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Bacillus subtilis Spore Coats: Complexity and Purification of a Unique Polypeptide Component

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Extensively washed, dormant spores of Bacillus subtilis were disrupted with glass beads in buffer at pH 7 in the presence of protease inhibitors. Approximately 31% of the total spore protein was soluble, and another 14% was removed from the insoluble fraction by hydrolysis with lysozyme and washing with 1 M KCl and 0.1% sodium dodecyl sulfate. The residual spore integuments comprised 55% of the total spore proteins and consisted of coats and residual membrane components. Treatment of integuments with sodium dodecyl sulfate and reducing agents at pH 10 solubilized 40% of the total spore protein. Seven low-molecular-weight polypeptide components of this solubilized fraction comprised 27% of the total spore protein. They are not normal membrane components and reassociated to form fibrillar structures resembling spore coat fragments. The residual insoluble material (15% of the total spore protein) was rich in cysteine and was probably also derived from the spore coats. A solubilized coat polypeptide of molecular weight 12,200 has been purified in good yield (4 to 5% of the total spore protein). Five amino acids account for 92% of its total amino acid residues: glycine, 19%; tyrosine, 31%; proline, 23%; arginine, 13%; and phenylalanine, 6%.

Several models have been elaborated over the past few years to explain the regulation of gene expression during bacterial sporulation. These include catabolite repression (28), sequential gene induction (7), modification of RNA polymerase-promotor specificity (23), and changes in elements of the cell's translational machinery (20). Changes in RNA polymerase structure (22) and in the stringency of ribosomal structural requirements (20, 35) may occur during sporulation, but the role of modifications in the machinery of RNA and protein synthesis in controlling sporulation-specific gene expression is difficult to assess in the absence of probes for directly assaying the transcription and translation of such genes.

In our continuing efforts to interpret the regulatory events operating during bacterial sporulation, we have chosen to characterize the bacterial spore coat. Coats have been reported to contain 50 to 80% of the bacterial spore protein (4, 31, 34) and are selected for study because they represent a sporulation-specific product which is produced in abundance. Previous investigations of the structural components found in bacterial coats (4, 13, 17, 34) have indicated that they consist predominately of protein, with minor amounts of carbohydrate. Proteins extracted from intact spores of Bacillus cereus have been shown to migrate as two major species during electrophoresis in 15% polyacrylamide gels, and these two polypeptides are considered to be very similar or identical in primary structure (3–5, 9). A more heterogeneous protein composition has been suggested for spore coats of Bacillus subtilis (13, 31), although Munoz and Doi (J. Biol. Chem., in press) found a component of about 13,000 daltons to predominate in extracts of whole B. subtilis spores.

Spore coats become visible as morphological structures during the latter half of the developmental period, and pulse-labeling experiments have indicated that synthesis of coat protein precedes assembly by 2 to 3 h (31). This suggests the existence of precursors of the assembled coat proteins, and this has been confirmed by demonstrations of the presence, early in sporulation, of soluble polypeptides that are precipitated by antisera raised against mature coat components (5, 9; Munoz and Doi, in press; N. K. Pandey and A. I. Aronson, personal communication). The detailed analysis of the regulatory events involved in synthesis and assembly of spore coats requires characterization of the final product, so that their precursors can be recognized and used in developing quantitative assays for their synthesis. In this paper we report the resolution of several low-molecular-weight polypeptides from spore coats of B. subtilis. These

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low-molecular-weight polypeptides (9,000 to 16,000) represent 65% of the protein we have been able to solubilize from spore coats. One of these proteins, whose apparent molecular weight is 12,200, has been purified and characterized.

**MATERIALS AND METHODS**

Bacterial strains and procedures for growth and spore isolation. Cells of *B. subtilis* strain 168, from an overnight culture in brain heart infusion broth (Difco), were used as inoculum for 2X nutrient broth (Difco) medium (21), and growth and sporulation were carried out at 37°C as previously described (11). After 2 to 3 days, spores were isolated and freed of residual cells and cell debris essentially as described by Spudich and Kornberg (31). This involves hydrolysis with lysozyme (0.1 mg/ml) for 10 min at 37°C, followed by washing with 1 M NaCl, 0.14 M NaCl, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and water. All solutions contained protease inhibitors (see below). The final preparations were estimated to be greater than 99% refractile spores by phase-contrast microscopy. Coats are defined, in this paper as in others in the field (cf. 3), as that spore fraction remaining insoluble after breakage, lysozyme hydrolysis, and extensive washing of spores. This fraction, which could more accurately be called integuments, contains membrane components as well as "true" coat components (see Discussion).

Spores, stored in deionized water at 4°C, were centrifuged and resuspended in 0.05 M potassium phosphate buffer, pH 7.0. This buffer (buffer A), and all other solutions used in preparing spore coats, contained 10 mM ethylenediaminetetraacetate and 5% (vol/vol) of a 6-mg/ml ethanol solution of phenylmethyl sulfonfluride. Spores were suspended in buffer A and broken in a Bronwill model 2876 cell disruptor, using equal volumes of glass beads (120-μm diameter) and spore suspension. After 3 min of treatment, using liquid CO2 for cooling, greater than 99% breakage was achieved. Glass beads were allowed to settle for 10 min, and the supernatant, containing spore coats and soluble protein, was removed by pipetting. The beads were washed with 4 volumes of buffer A, giving greater than 90% recovery of input material. The suspension was centrifuged at 20,000 × g for 60 min, and the pellet was washed twice with buffer A. The pellet of coats was suspended in buffer A containing 50 μg of lysozyme per ml and incubated for 15 min at 37°C to remove cortex peptidoglycan. The spore coats were then washed as follows: (i) once with buffer A; (ii) twice with 1 M KCl in water; (iii) twice with 0.14 M KCl in water; (iv) once with 0.1% SDS in water; and (v) five times with deionized water. All procedures were carried out at 4°C except for the SDS wash, which was done at 15°C.

**Extraction of spores and spore coats**

With procedure A, purified coats or spores were suspended (10 mg of protein per ml) in 0.05 M sodium carbonate-bicarbonate buffer (pH 10), containing 1.0% SDS and 50 mM dithiothreitol, and incubated at 37°C for 20 min. After centrifugation at 20,000 × g for 20 min, the pellet was extracted in one-half the original volume of the same buffer. Coat preparations were also extracted by procedure B: 0.06 M tris(hydroxymethyl)-aminomethane(Tris)-hydrochloride buffer, pH 6.8, containing 3.0% SDS and 5% (vol/vol) 2-mercaptoethanol at 100°C (boiling-water bath) for 3 min. Initial extraction of coats involved two extractions by procedure A followed by centrifugation and two extractions as described for procedure B. The extraction of protein was monitored by the assay procedure of Lowry et al. (24) and by radioactivity solubilized. Coats were prepared from cells which had grown and sporulated in the presence of 5 μCi of L-[3H]leucine or 2.5 μCi of [35S]sulfate per ml. Insoluble material was solubilized by heating at 100°C in 0.1 N NaOH. After centrifugation, the supernatant was assayed by the method of Lowry et al. (24). This procedure was repeated until no further protein was detected in the supernatant. Other extraction conditions were attempted (guanidine-HCl, 6 M; urea, 8 M; guanidine thiocyanate, 6 M); however, the above protocols released the greatest amounts of coat protein and were reproducible. Extracts were stored at −20°C.

**Fractionation and characterization of spore coat proteins.** Protein extracted from spore coats was characterized by SDS-gel electrophoresis. Conditions of electrophoresis have been previously described (11), and the only modification for the present analysis was the use of 5 to 20% linear gradients of acrylamide for the running gel. Samples were applied to vertical slab gels of 13 or 30 cm in length along with bromphenol blue to mark the migration of the buffer front. Gels were stained for 30 min to 1 h at 60°C in a solution of 0.2% Coomassie brilliant blue in 50% methanol-10% acetic acid. Destaining was performed with 10% acetic acid at 60°C for 15 to 30 min.

Crude extracts of labeled spore coats were fractionated by column chromatography on Sephadex G-100. Extracts were dialyzed twice against 200 volumes of 0.05 M Tris-hydrochloride buffer (pH 8.8) containing 0.1% SDS (wt/vol). The column was equilibrated and run with the same buffer. Immediately prior to application, the coat protein extracts were made 0.1% (vol/vol) with 2-mercaptoethanol and heated in a boiling-water bath for 1 min. The effluent was monitored for absorbance at 280 nm and radioactivity. Appropriate protein fractions were pooled and lyophilized. This material was suspended in 0.05 M Tris-hydrochloride (pH 8.8) and dialyzed twice against 200 volumes of the same buffer. Samples were stored at −20°C.

One spore coat protein was purified by selective precipitation from the crude extract obtained by procedure A. The pH of the crude extract was lowered to 7.6, using 0.6 M Tris-hydrochloride buffer, pH 6.8, containing 1% SDS. The solution was then made 0.1 M with Na2SO4 and heated in a boiling-water bath until the solution temperature reached 80°C. Upon cooling, the solution became turbid, and the precipitate was collected by centrifugation at 20,000 × g for 20 min, washed three times with deionized water, and stored at −20°C. Selective precipitation could also be accomplished by dialyzing the crude extract against 0.05 M Tris-hydrochloride buffer, pH 7.6, containing 0.1 M Na2SO4.

Electron microscopy. Samples for thin-section electron microscopy were prefixed in 2% glutaralde-
hyde for 3 h at 4°C and then fixed by the method of Kellenberger et al. (16). After ethanol dehydration, sections were embedded in Spurr's low-viscosity embedding medium (Polysciences, Inc., Warrington, Pa.). Sections were cut on an LKB ultramicrotome, stained with 1% uranyl acetate and 1/10-diluted lead citrate (27), and examined with a Phillips 300 electron microscope at 60 kV.

Samples for negative staining were applied to Formvar-coated grids, stabilized with carbon, and then stained with 1% aqueous uranyl acetate. Small fragments of spore coats were prepared by sonically treating spores (10 Wilburson 2 in 2 ml of buffer A plus 2 g of 120-μm glass beads for 20 min. The coat fragments were collected by centrifugation (20,000 × g, 60 min), incubated for 30 min at 37°C with 50 μg of lysozyme per ml in buffer A, washed twice with water, and examined by negative staining.

Carbohydrate analysis. Assays for hexose, glucose, hexosamine, and muramic acid were performed on purified coat preparations and the residue remaining after extraction of coats. These assays were performed as described by Kotter et al. (18) for coats of Myxococcus xanthus.

Amino acid analysis. Amino acid analyses were performed on coat fragments and various fractions derived from them. Proteins in solution were precipitated by making the aqueous solution 90% (vol/vol) with acetone. After centrifugation and washing with 90% acetone, the pellets were dried by a stream of nitrogen through the tubes. Samples were hydrolyzed in constant-boiling-point HCl. Cystine and methionine were quantitated in samples which had been performic acid oxidized as described by Hirs (14) prior to acid hydrolysis. Acid hydrolysis was performed in sealed, evacuated tubes for 18 h at 110°C, and the HCl was then removed by vacuum evaporation over NaOH. The residue was suspended in lithium citrate buffer and analyzed on a Durrum D-500 amino acid analyzer (19).

Removal of SDS and aggregation of coat protein. The low-molecular-weight fraction from G-100 chromatography was lyophilized, resuspended in 0.05 M Tris-hydrochloride (pH 8.0), and dialyzed against the same buffer containing 0.1% SDS. The removal of free SDS was accomplished by dialysis against 0.05 M Tris-hydrochloride (pH 7.5). Solid urea (recrystallized from 70% ethanol) was then added to the protein solution to a concentration of 8 M. Protein was present at 5 mg/ml, and bound SDS was removed by using Dowex AG 1X-2 equilibrated in 0.05 M Tris-hydrochloride (pH 7.5) containing 8 M urea, as described by Steiner et al. (32). The resin was removed by passing the solution through a syringe equipped with a 26-gauge needle plugged with glass wool.

Synthesis and identification of di- and tri-trityrosine. Di- and trityrosine were synthesized as described by Gross and Sizer (12). Their mobilities were determined during: (i) ascending chromatography, using n-butanol-acetic acid-water (4:1:1); (ii) high-voltage paper electrophoresis, using 0.5% pyridine-5% acetic acid (pH 3.5); and (iii) high-voltage paper electrophoresis in 7% formic acid (pH 1.9).

Peptide analysis. Partial acid hydrolysis was performed using constant-boiling-point HCl in sealed, evacuated ampoules for 20 min at 100°C (boiling-water bath). Hydrolysates were vacuum vaporized over NaOH, dried twice from glass-distilled water, and dissolved in 0.5% pyridine-5% glacial acetic acid (pH 3.5). Samples were subjected to high-voltage paper electrophoresis (Whatman 3MM, 45 V/cm for 2 h) in pH 3.5 pyridine-acetic acid buffer (0.5% pyridine-5% glacial acetic acid). The dried paper was stained with fluorescamine (25), and peptide spots were visualized by examination with a UV lamp. Spots were cut from the paper and eluted with 20% (vol/vol) glacial acetic acid, and the peptides were recovered by vacuum evaporation. Amino acid analysis of the isolated peptides was performed by acid hydrolysis (constant boiling with HCl, 110°C for 2 h) and high-voltage paper electrophoresis in 7% formic acid (4,500 V, 2 h). Amino acids were detected by fluorescamine staining and identified by coelectrophoresis with standards. Tyrosine and peptides containing tyrosine were detected by specific staining with p-nitroso-β-naphthol (6).

RESULTS

Preparation of spore coats. The pellet from centrifugation of a 48-h culture contained 80 to 90% mature free spores, 10 to 20% residual cells, and cell debris. Treatment with lysozyme and extensive washing (see Materials and Methods) yielded spore preparations essentially free of cells and cell debris. Mature spores were hydrolyzed with lysozyme and washed with 1 M KCl and 0.1% SDS prior to breakage. After breakage of spores and removal of glass beads, the insoluble integuments were treated similarly. Protein was assayed by the Lowry procedure or estimated by following radioactivity in [3H]leucine or [35S]sulfate which had been incorporated into protein during growth and sporulation (Table 1).

Extraction of spores and spore coats. Table 2 shows the results of extraction of isolated coat fragments by various procedures. Direct extraction by procedure B released the maximum amount of Lowry assayable protein, [3H]leucine, and [35S]sulfur label from coat fragments. An insoluble residue, representing 30% of the Lowry assayable protein, 28% of the [3H]leucine, and 60% of the [35S]sulfur, remained after extraction and was only solubilized by acid, base, or enzyme-catalyzed hydrolysis. Pronase solubilized 30% of the [3H]leucine and 20% of the [35S]sulfur present in this insoluble residue.

Extraction of coats by procedure A seemed to remove a substantial portion of the protein solubilized by procedure B. Extraction by procedure A, followed by procedure B, released the same total amount of protein, [3H]leucine, and [35S]sulfur as procedure B alone. Furthermore, if extraction by procedure B was followed by procedure A, the latter failed to release significant amounts of protein, [3H]leucine, or [35S]sulfur.
Table 1. Distribution of protein, [3H]leucine, and [35S]sulfate during preparation of spore coats

<table>
<thead>
<tr>
<th>Prepn step</th>
<th>% Lowry protein</th>
<th>% [3H]leucine</th>
<th>% [35S]sulfate</th>
<th>% [35S] in protein (corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Soluble after breakage in buffer A</td>
<td>25</td>
<td>31</td>
<td>67.2</td>
<td>16</td>
</tr>
<tr>
<td>Trichloroacetic acid precipitated from above</td>
<td>18</td>
<td>3.6</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>Insoluble after breakage</td>
<td>75</td>
<td>69</td>
<td>32.4</td>
<td>84</td>
</tr>
<tr>
<td>Buffer A wash of insoluble pellet</td>
<td>2.8</td>
<td>2.2</td>
<td>0.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Lysozyme supernatant</td>
<td>5.3</td>
<td>1.7</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>1 M NaCl wash</td>
<td>2.4</td>
<td>0.26</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>0.14 M NaCl wash</td>
<td>1.7</td>
<td>0.54</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>0.1% SDS wash</td>
<td>4.7</td>
<td>0.58</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

*63.6% of the [35S] incorporated into whole spores was soluble after breakage and was not precipitated by cold 5% trichloroacetic acid. Of this, 61% was in the included peak after fractionation on Sephadex G-50 and was assumed to be sulfolactate. The residual 2.8% was in acid-soluble polypeptides, probably of the type described by Setlow (29). The values for [35S]sulfate incorporated have been corrected for the 61% in sulfolactate.

Table 2. Extraction of spore coats

<table>
<thead>
<tr>
<th>Extraction conditions</th>
<th>% of total integuments solubilized</th>
<th>Lowry protein</th>
<th>% [3H]leucine</th>
<th>% [35S]sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procedure B alone</td>
<td>69.6</td>
<td>71.3</td>
<td>40.4</td>
<td></td>
</tr>
<tr>
<td>Reextracted by procedure B</td>
<td>4.6</td>
<td>8.7</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequential extractions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procedure A</td>
<td>48.0</td>
<td>47.5</td>
<td>24.3</td>
<td></td>
</tr>
<tr>
<td>Reextracted by procedure A</td>
<td>5.2</td>
<td>7.7</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Procedure B after procedure A</td>
<td>16.2</td>
<td>12.3</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>Reextraction of above by procedure B</td>
<td>2.8</td>
<td>2.1</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>72.2</td>
<td>69.6</td>
<td>40.6</td>
<td></td>
</tr>
<tr>
<td>Residual insoluble</td>
<td>28</td>
<td>30</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

Attempts to solubilize substantial amounts of protein from intact spores were not successful. Only 6% of the total spore protein (determined by Lowry or [3H]leucine assay) was solubilized in 3 h of incubation by procedure A. This is not surprising in view of the fact that the function of the spore coat is to protect the spore from environmental damage. We expected small coat fragments to be more readily solubilized.

Electron microscopy. Thin-section electron microscopy of B. subtilis spores reveals a spore coat with two major morphological structures, the inner lamellar coat and the more densely staining outer coat (OC) (Fig. 1A). The outermost layer of the OC stains lightly and is sometimes separate from the bulk of the OC. Amorphous stained material lies between the unstained cortex and the inner lamellar coat. The spore coat fragments isolated by our procedures appear as intact coat structures (Fig. 1C), with no morphological evidence of damage due to isolation. The results of extracting whole spores by procedure A are shown in Fig. 1B. The inner lamellar coat is sometimes detached from the cortex, but most disruption is seen in the OC. However, most of the coat structure remains intact, consistent with the removal of only 6% of the spore protein (see above).

Extraction of coat fragments by procedure A followed by procedure B results in drastic alterations in coat structure. The inner lamellar coat is solubilized, as well as much of the OC (Fig. 1D), leaving a loose matrix, apparently derived from the OC, an inner component of unknown origin, and a thin outer layer. Examination of coats extracted by procedure A alone did not reveal selective extraction of morphological components, but rather a less complete solubilization of total coat material (data not shown).

Fractionation and characterization of solubilized coat proteins. Initial attempts at fractionating soluble coat protein on 15 or 20% polyacrylamide gels revealed two diffuse bands (extraction procedure A) and 3 diffuse bands (extraction procedure B) in the molecular weight range 9,000 to 16,000 (data not shown). The use of 5 to 20% gradient SDS-gels provided the resolution necessary to characterize these low-molecular-weight extracted polypeptides. Figure 2 shows the patterns observed by Coomassie brilliant blue staining.

The extract from procedure A consists of several minor polypeptide species of molecular weight greater than about 16,000 and seven major polypeptides of molecular weight less than 16,000, with four predominant species in the 9,000 to 13,000 range (slot A, Fig. 2). The short (13 cm) gradient slab gels were not entirely adequate for resolving the low-molecular-weight polypeptides. Figure 3 shows the results ob-
tained with a 30-cm gradient slab gel. Extraction by procedure B, after extraction by procedure A, gave a similar pattern of high- and low-molecular-weight polypeptides (slot B, Fig. 2) but in grossly different proportions.

Among the four low-molecular-weight species, the fastest and slowest species (about 8,000 and 13,000, respectively) were much more prevalent in this extract. It contains relatively little of the 12,200-dalton, tyrosine-rich protein (see below). Two polypeptides of 18,000 to 20,000 daltons in size were also more prevalent in this extract. Reextraction by procedure B after extraction by procedures A and B (slot H, Fig. 2) gave a similar...
FIG. 2. Electrophoresis of fractions of solubilized coat protein in 5 to 20% gradient gels. (A) Protein solubilized from coat fragments by procedure A; (B) protein solubilized from coat fragments by procedure B after extraction by procedure A; (C) protein solubilized from coat fragments by procedure A after selective precipitation of the 12,200-dalton proteins; (D) the purified 12,200-dalton protein; (E) protein solubilized from intact spores by procedure A; (F) protein solubilized by a second extraction of spores as in (E); (G) protein extracted from coat fragments by procedure B alone; (H) protein solubilized from coat fragments by a second extraction with procedure B after extraction by both procedures A and B. Quantities of protein applied, in micrograms, are indicated above each slot. Molecular weight standards are bovine serum albumin, DNase, I, and lysozyme: 68,000, 31,000, and 14,500 daltons, respectively.

pattern. Direct extraction by procedure B without A (slot G, Fig. 2) gave essentially a mixture of the A and B patterns. Extraction of intact spores by procedure A removed four major polypeptides, three of which comigrated with 9,000- to 13,000-dalton polypeptides released from coat
procedure A indicates a molecular weight of about 12,200 (Fig. 2, slot D). It is the most intensely stained component of the seven low-molecular-weight bands solubilized by procedure A (Fig. 2, slot A), and little of it remains in the supernatant from precipitation (Fig. 2, slot C). Selective precipitation of this protein required lowering the pH to 7.7 and increasing the ionic strength by the addition of 0.1 M Na₂SO₄. Precipitation could be performed by dialysis against 0.05 M Tris-hydrochloride buffer, pH 7.7, containing 1% SDS and 0.1 M Na₂SO₄, or more rapidly and on a larger scale by titration of the crude extract to pH 7.7 with 0.6 M Tris-hydrochloride, pH 6.8, followed by the addition of Na₂SO₄ to 0.1 M and heating in a boiling-water bath to 80°C. Upon cooling, the solution became turbid, and phase-contrast microscopy revealed the presence of 0.1- to 1-μm-diameter spherical particles which were phase bright. This procedure removed 10% of the Lowry assayable protein from the crude extract or 15 to 20% of the low-molecular-weight fraction (see below).

The spherical particles were examined by thin-section electron microscopy. Figure 4A and B shows thin sections of aggregated and single particles. Figure 4C and D shows scanning electron micrographs of a mixture of spherical particles and B. subtilis spores.

The 12,200-dalton protein was soluble in 6 M guanidine-HCl or 8 M urea, in either 0.05 M ammonium acetate or 0.005 M Tris-hydrochloride, pH 7.7. Particles of the 12,200-dalton protein, suspended in water, were also solubilized by the addition of NaOH to pH 11. Lowering of the pH to 7 resulted in recovery of the intact molecule, as indicated by gel electrophoresis (data not shown).

The soluble proteins, from procedure A or B, were separated by gel filtration on Sephadex G-100 in the presence of SDS. The exclusion limit for G-100, under these conditions, was found to be about 18,000 daltons, based on the observation that lysozyme (reduced and denatured by heating in buffer plus 3% SDS and mercaptoethanol) was just included in the gel matrix and was clearly separated from the void volume. Figures 5 and 6 show the elution profiles of the crude extracts generated by extraction procedures A and B. The proteins with a molecular weight of less than 18,000 consistently represented about 65% of the optical density, [%H]leucine counts, and Lowry assayable protein applied to the column. Figure 3 shows the gel electrophoresis patterns of the materials isolated by column chromatography. The protein present in the void volume consists of species larger than 20,000 daltons (Fig. 3, slot A) and did not dissociate to lower-molecular-weight material, even

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**Fig. 3.** Electrophoresis of coat protein, solubilized by procedure A, after fractionation by chromatography, using Sephadex G-100. The gel, a 5 to 20% linear gradient of acrylamide, was 30 cm long. (A) Proteins from the void volume; (B) the included proteins; (C) the mixture prior to fractionation on Sephadex G-100; (D and G) as in Fig. 2. The positions of bovine serum albumin (68,000 daltons) and lysozyme (14,500 daltons) in this gel system are shown for reference.

fragments (Fig. 2E). A major component of this extract, molecular weight about 30,000, was apparently a minor component of extracts of coat fragments. A second extraction by procedure A failed to release much additional protein (slot F, Fig. 2).

The low-molecular-weight polypeptides (<16,000) represent the most predominant solubilized polypeptides visualized by Coomassie brilliant blue staining of gels. Therefore, we focused our attention on characterizing these components.

We have been able to purify one of these proteins by selective precipitation from crude extracts isolated by procedure A. The mobility of this protein in 5 to 20% gradient gels after solubilization in the pH 10 buffer used in pro-
though they were heated to 100°C for 3 min in 0.06 M Tris-hydrochloride (pH 6.8), containing 3% (wt/vol) SDS and 5% (vol/vol) 2-mercaptoethanol. In addition, the protein of greater than 20,000 daltons migrated as an even more heterogeneous population on 30-cm gradient gels (cf. Fig. 2, slot C, and Fig. 3, slot A).

The low-molecular-weight proteins isolated by column chromatography ran as seven discrete species on gel electrophoresis, all between 9,000 and 16,000 daltons. The fourth band from the bottom (Fig. 3, slot B) represents the 12,200-dalton protein.

**Amino acid analysis.** Table 3 shows the results of amino acid analysis of various coat fractions. The residual insoluble fraction after extraction by procedures A and B was characterized by a high content of methionine plus cysteine. Hydrolysates of unoxidized samples of the residue also contained two unknown components comprising less than 5% of the total ninhydrin-reacting material. One eluted near taurine and was also observed as a very minor component in hydrolysates of intact coats. The second eluted near tryptophan and was present in an amount which precluded detection in hy-
drolysates of intact coats. The amino acid composition of protein extracted from intact spores was significantly different from that of protein extracted from coat fragments.

The amino acid composition of the 12,200-dalton protein was determined by averaging the results of three separate analyses. Overloading an SDS-gel with preparations of this protein failed to reveal any contaminating polypeptides by Coomassie brilliant blue staining, although this does not rule out minor contamination by a heterogeneous population of other polypeptides from crude coat extracts.

![Graph](image_url)

**Fig. 5.** Fractionation of protein solubilized from coat fragments by procedure A. The solubilized protein was applied to a column of Sephadex G-100 and eluted with 0.05 M Tris-hydrochloride, pH 8.8, containing 0.1% SDS. Arrow A marks the void volume as determined with blue dextran 2000. Arrow C represents the sum of void and included volumes, as determined by elution of free [3H]leucine. The 280-nm-absorbing material eluting at C is 2-mercaptoethanol, which was present in the protein solution at the time of application to the column. Arrow B shows the position of elution of denatured lysozyme. Symbols: Absorbance at 280 nm (○); radioactivity (△).

**Fig. 6.** Fractionation on Sephadex G-100 of protein solubilized from coat fragments by procedure B. The coat fragments had been previously extracted by procedure A. Conditions and symbols are the same as those given in the legend to Fig. 5.

**Table 3.** Amino acid analysis of coat fractions expressed as moles percent

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Coat fragments</th>
<th>Soluble by procedure A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Soluble by procedure B preceded by A</th>
<th>Insoluble after procedures A and B</th>
<th>12,200-dalton protein</th>
<th>Solubilized from spores by procedure A</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASX&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.9</td>
<td>8.3</td>
<td>4.9</td>
<td>10.0</td>
<td>1.25</td>
<td>11.1</td>
</tr>
<tr>
<td>THR</td>
<td>7.5</td>
<td>4.9</td>
<td>4.8</td>
<td>5.5</td>
<td>0.38</td>
<td>5.1</td>
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<tr>
<td>SER</td>
<td>6.0</td>
<td>5.6</td>
<td>7.1</td>
<td>9.0</td>
<td>0.99</td>
<td>9.5</td>
</tr>
<tr>
<td>GLY&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.4</td>
<td>9.2</td>
<td>4.4</td>
<td>6.5</td>
<td>2.18</td>
<td>10.3</td>
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<tr>
<td>GLY</td>
<td>15.1</td>
<td>13.4</td>
<td>20.3</td>
<td>11.7</td>
<td>19.11</td>
<td>6.3</td>
</tr>
<tr>
<td>ALA</td>
<td>6.2</td>
<td>8.1</td>
<td>7.7</td>
<td>5.4</td>
<td>0.96</td>
<td>5.9</td>
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<td>VAL</td>
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<td>4.9</td>
<td>3.6</td>
<td>4.0</td>
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<td>ILE</td>
<td>3.0</td>
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<td>2.7</td>
<td>2.9</td>
<td>0.45</td>
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<td>LEU</td>
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<td>3.1</td>
<td>3.9</td>
<td>0.89</td>
<td>4.0</td>
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<tr>
<td>TYR</td>
<td>5.5</td>
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<td>8.9</td>
<td>8.3</td>
<td>32.2</td>
<td>4.4</td>
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<tr>
<td>PHE</td>
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<td>7.5</td>
<td>4.6</td>
<td>6.0</td>
<td>4.0</td>
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<tr>
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<td>2.0</td>
<td>10.2</td>
<td>0.52</td>
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<tr>
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<td>2.2</td>
<td>5.7</td>
<td>0.18</td>
<td>6.7</td>
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<tr>
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<td>6.1</td>
<td>2.6</td>
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<td>PRO&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>16.5</td>
<td>9.2</td>
<td>24.2</td>
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</tr>
<tr>
<td>MET&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.83</td>
<td>0.91</td>
<td>0.31</td>
<td>0.01</td>
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<tr>
<td>CYSc</td>
<td>2.1</td>
<td>1.1</td>
<td>0.74</td>
<td>4.2</td>
<td>0.01</td>
<td>1.6</td>
</tr>
</tbody>
</table>

% Total protein 100.0 46.5<sup>a</sup> 18.0 28.0 7.5

<sup>a</sup> The 12,200-dalton protein was removed prior to amino acid analysis.

<sup>b</sup> Determined as aspartic acid and glutamic acid after hydrolysis.

<sup>c</sup> Determined as methionine sulfone and cysteic acid after performic acid oxidation and hydrolysis.
Carbohydrate analysis. The hexosamine and hexose contents, in moles per 100 mol of amino acid residues, were <0.05 and 1.12, respectively, for the total coat fragments and 0.12 and 2.0, respectively, for the insoluble fraction after extraction by procedures A and B. Neither fraction contained detectable muramic acid (<0.15 mol/100 mol of amino acid). The fact that only 84% of the dry weight of coats was accounted for by protein is probably due to the presence of glass fragments in the coat preparation. The percent dry weight as protein in the insoluble residue was experimentally determined to be 59% and was expected to be 62%, based on 84% of the dry weight of coats being protein and 70% of this protein being extracted; thus, all of the nonprotein components are in the insoluble fraction.

Removal of SDS and reaggregation of coat proteins. Initial attempts at removing SDS by passage of a coat protein solution over a small (2 ml) column of Dowex AG 1-X2 resulted in a 70% loss of material but equal recovery of all polypeptides in the buffer urea effluent (data not shown). Recoveries of greater than 80% were obtained by adding a small amount of resin to coat proteins in buffered urea and removing the beads with a syringe plugged with glass wool. This procedure was used to remove SDS from the low-molecular-weight proteins solubilized by procedure A and after fractionation on G-100. The solution was then subjected to dialysis against buffer without denaturants. Aggregates formed which, after centrifugation (20,000 × g, 30 min), contained 80% of the total [3H]leucine present. Figure 7 shows two types of aggregates present after removal of urea by dialysis, as well as structures observed in sonically treated preparations of spore coats. Fibers of similar dimensions and orientation were present in both preparations, in addition to spherical particles. Components in the reaggregated preparation revealed what appears to be assembly intermediates with a definite symmetry (Fig. 7C). Clusters of annular particles of about 4 nm in diameter form loosely packed aggregates.

Peptide analysis and estimation of di- and trityrosine content. The insoluble 12,200-dalton protein was not susceptible to hydrolysis with trypsin, nor were any cleavage products observed when the protein was solubilized in 2 M urea and incubated with trypsin. Partial acid hydrolysis generates reproducible peptide fragments as shown in Fig. 8. The most intensely staining peptides (P1, P2, P3, P4, and P5) also stained positively for tyrosine. Peptides 1 through 4 and 8 all contained tyrosine and proline as major constituents (determined by hydrolysis and paper electrophoresis in 7% formic acid), and all but P5 contained phenylalanine. Peptides 2, 4, and 8 also contained glycine as a major amino acid, and peptides 4 and 8 contained arginine, but these peptides contained only traces of other amino acids. In addition, we observe one unknown fluorescently-stained spot in complete hydrolysates of peptide 1 and of the 12,200-dalton protein. It migrates just behind tyrosine in 7% formic acid. Figure 8 also shows the results of paper electrophoresis (7% formic acid) of a complete acid hydrolysate of the 12,200-dalton protein as well as of the di- and trityrosine reaction mixtures. Di- and trityrosine are stable to acid hydrolysis and were not detected in hydrolysates of the 12,200-dalton protein, intact coats, or the residue from coats after extraction at pH 10 with SDS and dithiothreitol.

Di- and trityrosine were prepared from L-tyrosine as described by Gross and Sizer (12). Assuming that the conversion of tyrosine to either di- or trityrosine was complete, these could be present in the reaction mixture at maximum values of 2.7 and 1.8 mM, respectively. We could easily detect these compounds in 1 μl of the reaction mixture, in the presence of absence of coat protein hydrolysates, after paper electrophoresis (pH 3.5, or 7% formic acid) or paper chromatography (in n-butanol-acetic acid-water, 4:1:1), by their characteristic UV-stimulated blue fluorescence. On the basis of this conservative estimate of detectability, we find that there is less than 1 mol of dityrosine in the hydrolysate of 360,000 g of total coat fragments, 120,000 g of the insoluble coat residue, 60,000 g of the 12,200-dalton protein, or 80,000 g of the high- and low-molecular-weight fractions isolated by G-100 chromatography.

DISCUSSION

Previous attempts at characterizing the spore coat proteins of B. subtilis have been difficult to evaluate, either because small amounts of protein were solubilized or because procedures used for fractionation were inadequate (13, 17, 31). Spore coats were shown to consist mainly of protein and to be relatively rich in glycine, tyrosine, and cysteine. We were not able to extract representative quantities of coat protein from intact spores. However, we have consistently been able to solubilize 70% of the protein present in the pellet generated by breakage of spores with glass beads. The incorporation of 2 to 8 M urea in the extraction procedures did not seem to affect the results. Gel electrophoresis of the soluble extracts demonstrates a consistent pat-
tern containing many protein components. The seven most abundant species are of low molecular weight (approximately 9,000 to 16,000).

The distribution of protein during the isolation of spore integuments and the partial solubilization and fractionation of their coat and membrane components is summarized in Fig. 9. These data are used in estimating the proportions of the total spore protein present in different physiological components (Table 4).
acid-soluble species that are normally degraded to amino acids during germination. They are soluble after proteolysis, unless spores are broken in the dry state, only fragments of these acid-soluble, labile spore proteins are recovered. We have characterized the similar proteins of *B. subtilis* spores (C. W. Johnson and D. J. Tipper, unpublished observations) and find that, under the breakage conditions used here, fragments that do not precipitate with trichloroacetic acid are produced. The acid-soluble proteins comprise 15 to 18% of the total spore protein and have only 70% of the leucine content of total spore proteins, so they account for most of the 13% trichloroacetic acid-soluble [3H]leucine (Table 1), which is estimated to correspond to 15% of the total spore protein (Fig. 9, Table 4).

The distribution of 35S label is drastically different from that of [3H]leucine (Table 1) in that 64% is acid soluble after breakage. The acid-soluble proteins have a low cysteine and methionine content and can account for only 2.6% of the total 35S. The difference, 61% of the total spore 35S, is most probably sulfolactic acid, as demonstrated by Nelson et al. (26). Germination of 35S-labeled *B. subtilis* spores results in the release of a similar fraction of the label into the medium (data not shown). Table 1 also shows the distribution of the 39% of the total spore 35S assumed to be in protein, during spore breakage. The high ratio of 35S to 3H in the integuments is due to the coat fraction that resists all solubilization and which has a high cysteine content (Table 3). It contains 15% of the spore protein and leucine but 33% of the total 35S in spore protein (Table 2, Fig. 9).

Spores were treated with lysozyme and were washed extensively (31) before breakage to remove residual vegetative cells, sporangia, and adherent vestiges of sporangial membrane. Some of the proteases produced during sporulation adhere tightly to the exterior of spores but are completely removed by washing with 1 M salt (33). The presence of ethylenediaminetetraacetate and phenylmethyl sulfonlfyl fluoride during spore breakage should also minimize artefacts. SDS-gel patterns of extracted proteins have been highly reproducible, and if any artefactual proteolysis occurs during isolation and extraction, it must also be highly reproducible.

The integuments derived from breakage were hydrolyzed with lysozyme to remove cortex and cell wall primordium peptidoglycans. Subsequent washing with 1 M KCl and 0.1% SDS was used to remove loosely bound protein. In control experiments it was found that lysozyme binds to integument preparations and could not be eluted with buffer or 1 M salt. Its removal required

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**Fig. 8. High-voltage paper electrophoresis of partial and complete hydrolyzates of the 12,200-dalton coat protein.** (A) Fluorescamine-staining peptides generated by partial acid hydrolysis and separation at pH 3.5, 4,500 V, for 2 h; (B) separation of a complete acid hydrolyzate of the 12,200-dalton protein. Amino acids were identified by comigration with standards. The positions of tyrosine, ditryosine, and trityrosine are also shown. Conditions for (B) are 7% formic acid, 4,500 V, for 2 h.
TABLE 4. Estimated distribution of spore proteins between fractions shown in Fig. 9a

<table>
<thead>
<tr>
<th>Origin</th>
<th>Fraction (Fig. 9)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>True coat polypeptides</td>
<td>H(15) + J(27) + some of K (4)</td>
<td>46</td>
</tr>
<tr>
<td>Membrane proteins</td>
<td>Most of G(11) + most of K (9)</td>
<td>20</td>
</tr>
<tr>
<td>Acid-soluble proteins</td>
<td>E (19) + D (16) + some of G (3)</td>
<td>15</td>
</tr>
<tr>
<td>Ribosomes, soluble enzymes, etc.</td>
<td></td>
<td>19</td>
</tr>
</tbody>
</table>

a The distribution of protein in fractions G and K between membrane and coat is purely conjecture.

0.1% SDS at 15°C. These combined procedures removed a considerable fraction (14%) of the total spore protein (Table 1, Fig. 9). The pattern obtained on SDS-gel electrophoresis resembled that seen on electrophoresis of solubilized B. subtilis membranes (11; R. C. Goldman, unpublished observations), so that much of this 14% probably is derived from membrane proteins (Table 4). This fraction contained none of the low-molecular-weight spore coat polypeptides subsequently solubilized; however, we cannot rule out the loss of specific coat components of higher molecular weight during the preparation of integuments. These integuments appear to be morphologically intact (cf. Fig. 1C with Fig. 1A).

Mature spores of B. subtilis do not contain a loosely fitting exosporium, as is seen, for example, in B. cereus spores (15). Nevertheless, most bacterial endospores are surrounded by an exosporium, and B. subtilis may be an exception only in that the exosporium fits tightly to the OC and is not clearly visible until the coats have been partially solubilized (4, 30). The outermost layer of the intact spores and integuments shown in Fig. 1 does seem to stain less densely than the bulk of the OC layer and occasionally seems to be separate from it. Sousa et al. (30) saw a layer they interpreted as being exosporium in preparations resembling those shown in Fig. 1B. This may be the source of the residual, outer linear structure seen in Fig. 1D and an impediment to the extraction of coat proteins from whole spores.

The washed spore integuments contained 55% of the spore protein, and Fig. 9 shows the distribution of this material between the low- and high-molecular-weight solubilized fractions and the residual insoluble material. Three-quarters could be solubilized, and 65% of this was found...
in the low-molecular-weight fraction that gave a consistent pattern of seven bands in the 9,000
to 16,000-dalton range. These bands are not seen in membrane preparations of sporulating B. sub
tilis cells (11) and are presumed to be unique spore coat components.

The low-molecular-weight proteins extracted by procedure A remain soluble when SDS is
removed and exchanged for urea. Removal of urea by dialysis results in the formation of aggre
gates which contain most of the protein and which resemble structures observed when spore
coats are fragmented by sonic treatment with glass beads. This confirms coat origin and sug
jects that their complexity is not artefactual. It also suggests that spontaneous aggregation of
mature coat protein may play a role in the formation of coat structures.

The proportion of any of the integument frac
tions which is derived from coat structures is a matter of conjecture: washing of the integu
ments with 1 M salt and 0.1% SDS will certainly leave residual membrane components, and coat
assembly may trap sporangial proteins nonspecifically. Coats may contain specific proteases func
tional in germination, as well as adsorbed activities which are destroyed during heat activa
tion (33). It has been estimated that in a mature sporangium, 40% of the total protein is
present in the spore (31) and 8% in spore mem
branes (11). Thus, 20% of the total spore protein is estimated to be in spore membranes, and this
could represent 28% of the protein present in our coat preparations. We have found that 25%
of the protein solubilized from our coat prepara
tions migrates during gel electrophoresis as a heterogeneous mixture of proteins of greater
than 16,000 in molecular weight (fraction K, Fig. 9).

Most of this material is probably derived from spore membranes, although some may rep
resent protein nonspecifically trapped during coat assembly or protein functionally related to
coat assembly. This is the reasoning used in estimating the proportion of membrane proteins in
fractions G and K (Table 4).

Fraction K could also contain high-molecular
weight aggregates of the low-molecular-weight coat proteins which resist dissociation by treat
ment with SDS and mercaptoethanol during 3
min in a boiling-water bath. Aronson and Fitz
James (3) have considered the possibility of lysinoalanine formation from cysteine and lysine
during extraction of coat protein at high pH.

Initial studies on this reaction (8) showed that
this reaction did not occur at either pH 10.6
during 6 h or pH 7.0 in 0.1 M Tris-hydrochloride
(100°C for 30 min). Our extraction procedures
(pH 10, 37°C for 20 min, and pH 6.8, 100°C for
3 min) solubilized protein which, upon amino
acid analysis, did not reveal the production of
lysinoalanine. It seems unlikely that the high
molecular-weight proteins in our solubilized pro
tein fractions arose from artefactual cross-link
ning of this type.

The insoluble fraction (H, Fig. 9) derived from
integuments is presumed to be of coat origin on
the basis of morphology (Fig. 1D) and its unus
ually high cysteine content (Table 3). We thus
estimate (Table 4) that of the protein in mature
spores of B. subtilis, almost half is in coats, the
rest being divided almost equally between mem
brane and "soluble" proteins (including ribo
somes) and the acid-soluble proteins. Similar
data have been derived from the use of different
fractionation techniques designed for the recov
ery of intact acid-soluble proteins (Johnson and
Tipper, unpublished observations). The greatest
margin for error is in the assignment of high
molecular-weight, detergent-solubilized proteins
between soluble, membrane, and coat fractions.

The insolubility of fraction H is unexplained.

Aronson and Pandey (5) found considerable
quantities of dityrosine in the acid hydrolysates of
a similar B. subtilis spore coat fraction. They
subsequently found this to be an artefact of per
formic acid oxidation and detected quantities of
dityrosine slightly above our detectability lim
its in the unoxidized and unhydrolyzed material
(Aronson and Pandey, personal communica
tion). Di- and trityrosine have been identified as
cross-links in resilin isolated from certain insects
(1), and their formation can result in cross
linked, insoluble protein aggregates. We do find
two unknown products in acid hydrolysates of
fraction H, but they do not comigrate with di
or trityrosine, and our upper limits on the pre
sence of the latter components in coat fractions
would seem to minimize the potential of such
cross-links for contributing to the structure of
spore coats. We have previously demonstrated
that aminosuccinyl lysine could not be detected in
B. subtilis coat hydrolysates, eliminating a
second type of intramolecular cross-link (34).

The peculiar solubility properties and amino
acid composition of the 12,200-dalton protein
clearly differentiate it from the bulk of the spore
proteins and also from the other low-molecular
weight proteins of presumed coat origin (fraction
J). We have prepared antisera to the 12,200
dalton protein (Goldman and Tipper, unpub
lished observations). This serum aggregates
spores and integument preparations, forms a
single strong precipitin band in immunodiffusion
gels against the 12,200-dalton protein, and fails
to form a band against fraction L, the super
nantant from precipitation of the 12,200-dalton pro

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tein (see slot D, Fig. 2). This confirms the uniqueness of the 12,200-dalton protein. Selective precipitation of this 12,200-dalton protein from crude extracts results in spherical aggregates of about 1-µm diameter when examined by light microscopy and thin-section and scanning microscopy. Thin sections usually show a granular structure without obvious regularity, but one tangential section showed a periodic structure. Optical rotation has indicated hexagonal symmetry (R. Goldman, unpublished observation). Although we have not investigated the precise structural conformation of this mode of aggregation, it appears to represent specific aggregation of the molecules rather than amorphous precipitation. The recovered precipitate of the 12,200-dalton protein accounts for 12 to 15% of the protein solubilized by procedure A, or about 20% of the low-molecular-weight fraction, consistent with the heavily stained band of 12,200-dalton proteins seen in gels of this low-molecular-weight fraction (Fig. 2B). It thus accounts for about 4% of the total spore protein and 9% of the total coat protein.

Five amino acids, glycine, tyrosine, proline, arginine, and phenylalanine, account for 92% of the total amino acid residues of the 12,200-dalton protein, which should contain about 100 amino acid residues. The content of several other amino acids is consistently less than 1 mol%. This suggests either microheterogeneity (e.g., due to aminopeptidase action) or minor contamination with polypeptides which comigrate in SDS-gels or are not detectable at the concentrations used (e.g., see Fig. 2D). A B. subtilis spore coat fraction relatively rich in tyrosine was previously reported by Kondo and Foster (17).

Aronson and Pandey (5) have observed a similar pattern of low-molecular-weight polypeptides in the solubilized fraction from B. subtilis spore integuments. They find that this fraction has a high tyrosine content.

The unique amino acid composition of the 12,200-dalton protein suggests the presence of repeating sequences. These could consist of homogeneous runs of the abundant amino acids or heterogeneous repeats. We find that the major tyrosine-containing peptides, generated by partial acid hydrolysis, also contain the other abundant amino acids, suggesting that homogeneous runs do not predominate. In addition, we have observed one as yet unidentified component in complete hydrolysates of this protein.

The following structural proteins from various sources have been characterized by high concentrations of certain amino acids (10): (i) collagens by large amounts of glycine (33%) and proline plus hydroxyproline (22%); (ii) silk fibroins by glycine, alanine, and serine (totaling 50% of the amino acid residues); (iii) sericins by serine (30%) and glycine (14 to 27%); (iv) resilins by glycine (30 to 42%); (v) elastin by glycine (30 to 35%), alanine (20 to 25%), and proline (10 to 15%). Most of these proteins are also deficient in sulfur-containing amino acids. Andreyeva et al. have reported that the synthetic polymer Gly-Pro-Tyr can form the collagen triple helix (2). The 12,000-dalton protein we have purified contains enough glycine, tyrosine, and proline so that 60% of the molecule could have a collagen-like structure. Regardless of the actual conformation of this 12,000-dalton component of spore coats, its relative abundance, unique amino acid composition, and high degree of insolubility strongly infer a structural role in spore coats of B. subtilis.

Munoz and Doi (in press) have investigated the proteins solubilized by SDS-urea-dithiothreitol at pH 9.8 and room temperature from whole spores of B. subtilis 168. These conditions, essentially the procedure A we used to extract whole spores, with the addition of urea, were reported to result in solubilization of 25% of the spore protein. The extract contained major components with mobilities on 12% polyacrylamide-SDS gels corresponding to molecular weights of 13,500 and 25,000. Gradient gel fractionation of our whole spore extracts (Fig. 2E and F) showed a component at 30,000 and a major component running between lysozyme (14,500 daltons) and the 12,200-dalton protein. The faster moving component is presumably identical to the 13,500-dalton protein of Munoz and Doi, which they purified (in press). It has a high content of alanine and leucine but a relatively low content of glycine, tyrosine, and proline, so it is clearly different from the tyrosine-rich, 12,200-dalton protein. It appears that extraction of whole spores solubilizes a fraction of the solubilizable spore coat polypeptides, mainly from the OC and greatly enriched in the 13,500-dalton protein. The 12,200-dalton species may reside in the inner lamellar coat, since neither is extensively solubilized by this procedure (Fig. 1B and Fig. 2, slot E). Thus, the low-molecular-weight fraction of our coat polypeptides contains at least two unique components, and at least one component (25,000 to 30,000 daltons) of the higher-molecular-weight fraction may also be derived from coats. The insoluble residue from extraction of coats has an amino acid composition clearly different from that of the soluble fractions, and it seems that B. subtilis spore coats consist of several unique polypeptides. Both Aronson and Pandey (5; personal commu-
nification) and Munoz and Doi (in press) find that antisera prepared against B. subtilis coat components precipitate soluble, high-molecular-weight species from sporulating cells. These are presumed to be precursors, and it is possible that several of the different polypeptides of mature spores are derived by cleavage of a common precursor. Further fractionation, purification, and characterization of both the mature coat components and the putative precursors will be required to determine their true interrelation-ship and complexity. We intend to use the antiserum specific for the 12,000-dalton protein to investigate the structure, synthesis, and maturation of its precursors during sporulation.

ACKNOWLEDGMENTS

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LITERATURE CITED

13. Hiragi, Y. 1972. Physical, chemical and morphological studies of spore coat of Bacillus subtilis. J. Gen. Micro-
bol. 72:87-99.
23. Losick, R., and A. L. Sonenshein. 1969. Change in the template specificity of RNA polymerase during sporula-
35. Tipper, D. J., C. W. Johnson, C. L. Ginther, T. Leighton, and G. Wittmann. 1977. Erythromycin resistant mutations in Bacillus subtilis cause temperature sen-