginning at only 3 to 4 h post-initiation of germination (Fig. 2). At 8 h post-initiation of germination, 11 nmol of 35SO4²⁻ had been incorporated per 10⁶ cysts.

The results of the previous experiment suggest that, although protein synthesis began immediately on germination (5), the capacity to synthesize S-containing amino acids from inorganic precursors was not fully operational in germinating cysts until 3 to 4 h into the process. We therefore tested for protein turnover during this time. When cysts prelabeled with [14C]leucine were germinated, ca. 7% of the total label became TCA soluble (presumably as the intact amino acid) in 8 h.

The release of TCA-soluble label from cysts subjected to conditions promoting germination was completely abolished by the inclusion of 1 mM HgCl₂ in the media, and 10 mM KCN inhibited the release of labeled amino acids by ca. 90%. In neither case did the cysts germinate. The addition of 10 mM NaF, 100 µg of chloramphenicol, 10 mM NH₄⁺, and 1 mg of Casamino Acids per ml to the germination media did not appreciably affect the extent of turnover or germination (Table 2).

DISCUSSION

Encystment in Azotobacter vinelandii, like sporulation in Bacillus species, leads to the formation of metabolically dormant cells. Certain similarities in the two differentiation processes and the importance of protein turnover in sporulation prompted us to look at turnover in
A. vinelandii during encystment and germination

Protein turnover has been demonstrated by using a pulse-chase procedure during exponential growth, encystment, and germination phases of the life cycle of A. vinelandii. During exponential growth, the protein turnover rate was 1.4 and 1.8% per h for cells with $t_9$ of 100 and 200 min, respectively. These values compare favorably with turnover rates in Escherichia coli (9) and with the results obtained for other bacteria (1). It is significant that the rate of protein turnover in A. vinelandii is relatively unaffected by growth rate, which is controlled in this organism by the N source rather than by the C source. The physiological rate, percentage of turnover per generation, is therefore directly proportional to the generation time. The respective rates for cells growing at 100 and 200 min per generation were 2.3 and 5.7% protein turnover per generation (Table 1). The maximal cell protein produced by cultures growing in NH$_4^+$ was 1.0 mg/ml of culture, and for N-fixing cells the value was 0.7 mg/ml of culture. In both cases, 70% of the total protein was produced during exponential growth. The extent of turnover during exponential growth at $t_9$ of 100 and 200 min was therefore 1.6 and 4%, respectively, of the total protein produced in the A. vinelandii cultures. This is in sharp contrast to the extent of turnover occurring during encystment when 50% of the protein present is degraded and resynthesized.

The capacity of A. vinelandii to fix N$_2$ is low within an hour after its induction to encystment (3, 12). However, during encystment a number of new proteins are synthesized, including those found in the exine, intine, and the various enzymes involved in gluconeogenesis (3). It would appear that turnover of preexisting protein is the only means by which the cell can supply the necessary amino acids for the requisite synthesis. This turnover appears to be the result of the specific induction of encystment by BHB and not due to a shift down to acetate metabolism per se because only limited turnover occurs when glucose-grown cells are actually shifted to acetate as a carbon source.

The fact that significant turnover was not observed in rifampin-treated cells which were induced to encyst suggests that a new proteolytic enzyme(s) may be synthesized on encystment and that the addition of rifampin blocks transcription of any new mRNAs. The role of rifampin is general since the antibiotic blocks encystment. An analogous situation occurs in sporulation in which the addition of chloramphenicol (an inhibitor of protein synthesis) at the initiation of sporulation blocks turnover (15).

Germination and protein turnover of cysts were characterized by their sensitivity to KCN.

![FIG. 1. Protein turnover and the loss of nitrogenase activity during encystment of A. vinelandii 12837. Cells were labeled with 0.1 μCi of $[^{14}$C]leucine per ml during exponential growth, washed, and induced to encyst in 0.2% BHB over a 120-h period with concurrent chaging with 4 mM leucine. The data presented are the percent TCA-insoluble radioactivity remaining versus the hours of incubation in a BHB-containing medium. Nitrogenase was assayed by acetylene reduction (15). Symbols: ○, percent TCA-insoluble radioactivity remaining; ▲, specific activity of nitrogenase in nanomoles of ethylene produced per microgram of cell protein per hour.](http://jb.asm.org/)

![FIG. 2. Time course for the incorporation of $[^{3}$H]methionine (▲) and $[^{35}$S]SO$_4^{2-}$ (○) into germinating cysts relative to that occurring in cysts which are 8-h postinduction of germination (vegetative cells) (5). Duplicate 1-ml samples were taken at the indicated times, treated with TCA, washed, dried, and counted. The methionine and sulfate uptakes corresponding to relative incorporation of 1.0 are 20 pmol and 11 nmol per $10^6$ cysts, respectively. Also shown is the induction of N-fixing ability in the germinating cysts (●) estimated by acetylene reduction and expressed as a fraction of the specific activity in vegetative cells (0.3 nmol of acetylene reduced per μg of cell protein per h).](http://jb.asm.org/)
This respiratory poison functions in cysts to block energy production which is necessary for the synthesis or expression of the protease. To initiate germination, cysts require a utilizable substrate and aerobic conditions and then respond with immediate \( O_2 \) uptake and synthesis of RNA and proteins (5). This situation is somewhat different from that occurring in Bacillus megaterium spores, which are able to initiate germination anaerobically. Early in the process a specific protease, system I, whose expression is insensitive to KCN, degrades specific basic proteins. The formation of a proteolytic system which appears later in spore germination can, however, be blocked with KCN (13).

The addition of 10 mM NaF to A. vinelandii cysts has almost no effect on germination or protein turnover and suggests that fluoride-sensitive enzymes such as enolase play little role in cyst germination. The energy for germination in these studies is derived from glucose oxidation (5) and not from a stored substrate. High concentrations of 3-phosphoglyceric acid are found in bacterial spores, and its metabolism generates ATP required early in germination for both RNA and protein synthesis (7, 8).

Chloramphenicol at 100 µg/ml of culture blocks protein synthesis in vegetative cells of A. vinelandii, but resistance to the antibiotic is rapidly gained during encystment (3). It would appear that germinating cysts are also resistant to this antibiotic. This may be due to their unique membranes which are almost completely devoid of phospholipids, containing instead \( n \)-alkylresorcinols and \( n \)-alkylpyrones (10). A. vinelandii cysts do not germinate in solutions of Casamino Acids or ammonium ion (5), nor do these compounds enhance germination rate and subsequent protein turnover in the presence of a germinant such as glucose. Since cysts are permeable to the three amino acids used in our turnover and uptake studies, it is likely that all normal amino acids can be taken up during germination. We suggest that amino acids are not metabolized as energy sources by germinating cysts and that protein turnover, even in the presence of 0.1% Casamino Acids, may be due to a need to remove certain cyst-specific proteins from the differentiating cells.

The cysts' lack of initial capacity to incorporate sulfate into acid-insoluble material, presumably protein, is similar to the situation which occurs in bacterial endospores but differs in time scale. In spore germination, amino acid biosynthetic enzyme activities are detected within 20 to 60 min post-initiation of germination (14).

The incorporation of \( ^3 \)H-methionine by germinating cysts was 20 pmol/10^8 cysts based on 15% efficiency for counting tritium. This low value is in sharp contrast to uptake values for sulfate in this study, 11 nmol/10^8 cysts, and for leucine, 10 nmol/10^8 cysts, which had been reported previously (5). The low uptake of methionine can be accounted for as follows. The labeled amino acid was of high specific activity and its resulting low concentration in the germination medium limited uptake. Furthermore, the attenuation of the low-energy \( ^3 \)H electrons within the cysts would reduce further the apparent uptake. However, these considerations do not negate the conclusion that methionine is incorporated into TCA-insoluble cell material almost immediately at the onset of germination, whereas sulfate reduction and incorporation in A. vinelandii only appear after 180 min.

Protein turnover occurs during differentiation in A. vinelandii and supplies amino acids for protein synthesis during times when \( N_2 \) fixation and certain amino acid biosyntheses may be absent. Protein turnover during these times in A. vinelandii and in the bacilli is further evidence of a basic similarity of the differentiation patterns in these two organisms (11). There are, however, differences between the two systems. Encysting A. vinelandii do not secrete proteases as do most sporulating bacilli (1). The bacilli utilize stored 3-phosphoglyceric acid, a glycolytic intermediate, for ATP generation during germination, whereas A. vinelandii does not. It remains to be seen whether the turnover occurring during the germination of A. vinelandii cysts is general or is due to the degradation of unique proteins as is the case in the germination of B. megaterium spores (13).
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LITERATURE CITED