Identification and Localization of the Major Proteins Degraded during Germination of Bacillus megaterium Spores*

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PETER SETLOW

From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032

Of the 15 to 20% of total spore protein which is degraded during germination of spores of Bacillus megaterium, >80% is derived from a protein fraction soluble in dilute acids such as acetic acid that contains two major proteins, termed A and B. These two proteins are low or absent in germinated spores, log phase cells, and early stationary phase cells, but both are synthesized and appear in parallel late in sporulation after the appearance of nonrefractile forespores but before the appearance of refractile spores. These proteins accumulate during the time that the developing spor forming is resistant to ultraviolet light but well before acquisition of heat resistance. Both proteins are located in the core of the dormant spore, but analysis of ruptured spores has revealed no binding of protein A or B to ribosomes, membranes, or DNA.

In a number of different organisms the breaking of dormancy or the activation of metabolically quiescent stages of growth has been shown to be accompanied by rapid proteolysis. The fertilization of sea urchin eggs (1), seed germination (2), and fungal spore germination (3) are all accompanied by a burst of protein degradation. Major functions which have been ascribed to this proteolysis include activation of enzymes or enzyme systems (1) and generation of amino acids for protein synthesis by the developing organism (2, 3).

Spores of Bacillus species are one of the most quiescent stages of growth known, since these bacterial spores carry out no detectable energy metabolism or macromolecular biosynthesis (4-6). However, these dormant spores can be converted into a growing cell via the process of spore germination, and within minutes after germination has been initiated, energy metabolism and RNA and protein synthesis begin (6-8). As was described above for activation of other quiescent systems, spore germination in Bacillus species is also accompanied by rapid protein degradation (9). During the first 20 min of germination of Bacillus megaterium spores 15 to 20% of the dormant spore protein is degraded to free amino acids (9). These amino acids generated by proteolysis are essential for protein synthesis early in germination, since biosynthesis of a number of amino acids is absent at this time due to the absence of biosynthetic enzymes (8). Interestingly, the majority of the dormant spore proteins degraded during germination appear to be of low molecular weight and possibly located in the spore core (9). A preliminary report has also indicated that only a few proteins are degraded during germination and that these species may be unique to the dormant spore (10). This communication describes in detail the identification of the major proteins in dormant spores of B. megaterium which are degraded during spore germination, the synthesis and appearance of these proteins during sporulation, and the location of these proteins in the dormant spore. The accompanying paper describes the purification and physical and chemical properties of these unique dormant spore proteins (11).

MATERIALS AND METHODS

Sources of Chemicals and Spores—L-[2,3-3H]Valine, L-[G-3H]serine, [methyl-3H]thymidine, and [35S]sulfate were purchased from New England Nuclear; chloramphenicol, chymotrypsin, chymotrypsigen, insulin, lysozyme, pepsin, and trypsin were purchased from Sigma; and RNAse was obtained from Worthington Biochemica.

Unless otherwise noted, all work was carried out with Bacillus megaterium QM B1551, ATCC number 12872, which was originally obtained from Dr. Hillel Levinson (United States Army Natick Laboratories, Natick, Mass.). Bacillus cereus T was originally obtained from Dr. Harlyn Halvorson (Brandeis University, Waltham, Mass.). Unless otherwise noted, cells were grown and spores were prepared at 30º in supplemented nutrient broth, harvested, washed, and stored as previously described (12). A few cases spores were prepared in either the minimal medium of Spizizen (13) supplemented with 0.1% Casamino acids or in the sucrose-salts medium described by Slepecky and Foster (14). All spore preparations were >96% refractile and were free of vegetative cells and cell debris as observed in the phase contrast microscope. Spores labeled in protein with 35S or in DNA with [3H]thymidine were prepared in a similar manner as previously described (9, 15).

Spores containing protein labeled with [3H]valine were prepared and harvested in an identical manner, except that the growth medium contained 2 mM [3H]valine (2.5 µCi/ml) from early log phase. Samples of the growth medium were taken at the time of spore harvest and analyzed by paper chromatography (see below). Approximately 55% of the label remained in valine at this time, suggesting that all spore proteins were probably labeled. The label in the dormant spores was <1% free valine as determined by paper chromatography, although chromatography of acid hydrolysates (6 N HCl, 24 hours, 105º) of labeled spores showed that ~95% of the label co-chromatographed with

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valine. Approximately 3\% of the label in these spores was in some low molecular weight compound (included on Sephadex G-10) which was not valine.

**Spor Germination**—Standard conditions for germination were as follows unless otherwise noted. Dormant spores (20 mg dry weight/ml) were heated in water for 10 min at 60°C and then cooled. Germination was carried out at 30°C at a spore concentration of 500 μg dry weight/ml and unless otherwise noted was in the complete nutrient medium (19) without added Caaamino acids. Under these conditions initiation of germination was >95\% complete in 15 min as judged in the phase contrast microscope, and addition of chloramphenicol did not affect the rate of initiation of germination.

**Measurement of Solubilization of Radioactivity from \[^{3}H\]Valine-labeled Spores**—\[^{3}H\]Valine-labeled spores were germinated under standard conditions with 2.5 mM valine in the germination medium to prevent pelletization of \[^{3}H\]valine produced by proteolysis (9). Samples (0.6 ml) were added to 0.1 ml of 35\% trichloroacetic acid and after 30 min at 4°C samples were centrifuged and 0.5 ml of the supernatant fluid counted in 5 ml of a toluene scintillation fluid containing 33\% Triton X-100. The percentage of radioactivity solubilized was determined by counting a sample from the same culture prior to centrifugation but after rupture of spores with lysozyme (9) to eliminate quenching of \[^{3}H\]valine by proteins. The data were corrected for the small amount of acid-soluble radioactivity present in dormant spores. Unless otherwise noted values for the percentage of total protein degraded were determined after 40 min of germination.

**Extraction of Coat Proteins from Dormant Spores**—Three different procedures, designated procedures 1, 2, and 3, were used to extract coat proteins from dormant spores. In procedure 1, spores were extracted with 2 M urea and 0.1 M sodium dodecyl sulfate (9) at 37°C for 90 min as previously described (17) and washed five times with 50 mM Tris-HCl (pH 8.0). In procedure 2, spores were extracted with 2 M urea, 1.0\% sodium dodecyl sulfat, 0.1 M 2-mercaptoethanol, and 30 mM Tris-HCl (pH 8.0) for 80 min at 24°C as previously described (17). The treated spores were then washed twice with 4 M urea and four times with 50 mM Tris-HCl (pH 8.0). In procedure 3, spores were extracted with 0.1 M NaCl, 0.5\% sodium dodecyl sulfate, and 0.1 M dithiothreitol (pH 11.0) at 37°C for 120 min as described by Vary (18). The treated spores were then washed twice with 4 M urea and four times with 50 mM Tris-HCl (pH 8.0).

**Isolation of Acetic Acid-soluble Fraction from Cells or Spores**—Lyophilized cells or spores (50 to 100 mg dry weight) were dry ruptured in a dental amalgamator (Wig-L-Bug) with glass beads (~100 mg dry weight) as the abrasive as described by Sacks and Bailey (19). In a few cases glucose crystals were used as the abrasive (12). Ten 1-min periods of shaking in the amalgamator with cooling in between sufficed to rupture >90\% of all spores or cells. The dry powder was then suspended in 4 ml of cold 3\% acetic acid, incubated at 4°C for 30 min, and centrifuged (10 min, 15,000 g). The pellet was re-extracted with an additional 3 ml of 3\% acetic acid, and both supernatant fluids were pooled and dialyzed in acetylated tubing for 36 hours at 4°C against two 1-liter changes of 1\% acetic acid. The dialyzed sample was then lyophilized and the dry residue dissolved in 1 to 2 ml of water prior to analysis. In a few cases the acetic acid-soluble fraction was further purified by passage through a column of DEAE-cellulose equilibrated in 1\% acetic acid. There was no significant loss of protein from the acetic acid-soluble fraction of either spores or cells going in the pooled supernatant fluid to the final solution, even when the DEAE pass-through step was used (11).

**Electrophoretic Procedures**—Electrophoresis on sodium dodecyl sulfate acrylamide gels (10%) was carried out as described by Weber and Osborn (20) using a pyronin Y dye marker. Disc gel electrophoresis was carried out with 7.5\% acrylamide gels using the acid buffer system of Reisfeld et al. (21) and bromocresol green as a dye marker. Gels were stained with Coomassie blue, and the relative intensities of protein bands determined as described previously (10). When gels were to be analyzed for radioactivity, the runs were stopped when the dye marker was ~1 to 3 cm from the bottom of the gel and a 1 cm plug of 10\% acrylamide gel was cut out of the top of the gel. From an aliquot (2 mM) of the gel the protein were collected, and 0.5 ml of 10\% sodium dodecyl sulfate added to each sample which was counted in Triton-toluene scintillation fluid.

**Measurement of Morphological Changes and Resistance to Heat and Ultraviolet Light**—The appearance of nonrefracrere forespores or retractive spores was quantitated by scoring approximately 100 cells in the phase contrast microscope. The appearance of heat-resistant forms during sporulation was measured by diluting samples of a sporulating culture 1/100 in sterile water and heating for 20 min at 65°C. After cooling in ice samples of the heated culture were spread on nutrient agar plates, incubated overnight (12 to 14 hours), and colonies counted. Log phase cells and early sporulating cells were killed by this heat treatment (<0.001% survival) while suspensions of dormant spores showed no loss in viable count.

Resistance to ultraviolet light was determined in a similar fashion. Samples of sporulating cultures were diluted 1/100 in sterile water and 3-ml samples were placed in a glass Petri dish (9 cm in diameter). Irradiation was carried out for 1 min with a short wavelength ultraviolet lamp (model UV5-11, Ultraviolet Products Inc., Sunland, California) at a height of 5 cm with vigorous stirring. Samples of the irradiated culture were spread on nutrient agar plates and analyzed as described above. The irradiation procedure killed >99.9\% of log phase cells and early sporulating cells, but spores showed only a 20 to 30% loss in viability.

**β Attenuation Analysis**—β Attenuation analysis was carried out using minor modifications of the procedure of Leanz and Gilvarg (16). Labeled spores were incubated in water at 4°C or in germination medium at 30°C. After 30 min the spores in germination medium were ruptured by addition of lysozyme (1 mg/25 mg dry weight of spores). Identical amounts of intact dormant spores and ruptured germinated spores were then counted in Triton-toluene scintillation fluid, and the β attenuation calculated as 100\% x (1 minus the ratio of counts in whole spores over counts in disrupted spores).

**Labeling of Sporulating Cells and Analysis of Spores Produced**—Two different procedures were used for labeling sporulating cells. In one procedure cells were grown in supplemented nutrient broth, and at various times in sporulation \[^{3}H\]valine (2 mM, 10 mCi/mmol) was added to 25 ml of culture. These cultures were allowed to sporulate, and the spores harvested, washed, lyophilized, and the specific radioactivity determined on a water suspension of each spore sample as counts per min per 1.0 optical density unit at 600 nm. The percentage of total dormant spore protein synthesized by the time (t) of \[^{3}H\]valine addition was calculated as:

\[
\frac{1 - \left( \frac{\text{Specific radioactivity of spores labeled at time } t}{\text{Maximum specific radioactivity of spores}} \right) x 100\%}{\text{Fraction of label in the acetic acid-soluble fraction of uniformly labeled spores}}
\]

The value for maximum specific radioactivity was obtained from spores from cultures labeled at the end of log phase growth which is prior to synthesis of spore proteins (22). The dry spores obtained in this experiment were further analyzed by dry rupture and preparation and quantitation of the acetic acid-soluble fraction as described above. Since analysis on disc gel electrophoresis at low pH revealed that for all samples tested >90\% of the radioactivity in the acetic acid-soluble fraction was in proteins A and R, the percentage of the maximum amount of proteins A and R synthesized prior to the time (t) of \[^{3}H\]valine addition was calculated as:

\[
100\% \times \frac{1 - \left( \frac{\text{Fraction of total spore protein synthesized after time } t}{\text{Fraction of label in the acetic acid-soluble fraction of spores labeled at time } t} \right) x \text{Fraction of protein in the acetic acid-soluble fraction of uniformly labeled spores}}}{\text{Fraction of label in the acetic acid-soluble fraction of uniformly labeled spores}}
\]

The value for the fraction of label in the acetic acid-soluble fraction of uniformly labeled spores was obtained with spores labeled at the end of log phase as described above.

The percentage of label solubilized during germination was also determined on each spore crop as described above, and the percentage of the maximum amount of protein degraded during germination which was synthesized prior to addition of valine at time t calculated as:

\[
100\% \times \frac{1 - \left( \frac{\text{Fraction of total spore protein synthesized after time } t}{\text{Fraction of label in uniformly labeled spores solubilized upon germination}} \right) x \text{Fraction of label in spores labeled at time } t \text{ which was solubilized upon germination}}}{\text{Fraction of label in uniformly labeled spores solubilized upon germination}}
\]

The second procedure for labeling sporulating cells was to add a small amount of high specific activity radioactive amino acid (50 to 100
Dormant Spores—The nuclear fraction was prepared from dormant dry-ruptured, and suspended in 3 ml of 0.1 mM Tris-HCl (pH 8.0) and 5 mM EDTA. After 30 min at 4°C aliquots were counted before and after centrifugation (10 min, 15,000 × g) to determine the percentage of label in insoluble protein. The supernatant fluid was used to 26 ml of a sporulating culture, after 30 min add unlabeled amino acid to 2 ml to allow the culture to sporulate, and harvest, clean, and lyophilize the spores produced. The dry spores were either dry-ruptured and the acetic acid-soluble fraction isolated, or they were extracted by procedure 3 (see above) to solubilize outer coat proteins. Aliquots of the latter extraction mix were counted before and after centrifugation (10 min, 15,000 × g) to determine the percentage of label solubilized. The extracted spores were then washed, lyophilized, dry-ruptured, and suspended in 0.1 mM Tris-HCl (pH 8.0) and 5 mM EDTA. After 30 min at 4°C aliquots were counted before and after centrifugation to determine the percentage of label as soluble and insoluble protein and as acetic acid-soluble protein. The acetic acid-soluble fraction was then dialyzed in acetylated tubing for 24 hours against 1% acetic acid to remove low molecular weight solutes (2 to 5% of total radioactivity), and all values were corrected for label removal by dialysis.

Separation and Analysis of Proteins A and B in Various Fractions of Dormant Spores—The nuclear fraction was prepared from dormant spores by a modification of the procedure of Chambon et al. (23). Spores were extracted and washed using procedure 1 (see above) and suspended at 10 mg/ml dry weight in 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride (Buffer A). Spores were then lysed by addition of lysozyme to 300 μg/ml and incubation at 37°C for 15 min. There are no free amino acids generated during lysis so most proteolysis appears blocked (9). The lysate was centrifuged (30 min at 78,000 × g) and the supernatant fluid saved (S-1 fraction). The protein in the supernatant fluid was then precipitated with Teflon pestle in a volume of Buffer A equal to the volume of S-1 removed and recentrifuged (15 min, 78,000 × g). Both the supernatant fluid (S-2) and the pellet (P-1) were saved. S-1 and S-2 were made 3% in acetic acid, incubated for 30 min at 4°C, and centrifuged (10 min, 15,000 × g). P-1 was also extracted with a volume of 3% acetic acid equal to the volume of S-1. All three supernatant fluids were dialyzed in acetylated tubing to remove low molecular weight solutes, lyophilized, and dissolved in a small volume of water. The relative amounts of proteins A and B in each fraction were then determined by disc gel electrophoresis at low pH as described above. Spores (100 mg) were also disrupted by dry rupture in the dental amalgamator using glass beads as the abrasive. This procedure is less harsh than rupture with glass beads as the abrasive and allows isolation of intact ribosomes.1 The dry powder was suspended in 10 ml of 50 mM Tris-HCl, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, and 1 mM EDTA. After incubation at 4°C for 30 min the mix was centrifuged (2 hours, 100,000 × g) and both the supernatant and pellet fractions extracted with 3% acetic acid, further purified, and analyzed for proteins A and B as described above.

Other Methods—Protein was measured by the Lowry procedure (24) and dipicolinic acid was measured colorimetrically as described by Bultman and Field (25). DNA was assayed using diphenylamine and RNA using orcinol (26). Dialysis tubing was acetylated in acetylated tubes and RNA using orcinol (26). Dialysis tubing was acetylated with acetic anhydride in anhydrous pyridine for 4 hours at 24°C as described by Craig (27). Paper chromatography of [3H]valine was carried out on Whatman No. 1 paper with butanol/acetic acid/water (10/2/5) as the solvent.

Results

Identification of Proteins Degraded during Germination

Several Unique Dormant Spore Proteins are Species-degraded during Germination—Previous work has shown that most of the 15 to 20% of total spore protein degraded during germination can be accounted for by low molecular weight spore protein which is soluble in dilute acetic acid (9). Indeed, rupture of dormant spores in the dry state to preclude enzyme action followed by immediate extraction of the powder with dilute acetic acid revealed that the acid-soluble fraction comprised >20% of dormant spore protein as determined either by Lowry (24) analysis or with spores labeled in protein with [3H]valine (Table I). However, only 1 to 3% of the total protein of germinated spores, log phase cells, or early stationary phase cells was found in the acid-soluble fraction (Table I).

Surprisingly, disc gel electrophoresis at low pH revealed only two major protein bands, termed proteins A and B, in acetic acid extracts of dormant spores (Fig. 1). These acetic acid-soluble proteins had low molecular weights, since only a few diffuse bands migrating between the positions of insulin and lysozyme were observed on sodium dodecyl sulfate acrylamide gel electrophoresis (Fig. 1). The pattern of protein bands seen on the stained gels was indeed an accurate reflection of the true protein pattern, since electrophoresis of the acetic acid-soluble fraction from dormant spores labeled with [3H]valine revealed only two major peaks of radioactivity (Fig. 2). These peaks coincided with the stained protein bands and accounted for >80% of the radioactivity found on the gel. In addition, there was ~96% recovery of the radioactivity applied to the gel.

Despite the presence of large amounts of proteins A and B in dormant spores, these species were not detected in acetic acid extracts of germinated spores, log phase cells, or early stationary phase cells (Fig. 1). Furthermore, extraction of dry-ruptured spores or cells with either 0.9 mM H₂SO₄ or 66% acetic acid plus 0.1 mM MgCl₂ followed by dialysis against 1% acetic acid

<table>
<thead>
<tr>
<th>Stage of growth</th>
<th>Fraction</th>
<th>Protein in each fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant spores</td>
<td>Acetic acid-soluble</td>
<td>21</td>
</tr>
<tr>
<td>Dormant spores</td>
<td>Removed by extraction</td>
<td>35</td>
</tr>
<tr>
<td>Dormant spores</td>
<td>Insoluble</td>
<td>19</td>
</tr>
<tr>
<td>Dormant spores</td>
<td>Soluble</td>
<td>46</td>
</tr>
<tr>
<td>Germinated spores</td>
<td>Acetic acid-soluble</td>
<td>2.6</td>
</tr>
<tr>
<td>Germinated spores</td>
<td>Removed by extraction</td>
<td>38</td>
</tr>
<tr>
<td>Germinated spores</td>
<td>Solubilized during germination</td>
<td>22</td>
</tr>
<tr>
<td>Germinated spores</td>
<td>Insoluble</td>
<td>17</td>
</tr>
<tr>
<td>Germinated spores</td>
<td>Soluble</td>
<td>26</td>
</tr>
<tr>
<td>Log phase cells</td>
<td>Acetic acid-soluble</td>
<td>1.7</td>
</tr>
<tr>
<td>Early stationary phase cells</td>
<td>Acetic acid-soluble</td>
<td>2.1</td>
</tr>
</tbody>
</table>

1 P. Setlow, unpublished results.
FIG. 1. Acrylamide gel patterns of acetic acid-soluble proteins from cells and spores. In all cases the acetic acid-soluble fractions were prepared and electrophoresis was carried out as described under "Materials and Methods." Gels A to E were run at low pH under nondenaturing conditions. They are: A, extract from 0.1 mg of dormant spores; B, extract from 0.4 mg of germinated spores; C, extract from 0.3 mg of Bacillus megaterium spores prepared in the medium of Spizizen (13); D, extract from 0.3 mg of Bacillus cereus spores; and E, extract from 0.2 mg of B. megaterium spores opened with lysozyme in Buffer A as described under "Materials and Methods." The arrows for Gel E point to bands for lysozyme (L) and to several new bands which may be modified forms of protein B. Gel F was run with sodium dodecyl sulfate and 0.2 mg of the acetic acid extract from dormant spores was applied. Arrows refer to the positions of lysozyme (L) and insulin (I) run in a parallel gel.

also failed to reveal protein A or B in stages of growth other than the dormant spore. This was the case even when disc gel electrophoresis was carried out at low pH in the presence of 8 M urea. It should be noted, however, that very low levels of protein A or B (<4% of the level in dormant spores) might not have been detected in germinated spores or cells.

The findings mentioned above suggested that proteins A and B were the major species degraded during germination. This suggestion was strengthened by electrophoretic analysis of proteins from [H]valine-labeled spores from which the majority of spore coat proteins had been removed, since spore coat proteins are probably not degraded significantly during germination (9) and see below). Rupture and subsequent fractionation of these treated spores revealed that only the soluble proteins showed any significant decrease upon germination, and the magnitude of this decrease accounted almost completely for the proteolysis which took place during germination (Table I). Furthermore, sodium dodecyl sulfate acrylamide gel electrophoresis showed that the soluble fraction of dormant spores contained a large amount of low molecular weight protein migrating at the position of proteins A and B (between insulin and lysozyme (Fig. 1)) which was almost completely absent in germinated spores (Fig. 3A). The amount of this excess low molecular weight protein in dormant spores was 18% of total spore protein. There was, however, no change in the gel pattern of the insoluble proteins upon germination, and most of these proteins were slightly larger than lysozyme (Fig. 3B).

Lability of Proteins A and B—If proteins A and B are the species rapidly degraded during spore germination, one might expect them to be rather labile. Indeed, the yields of both proteins A and B and total acetic acid-soluble protein were significantly reduced when spores were broken using methods such as sonication or lysozyme rupture which allowed spore...
enzymes to act (Table II). When lysozyme rupture was carried out in the presence of high concentrations of the protease inhibitors, EDTA, and phenylmethylsulfonyl fluoride, the recovery of protein A was almost complete, but essentially no protein B was detected. However, under these conditions, the yield of the acetic acid-soluble fraction was not decreased, rather several new diffuse electrophoretic bands were observed in the acetic acid-soluble fraction (Fig. 1). Presumably these new bands were the result of limited proteolysis of protein B during spore lysis. Interestingly, dry rupture of spores followed by suspension of the powder in buffer led to little or no loss in protein A or B.

Proteins A and B were labile not only during rupture and extraction of dormant spores but have also been reported to be rapidly degraded by sonic extracts of dormant and germinated spores (28). Indeed, the reported rate of degradation of these unique proteins by spore extracts was slightly faster than the rate of their degradation in vivo. Furthermore, both the purified A and B proteins are very sensitive to proteolysis by a number of purified proteases (11).

Presence of A and B Proteins in Spores Prepared in Different Media and in Bacillus cereus T—The presence of such a large amount of proteins A and B in Bacillus megaterium made it of obvious interest to determine the levels of these or similar proteins in other types of spores. In B. megaterium spores prepared in several growth media, including a rich (supplemented nutrient broth) and a poor (Slepecky and Foster (14)) medium, and in B. cereus T, the acetic acid-soluble fraction always comprised 15 to 20% of total spore protein (Table III). Where tested this value was in good agreement with the magnitude of proteolysis during germination (Table III). Disc gel electrophoresis also revealed that the A and B proteins were the major species present in the acetic soluble fraction of B. megaterium spores prepared in all growth media, while similar proteins were also present in B. cereus T (Fig. 1).

**Table II**

**Yield of acetic acid-soluble protein and proteins A and B with different extraction procedures**

<table>
<thead>
<tr>
<th>Extraction procedure</th>
<th>Acetic acid-soluble fraction</th>
<th>Proteins A and B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry rupture and extraction with acid</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Lysozyme rupture with Mg&lt;sup&gt;2+&lt;/sup&gt; present</td>
<td>37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lysozyme rupture with EDTA present</td>
<td>91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>~89&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sonication</td>
<td>42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Dry rupture with glucose suspension in Buffer A with Mg&lt;sup&gt;2+&lt;/sup&gt;, and extraction with acid</td>
<td>97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> These values set at 100%.
<sup>b</sup> Determined with [3H]valine-labeled spores.
<sup>c</sup> The recovery of protein A was 100%, but recovery of protein B was <10%; the value for total recovery refers to recovery of both protein A and the new electrophoretic bands appearing under these conditions which are presumably modified forms of protein B.

**Synthesis and Appearance of Proteins A and B during Sporulation**

**Time of Appearance of Proteins A and B**—The presence of proteins A and B in dormant spores and their absence from both log phase cells and early stationary phase cells indicated that these proteins must appear late in sporulation. Indeed, both proteins A and B appeared in parallel only after the appearance of nonrefractile forespores but prior to the appearance of refractile spores (Fig. 4). The accumulation of proteins A and B took place during the same period as the acquisition of resistance to ultraviolet light by the developing spore, but 1 to 2 hours before the acquisition of heat resistance and the accumulation of dipicolinic acid (Fig. 4).

**Table III**

**Analysis of different spore preparations for acetic acid-soluble protein and proteolysis during germination**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth medium</th>
<th>Acetic acid-soluble protein</th>
<th>Protein degraded during germination</th>
<th>% of total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. megaterium</td>
<td>Supplemented nutrient broth</td>
<td>18</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>B. megaterium</td>
<td>Spizizen</td>
<td>17</td>
<td>13&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>B. megaterium</td>
<td>Slepecky and Foster</td>
<td>16</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>B. cereus T</td>
<td>Supplemented nutrient broth</td>
<td>18</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Only 75% of the spores germinated.
<sup>b</sup> Only 40% of the spores germinated.

**Table IV**

**Absence of proteins A and B from mother cell during sporulation**

<table>
<thead>
<tr>
<th>Time in sporulation</th>
<th>Mother cell</th>
<th>Spore plus mother cell</th>
<th>% of maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>97&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>97&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> These numbers refer to the arrows in Fig. 5.
<sup>b</sup> Maximum amount of proteins A and B set at 100%.
Fig. 4. Appearance of proteins A and B and various dormant spore properties during sporulation. Cells were grown in supplemented nutrient broth, 50-ml samples harvested by centrifugation (10 min, 15,000 × g), the pellets frozen in Dry Ice-ethanol and lyophilized. The cells were dry-ruptured, the acetic acid-soluble fraction isolated, and proteins A and B quantitated as described under "Materials and Methods." The percentages of nonrefractile forespores, refractile heat-resistant forms, and ultraviolet-resistant forms were determined as described under "Materials and Methods." Samples (20 ml) were also centrifuged (10 min, 15,000 × g), washed once with 20 ml of 0.15 M NaCl, the pellets extracted with boiling water (3 ml, 10 min), and the supernatant fluid analyzed for dipicolinic acid. A, Absorbance; O, nonrefractile forespores; Δ, ultraviolet-resistant forms; □, Band A; ■, Band B; ○, refractile spores; ●, dipicolinic acid; ▲, heat-resistant forms.

The accumulation of proteins A and B only after forespores were present suggested the possibility that they might be unique only to the dormant spore and the developing forespore and would not be found in the mother cell. Indeed, when sporulating cells were treated with dilute acid to inactivate proteolytic enzymes, and both the mother cell and the whole cell (spore plus mother cell) analyzed for proteins A and B, <5% of proteins A and B were found in the mother cell (Table IV). This finding also raises the possibility that proteins A and B might actually be synthesized within the developing forespore.

Time of Synthesis of A and B Proteins—Despite their accumulation late in sporulation, it was possible that proteins A and B were merely breakdown products of some precursor protein synthesized at an earlier time in sporulation. This possibility is one which must be considered in view of the massive amount of protein turnover which occurs during sporulation. However, the synthesis of proteins A and B also occurred only after the appearance of refractile spores (Fig. 5), indicating that if there is a precursor to protein A or B it must be processed very rapidly.

As was mentioned above for the appearance of proteins A and B, both proteins also appeared to be synthesized in parallel, since analysis of spores from cultures pulse labeled at several times in sporulation showed no difference in the ratios of radioactivity in the A and B proteins (Fig. 5). In addition, the synthesis of proteins A and B occurred during the same period as synthesis of most (but not all) of the proteins which were subsequently degraded during sporulation (Fig. 5). In this latter experiment there was also good quantitative agreement between the percentage of total spore radioactivity in the acetic acid-soluble fraction and the percent solubilized during germination.

Location of Proteins A and B in Dormant Spore

Proteins A and B are Located in Spore Core—The synthesis of proteins A and B prior to the appearance of refractile spores suggested that these proteins were located in the spore core, since in several Bacillus species, this has been reported to be the period when spore core proteins are synthesized but several hours prior to synthesis of spore coat proteins (22). Indeed, when synthesis of acetic acid-soluble protein (predominantly proteins A and B), soluble but acetic acid-insoluble protein (predominantly spore core protein (22)), insoluble protein (predominantly spore coat protein (22)), and protein removed from spores by detergent treatment (spore coat protein) were examined during sporulation, peak synthesis of the protein fractions characteristic of the spore coat (insoluble and removed by detergent) occurred 2 to 4 hours after peak synthesis of the acetic acid-soluble protein (Fig. 6). However, peak synthesis of soluble but acetic acid-insoluble protein (spore core) occurred only slightly before peak synthesis of proteins A and B. In this experiment up to 50% of the radioactive pulse incorporated into dormant spores was found in the acetic acid-soluble fraction, and ~80% of these counts were in proteins A and B as determined by disc gel electrophoresis.

The availability of 3H-labeled spores containing a high percentage of the label in proteins A and B also allowed estimation of the location of these proteins using β attenuation analysis (16). Using this technique the β attenuation for spores labeled predominantly (50%) in proteins A and B was essentially identical with that for spores labeled in DNA as a marker for the spore core but much lower than that for spores labeled in coat proteins (Table V).

The location of proteins A and B in the spore core was further suggested by analysis of dormant spores treated with detergents to remove varying amounts of spore coat protein. Despite removal of up to 43% of total spore protein, neither the absolute
Fig. 5. Synthesis during sporulation of proteins A and B and the proteins degraded during spore germination. Cells were grown in supplemented nutrient broth, and at the times indicated by the points [3H]valine (2 mM, 3·10^3 cpm/nmol) was added to 25 ml of cells. These cells were then allowed to sporulate, and the spores harvested and washed. The percentage of spore protein synthesized prior to addition of valine, the percentage of the maximal amount of proteins A and B synthesized prior to addition of valine, and the percentage of the maximal amount of protein degraded during germination synthesized prior to addition of valine were determined and calculated for each spore crop as described under “Materials and Methods.” The ratio of the amount of protein A synthesized to protein B synthesized was determined by analysis of spores pulse labeled with [3H]serine at various times in sporulation and then chased with an excess of unlabeled serine (see “Materials and Methods”). The lyophilized spores were ruptured, the acetic acid fraction isolated, run on acrylamide gel electrophoresis at low pH, and 2 mM fractions of the gel counted. Values given are the ratio of counts in protein A to those in protein B. All time points are for the midpoint of the pulse period. C, ratio of A protein to B protein synthesized; O, total spore protein; △, protein degraded during germination; •, A and B proteins; ●, refractile spores.

TABLE V

<table>
<thead>
<tr>
<th>Component</th>
<th>% β Attenuation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coat proteins</td>
<td>3.2</td>
</tr>
<tr>
<td>Proteins A and B</td>
<td>25</td>
</tr>
<tr>
<td>DNA</td>
<td>24</td>
</tr>
</tbody>
</table>

Lack of Association of Proteins A and B with Several Components of Spore Core—If, as seems likely, proteins A and B are located in the spore core one would like to determine whether these proteins are bound to any core component. This is of special interest since both proteins are basic and bind to B. megaterium DNA in vitro (11). However, high speed centrifugation of a suspension of dry ruptured spores left >90% of both proteins A and B in the supernatant fraction and separated from the bulk of spore RNA which sedimented presumably as ribosomes (Table VII). Similarly, fractionation of gentle lysates of detergent-treated spores revealed that protein A was also not bound to any sedimenting material, including the great bulk of the spore DNA (Table VII). Unfortunately >95% of protein B was modified, presumably by proteolysis, using the latter procedure (Fig. 1, Table II),
TABLE VI
Effect of various extraction procedures on yield of proteins A and B and magnitude of proteolysis during germination

<table>
<thead>
<tr>
<th>Extraction procedure</th>
<th>Protein removed</th>
<th>Proteolysis during germination</th>
<th>Yield of proteins A and B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>23</td>
<td>100</td>
</tr>
<tr>
<td>Procedure 1</td>
<td>21</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Procedure 2</td>
<td>26</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Procedure 3</td>
<td>42</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Procedure 3 + chymotrypsin or trypsin</td>
<td>44 (42-45)*</td>
<td>86 (88-90)*</td>
<td></td>
</tr>
</tbody>
</table>

*Values are for the percentage of total protein present in the dormant spore before extraction.

**Spores extracted by procedure 1 will no longer germinate.

Values in parentheses are the range of values for the three different procedures.

TABLE VII
Location of DNA, RNA, and proteins A and B in various fractions of dormant spores

Preparation and analysis of spore fractions was carried out as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Proteins A and B</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spores ruptured with lysozyme</td>
<td>&gt;90*</td>
<td>96</td>
<td>3</td>
</tr>
<tr>
<td>S-1</td>
<td>&gt;90*</td>
<td>96</td>
<td>3</td>
</tr>
<tr>
<td>P-1</td>
<td>&lt;5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Supernatant</td>
<td>&gt;90</td>
<td>94</td>
<td>21</td>
</tr>
<tr>
<td>Pellet</td>
<td>&gt;10</td>
<td>6</td>
<td>79</td>
</tr>
</tbody>
</table>

*Although >90% of protein A was found intact in S-1, protein B appeared modified during the lytic procedure. However, >90% of the putative modified protein B was also found in S-1.

Although the modified protein also did not sediment under these conditions.

DISCUSSION

It seems clear that proteolysis during germination of *B. megaterium* spores is primarily the result of degradation of proteins A and B. This is indicated by (a) the quantitative agreement between the amount of these proteins in dormant spores and the amount of proteolysis during germination; (b) the inability to detect these proteins in germinated spores even when extractions were carried out with sodium dodecyl sulfate solutions; (c) the synthesis of proteins A and B at the same time as synthesis of much of the proteins degraded during germination; (d) the removal of up to 45% of total spore protein with no significant effect on either proteolysis during germination or the amount of proteins A and B in dormant spores; (e) the absence from dormant spores of significant amounts of endoprotease active on high molecular weight "average" proteins such as enzymes (17), casein, or hemoglobin (28); and (f) the rapid degradation of proteins A and B by spore extracts (28). Calculation of the percentage of total proteolysis during germination due to degradation of proteins A and B is a little uncertain, but using the difference in the magnitude of the acetic acid-soluble fraction of dormant and germinated spores (Table I) and a value of 80% for the percentage of total protein in the acetic acid-soluble fraction as proteins A and B (Fig. 2), it can be calculated that proteins A and B make up 10 to 15% of total spore protein and contribute 60 to 75% of the total protein degraded during germination (9). The degradation of small amounts of other dormant spore proteins cannot be ruled out, and indeed some degradation of other proteins including those of high molecular weight has been suggested to occur during germination (9) (Fig. 3). It also seems clear that proteins A and B are not located in spore coat. This is suggested by the synthesis of both proteins during the period when spore core proteins are made, the lack of extraction of these proteins by procedures known to solubilize spore coat proteins, and by β attenuation analysis of radioactive spores labeled primarily in proteins A and B. In addition, it is likely that these proteins are also absent from the spore cortex, since they are not destroyed by proteolytic enzymes in the intact spore even when spore coat proteins have been removed and the spore cortex becomes accessible to lysozyme (17). Proteins A and B are, however, rapidly degraded by proteolytic enzymes in vitro (11). All of the data are, therefore, consistent with a location of proteins A and B in the spore core where they must comprise 30 to 50% of the total protein (9). As yet the data do not permit localization of these unique proteins within the spore core itself, since association of protein A or B to DNA, ribosomes, or membranes could not be demonstrated in ruptured spores. However, it is certainly possible that this result was an artifact, and that extraction of spores must be carried out under different conditions to reveal association of proteins A and B with spore components. Furthermore, the rapid proteolysis of protein B during preparation of spore lysates prevented assessment of the binding of this protein to spore DNA, and this still remains a possibility.

One essential function has previously been ascribed to the proteins degraded during germination, that of generating amino acids for protein synthesis early in germination (8). This may, indeed, be the only function of proteins A and B, i.e. to serve as an amino acid depot as do proteins such as gliadin, legumin, and zein in plant seeds (29). However, the rather unique properties of proteins A and B (low molecular weight and high isoelectric point (11)) and the massive amount present in the spore core make it very tempting to propose that they also function in maintaining some property of the dormant spore. Such proposals are especially appealing because rapid degradation of proteins A and B during germination would thereby be a rapid means for catalyzing the loss of specific dormant spore properties.

One such additional function for proteins A and B in the spore is suggested by the simultaneous acquisition by the developing spore of both proteins A and B and resistance to ultraviolet light. This high ultraviolet resistance of dormant spores is thought to be the result of a different conformation of DNA in dormant spores as compared to cells or germinated spores (30, 31). Furthermore, the location and electron micro-
scopic appearance of DNA in the developing spore has been reported to change during the period of acquisition of ultraviolet resistance (32). Consequently, it can be envisaged that accumulation of large amounts of proteins A and B, either alone or in concert with other spore components, results in an alteration in the conformation of spore DNA and resistance to ultraviolet light. Such a proposal also gains some credence from the fact that both proteins A and B bind to B. megaterium DNA in vitro, albeit rather weakly (11). However, it should be remembered that both of these proteins are present at extremely high concentrations (13 to 17 mg/ml total) in the spore core assuming dormant spores have a water content of 70% (33).

Whatever the function of proteins A and B in the dormant and germinating spore, it is clear that one of the major questions concerning these unique spore proteins is the mechanism whereby they remain intact in the dormant spore despite the presence of degradative enzymes which can act rapidly in the first minutes of spore germination. Possibly, answers to this question will give insight not only into the control of proteolysis but also into the mechanisms for maintaining spore dormancy and initiating spore germination.

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REFERENCES
Identification and localization of the major proteins degraded during germination of Bacillus megaterium spores.

P Setlow


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