Protein Metabolism during Germination of Bacillus megaterium Spores

II. DEGRADATION OF PRE-EXISTING AND NEWLY SYNTHESIZED PROTEIN*

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SUMMARY

Two distinct proteolytic systems have been detected during germination of Bacillus megaterium spores: one degrading a unique class of dormant spore proteins and the other degrading primarily protein synthesized during germination. Proteolysis of dormant spore protein began by the 3rd min of germination and by 25 min had degraded 15 to 20% of the pre-existing protein to free amino acids. This reaction was not significantly (<20%) different with or without amino acids or a carbon or nitrogen source in the germination medium, or when RNA synthesis, protein synthesis, or energy metabolism were inhibited. Spore coat proteins and most enzymes were not degraded in this process, rather the major substrates were a unique class of low molecular weight (6,000 to 12,000) proteins which were soluble in acetic acid.

Proteins synthesized early in germination (0 to 12 min) were also degraded rapidly (20% per hour). However, proteins synthesized later in germination (90 to 100 min) were degraded more slowly (~4% per hour). At all times tested proteolysis of newly synthesized protein was identical in the presence or absence of amino acids or chloramphenicol in the medium, but was abolished by inhibitors of energy metabolism. Most proteins degraded in this process had molecular weights greater than 12,000 and were insoluble in acetic acid.

Growing cells and different proteins are degraded in growing and stationary phase cells (5, 6). In Escherichia coli the increased proteolysis induced by starvation for a required amino acid is due in part to the accumulation of one or more species of uncharged tRNA (9). Prokaryotes undergoing differentiation also show high rates of protein turnover. During sporulation in Bacillus species the rate of protein turnover is 5 to 10 times that in growing cells (7, 8), and a large number of enzymes are degraded rapidly (10). One of the early events in bacterial sporulation is, in fact, the synthesis of an intracellular protease, and mutants deficient in this protease show little protein turnover and fail to sporulate (11, 12).

Rapid protein degradation has also been observed in prokaryotes and eukaryotes during conversion of a metabolically quiescent or dormant stage of an organism into a more active one. Fertilization of sea urchin eggs and germination of seeds results in greatly increased rates of metabolism and macromolecular synthesis, accompanied by a burst of protein degradation (13, 14). Functions ascribed to this rapid proteolysis include providing amino acids for new protein synthesis and activation of enzymes or enzyme systems.

Spores of Bacillus species represent one of the most quiescent growth stages known for any organism. These dormant spores carry out no detectable macromolecular synthesis and are devoid of endogenous metabolism (15, 16). However, these processes resume during the first minutes of spore germination (17, 18). The preceding communication established that rapid production of amino acids by proteolysis also accompanies germination, and that these amino acids are necessary for early protein synthesis (19). This communication describes in detail some of the various proteolytic reactions accompanying germination.

MATERIALS AND METHODS

Sources of Reagents and Spores—Sources of tritium-labeled amino acids, [α-32P]ATP, tRNA-C*C, [14C]cyanate-labeled hemoglobin, and Bacillus megaterium tRNA have been described previously (20, 21). Sources of other labeled and unlabeled compounds were described in the preceding paper (19). All work was carried out with B. megaterium QM B1551, and growth, harvesting, characterization, and storage of unlabeled spores and [14C]leucine-synthesizing spores was also as described previously (10). Spores labeled with 35S were prepared in an identical manner with addition of 1 mCi of [35S]sulfate per 100 ml of growth medium.

Spore Germination—Standard conditions for spore germination were as follows unless otherwise noted. Dry spores (20 mg per ml in water) were heated for 10 min at 60° and then cooled. Germin-

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nation was at 30° at a spore concentration of 500 μg per ml in the complete medium of Spizizen which contains glucose, citrate, ammonium sulfate, and salts (22). Some experiments also utilized a glucose medium: 50 mM KPO₄ (pH 7.4), 100 mM glucose; or a KBr medium: 50 mM KPO₄ (pH 7.4), 50 mM KBr. In all media germination was >95% complete in 15 min as seen in the phase-contrast microscope, and the rate of initiation of germination was not affected significantly by chloramphenicol, actinomycin D, or NaN₃ plus KCN.

Preparation and Assay of Spore Extracts—Extracts of dormant spores (10 to 20 mg per ml) were prepared by lysozyme treatment (19) for 10 min at 37° in 50 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride after pre-treatment of spores with 8 mM urea-10% β-mercaptoethanol at pH 5.0 as pre-described (10). Extracts of germinated spores were prepared similarly after pretreated spores had germinated for 40 min (germination >90%) under standard conditions plus 1 mM alanine. The alanine is necessary for rapid germination of pretreated spores.1 Tryptases were sonicated briefly to reduce the viscosity and a portion was analyzed for RNA (see below). The extract was centrifuged (10 min, 15,000 × g) and 25 μl of [³⁵S]hemoglobin (5 mg per ml, 10⁶ cpm per ml) added to the supernatant fluid which was then desalted on a small column (25 × 1.0 cm) of Sephadex G-25 coarse equilibrated in 0.1 M KCl, 10 mM Tris-HCl (pH 7.4), and 0.2 mM phenylmethylsulfonyl fluoride. Peak fractions were pooled and the dilution of the extract on the column determined from the concentration of [³⁵S]hemoglobin. The absence of degradation of a number of proteins added to dormant spores just prior to lysozyme rupture indicated that no significant proteolysis occurred during spore lysis, and this was further suggested by the absence of free amino acid production during lysis.2

In several experiments it was essential to know the exact amount of dormant or germinated spores in extracts. Since there is no detectable change in RNA level through 50 min of germination in the presence or absence of chloramphenicol (22), RNA determinations were used to normalize data from different extracts to the same spore concentration. Generally this correction was small (±20%).

Isoenzyme-1 tRNA synthetase was assayed as described by Vold (24), tRNA nucleotidyltransferase according to the procedure of Deutscher (25), and endoase according to the method of Witt and Witz (26). Other assays were described in the preceding paper (19).

Protein Level in Spores—Samples of spores were centrifuged (2 min, 15,000 × g) and the pellet washed with 5 ml of 5% trichloroacetic acid. The pellet was then suspended in 2 ml of 5% trichloroacetic acid, heated at 90° for 15 min as previously described (8), and the protein then determined by the Lowry procedure (27) with bovine serum albumin as the standard. For quantitative amino acid analysis of spore proteins dormant spores (10 mg dry weight per ml) were heated at 60° as described above, and split into two fractions. One fraction (2 ml) was analyzed for RNA and then acid-hydrolyzed, and the other (5 ml) was germinated under standard conditions in the glucose medium plus chloramphenicol (100 μg per ml). After 40 min the spores were centrifuged, washed twice with 15 ml of H₂O, suspended in 1 ml of H₂O, analyzed for RNA, and then acid-hydrolyzed. The acid hydrolysates were subjected to amino acid analysis as described previously (19).

Measurement of Proteolysis—Degradation of dormant spore protein was assessed by measuring the decay of radioactive [³⁵S]leucine-labeled spores from an acid-insoluble form to an acid-soluble form as described previously (10). Unlabeled leucine (2.5 mM) was always added to the germination medium to prevent reutilization of the leucine generated by proteolysis. Solubilization of radioactivity from [³⁵S]-labeled spores was measured similarly with cystine (0.5 mM) and methionine (0.5 mM) to the medium. All data have been corrected for the presence of a small amount (1 to 3%) of trichloroacetic acid-soluble radioactivity in dormant spores.

Degradation of protein synthesized during germination was measured following a short (10 min) pulse of [³⁵S]leucine (3 nm, 20 to 70 Ci per mmol). The labeled spores (5 ml) were passed through a membrane filter (2.4 cm, 0.22 μm) and washed with 4 ml of warm (30°) medium containing 1 mM isoleucine. The washed spores were then suspended in 5 ml of prewarmed medium consisting of 2.5 mM isoleucine in the germination exudate from an unlabeled culture centrifuged at the time the labeled cells were filtered. Samples were then taken and analyzed for acid-insoluble and -soluble radioactivity as described previously (10). The amount of degradation in the hour following the pulse was determined from measurements of acid-soluble radioactivity at 0, 10, 20, 30, and 60 min. The pulse, and averaging the results of two or more experiments. Initial rates of degradation were determined similarly from values at 0, 10, and 20 min, and these rates were relatively linear. Values were corrected for acid-soluble radioactivity present at zero time.

In one experiment the labeled amino acid was removed by passage through a Sephadex column. Spores were germinated under standard conditions at 1 mg per ml, pulse-labeled from 0 to 12 min, and an aliquot (2 ml) passed through a column (25 × 1.0 cm) of Sephadex G-25 (coarse) equilibrated at room temperature in 12-min germination exudate supplemented with 2 mM isoleucine. Spores were eluted in 4 ml after 4 min, and sampling for acid-soluble and -insoluble radioactivity was begun.

RESULTS

Degradation of Dormant Spore Protein during Germination

Magnitude of Proteolysis—Although protein synthesis begins by the 3rd min of germination of B. megaterium spores (19), the protein content of the spore as measured by the Lowry procedure decreased about 20% during the first 15 min of germination (Fig. 1). This rapid decrease in protein was followed by a slow increase, with the original protein level of the dormant spore being attained at about 80 min, with the first cell division taking place between 180 to 200 min.3

The loss of spore protein after 40 min of germination was measured by a variety of procedures (Table I) and ranged from a high of 23% (Lowry) to a low of 6.8% (¹⁴C in protein). Probably the most accurate value was that obtained by quantitative amino acid analysis of dormant and germinated spore protein, which was only slightly higher than that obtained from analysis of the amount of free amino acid released in germination (Table I) (19).

The apparent discrepancy in the value obtained by measuring the decrease in protein content was due to unequal distribution of sulfur in spore proteins (see below).

Kinetics and Identity of Proteolytic Products—Although the fall in spore protein content appeared slower than the rapid decrease in absorbance which accompanies initiation of germination, accurate kinetic measurements were difficult to perform by the Lowry procedure. Therefore, further studies measured conversion of [³⁵S]leucine in spore protein into a form which was soluble in trichloroacetic acid (Fig. 1). The close correlation between the fall in spore protein content and the rapid solubilization of [³⁵S]leucine in protein (Fig. 1) suggested that these events were indeed the result of rapid proteolysis accompanying germination.

Examination of the kinetics of [³⁵S]leucine solubilization at early times in germination (Fig. 1) also revealed that production of acid-soluble material was slightly slower than the fall in absorbance. Identical results were obtained in this experiment when

1 P. Setlow, unpublished results.
had only a slight inhibitory effect (17). Proteolysis was also abolished by HgCl₂ which is known to prevent spore germination. 

Similarly, abolishing AT₁ production by addition of KCN and NaF but was unaffected by phenylmethylsulfonyl fluoride and EDTA but was abolished by HgCl₂ which is known to prevent spore germination (18). The magnitude and time course of proteolysis were also unaffected by phenylmethylsulfonyl fluoride and 1-OAT but was present as free leucine through the first 40 min (Fig. 1), and the ble radioactivity released in this experiment showed that >93% of radioactivity from 36S-labeled spores was as described under "Materials and Methods." 

**Table I**

**Amount of protein degraded during germination**

Protein determination by amino acid analysis or solubilization of radioactive activity from ³⁵S-labeled spores was as described under "Materials and Methods." In a parallel culture, samples (40 ml) were centrifuged and protein determined as outlined under "Materials and Methods." 

<table>
<thead>
<tr>
<th>Method of protein determination</th>
<th>Protein degraded after 40 min of germination</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowry</td>
<td></td>
<td>23a</td>
</tr>
<tr>
<td>[³H]Leucine in protein</td>
<td></td>
<td>18b</td>
</tr>
<tr>
<td>³⁵S in protein</td>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>Quantitative amino acid analysis</td>
<td></td>
<td>15c</td>
</tr>
<tr>
<td>Quantitative analysis of amino acids released in germination</td>
<td></td>
<td>15c</td>
</tr>
</tbody>
</table>

a Calculated from data in Fig. 1.

b Calculated from data in Fig. 2.

c Calculated from data of Setlow and Primus (19).

spores were extracted with trichloroacetic acid or boiling 80% propanol-1 which is known to extract small molecules from both dormant and germinated spores (17). Analysis of the acid-soluble radioactivity released in this experiment showed that >93% was present as free leucine through the first 40 min (Fig. 1), and that there was no indication of any intermediate degradation products.

**Effect of Inhibitors and Different Media on Proteolysis**—The rapid proteolysis of dormant spore protein was not inhibited by actinomycin D or chloramphenicol, suggesting that the proteolytic system pre-existed in the dormant spore (Table II). Similarly, abolishing ATP production by addition of KCN and NaF had only a slight inhibitory effect (17). Proteolysis was also unaffected by phenylmethylsulfonyl fluoride and EDTA but was abolished by HgCl₂ which is known to prevent spore germination (18). The magnitude and time course of proteolysis were also identical in the complete medium with or without all 20 amino acids (Table II), and there was no effect of removal of a nitrogen source, a carbon source, or both from the germination medium.

**Spore Coat Proteins and Most Enzymes Are Not Degraded**—The large amount of rapid proteolysis accompanying germination suggested that dormant spores might contain a large amount of unique, labile proteins. Spore coat proteins initially seemed likely candidates for those degraded in germination because they comprise ~50% of the total spore protein and the proteolytic products are found outside the spore (19, 31, 32). However, spores from which some coat proteins were removed by treatment with urea-sodium dodecyl sulfate (32) showed the same amount of proteolysis as untreated spores (Table III).

In addition to the urea-sodium dodecyl sulfate proteins, spore coats also contain other proteins which are not removed by urea-sodium dodecyl sulfate. This protein fraction comprises 30 to 40% of total spore protein, is extremely insoluble and is rich in cystine (31, 32). The constant amount of the insoluble protein fraction in both dormant and germinated spores suggested that these coat proteins were also not degraded in germination (Table III). This was further suggested by the 3-fold higher degradation of protein-bound leucine compared to protein-bound sulfur (Table III); presumably most of the sulfur is present in the insoluble, cystine-rich coat proteins.

Assay of seven different dormant spore enzymes also showed that enzymes were not degraded indiscriminantly (Table IV). After 40 min of germination in the presence of chloramphenicol which prevented new protein synthesis, only one enzyme, TRNA nucleotidyltransferase, showed decreased activity. It should be emphasized that extracts for assay of these enzymes were prepared under conditions which blocked proteolysis (see "Materials and Methods").
TABLE III

Distribution of labeled spore proteins in various fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[3H]Leucine-labeled</th>
<th>35S-labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stripped off by urea-sodium dodecyl sulfate</td>
<td>Dormant</td>
<td>Germinated</td>
</tr>
<tr>
<td>Solubilized on germination</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Insoluble</td>
<td>26 (28)</td>
<td>25 (27)</td>
</tr>
<tr>
<td>Soluble but acetic acid-insoluble</td>
<td>31 (55)</td>
<td>26 (50)</td>
</tr>
<tr>
<td>Acetic acid-soluble</td>
<td>16 (17)</td>
<td>4 (17)</td>
</tr>
</tbody>
</table>

TABLE IV

Absence of degradation of most enzymes during germination

Extracts of dormant and germinated spores were prepared, standardized, treated, and assayed as described under "Materials and Methods." Values in parentheses give the range found in different experiments.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity in dormant spore</th>
<th>(Specific activity in germinated spore/specific activity in dormant spore) × 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[μmol/min/g dry spore]</td>
<td></td>
</tr>
<tr>
<td>Alanine dehydrogenase</td>
<td>10.6</td>
<td>101 (99-102)</td>
</tr>
<tr>
<td>Arginase</td>
<td>16.4</td>
<td>101 (101-106)</td>
</tr>
<tr>
<td>Arginine decarboxylase</td>
<td>0.17</td>
<td>109 (87-146)</td>
</tr>
<tr>
<td>Enolase</td>
<td>147</td>
<td>95 (92-97)</td>
</tr>
<tr>
<td>Isoleucyl-tRNA synthetase</td>
<td>0.052</td>
<td>103 (97-108)</td>
</tr>
<tr>
<td>Leucine dehydrogenase</td>
<td>4.9</td>
<td>100 (96-103)</td>
</tr>
<tr>
<td>tRNA nucleotidyltransferase</td>
<td>0.000</td>
<td>45 (43-47)</td>
</tr>
</tbody>
</table>

Nature of Proteins Degraded—The nature of the proteins degraded in germination was established by rupture and subsequent fractionation of spore proteins into insoluble, soluble, and acetic acid-soluble fractions. The only fraction of 35S-labeled or [3H]leucine-labeled dormant spore protein which showed a large decrease on germination was the acetic acid-soluble fraction, although the soluble but acetic acid-insoluble protein fraction also decreased slightly (Table III). Similar results were obtained when spores were opened either by dry rupture, or by lysozyme following urea-sodium dodecyl sulfate treatment (Table III). A large amount of acetic acid-soluble protein was also observed in dormant spores when dry-ruptured spores were extracted immediately with acetic acid.

Size of Protein Degraded—The acid solubility of much of the protein (or proteins) degraded in germination suggested that it might be of low molecular weight. Sephadex G-50 gel filtration of soluble proteins extracted by dry rupture revealed a significant amount of low molecular weight protein present in dormant spores but absent from germinated spores (Fig. 2, note semilog scale). The excess low molecular weight material in the dormant spore amount to ~21% of total spore protein. Similar results were obtained if soluble proteins were prepared from urea-sodium dodecyl sulfate-treated spores using lysozyme rupture and then run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). In this case the low molecular weight protein was again ~21% of total spore protein. In both experiments the molecular weight of the dormant spore protein which disappeared on germination was between that of insulin and lysozyme, or between 6,000 and 12,000.

Although it is clear that the majority of the dormant spore pro-
nucleotidyltransferase during germination. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of proteins from dormant and germinated spores. [3H]-Leucine-labeled spores (25 mg per ml, 4 x 10⁴ cpm per ml) were treated with urea-sodium dodecyl sulfate, washed, suspended in 3 ml of 50 mM Tris- HCl (pH 8.0), 10 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and split in two fractions. One fraction (1.5 ml) was left in ice while the remainder (1.5 ml) was germinated under standard conditions with addition of alanine (1 mM) and chloramphenicol (100 µg per ml). After 40 min the spores were centrifuged, washed once with water, and suspended in 1.5 ml of 50 mM Tris- HCl (pH 8.0), 10 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride. Lysozyme (~1.7 mg) was then added to both samples, and after 10 min at 37° the solutions were chilled, sonicated briefly, and then centrifuged (10 min, 15,000 X g) after addition of sodium dodecyl sulfate polyacrylamide gels (10 ma per gel) with a pyronin Y dye marker. After completion of the run a 1-cm plug of 10% acrylamide gel was collected, and 0.5 ml of 10% sodium dodecyl sulfate added to each sample, which was then counted in Triton-toluene scintillation fluid. Counts have been corrected for relative spore concentration as described under “Materials and Methods.”

![Table V](https://example.com/tablev.png)

**Table V**

Degradation of protein synthesized during germination

Spores were germinated under standard conditions, pulse-labeled, washed, resuspended, and protein degradation determined as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>Amino acid used</th>
<th>Time in germination when amino acid pulse given</th>
<th>Protein degraded in % following pulse</th>
<th>Initial rate of protein degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine (3 nM)</td>
<td>2-12</td>
<td>23</td>
<td>51</td>
</tr>
<tr>
<td>Isoleucine (20 µM)</td>
<td>2-12</td>
<td>25</td>
<td>57</td>
</tr>
<tr>
<td>Leucine (60 µM)</td>
<td>2-12</td>
<td>21</td>
<td>47</td>
</tr>
<tr>
<td>Tyrosine (10 µM)</td>
<td>2-12</td>
<td>20</td>
<td>46</td>
</tr>
<tr>
<td>Isoleucine (3 nM)</td>
<td>20-30</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>Isoleucine (3 nM)</td>
<td>50-60</td>
<td>8.7</td>
<td>17.7</td>
</tr>
<tr>
<td>Isoleucine (3 nM)</td>
<td>90-100</td>
<td>5</td>
<td>10.8</td>
</tr>
<tr>
<td>Isoleucine (3 nM)</td>
<td>150-160</td>
<td>4</td>
<td>8.7</td>
</tr>
<tr>
<td>Isoleucine (3 nM)</td>
<td>log phase cells (10 min pulse)</td>
<td>4</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Proteins degraded during germination are of low molecular weight, it is possible that small amounts of larger proteins are also degraded as was suggested by the decrease in activity of tRNA nucleotidyltransferase during germination. Sodium dodecyl sulfate polyacrylamide gel electrophoresis clearly showed that there was no massive degradation of large proteins (>12,000 mol wt) (Fig. 3); however, loss of a small amount (~10%) of large protein would probably not have been detected using this procedure.

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Kinetics of degradation of protein synthesized during germination. Spores were germinated under standard conditions and procedures for pulse-labeling, washing, and determination of protein degradation are given under “Materials and Methods.” All data points are the average of five separate experiments.

**Degradation of Protein Synthesized in Germination**

**Magnitude and Kinetics of Degradation**—In addition to the degradation of dormant spore protein during germination, rapid degradation of some enzymes synthesized in germination has also been suggested (33). Protein synthesized early in germination does turn over rapidly with 20 to 25% of the protein synthesized from 0 to 12 min degraded in the following hour (Table V). However, the rate of turnover of proteins synthesized later (90 to 100 min) in germination was ~5-fold lower, and was similar to the rate of turnover of proteins synthesized in logarithmic growth (Table V). The 5-fold difference in the rates of turnover of proteins synthesized early and late in germination was observed comparing either initial rates of degradation or the amount of degradation per hour (Table V). It should be pointed out that since the rate of protein synthesis is constant from 3 to 100 min of germination (19), the amount of protein synthesized in a 10-min period is the same both early and late in germination.

The kinetic data of proteolysis were similar at all pulse-labeling times tested, with turnover being most rapid in the 10 to 20 min following the pulse and then gradually slowing to a constant rate of 3 to 4% per hour by 50 min (Fig. 4). The initial bursts of proteolysis observed in this experiment suggest that the true rates of proteolysis may actually be higher than the values observed due to the rather long pulse time (10 min) used.

**Rapid Degradation of Newly Synthesized Protein Is not Artifact**—It was essential to prove that rapid turnover of proteins synthesized early in germination was not an artifact, since proteolysis in *E. coli* can be stimulated by collecting cells on a filter (3). However, this was not the case in germinating spores, since several modifications in the filtering and washing procedure had no significant effect on the data. Proteolysis was unchanged if: (a) different amino acids or different concentrations of isoleucine were used in the pulse label (Table V); (b) the filter was not boiled in water; (c) the filtering time was reduced 2-fold (from 100 to 50 s) by use of a larger filter; (d) the filtering procedure was not used, but protein synthesis was stopped by addition of chloramphenicol and proteolysis measured as the decrease in...
TABLE VI
Effect of several compounds on degradation of protein synthesized during germination

Spores were germinated under standard conditions, pulse-labeled, washed, and resuspended in the standard medium with changes as noted. Proteolysis was determined as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>Alteration in chasing medium</th>
<th>Protein degraded in hour following pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-12 min version 150-160 min version</td>
</tr>
<tr>
<td>None</td>
<td>23</td>
</tr>
<tr>
<td>+ Chloramphenicol (100 µg/ml)</td>
<td>19</td>
</tr>
<tr>
<td>+ NaF (10 mM)</td>
<td>24</td>
</tr>
<tr>
<td>+ KCN (10 mM) or KCN (10 mM) + NaF (10 mM)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>+ Dinitrophenol (100 µM)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>+ All 20 amino acids (100 µM each)</td>
<td>19</td>
</tr>
<tr>
<td>Glucose medium plus isoleucine instead of germination oxidate</td>
<td>21</td>
</tr>
<tr>
<td>+ Phenyldimethylsulfonyl fluoride (0.2 mM)</td>
<td>24</td>
</tr>
<tr>
<td>Without isoleucine chase</td>
<td>6</td>
</tr>
</tbody>
</table>

acid-insoluble radioactivity; or (c) the labeled amino acids were removed by gel filtration on Sephadex chromatography. It should be noted that in the latter experiment the spores were never collected on a filter and were exposed constantly to fresh oxygenated media as they moved through the column.

Degradation Products and Effect of Inhibitors—Since turnover of newly synthesized protein occurred concomitantly with degradation of dormant spore protein, it was of interest to compare these two processes. A number of similarities were observed: (a) the degradation products of newly synthesized protein were free amino acids, since the products were re-incorporated into acid-insoluble material in the absence of excess unlabeled amino acid (Table VI); and (b) degradation of newly synthesized protein was not affected by substitution of the glucose medium for the complete medium or by addition of amino acids, chloramphenicol, NaF, or phenylmethyisulfonyl fluoride (Table VI). However, in contrast to proteolysis of most dormant spore protein, degradation of newly synthesized protein was abolished (>85%) by inhibitors of energy metabolism (Table VI). All compounds tested had similar effects on the turnover of proteins synthesized in the first 12 min of germination or between 150 and 160 min when cells had entered log phase growth (Table VI).

Size of Protein Degraded—The difference in requirements for metabolic energy between proteolysis of newly synthesized and pre-existing protein suggested that there might also be a difference in the protein (or proteins) degraded in the two reactions. Indeed, although the protein synthesized from 2 to 12 min was >85% soluble, it was >92% insoluble in acetic acid before and after a 60-min chase (data not shown); sodium dodecyl sulfate acrylamide gel electrophoresis also revealed no large amount of low molecular weight (<12,000) protein (Fig. 5). Similarly, sodium dodecyl sulfate acrylamide gel electrophoresis of identical amounts of radioactive newly synthesized protein isolated before or after a 80-min chase showed that low molecular weight newly synthesized proteins were not degraded preferentially, although it is apparent that some newly synthesized proteins are degraded more extensively than others (Fig. 5).

DISCUSSION

Protein degradation during spore germination appears to be catalyzed by a minimum of two distinct systems which I have termed Systems I and II. System I rapidly degrades a unique class of acid-soluble, low molecular weight dormant spore proteins, and this process is relatively insensitive to inhibition of energy metabolism. In contrast, System II degrades primarily higher molecular weight (>12,000) proteins and shows a requirement for active energy metabolism. These properties of System II, and its resistance to inhibition by chloramphenicol are similar to those of the system responsible for the rapid turnover that occurs during sporulation in Bacillus species (8, 34).

Although System II has been characterized only by its ability to degrade proteins synthesized during germination, this does not imply that some dormant spore proteins are not attacked by System II. Indeed the decrease in activity of tRNA-nucleotidyltransferase, and the slight inhibition (~20%) by KCN of degradation of dormant spore protein during germination may reflect the action of System II. The absence of a large amount of degradation of dormant spore proteins by System II is probably due to the small amount of dormant spore protein available for attack. Presumably coat proteins can not be attacked by System II, and this leaves only the soluble but acid-insoluble proteins which make up only 30% of total spore protein. Degradation of 10 to 20% of this protein fraction by System II would be small compared to the degradation of 15 to 20% of total spore protein by System I.

The work described in this communication has differentiated Systems I and II only on the basis of their substrates and energy requirements. Clearly, the next step is the characterization of the enzymes involved in the two systems and the determination whether the enzymes for each system are distinct or if some are common to both. It is also possible that analysis of the enzymes involved in the two systems may provide information on the
energy requirement seen for System II. Although such a requirement has been observed previously for intracellular proteolysis in both prokaryotes and eukaryotes, it is not fully understood.

Knowledge of the enzymes involved in System II might also aid in explaining the rapid degradation of the first proteins synthesized during germination. For instance, it is possible that there is some decrease in the intrinsic activity of System II during germination. However, it is also possible that the first proteins synthesized in germination might be very susceptible to System II; indeed it is known that different proteins are made at early and late times in germination (36). In contrast to the uncertainty concerning the rapid action of System II early in germination, a major cause of the rapid action of System I is the presence of a large amount of labile protein in dormant spores. Furthermore, the exhaustion of the pool of this protein explains the rapid decrease in the rate of degradation of dormant spore protein after 20 min of germination.

The identification of a large pool of labile low molecular weight protein in dormant spores is certainly one of the more significant findings of this study. Work described in the previous paper indicated that one function of this labile protein is to provide amino acids during germination. However, it is very tempting to speculate that this protein may also serve some other function, possibly in the maintenance of spore dormancy. Clearly, the rapid degradation of such protein would be a simple way to catalyze the breaking of dormancy and the initiation of spore germination.

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