Spore Protease
SYNTHESIS AND PROCESSING OF PRECURSOR FORMS DURING SPORULATION AND GERMINATION*

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Bacillus megaterium Spore Protease

The protease which initiates the rapid protein degradation during germination of Bacillus megaterium spores was synthesized during sporulation as a Mr = 40,000 polypeptide (P1s) which was found in the developing forespore. P1s was processed during sporulation to a Mr = 41,000 species (P1a) 2–3 h after P1s synthesis and at the time of or slightly before accumulation of dipicolinic acid. P1a was the predominant form of the protease in the dormant spore, with smaller amounts of unprocessed P1s. In the first minutes of spore germination P1a was processed (t1/2 < 10 min) to a Mr = 40,000 species (P4a), which appeared identical to the subunit of the purified active enzyme. The latter processing reaction did not require metabolic energy, but P4a disappeared completely during further germination (t1/2 ~ 40 min) in a reaction which did require metabolic energy. It seems possible that precursors P1a and P4a of the spore protease are involved in the regulation of the activity of this spore enzyme.

The first minutes of germination of spores of various Bacillus and Clostridium species are accompanied by the degradation of up to 25% of the spore’s protein (1). The major substrates for this proteolysis are two to three low molecular weight proteins (termed A, B, and C proteins in Bacillus megaterium) which are synthesized during sporulation (1). In B. megaterium the degradation of these proteins during germination is initiated by an amino acid sequence specific endoprotease which acts only on the A, B, and C proteins and analogous proteins from spores of other species (2, 3). Mutants have been isolated with low levels of this protease, and all analogues from spores of other species (2, 3). Mutants which are synthesized during sporulation (1) have been purified from spores of B. megaterium, and is a tetramer of Mr = 40,000 subunits; only the tetramer is enzymatically active (5). Use of a radioimmunooassay for the spore protease has demonstrated that: 1) the protease antigen disappears during spore germination in parallel with the loss in protease enzyme activity; 2) the protease antigen is absent from log phase and young sporulating cells; and 3) the protease antigen appears within the developing forespore midway through sporulation at about the time of or even slightly before synthesis of the enzyme’s substrates (5). Since the A, B, and C proteins are not attacked by the protease within the developing or dormant spore (1), there must be some regulatory mechanism such that this enzyme is inactive in the developing forespore and dormant spore yet becomes active upon spore germination. While many mechanisms could effect the required regulation, a frequently observed regulatory mechanism for proteolytic enzymes is their synthesis as an inactive precursor or zymogen which has a higher molecular weight than the active enzyme. Consequently, we undertook to determine the molecular weight of the spore protease subunit throughout sporulation and germination in order to detect possible zymogen forms.

MATERIALS AND METHODS

Bacterial strain—Most of the work described in this report was carried out with B. megaterium QM B1551 (originally obtained from H. S. Levinson, U. S. Army Natick Laboratories, Natick, MA). Some experiments also utilized a spontaneous streptomycin-resistant derivative of this strain, which grew and sporulated in the presence of streptomycin sulfate (100 μg/ml). The four protease mutant strains (B-2, B-41, C-1, and C-44) of B. megaterium QM B1551 were described previously (4), and spontaneous streptomycin-resistant derivatives of each of these strains were used throughout this work. These strains are designated B-2-1, B-41-2, C-1-5, and C-44-1.

Radiochemicals, Enzymes, and Immunological Reagents—[35S]Methionine (~1000 Ci/mmol) was obtained from ICN, and a uniformly labeled [14C]amino-acid mixture (>50 mCi/mg of atom carbon) was obtained from Amersham Corp. The fluor for enhancement of autoradiographic detection of 14C or 35S was obtained from New England Nuclear (ENHANCE). Trypsin was obtained from Worthington. The purified B. megaterium spore protease, the spore protease iodinated with [125I]-Bolton-Hunter reagent, the rabbit anti-spore protease γ-globulin, control rabbit γ-globulin, and goat anti-rabbit γ-globulin were prepared and stored as described previously (5). Freeze-dried cells of Staphylococcus aureus were used as a source of protein A for precipitation of rabbit γ-globulin and were obtained from the Enzyme Center as IgGserb. Peroxidase-coupled goat anti-rabbit γ-globulin was obtained from Cappel and normal goat serum from Gibco. Nitrocellulose paper for transfer of proteins from acrylamide gels was obtained from Millipore.

Growth, Sporulation, and Spore Germination—All bacterial strains were grown at 30 °C in supplemented nutrient broth (6). The medium for streptomycin-resistant strains also contained 100 μg/ml of streptomycin sulfate. Developing forespores were isolated from sporulating bacteria as described previously (7). Spores of all strains were harvested, washed, and stored as previously described (6). For germination spores (5–25 mg/ml) were first heat-shocked (60 °C, in 15 min) in water and cooled in ice. Germination was at 30 °C and 1–3 mg/ml of spores in 50 mM Tris-HCl (pH 7.4) and 50 mM glucose. In this medium the initiation of spore germination was 95% complete in 10 min as measured by release of the dormant spore’s DNA. Some experiments also used a KBr germination medium containing 50 mM KPO4 (pH 7.4) and 50 mM KBr.

Pulse-labeling and Pulse-Chase Experiments—Cells were grown in supplemented nutrient broth and were routinely pulse-labeled by addition of 2.5–5 μCi of the [35S]amino-acid mixture or 50–100 μCi of [3H]ethionine/ml of culture. After a labeling period of 20–60 min,

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the cells were centrifuged (5 min, 10,000 x g), washed with 5–10 ml of 0.15 M NaCl (pulse-labeling only) or with an equal volume of supernatant fluid from a parallel unlabeled culture (pulse-chase). The washed pellet was either frozen and lyophilized (pulse-labeling only) or resuspended in an equal volume of prewarmed supernatant fluid from an unlabeled culture which was made either 0.1% in casein amino acids (l-[14C]amino-acid-labeling) or 0.5 mM in methionine (l-[35S]methionine-labeling). The resuspended cultures were incubated at 30 °C, and at various times aliquots were centrifuged (5 min, 10,000 x g) and the pellet was frozen and lyophilized. Protein synthesis was measured by adding a mixture of 10 μl of culture to 1 ml of cold 5% trichloroacetic acid, and acid-insoluble radioactivity determined as previously described (8).

Cell or Spore Breakage and Extraction—Samples of cells or spores from 1-10 ml of culture were centrifuged, and the pellets were frozen and lyophilized. The dry cells or spores were broken by dry rupture in a dental amalgamator with glucose crystals (75 mg) as the abrasive; rupture was carried out for 1 min periods with cooling in between. Complete disruption of cells required 1–3 min, germinated spores required 2–5 min, and dormant spores required 4–6 min. The dry powder was extracted with 0.3–2.5 ml of cold buffer containing 20% glycerol, 50 mM Tris-HCl (pH 7.4), 3 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A). Previous work has shown that wild type spore protease antigen is stable in this buffer even when extracted from sporulating cells (5). After 30 min of extraction the mixture was centrifuged for 2 min in an Eppendorf microcentrifuge and the supernatant fluid stored frozen at −80 °C. In one experiment spores (50 mg) were broken in a dental amalgamator for only 2 min. The dry powder was extracted for 1 min with 1 ml of cold buffer containing 50 mM Tris HCl (pH 7.4), 20% glycerol, and 5 mM CaCl2 (buffer B), the extract was centrifuged, and the supernatant fluid was dialyzed for 4 or 16 h against cold buffer B.

Immunological Procedures—Immunoprecipitation of protease antigen was routinely carried out on 1 ml of extract from 2 ml of culture. The extract was diluted 1:1 with a buffer containing 25 mM KPO4 (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml of bovine serum albumin (buffer C), and 4 μl of nonimmune γ-globulin was added. After 2 h at 4 °C the nonimmune γ-globulin was precipitated by addition of 30 μl of reconstituted IgGorb; after 30 min at 4°C the precipitate was removed by centrifugation and discarded. The supernatant fluid was treated with 4 μl of nonimmune γ-globulin overnight at 4°C, and again the γ-globulin was removed by precipitation with IgGorb. The supernatant fluid was split in half; one half received nonimmune γ-globulin (2 μl) while the other half received anti-protease γ-globulin (2 μl). Preliminary experiments with extracts to which trace amounts of 32P-protease were added showed that 2 μl of immune γ-globulin was sufficient to precipitate only 1/2 times the maximum amount of protease antigen present in an extract from 1 ml of sporulating cells. The latter value was only slightly higher than the comparable value for the free dormant spores as noted previously (5). After incubation of the mixtures at 18°C at 4°C, the control and immune γ-globulin was precipitated by addition of IgGorb (15 μl), and the pellets were washed 4 times with 500 μl of buffer C. The final pellets were suspended in 40 μl of 2% SDS and sonicated briefly in an ultrasonic bath, and after 10 min at room temperature the mix was centrifuged and the supernatant fluid was either analyzed immediately or stored at −80 °C.

Spore protease antigen was detected on SDS gels following electrophoretic transfer of the proteins to nitrocellulose paper (immunoblotting) as described by Towbin et al. (9). We routinely applied extract from 0.2-0.5 ml of culture to the gels and used a 1/50 dilution of our immune γ-globulin. The radioimmunoassay of the spore protease was carried out as described previously (5).

Analysis of Tryptic Peptides of Spore Protease—Immunoprecipitates from extracts of sporulating cells, dormant spores, or spores germinated with KCN which had been pulse-labeled during sporulation with [35S]methionine or pulse-labeled and then chased were run on SDS gels, and the gels were stained, dried, and autoradiographed without ENHANCE. Protease bands were identified from the autoradiogram by the stained bands and cut out, the peptides were suspended in water for a few minutes, and the paper backing was removed from the gel. The gel slices were macerated with 0.5 ml of methanol, centrifuged, washed 2 times with 1 ml of methanol, and lyophilized. The dried gel fragments were suspended in 1 ml of 0.1 M NH4HCO3 (pH 8.1) containing 4 μg/ml trypsin and incubated for 3 h at 37 °C. The suspension was then passed through a Swinex filter, the filtrate was lyophilized, and the residue was dissolved in 0.5 ml of H2O and lyophilized. In a separate experiment purified spore protease (200 μg) was run on an SDS gel, stained, and dried, and the protease band was cut out and mixed with a labeled band, processed, and digested in 3 ml with trypsin (12 μg) as described above. An area of the gel equivalent to the area of the protease bands (but containing no protein) was also cut out and processed as described above.

Lyophilized trypsic digest was dissolved in 100 μl of 88% HCOOH, and spore protease peptides were resolved by HPLC using 20 mM KPO4 (pH 2.5) as described previously but with a 50-min linear gradient of from 0–50% acetonitrile (10). The optical density was monitored at 214 nm, and fractions were collected over 30-s intervals, dried, and counted as described previously (11).

Other Methods—Protein was routinely determined by the method of Lowry et al. (12); the protein concentration of purified spore protease preparations was measured by the method of Bradford (13). DPA was extracted and measured as described previously (7, 14). SDS-acrylamide gel electrophoresis was carried out with a 10% acryl-amide lower gel as described by Laemmli (16) using 1.5-mm slab gels with 4-mm wells. Gels were stained overnight in 0.4% Coomassie blue, 25% 2-propanol, and 10% acetic acid, and processed for autoradiography with ENHANCE as described in the technical literature accompanying this reagent. Molecular weights of protease precursors were assigned by comparison of their mobilities on SDS gels to those of bovine serum albumin, ovalbumin, and the purified protease's subunits (5). The amount of protease antigen on a gel which was autoradiographed or immunoblotted was determined by scanning the autoradiogram or immunoblot with a densitometer and cutting out and weighing the appropriate peaks. For autoradiograms the intensities of bands from the immune precipitates were corrected for radioactivity in the equivalent region of the gel from the parallel nonimmune precipitate. For immunoblots, all gels were run with protein from equivalent volumes of culture of cells and/or spores. Each sample was run in duplicate and each was scanned. The values presented are averages of the duplicates.

![Fig. 1. Autoradiogram of SDS gel of control and immune precipitates from cells pulse-labeled during sporulation. Cells were grown in supplemented nutrient broth and at various times 2 ml of culture was pulse-labeled for 20 min with [14C]amino-acids, protein synthesis was measured, and the cells were harvested, washed, lyophilized, broken, extracted in 2 ml of buffer A and immunoprecipitated as described under "Materials and Methods." Samples (10 μl) of the solubilized protein from paired immune and nonimmune precipitates were then run on an SDS gel, stained, and autoradiographed.](http://www.jbc.org)
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![Graph showing rate of P46 synthesis and protease antigen accumulation during sporulation](image)

Fig. 2. Rate of $P_{46}$ synthesis and protease antigen accumulation during sporulation. Cells were pulse-labeled and analyzed as described in Fig. 1; the autoradiogram was scanned and quantitated as described under "Materials and Methods." The amount of $P_{46}$ synthesized in pulse-labeling period c was set at 100%. At various times unlabeled samples were also taken, extracted, and analyzed for DPA and for total spore protease antigen using the radioimmunoassay. The arrows labeled I and 2 give the times for pulse-labeling of the samples which were analyzed in Fig. 3.

**RESULTS**

**Analysis of Spore Protease During Sporulation**

Pulse-labeling, Pulse-Chase, and Immunoblot Analyses—Previous work has indicated that the spore protease antigen accumulates during sporulation 2–3 h before accumulation of DPA (5). Analysis of immunoprecipitates from cells pulse-labeled during growth and sporulation revealed a single labeled protein (termed $P_{46}$) precipitating specifically with protease antibody (Fig. 1). $P_{46}$ was synthesized most rapidly early in the period of protease antigen accumulation (Figs. 1 and 2) and was found only in the developing forespore (data not shown). Strikingly, the molecular weight of $P_{46}$ was 46,000 and immunoprecipitation of a pulse-labeled $M_r = 40,000$ species (the size of the purified protease's subunits (termed $P_{41}$)) was not observed (Fig. 1).

Analysis of immunoprecipitates from cells pulse-labeled at two different times during $P_{46}$ synthesis followed by a chase during the remainder of sporulation indicated that $P_{46}$ was chased into a $M_r = 41,000$ species (termed $P_{40}$) in the dormant spore, with only small amounts of $P_{46}$ remaining (Fig. 3). Analysis of the levels of $P_{46}$ and $P_{41}$ during a pulse-chase experiment was consistent with a precursor-product relationship, with $P_{46}$ processing ending only 5 h after its synthesis (Fig. 4). In the latter experiment the chase was indeed effective, since total acid-insoluble counts decreased immediately following initiation of the chase (Fig. 4). Also demonstrated in the latter experiment was that >80% of the labeled $P_{46}$ and $P_{41}$ was in the developing forespore (data not shown).

Immunoblot analysis of SDS gels of unlabeled extracts prepared throughout growth and sporulation was somewhat more complicated and gave a number of bands even with extracts from log phase cells (Fig. 5, lane a). However, all of the bands seen with the latter extract were also seen when nonimmune $\gamma$-globulin was substituted for anti-protease $\gamma$-globulin (data not shown) with the exception of the most predominant band (arrow 1). A protein corresponding to this latter band is a minor contaminant in our protease prepara-

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**Fig. 3. Autoradiogram of SDS gel of control and immune precipitates from pulse-labeled and pulsed and chased sporulating cells.** At two times in sporulation 3 ml of cells were pulse-labeled with $^{14}C$]amino-acid. After 30 min 2 ml of cells were harvested, while the remaining 1 ml was chased until formation of free spores and then harvested as described under "Materials and Methods." The cells or spores were broken, extracted, immunoprecipitated, run on an SDS gel and autoradiographed as described under "Materials and Methods." For samples in groups 1 and 2, the mid-points of the pulse-labeling periods were at the arrows labeled f and 1, respectively, in Fig. 2. Lanes a and b were from pulse-labeled cells; lanes c and d were from pulse-chased spores. Lanes a and c were from nonimmune precipitates; lanes b and d were from immune precipitates. The arrow $P_{46}$ gives the position of the purified protease subunit; the arrows b and c give the position of bovine serum albumin and ovalbumin markers.
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Fig. 4. Kinetics of change in P46 and P41 after pulse-labeling of P46 and chasing. Cells (15 ml) were pulse-labeled for 40 min with [35S]methionine at approximately the high point of P46 synthesis. The label in the entire sample was then chased (see "Materials and Methods"), and aliquots (2 ml) were taken immediately after beginning the chase (at 3.55 h) and at various times thereafter. The samples were centrifuged, the pellets were washed, lyophilized, extracted, immunoprecipitated, run on an SDS gel, and autoradiographed, and the autoradiogram was scanned and the data were analyzed as described under "Materials and Methods." The value for P46 at the initiation of the chase was set at 100. At various times acid-insoluble radioactivity and DPA were also measured. The arrow gives the time when 50% of maximum DPA had been accumulated in the culture.

Presumably there are antibodies to this spore in our anti-spore protease y-globulin, resulting in its detection on immunoblot analysis which is carried out with excess antibody. However, the level of this interfering species was constant throughout growth and sporulation, and it was not detected in immunoprecipitates of pulse-labeled cells (Figs. 1 and 5). In contrast, P46 did not appear until early in sporulation, whereupon it declined as P41 appeared (Figs. 5 and 6). These data were again consistent with the assumption that P46 is a precursor to P41, with the appearance of P41 being slightly before the developing spore accumulated DPA. Greater than 80% of the P46 and P41 was found in the forespore (Fig. 5, compare lanes e and e-F). Radioimmunoprecipitation of the extracts from this experiment also showed that accumulation of protease antigen paralleled accumulation of P46 plus P41, and thus that both P46 and P41 are antigenically related to the spore protease (Fig. 6).

Analysis of Protease Mutants—While the data given above were consistent with P46 and P41 being precursors of the spore protease, it was essential to prove this point conclusively. Consequently, we analyzed spore protease mutants for a defect in either P46 or P41, or both. Pulse-labeling of the best protease mutant (C-1-5) in parallel with the streptomycin-resistant wild type strain, followed by extraction and analysis of immunoprecipitates revealed only 10-15% of the wild type level of P46 in immunoprecipitates from mutant C-1-5 (data not shown). However, the rate of total protein synthesis was identical in the two strains (data not shown). In contrast to the immunoprecipitation data, radioimmunoassay of extracts of spores of C-1-5 (as well as those from the other protease mutants) indicated that they contained as much protease antigen as did wild type spores (Table 1). This suggested that the low level of labeled P46 in immunoprecipitates from C-1-5 did not reflect a low rate of P46 synthesis, but rather that antigenic determinants necessary for immunoprecipitation of labeled mutant P46 were labile and were lost during the long (1½ days) incubation of samples for immunoprecipitation prior to the addition of immune serum. That spore protease antigen was indeed altered in mutant C-1-5 was shown more dramatically by immunoblot analysis, since spores of C-1-5 contained only 25% of the level of P41 found in the wild type or other protease mutant spores, but contained a 3 to 4-fold higher level of P46 (Fig. 7). Furthermore, pulse-chase experiments with mutant C-1-5 revealed that only a small amount of the labeled P46 detected in immunoprecipitates was chased into P41 in dormant spores (data not shown).

Comparison of Tryptic Peptides from P46, P41, and P49—As a final proof of the interrelationship of P46, P41, and P49 we compared the [35S]methionine-labeled tryptic peptides from P46, P41, and P49, on HPLC, and compared this pattern with the peptide profile obtained from a digest of purified unlabeled P46. The labeled peptides from pulse-labeled P46 and from chased P41 were identical except for two peaks early in the pattern from P46 which were not in P41 (Fig. 8, peaks noted with asterisks). The labeled peptide map of P46 remaining after a 5-h chase (after P46 processing was complete) was...
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SDS gels was slight, extracts from spores germinated for only 7-10 min contained approximately equal amounts of P41 and P40, and these two species were resolved on SDS gels (lanes 3 and 7, Fig. 10). The detection of two bands in these extracts indicates that P41 and P40 are distinct species. The conversion of P41 and P40 was detected either by immunoblot analysis (Fig. 10), or by analysis of immunoprecipitates from spores which initially contained labeled P46, and which were then germinated (data not shown). The conversion of P41 to P40 was rapid (it was at least half complete in 10 min (Fig. 10)) and took place even when ATP production during germination was blocked with cyanide (lanes 6, 7, and 8, Fig. 10) or by germination in KBr plus NaF (Ref. 16 and data not shown). P40 once formed, was labile, and disappeared with a t1/2 of about 40 min in a reaction which required metabolic energy, since P40 was stable in spores germinated in KCN (Fig. 10). Previous work has shown that both protease antigen and spore protease activity disappear in parallel during germination in an energy-requiring process (5). The conversion of P41 to P40 took place not only in vivo during spore germination, but also in vitro. Fresh extracts of dormant spores contained predominantly P41, but dialysis overnight at 4 °C in buffer B resulted in almost complete conversion to P40 (lanes 9, 10, and 11, Fig. 10). Again, analysis of a sample at an intermediate time resolved both P41 and P40 (lane 10, Fig. 10). The conver-

**Table I**

| Strain                             | Relative level of protease antigen |
|------------------------------------|-----------------------------------|
| Wild type                          | 100*                              |
| Wild type, streptomycin-resistant  | 97                                |
| B-2-1                              | 100                               |
| B-41-2                             | 120                               |
| C-1-5                              | 91                                |
| C-44-1                             | 88                                |

* Value set at 100.

identical to the pattern from pulse-labeled P46 and the labeled peptide maps of P41 and P40 (the latter from spores germinated 20 min with KCN, see below) were also identical (data not shown).

Comparison of the labeled peptides from pulse-labeled P46 with the peptide profile from the purified protease (P40) also showed all major radioactive peaks corresponded to P40 peptides, with two exceptions (Fig. 9, peaks noted with asterisks). These two exceptions were the two labeled peaks from P46 which were also not found in labeled P41 (Figs. 8 and 9). Presumably they are derived from the part of the molecule cleaved off in conversion of P46 to P41.

**Analysis of Spore Protease during Germination**

Strikingly, the first minutes of spore germination were accompanied by the conversion of P41 to a M₉ = 40,000 species (P40) with a mobility identical to that of the purified enzyme (Fig. 10). While the difference in mobility of P41 and P40 on SDS gels was slight, extracts from spores germinated for only 7-10 min contained approximately equal amounts of P41 and P40, and these two species were resolved on SDS gels (lanes 3 and 7, Fig. 10). The detection of two bands in these extracts indicates that P41 and P40 are distinct species. The conversion of P41 and P40 was detected either by immunoblot analysis (Fig. 10), or by analysis of immunoprecipitates from spores which initially contained labeled P46, and which were then germinated (data not shown). The conversion of P41 to P40 was rapid (it was at least half complete in 10 min (Fig. 10)) and took place even when ATP production during germination was blocked with cyanide (lanes 6, 7, and 8, Fig. 10) or by germination in KBr plus NaF (Ref. 16 and data not shown). P40 once formed, was labile, and disappeared with a t₁/₂ of about 40 min in a reaction which required metabolic energy, since P40 was stable in spores germinated in KCN (Fig. 10). Previous work has shown that both protease antigen and spore protease activity disappear in parallel during germination in an energy-requiring process (5). The conversion of P41 to P40 took place not only in vivo during spore germination, but also in vitro. Fresh extracts of dormant spores contained predominantly P41, but dialysis overnight at 4 °C in buffer B resulted in almost complete conversion to P40 (lanes 9, 10, and 11, Fig. 10). Again, analysis of a sample at an intermediate time resolved both P41 and P40 (lane 10, Fig. 10). The conver-

**Fig. 7. Immunoblot analysis of protease antigen from wild type and protease mutant spores.** Equivalent amounts (25 μg) of spores of the wild type (lane a), the streptomycin-resistant wild type (lane b), mutant C-1-5 (lane c), mutant B-2-1 (lane d), mutant C-44-2 (lane e), and mutant B-41-2 (lane f) were broken and extracted with 300 μl of buffer A, and samples (15 μl) were run on a SDS slab gel and immunoblotted. The letter o gives the position of an ovalbumin marker.
FIG. 8. HPLC analysis of the $^{[35]S}$methionine-containing tryptic peptides of P46 and P41. Cells (10 ml) were pulse-labeled with 1 mCi of $^{[35]S}$methionine slightly before the time for peak synthesis of P46. After 60 min, 5 ml were harvested immediately while 5 ml were chased for 5 h with unlabeled methionine. The cells were broken and extracted with 2.5 ml of buffer A, and the samples were processed for immunoprecipitation. In this experiment, the whole sample was treated with immune $\gamma$-globulin (10 pl). The solubilized protein from the immunoprecipitates was run on four 4-mm lanes of a 1.5-mm SDS slab gel, stained, and autoradiographed. Labeled P46 and P41 were cut out, processed, and digested, and the tryptic peptides were resolved by HPLC and the $^{[35]S}$methionine counted for 20 min as described under "Materials and Methods." All counts have been corrected for background (16 cpm).

The identification of P46 as a spore protease precursor was made initially by its immunoprecipitation from extracts of

FIG. 9. Comparison of tryptic peptides from P46 with $^{[35]S}$methionine-containing tryptic peptides from P41. 280 $\mu$g of purified spore protease (P40) were run on 10 4-mm lanes of a 1.5-mm SDS slab gel, and stained. The stained bands were cut out and mixed with the P46 band from 5 ml of pulse-labeled cells obtained as described in the legend to Fig. 8. The mixture was then processed and digested with trypsin, and peptides were resolved by HPLC and counted as described under "Materials and Methods." The optical density tracing at 214 has been corrected for absorption due to impurities in the buffers and gel itself. This correction was minor.

sion of P41 to P46 in spore extracts is presumably the reason that P41 is not found in preparations of the purified spore protease. In contrast to P41 and P46, any P46 present in the dormant spore did not disappear rapidly during germination or in dormant spore extracts (Fig. 10). This was true both with wild type spores (Fig. 10) and with spores of C-1-5 which contained levels of P46 3-4 times higher than wild type spores (data not shown).

DISCUSSION

The data presented in this communication indicate that the B. megaterium spore protease is synthesized during sporulation as a $M_r = 46,000$ polypeptide (P46). P46 is processed to a $M_r = 41,000$ (P41) form 2-3 h later and the dormant spore contains predominantly P41 with small amounts of unprocessed P46. In the first minutes of spore germination P41 is converted to a $M_r = 40,000$ species (P40) which appears identical with the subunits of the active enzyme purified from germinated spores. P40 then disappears during further germination in an ATP-dependent process. However, any P46 present in dormant spores is not significantly altered during germination.

The identification of P46 as a spore protease precursor was made initially by its immunoprecipitation from extracts of
cells pulse-labeled or pulsed and then chased during sporulation. While the data from these experiments are certainly consistent with a precursor-product relationship between P_{so}, P_{ti}, and P_{so} alone they do not conclusively prove it. However, three other lines of evidence also indicate the inter-relationship of P_{so}, P_{ti}, and P_{so}.

1) The radioimmunoassay, which utilizes iodinated P_{so} as the labeled antigen, showed that accumulation of protease antigen during sporulation paralleled accumulation of P_{so} plus P_{ti}, and that an extract which contained high levels of P_{so} but no P_{ti} or P_{so} had almost as much antigen as an extract containing high P_{ti} levels but little P_{so}.

2) Analysis of the protease mutant C-1-5 has shown that during sporulation C-1-5 synthesizes only 15% of the immunoprecipitable P_{so} of cells carrying the wild type protease gene. C-1-5 does synthesize as much protease antigen as do wild type cells, but presumably this antigen is labile and is lost during the 1 1/2 days of incubation of samples for immunoprecipitation prior to addition of immune serum. Additionally, spores of C-1-5 contain approximately one-fourth the P_{ti} and 4 times the P_{so} of wild type spores—presumably because of a defect in the P_{so} to P_{ti} conversion caused by a mutation in either the protease gene or in the gene for a processing enzyme (which could be the protease itself (see below)).

3) The final evidence for the inter-relationship between P_{so}, P_{ti}, and P_{so} was the good agreement between the [35S]methionine-containing tryptic peptide maps of P_{so}, P_{ti}, and P_{so} and between the tryptic peptide pattern of unlabeled P_{so} and the methionine-containing tryptic peptide map of P_{so}. Also shown in these latter experiments was that the small amount of P_{so} which is not processed during sporulation had an identical peptide map to that of P_{so} newly synthesized in sporulation.

While the precursor-product relationship between P_{so}, P_{ti}, and P_{so} seems firmly established, a number of questions remain concerning this system. 1) Why is there a 2–3 h delay in processing of P_{so} to P_{ti}? P_{so} is processed to P_{ti} only at or slightly before the time of DPA accumulation during sporulation, but residual dormant spore P_{so} is not processed to P_{ti} during spore germination. This suggests that the P_{so} to P_{ti} conversion requires intracellular conditions which exist only at the time of DPA accumulation. These conditions might include a rapidly decreasing water activity in the spore which is thought to take place at about the time of DPA accumulation (17), a decrease in the intrasporal pH which also must take place late in sporulation (18), or a decrease in the content of free divalent cations (19). Alternatively the P_{so} to P_{ti} conversion may require a processing enzyme which is synthesized only very late in sporulation and which is then lost. 2) Are the changes in going from P_{so} to P_{ti} to P_{so} all due to proteolysis? It seems most likely that the conversion of P_{so} to P_{ti} is due to proteolysis, since HPLC revealed two extra methionine-containing tryptic peptides in P_{so} as compared to P_{ti}, but the nature of the P_{ti} to P_{so} conversion is not as clear. The separation of P_{ti} and P_{so} on SDS gels clearly indicates that they are different species. However, since the [35S]methionine-labeled peptide maps of P_{ti} and P_{so} were identical, we have as yet no direct information on the nature of the difference between P_{so} and P_{so}. It is of course possible that the P_{ti} to P_{so} conversion is proteolytic, but alters no methionine-containing peptides. The conversion of P_{ti} to P_{so} in the absence of ATP production during germination rules out most covalent modifications as causing the P_{ti} to P_{so} shift. However, it is possible that the P_{ti} to P_{so} conversion involves removal of a prosthetic group from P_{so}. Clearly, it will be necessary to directly demonstrate that P_{so} loses some primary structure upon conversion to P_{so}. 3) Could the spore protease be involved directly in its own processing in the P_{so} to P_{ti} and/or the P_{ti} to P_{so} conversions? Consistent with this possibility is the aberrant conversion of P_{so} to P_{ti} in mutant C-1-5. However, this could also be because the mutant P_{so} is recognized poorly by a processing enzyme. Evidence against an autoconversion model is that P_{so} to P_{ti} conversion is apparently normal in three of the four protease mutants, and that P_{so} to P_{so} conversion is at most only slightly slowed in mutant C-1-5. However, the protease mutants almost certainly do have enzymatic activity in vivo (4), and consequently could be sufficiently active to catalyze their own processing. In view of the precedents for autoactivation of proteases, this is a question that merits further study. 4) The fourth, and possibly the most interesting question concerning the various protease precursors, is: What is their biological significance? While it...
is certainly possible that the precursors have no significant function, it is more attractive to imagine that they have some regulatory role. This is particularly attractive, since the spore protease must be inactive in the developing forespore and dormant spore, and become active only upon germination. Consequently, if P₄₁ and/or P₆₆ were catalytically inactive, or were unable to form an enzymatically active tetramer (5), this would explain the lack of protease action in developing and dormant spores. However, it would then imply that the system(s) involved in the P₆₆ to P₄₁ and P₄₁ to P₄₀ conversions must themselves be very tightly regulated. This would further suggest that the understanding of the control of these conversion processes may bring us one step closer to an understanding at the molecular level of the controls involved in the onset and maintenance of the enzymatic dormancy of the bacterial spore.

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