Alterations of spore coat processing and protein turnover in a Bacillus cereus mutant with a defective postexponential intracellular protease

(defective serine protease/spores deficient in coat protein)

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ABSTRACT A mutant with an alteration in the major intracellular serine protease produced by postexponential Bacillus cereus was isolated by screening mutants defective in spore germination. The purified enzyme from the mutant is more labile to heat and alkaline pH than the protease from the wild type. Protease activity appears at the same time as in the wild type but only reaches 50% of the specific activity and decays more rapidly during sporulation. Coincident with the decay is a decrease in the rate of protein turnover. Generation of amino acids by turnover seems to be important for sporulation because the number of spores produced by the mutant is increased 4- to 10-fold by addition of casamino acids. As anticipated, the mutant produces spores that germinate poorly but, surprisingly, these spores are very deficient in coat protein. Coat antigen is present in cell extracts of mutant and wild type, however, both as large molecules not found on mature spores and as spore coat protein monomers. The large molecules rapidly disappear in a pulse chase experiment in the wild type with some increase in the coat monomers. In mutant extracts, however, this large coat antigen is slowly and improperly processed.

Shortly after the end of exponential growth, sporulating Bacilli produce a variety of intracellular and extracellular proteases. In most cases, the secreted activity is primarily a metal protease that seems to be dispensable for sporulation because mutants producing little or undetectable amounts of this activity sporulate well (1-3). Bacillus subtilis also secretes a serine protease that may have an intracellular function (4), although there is some question of both the proposed function (5) and the pleiotropic effects of such mutations (6). Intracellular activity that appears to be due primarily to one or a few different enzymes on the basis of activity profiles after gel electrophoresis of crude extracts (refs. 7 and 8; Cheng, Aronson, and Fitz-James, unpublished data) is inhibited completely by serine protease inhibitors. The functions of these intracellular enzymes are not known although roles in protein turnover (9, 10) and possibly in enzyme modification (11, 12) have been considered. The relation, if any, to proteases in exponentially growing cells has not been determined.

Initially, to find mutants with altered intracellular protease, we screened temperature-sensitive sporulation mutants (Cheng et al., unpublished data). One of these contained a protease more heat labile than the wild type, and one of its major phenotypic alterations was production of spores that germinated poorly. We have exploited this property to enrich for conditional mutants with this defect. Among these, a mutant with an altered intracellular protease, the major protease activity in these cells, has been isolated and analyzed. The mutation results not only in a decreased rate of protein turnover, but in the inability to process precursors of spore coat protein properly.

MATERIALS AND METHODS

Growth and Mutant Isolation. Bacillus cereus T and various mutants were grown in a yeast extract glucose medium (GTris, ref. 1) or a minimal medium (CDGS, ref. 1) in Erlenmeyer flasks (volume of media was 10-20% of the flask volume) on New Brunswick rotary shakers at 30° or 38°. Growth was monitored in a Coleman 8 colorimeter equipped with a 650 nm filter.

The protease mutant, G8, was isolated by first treating spores with ethyl methane sulfoxite (Eastman Kodak, ref. 13). The spores were washed and incubated in GTris at 38° for 72 hr. The harvested spores were suspended in 0.1 M sodium phosphate, pH 6.8, to about 10⁹/ml and heated at 70° for 15 min. Germination was then initiated by addition of 10 mM L-alanine and 1 mM adenosine. After incubation at 27° for 30 min, a time sufficient for all wild-type spores to initiate germination, the suspension was centrifuged, washed once with 0.1 M sodium phosphate, pH 6.8, and layered over 40% Renografin (aqueous solution from stock of 66% meglumine diatrizoate-10% sodium diatrizoate; Squibb) in an SW50.1 tube. After centrifugation at 60,000 X g for 45 min at 20° in a Spinco L-200 centrifuge, the dense ungerminated spores were collected from the pellet and inoculated into GTris. After a 72-hr incubation at 28°, the germination and centrifugation procedures were repeated. This time, the germinated spores remaining on top of the 40% Renografin were collected and reinoculated into GTris, and a second cycle of enrichment at 38° and 28° was done. The final preparation of germinated spores was inoculated into GTris and incubated at 38° for 3 days; the spores were plated on GTris at 38°. Colonies appearing 4-5 hr later than the wild type but quickly reaching wild-type colony size were picked, purified by single colony isolation, and grown to the postexponential phase (10-12 hr in Fig. 1) to screen for heat-labile intracellular protease (Cheng et al., unpublished data).

Revertants of G8 were selected by growing a culture in GTris at 38° for 48 hr. The spores were harvested, suspended in 0.05 M Tris-HCl, (Sigma), pH 7.4, and incubated with 100 μg/ml of lysozyme (EC 3.2.1.17, Worthington) for 10 hr at 30°. The suspension was then heated at 75° for 30 min to enrich for lysozyme-resistant spores and plated on GTris agar. After 5 days at 30°, 300 μg of sterile lysozyme was added to each plate, which was incubated at 30° for 1 hr. Lysozyme-resistant spore colonies are easily detected (see Fig. 3), and at least 50 were picked for further analysis.

Measurement of Sporulation Frequency. Cells growing in GTris at 28° until the end of exponential growth (8-9 hr in Fig. 1) were centrifuged, washed once with a replacement medium consisting of GTris medium (minus glucose and yeast extract) supplemented with sodium glutamate, resuspended to about the same cell concentration in the replacement medium, and
for 90 min. The suspension was centrifuged as above and the supernatant fluid combined with the original extract. This preparation was dialyzed for 10 hr at 27° against 500 ml of antibody buffer. After dialysis, deoxycholate was added to 0.5% and the suspension was centrifuged at 15,000 × g for 20 min. Supernatant fluid (0.2 ml) was mixed with varying amounts of coat antibody (15) or control serum and incubated at 37° for 1 hr and then at 4° for 14 hr. Twenty microfilters of goat anti-
sodium dodecyl sulfate-urea gels and processing of the gels for radioactivity
were essentially described as by Laemmli (18), with the inclusion of 6 M urea in the separating and spacer gels (17).

Analytical Procedures. Germination studies were as previously described, using both L-alanine-adenosine and calcium dipicolinate (16).

Quantitation of spore coat protein involved extraction of a known number of spores with reagents as described above, precipitation of sodium dodecyl sulfate with saturated KCl, precipitation of the protein with 15% trichloroacetic acid, dissolution of the pellet in 0.2 M NaOH, and use of the Lowry procedure (14).

For the preparation of labeled extracts, 75 ml of cells were incubated at 38° in GTris until 4 hr after the end of exponential growth (13 hr in Fig. 1). At this time either 10 Ci/mmol of L-[^14]C]leucine (300 mCi/mmol; Schwarz/Mann) or 300 mCi of L-[4,5-3H]leucine (59 Ci/mmol; Schwarz/Mann) was added and incubation continued for 7 min. Twenty milliliters were removed, immediately centrifuged and washed, and cell extracts were prepared as described above. A 2000-fold excess of unlabeled L-leucine was added to the remaining culture (80-90% of the radioactive amino acids were incorporated in 7 min) and incubation was continued to provide samples for 15- and 30-min chase points. In all cases, differentially labeled samples were crossmixed prior to washing. Antibody precipitation was as previously described.

RESULTS

Growth and Protease Production. One hundred ten mutants with a conditional germination defect were screened for temperature-sensitive intracellular protease, and three had some alteration. The one with the most labile activity, G8, was selected for further study. This mutant grew in an enriched medium, as did the wild type (Fig. 1), and produced extracellular metal protease at the same time and amount as is shown for the wild type in Fig. 1. Although the intracellular protease appeared at the same time as the wild type, the maximum specific activity was only about 50% that of the wild type and the activity decayed more rapidly (Fig. 1). In both mutant and wild type, there is probably little if any of this activity in mature spores. A rather unique proteolytic enzyme seems to function in the extensive protein turnover found in germinating spores (P. Setlow, personal communication).

Altered Intracellular Protease in G8. Most of the protease activity of G8 in crude extracts or purified preparations was heat labile (Fig. 2). During the last step of purification on DEAE-cellulose (Y. E. Cheng, Ph.D. dissertation, Purdue University, 1976) a second minor (3% of the total) intracellular serine protease was uncovered. This activity has the same heat stability as the wild-type enzyme (IP2 in Fig. 2). The major protease of G8 was also very readily inactivated by incubation
Fig. 2. Heat inactivation of purified proteases prepared from wild type and mutant G8. Enzymes purified about 100-fold after elution from DEAE-cellulose columns (Cheng et al., unpublished data) were heated at 53°C for the times indicated in 50 mM Tris-HCl-2 mM CaCl₂, pH 7.5. Assays were as described in Materials and Methods. (O—O) Wild-type IP₁ (major intracellular protease); (● —●—●) wild-type IP₂; (X—X) G8 IP₁; (▲—▲—▲) G8 IP₂.

above pH 7, whereas the wild-type enzyme was completely stable to at least pH 9.0.

Effects of Protease Mutation on Spore Formation. The properties of G8 and examples of the two classes of revertants are summarized in Table 1. Although G8 was originally selected for conditional spore germination, it was also found to be temperature sensitive for spore production, especially in a minimal medium. The capacity to form spores (i.e., heat-resistant structures) may be almost completely alleviated by addition of amino acids to a resuspension medium (Table 2). Spores produced by G8 in this replacement medium have the same properties as the mutant spores produced in enriched medium (Table 1).

Higher concentrations of casamino acids do not result in a greater yield of G8 spores. Neither individual nor other mixtures of amino acids have been tested. These results suggest a role for this protease in providing amino acids via turnover. Indeed, the rate of protein turnover (as measured by loss of [14C]leucine from the acid-insoluble fraction of cells labeled for 60 min at various times) in G8 decreased from 7 to 10% per hr for the first 3-hr postexponential period to negligible values 4–5 hr after the end of exponential growth (13–14 hr in Fig. 1), whereas the wild type sustained a rate of 7–10% per hr from 9 to 15 hr (Fig. 1). There is obviously no direct correlation between the amount of the intracellular protease (at least as measured in Fig. 1) and the rate of turnover.

Two other unexpected phenotypic properties of sporulating G8 were the slow release of spores from the mother cell and production of lysozyme-resistant spores (Fig. 3). The latter property has been correlated with spores containing little coat or defective packing of the coat (16, 17). In the case of G8, a coat defect was confirmed by electron microscopic studies (Cheng et al., unpublished data) and decreased amount of coat protein extractable from mature spores (Table 2).

Isolation of Revertants. The lysozyme sensitivity of G8 spores provided a basis for selecting revertants, as outlined in Materials and Methods. A class of partial revertants, as exemplified by G8RI in Table 1, were temperature sensitive for many properties, especially production of lysozyme-resistant spores. The second class of revertants, represented by G8RII in Table 1, regained all of the phenotypic properties of the wild

Table 1. Summary of properties of G8, revertants, and wild type

<table>
<thead>
<tr>
<th>Strain</th>
<th>EP*</th>
<th>Half-life of IP₁ at 53°C (min)</th>
<th>No. of spores (x10⁶)</th>
<th>Lysozyme from mother cell</th>
<th>Germination rate</th>
<th>% Spores/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>+</td>
<td>&gt;120</td>
<td>205/240</td>
<td>20</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G8</td>
<td>+</td>
<td>13</td>
<td>205/76</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G8RI</td>
<td>+</td>
<td>15–46</td>
<td>200/130</td>
<td>6</td>
<td>tS</td>
<td>-</td>
</tr>
<tr>
<td>G8RII</td>
<td>+</td>
<td>&gt;120</td>
<td>220/250</td>
<td>20</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Amount and heat stability (60° for 10 min) of extracellular protease.
† Portions of the cell supernatants were assayed with Azocoll (Materials and Methods) at several times after 11 hr of growth (Fig. 1).
‡ Spores were quantitated by heating at 75°C for 30 min and plating, on GTris agar, 5-day-old CDG8 cultures grown at 28°C or 38°C.
§ Percent decrease of OD 650 nm per min at 37°C (16).
¶ Scored directly on GTris plates as in Fig. 3. Confirmed by direct examination of spores in a Zeiss phase microscope. tS means lysozyme-sensitive spores formed only if spores were produced at 38°C.
∥ Qualitative assessment by examination in phase microscope after a 4-day incubation in GTris at 30°C or 38°C.
Table 3. Coat content of spores and sporulating cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>g of coat protein/spore*, (10^3)</th>
<th>Crude extract</th>
<th>Extracted pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.60 ± 0.16</td>
<td>&lt;1</td>
<td>35-40‡</td>
</tr>
<tr>
<td>G8</td>
<td>0.40 ± 0.10</td>
<td>&lt;1</td>
<td>45-55</td>
</tr>
</tbody>
</table>

* Total spores were determined by counting duplicate samples in a Petroff-Hauser chamber. Extractable coat protein was determined as described in Materials and Methods.
† Extract was prepared at early stage IV (phase whitening or time of coat maturation, about 14–15 hr in Fig. 1), as described in Materials and Methods. Quantitation was as in ref. 15. A background value of 3% has been subtracted.
‡ These values are equivalent to 40–50 µg of mature coat protein (15).

type. Both classes were found with a frequency of 1/10^6–1/10^7, as would occur if G8 had a point mutation affecting the major intracellular protease.

Altered Spore Coat Maturation. The decreased coat content of G8 spores accounts for the lysozyme sensitivity and has been well correlated with slow germination rates (16). Because it was not readily apparent how a protease mutation could result in altered spore coat production, coat was assayed in extracts of sporulating cells (Table 3). By use of the binding of \(^{125}\text{I}-\text{labelled antibody} against coat protein (15), at least as much coat antigen was found in extracts of G8 as in the wild type. The defect thus appeared to be in the packing of coat monomers onto spores and suggested a role for the protease in processing coat precursors.

When labeled extracts of sporulating cells were treated with coat antibody, a prominent component of about 65,000 daltons was found in the precipitates of both wild type and G8 (II in Fig. 4a). There was also a component of about 48,000 daltons (II in Fig. 4a). The wild type had a third component of 12,000–13,000 daltons (III in Fig. 4a), which is the size of the major coat polypeptide found in extracts of mature spores (17). In addition to components I and II in antibody precipitates of G8, there were one or two major fractions moving with the dye front (IV in Fig. 4a). These latter fractions were found to a lesser extent in the wild type and in the precipitate of extracts treated with control serum (Fig. 4c) and are likely to result from nonspecific precipitation of polypeptide fragments.

Table 4. Distribution of radioactivity in spore coat antigens in a pulse-chase experiment*

<table>
<thead>
<tr>
<th>Fraction† (daltons)</th>
<th>7 min pulse</th>
<th>8 min chase</th>
<th>23 min chase</th>
<th>7 min pulse</th>
<th>8 min chase</th>
<th>23 min chase</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (65,000)</td>
<td>3,725</td>
<td>1,325</td>
<td>—§</td>
<td>4,000</td>
<td>2,405</td>
<td>1,525</td>
</tr>
<tr>
<td>II (48,000)</td>
<td>3,200</td>
<td>1,750</td>
<td>850</td>
<td>4,550</td>
<td>2,775</td>
<td>2,225</td>
</tr>
<tr>
<td>III (12,000–13,000)</td>
<td>1,600</td>
<td>2,450</td>
<td>2,925</td>
<td>1,150‡</td>
<td>1,825‡</td>
<td>2,000‡</td>
</tr>
<tr>
<td>Total cpm*</td>
<td>15,200</td>
<td>13,460</td>
<td>11,275</td>
<td>23,640</td>
<td>23,750</td>
<td>23,500</td>
</tr>
</tbody>
</table>

* Data from Fig. 4. Total cpm in extracts prior to antibody addition were adjusted to the same values. Five slices were combined for fractions I and III; seven for II.
† See Fig. 4a. Molecular mass estimated from gel with standards (bovine serum albumin, chymotrypsinogen, ribonuclease, and cytochrome c) run in parallel.
‡ Sum of slices 1–55. Radioactivity in 55–60 is probably nonspecific (Fig. 4c).
§ No discernible peak (Fig. 4c).
¶ There are no sharp peaks in this region for G8 preparations (Fig. 4). These values are the sum of five slices in that region.
extensive than the increase in III, implying that if there were a precursor product relationship only about 40% of the precursor is conserved. In G8, the decrease of components I and II is neither as extensive nor as rapid as in the wild type. The increase in the cpm in the region of component III (Table 4) may not be true coat monomers, as there is no well defined peak in this region of the gels (Fig. 4). A conditional revertant of G8, GSR18, did show a more definitive accumulation of component III after a 15-min chase (Y. E. Cheng, Ph.D. dissertation, Purdue University, 1976). In both G8 and GSR18, however, there was much radioactivity accumulating in fractions 20–35 (Fig. 4), in contrast to the wild type, suggesting improper cleavage of coat precursors.

**DISCUSSION**

An alteration in the major intracellular serine protease produced by sporulating *B. cereus* results in several interesting phenotypic changes. Because all of these properties are restored in revertants that occur at a frequency consistent with an initial point mutation, they are probably all directly or indirectly due to the altered protease.

During early sporulation, there is apparently sufficient protease present in G8 (Fig. 1) to sustain the normal rate of turnover (as measured by loss of radioactive amino acids from the trichloroacetic acid-insoluble fraction). Four to five hours after the end of exponential growth, however, the protease activity in G8 has decreased considerably (Fig. 1) and protein turnover is undetectable (Cheng et al., unpublished data). In addition, these cells accumulate many small peptides (Fig. 4). The turnover seems to be more important for providing amino acids than for removing inhibitory polypeptides because the sporulation frequency at 38°C of G8 may be increased 4- to 10-fold by addition of amino acids to a salts-glutamate sporulation medium (Table 2). The spores produced still are deficient in coat, are lysozyme sensitive, and germinate slowly. A requirement for amino acids during sporulation is consistent with the inactivation of many amino acid biosynthetic enzymes in sporulating cells (19, 20).

The reason for the slow release of spores from the mother cell is not known, but may be related to the inactivity in G8 of a lytic enzyme found in sporulating cells by Strange and Dark (21). A most intriguing finding is the presence of spore coat precursors and the apparent role of this protease in their processing. At the shortest labeling times studied, both 65,000- and 48,000-dalton components are seen. The latter is always a broader peak and may be more heterogeneous. There is not adequate data yet to determine whether there are two or more distinct precursors or whether the 48,000-dalton fraction is derived from the larger molecule. The only other well defined component in these antibody precipitates is one at 12,000–13,000 daltons, the size of most of the protein in coat extracted from spores (17). The pulse chase data summarized in Table 4 are consistent with about 40% of the 65,000-dalton component being converted to mature spore coat polypeptides. The remainder is presumably degraded to non-coat antigenic components or to amino acids.

Other lysozyme-sensitive spore mutants have now been found that accumulate a 65,000-dalton component. In these cases, the mutants appear to contain intracellular protease in the same amounts and with the same stability as the wild type, so lack of processing must be due to other alterations (Stelma and Aronson, unpublished data).

The overall evidence would certainly support a role for this protease in processing spore coat precursor. (4) The major molecular weight species are related antigenically and only the 12,000- to 13,000-dalton component is found on mature spores. (ii) The 65,000-dalton component decreases in a pulse chase experiment, and the preliminary evidence summarized in Table 4 is consistent with some conversion to the 12,000- to 13,000-dalton species. (iii) Alteration of this protease results in slower cleavage of the 65,000-dalton antigen and cleavage to products not seen in the wild type. (iv) This protease mutant accumulates spore coat antigen as in the control (Table 2) but very little mature 12,000- to 13,000-dalton spore coat protein is found in extracts or on spores. (v) Preliminary results with purified spore coat precursor and proteases from wild type or G8 confirm the abnormal processing by the latter activity. A direct role of this protease in coat processing is thus probable.

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