Purification and Properties of Some Unique Low Molecular Weight Basic Proteins Degraded during Germination of Bacillus megaterium Spores*

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The first 20 min of germination of dormant spores of Bacillus megaterium are accompanied by the rapid breakdown of 15 to 20% of the dormant spore protein to free amino acids, which in turn supports much of the protein synthesis by the germinated spore early in its development (1). There is evidence that only a select group of dormant spore proteins are degraded in this process, and the preceding communication established that two acetic acid-soluble proteins identified by disc gel electrophoresis comprise 60 to 75% of the protein degraded during germination (2). These proteins, termed A and B, were unique to the dormant and developing spore and appeared to be located in the spore core (2). This communication describes the purification and properties of these A and B proteins and also the purification and properties of an additional species, termed protein C, which is also degraded during spore germination.

Two major proteins, termed proteins A and B, and one minor species, termed protein C, have been purified to homogeneity from dilute acid extracts of dormant spores of Bacillus megaterium. These three species comprise ~80% of the protein in the dilute acid extracts and account for 60 to 75% of the protein degraded during spore germination. All three proteins have low molecular weights (7,000 to 10,000), high isoelectric points (>9.8), alanine as the NH₂-terminal amino acid, are more hydrophilic than most proteins, and all lack cysteine, cystine, and tryptophan. In addition all three proteins are extremely sensitive to a wide variety of proteolytic enzymes, much more so than “average” proteins such as serum albumin, lysozyme, and hemoglobin. These proteins also bind to both purified DNA and to a nuclear body from dormant spores. Although this binding gives little or no protection to proteins A and B from proteolysis, it does result in elevation of the melting temperature of the DNA by as much as 20°.

The first 20 min of germination of dormant spores of Bacillus megaterium are accompanied by the rapid breakdown of 15 to 20% of the dormant spore protein to free amino acids, which in turn supports much of the protein synthesis by the germinated spore early in its development (1). There is evidence that only a select group of dormant spore proteins are degraded in this process, and the preceding communication established that two acetic acid-soluble proteins identified by disc gel electrophoresis comprise 60 to 75% of the protein degraded during germination (2). These proteins, termed A and B, were unique to the dormant and developing spore and appeared to be located in the spore core (2). This communication describes the purification and properties of these A and B proteins and also the purification and properties of an additional species, termed protein C, which is also degraded during spore germination.

MATERIALS AND METHODS

Spores and Labeled Spores—All work described in this communication was carried out with Bacillus megaterium QM B1551, ATCC number 12872, originally obtained from Dr. Hillel Levinson (United States Army Natick Laboratories, Natick, Mass.). Spores were prepared at 30°C in supplemented nutrient broth, harvested, cleaned, and stored as previously described (3). Spores labeled with [³²P]phosphate were also prepared as described previously (4). All spore preparations used were free of cell debris and were >95% refractile as observed in the phase contrast microscope.

Sources of Chemicals, DNA, Enzymes, and Proteins—[³²P]Phosphate was purchased from New England Nuclear and sources of chemical reagents were described in the preceding communication (2). DNA was purified from log phase cells of Bacillus megaterium using the method of Miura (5). Contaminating ribonucleic acid was removed by ribonuclease digestion (6). Native B. megaterium DNA cellulose (>150 µg of DNA/ml packed column volume) was prepared as described by Brehm et al. (6). Bovine serum albumin, chymotrypsin, chymotrypsinogen, insulin, lysozyme, pronase, thermolysin, and trypsin were obtained from Sigma; hemoglobin, pancreatic ribonuclease, and T₄ ribonuclease were obtained from Worthington Biochemicals. The extracellular protease from sporulating B. megaterium was purified to homogeneity by a modification of the method of McCann et al. (7) for purification of a similar enzyme from the culture fluid of Bacillus subtilis. The nuclear fraction (S-2) of B. megaterium spores was prepared as described in the preceding paper (2) using a modification of the procedure of Chamov et al. (6).

Chemical Determinations—Performic acid oxidation of purified proteins was carried out as described by Hirs (9), and quantitative amino acid analysis was carried out after acid hydrolysis (to N HCl, 106°C, 24 hours) as described previously (1). Tryptophan was determined spectrophotometrically as described by Beaven and Holliday (10), and NH₂-terminal amino acids were identified according to the Edman procedure (11). Carbohydrate was assayed using the phenol sulfuric acid reagent (12), and protein was routinely measured using the Lowry procedure (13). The phosphate content of proteins A, B, and C was determined by purifying all three proteins from 100 mg of [³²P]labeled spores (10⁶ cpm/nmol) through the CM-cellulose column and assaying column fractions for [³²P] and protein.

Determination of Molecular Weight—Molecular weight determinations on sodium dodecyl sulfate acrylamide gels (12.5%) were carried out using the procedure of Weber and Osborn (14) with standards of insulin, lysozyme, and chymotrypsinogen. Molecular weight determinations by equilibrium sedimentation were carried out using a Beckman model F ultracentrifuge with a scanner attachment and measuring optical density at 273 nm. Equilibrium runs were carried out at protein concentrations of 0.4 to 0.8 mg/ml in either 1% acetic acid and 0.1 M NaCl or 0.01 M Tris-HCl, 1 mM EDTA, and 0.1 M NaCl. Runs were continued until there was no further change in the scanner tracing (~24 hours). Molecular weight determinations using Sephadex chromatography were carried out as described by Whitaker (15) using a
column (1.2 x 44 cm) of Sephadex G-50 equilibrated at 4° in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1 M NaCl. One-milliliter samples were applied, and 1-ml fractions were collected. The standards used were insulin, lysozyme, and chymotrypsinogen.

Measurement of Protein Degradation—Degradation of proteins by various proteases was carried out in 50-μl incubations of 0.05 M Tris-HCl (pH 8.0) (tryptic and chymotryptic) or 0.5 M Tris-HCl (pH 8.0) and 5 mM CaCl₂ (B. megaterium exoprotease, pronase, spore extracts, or thermolysin). The amount of protease was varied, but protein substrates were held constant at 0.8 mg/ml except for proteins A, B, and C which were assayed at 0.4 mg/ml. After incubation for 30 min at 37°, 1-μl samples were applied to cellulose acetate strips which were run on electrophoresis for 30 min at 40 volts/cm with 20 mM Tris-HCl (pH 8.0) as the buffer. The strips were stained with Coomassie blue, destained, and the minimum amount of protease required to alter the electrophoretic mobility of >90% of the substrate determined.

Measurement of DNA-Protein Binding—DNA-protein binding was measured using DNA-cellulose columns. DNA-cellulose columns (1 ml, flow rate ~3 ml/hour) were washed with 1 mM Tris-HCl (pH 7.0) and 0.2 mM EDTA at 4°. 100 to 500 μg of protein were applied, and the column was washed with NaCl solutions in 1 mM Tris-HCl (pH 8.0) and 0.2 mM EDTA.

Formation of DNA-Protein Complexes and Measurement of DNA Melting—DNA-protein complexes were formed using the gradient dialysis method of Shih and Bonner (16) and the final complex was dialyzed for 24 hours at 4° versus two changes of 1 mM Tris-HCl (pH 8.0) and 0.2 mM EDTA. Melting curves were determined in stopped cuvettes using a Gilford spectrophotometer with a jacketed cuvette holder. DNA concentrations were ~30 to 40 μg/ml and the temperature was raised no faster than 1°/5 min.

Other Methods—Disc gel electrophoresis was carried out at low pH as described by Reisfield et al. (17), and gels were stained, destained, and the relative intensities of bands in the gels determined as described previously (2). Fluorescence spectra were recorded on a Perkin-Elmer spectrofluorometer, and absorbance spectra on a Cary 14 spectrometer. Dialysis tubing was acetylated with acetic anhydride in pyridine for 4 hours at room temperature as described by Craig (18). Extracts of dormant spores were prepared by sonication as described previously (19). Measurement of the minimum isoelectric points was determined by following the direction of migration of the protein on cellulose acetate electrophoresis in 25 mM arginine buffer (pH 9.8).

RESULTS

Purification of Proteins A, B, and C

The observation that acetic acid extracts of dormant spores of Bacillus megaterium gave only two bands on disc gel electrophoresis (2) suggested that it should be relatively simple to purify these proteins in good yield and this was indeed the case. Unless otherwise noted, all steps in the following purification procedure were carried out at 4°.

Rupture and Extraction of Spore—Dormant spores (150 mg dry weight) were ruptured in the dry state in a dental amalgamator (Wig-L-Bug) with glass beads (100 mg dry weight) as the abrasive as originally described by Sacks and Bailey (20). Ten 1 min periods of shaking sufficed to rupture >90% of the spores. Spore rupture in the dry state was essential to prevent enzymatic destruction of the labile proteins A, B, and C (2). The dry powder from 1 g of spores was then extracted with 25 ml of 3% acetic acid, and after 30 min at 4° the mix was centrifuged (15 min, 15,000 x g), the pellet re-extracted with 15 ml of 3% acetic acid, and the supernatant fluids pooled.

Dialysis and CM-cellulose Pass-Through—The pooled supernatant fluids were dialyzed in acetylated dialysis tubing for 36 hours at 4° against two 1-liter changes of 1% acetic acid. It was essential to use acetylated tubing, since proteins A and C in particular were lost rapidly (t₁/₂ = 1.5 days) from untreated tubing. The dialyzed material was then passed through a column (2.4 x 10 cm) of CM-cellulose equilibrated at 4° in 1% acetic acid and the column washed with 1% acetic acid. Very little protein absorbed to this column, but a significant amount of material absorbing at 260 nm was removed. The pooled run through fractions from the CM-cellulose column were frozen and lyophilized.

CM-cellulose Chromatography—The dry powder from the previous step was dissolved in 8 ml of 5 mM Tris-maleate (pH 6.0) and applied to a column of CM-cellulose (2.3 x 18 cm) equilibrated at 4° in 5 mM Tris-maleate (pH 6.0). The column was washed with the same buffer until little protein was coming off, and protein was eluted with a linear gradient of 0 to 0.4 M NaCl (125 ml of each solution) in 5 mM Tris-maleate (pH 6.0) and 4-ml fractions were collected. Three distinct protein peaks were obtained, two major peaks, proteins A and B, and a minor peak, protein C (Fig. 1). The most concentrated fractions of each of these peaks were pooled, dialyzed against 1% acetic acid in acetylated tubing, lyophilized, and dissolved in a small volume of water. The ratios of the three proteins, when calculated from the CM-cellulose column profile and correcting for the different color yields in the Lowry (13) reaction, were 1 protein C/4 protein B/5 protein A.

![Fig. 1 (left). CM-cellulose chromatography of the DEAE pass-through fraction. The DEAE pass-through fraction prepared from 1 g dry spores was chromatographed on a CM-cellulose column as described in the text.](http://www.jbc.org/)

![Fig. 2 (right). Low pH acrylamide gels of purified proteins A and B. Proteins A and B purified by CM-cellulose chromatography were run on low pH acrylamide gels as described under "Materials and Methods": Gel A, 8 μg of purified protein A; Gel B, 16 μg of purified protein B.](http://www.jbc.org/)
Recovery and Purity of Proteins A, B, and C—The simple purification procedure described above gave milligram amounts of proteins A, B, and C with an overall recovery of ~45% based on the total protein initially present in the acetic acid-soluble extract (Table I). The major loss in this procedure was in saving only the purest fractions from the CM-cellulose column. Each of the proteins gave only a single major band in disc gel electrophoresis at low pH (Fig. 2) and were judged to be >95% pure. The minor band in the protein B preparation is probably a breakdown product of protein B, since it was not present immediately after purification but only appeared after prolonged storage. A gel for protein C is not shown in the figure, since on electrophoresis it co-migrated with protein A. All three proteins also gave only a single major band on disc gel electrophoresis at alkaline pH, and also on sodium dodecyl sulfate acrylamide gel electrophoresis, and cellulose acetate electrophoresis at pH 6.0, 8.0, and 10.2.

Physical and Chemical Properties of Purified Proteins

Physical Properties—As was not surprising considering their solubility in dilute acids, all three proteins had extremely low molecular weights as determined by several techniques (Table II), and these values agreed well with minimum molecular weights from amino acid analysis (see below). For all three proteins values obtained under denaturing and non-denaturing conditions were similar, indicating that the proteins are not dimers or higher oligomers.

Spectral Properties—The absorption spectra of proteins A and B showed no non-protein absorption (Fig. 3A), and the spectrum of protein C was identical with that of protein A.1 Spectra for both proteins showed maxima at 275 nm, suggesting the absence of tryptophan; the spectrum of protein B also showed several other peaks between 245 and 275 nm, suggesting a high ratio of phenylalanine to tyrosine which was in fact observed upon amino acid analysis. The fluorescence emission spectrum of all three proteins was similar to that shown for protein B (Fig. 3B). The emission maximum was at 305 nm, close to values for tyrosine in protein, and again there was no evidence for the presence of tryptophan or other non-protein components.

Amino Acid and Chemical Analyses—Amino acid analysis of all three proteins revealed rather atypical amino acid compositions, since all three proteins lacked cysteine, cystine, and tryptophan (Table III). In addition, protein A lacked histidine

While protein B lacked isoleucine, leucine, methionine, and proline (Table III). However, there was agreement between minimum molecular weights calculated from amino acid analyses and values obtained directly (Tables II and III). The amino acid analyses also ruled out the possibility that one of the three proteins was derived from another by proteolytic modification.

The absence of several hydrophobic amino acids from proteins A, B, and C suggested that they were rather hydrophilic. Indeed, when their hydrophilic character was calculated either as per cent polar residues according to the method of Capaldi and Vanderkooi (21) or as the mean residue hydrophilicity (H_{\text{MRES}}) according to the method of Bigelow (22), values for all three proteins (Table III) fell on the hydrophilic side of the spectrum of proteins for which these calculations have been made (21, 22). In fact, the values calculated for protein B indicate that this species is more hydrophilic than >98% of the proteins for which calculations have been made.

Surprisingly, in view of the high ratio of acidic to basic residues found by amino acid analysis, all three proteins had extremely high isoelectric points, all being higher than pH 9.8. This certainly indicates that many of the glutamate and aspartate residues in these proteins must be amidated. In addition, all three proteins contained less than 1 residue of

\[ P. Setlow, unpublished results. \]
Amino acid analyses were carried out as described under “Materials and Methods,” and values reported are averages of three separate determinations rounded off to the nearest integer. Values for residues/mol were calculated by assigning values of 1 to the averages of tyrosine and phenylalanine in protein A and histidine, arginine, and tyrosine in protein B and a value of 2 to the average of proline, methionine, tyrosine, and phenylalanine in protein C. Individual values varied by less than 15% from these average values.

| Amino acid | Protein A | Protein B | Protein C |
|------------|-----------|-----------|-----------|
| residues/mol |           |           |           |
| Lysine     | 4         | 8         | 6         |
| Histidine  | <0.2      | 1         | 1         |
| Arginine   | 2         | 1         | 3         |
| Aspartic acid | 6     | 11        | 11        |
| Serine     | 3         | 6         | 4         |
| Threonine  | 4         | 7         | 6         |
| Glutamic acid | 11      | 20        | 15        |
| Proline    | 2         | <0.2      | 2         |
| Glycine    | 9         | 5         | 10        |
| Alanine    | 12        | 12        | 13        |
| Valine     | 5         | 3         | 5         |
| Methionine | 2         | <0.1      | 2         |
| Isoleucine | 3         | <0.1      | 3         |
| Leucine    | 5         | <0.1      | 6         |
| Tyrosine   | 1         | 1         | 2         |
| Phenylyalanine | 1       | 3         | 2         |
| Cysteine   | <0.1      | <0.1      | <0.1      |
| Tryptophan | <0.1      | 0.1       | <0.1      |
| Total residues | 70     | 76        | 91        |
| Minimum molecular weight | 7101 | 8454 | 9649 |
| % Polar residues | 57.1 | 69.2 | 50.5 |
| $H_{\Phi}$ | 850 | 523 | 816 |

**TABLE IV**

**Sensitivity of proteins A, B, and C and other proteins to digestion by various proteases**

Protease sensitivity was determined as described under “Materials and Methods.” Mixtures of protein A or B with DNA were prepared by gradient dialysis as described under “Materials and Methods,” and values reported are averages of three separate determinations rounded off to the nearest integer. Values for residues/mol were calculated by assigning values of 1 to the averages of tyrosine and phenylalanine in protein A and histidine, arginine, and tyrosine in protein B and a value of 2 to the average of proline, methionine, tyrosine, and phenylalanine in protein C. Individual values varied by less than 15% from these average values.

| Protein substrate | Bacillus megaterium exoproteinase | Chymotrypsin | Proteinase | Thermolysin | Trypsin | Spore extract |
|-------------------|----------------------------------|--------------|------------|-------------|---------|--------------|
|                    | protease sensitivity-ratio of substrate/protein giving >90% digestion |              |            |             |         |              |
| Albumin           | 5                                | 500          | <10        | <200        | 500     |              |
| Hemoglobin        | 30                               | 35           | <10        | <200        | 100     | 0.03         |
| Lysozyme          | 5                                | 18           | 30         | <200        | 20      |              |
| Protein A         | 3,000                            | 250          | 700        | 22,500      | 2,500   | 1.6          |
| Protein B         | 2,800                            | 70           | 200        | 17,500      | 350     | 0.9          |
| Protein C         | 600                              | 15,000       | 1,800      | 3,000       | 1,500   | 1.2          |
| Protein A + DNA   |                                  | 15,000       | 3,000      |              |         |              |
| Protein B + DNA   |                                  | 15,000       | 1,500      |              |         |              |

**FIG. 4** DNA-cellulose chromatography of purified proteins A, B, and C. One-milliliter columns of Bacillus megaterium DNA-cellulose were prepared as described under “Materials and Methods,” and protein (~250 μg) was applied in 1 ml. The column was washed with 4 ml each of 1 mM Tris-HCl (pH 7.0), 0.2 mM EDTA (Buffer A), 20 mM NaCl in Buffer A, 50 mM NaCl in Buffer B, and 100 mM NaCl in Buffer C. Protein was located using the Lowry (15) reaction.
protein to DNA of 12/1 for protein A and 2.5/1 for protein B. Above these ratios of protein to DNA there was no significant increase in \( T_m \) and much of the DNA was precipitated. Interestingly, at intermediate protein to DNA ratios biphasic melting curves were not obtained as have previously been observed for melting curves of histone-DNA complexes (16).

The elevation in \( T_m \) due to binding of proteins A or B, or both, was observed not only with purified \( B. \ megaterium \) DNA, but also with a nuclear fraction prepared from dormant spores. This fraction contains approximately 1.5 times as much protein as DNA by weight (8), but had a \( T_m \) almost identical with that of naked DNA as has been reported previously (Fig. 5C) (25). However, binding of a mixture of proteins A, B, and C purified through the DEAE pass-through step to this nuclear fraction resulted in an increased \( T_m \). The maximum increase in the \( T_m \) was obtained at a protein to DNA ratio of 8/1 (Fig. 4); above this value much of the DNA was precipitated. Binding of either protein A or protein B alone to the nuclear fraction also gave an increased \( T_m \) with results similar to those for binding to naked DNA (data not shown).

The binding of proteins A, B, and C to DNA \textit{in vitro} with stabilization of the DNA suggested the possibility that this interaction might also alter the protease sensitivity of the protein. However, binding of either protein A or B to DNA had little effect on the sensitivity of these proteins to digestion by either trypsin or thermolysin (Table IV).

**DISCUSSION**

Since an important function of proteins A, B, and C is to serve as a source of amino acids during spore germination (1), it was not surprising that the purified proteins were extremely sensitive to proteolysis. This protease sensitivity, especially to nuclease proteases, is probably due to a combination of the absence of disulfide bonds, the low molecular weight, and the high hydrophilic character, since these properties would be expected to result in a rather flexible protein conformation with many of the peptide bonds accessible to proteolytic attack.

Another significant property of proteins A, B, and C is that although they contain 18 of the common amino acids (assuming both aspartate and glutamate residues are amidated), all three proteins lack cysteine, cystine, and tryptophan. Although there is no data on the amount of cysteine, cystine, or tryptophan generated by proteolysis, for tryptophan in particular it is known that degradation of pre-existing protein supplies all tryptophan for protein synthesis through 40 min of germination (1). Consequently, this need must be supplied by degradation of proteins other than A, B, and C, and, indeed, a second group of dormant spore proteins degraded during germination has been identified. 1 This new protein fraction makes up 3 to 4% of total dormant spore protein and contains amounts of tryptophan (but not cysteine or cystine) sufficient to support protein synthesis during germination. 1 The source of cysteine or cystine during germination is less clear, although one possible source is cysteine in disulfide linkage to spore coat proteins as has been reported in \textit{Bacillus cereus} spores (26).

Many of the properties of proteins A, B, and C which may be important for their \textit{in vivo} function also distinguish these species from the great majority of known bacterial proteins. There are, however, several types of bacterial proteins, including ribosomal proteins and proteins associated with bacterial nuclear fractions, which have properties similar to those of proteins A, B, and C, including low molecular weights, high isoelectric point values, and the absence of cysteine, cystine, and tryptophan (27-30). Therefore, it might be proposed that proteins A, B, and C are merely normal cellular constituents which have been amplified (>30-fold (9)) in the dormant spore. Although proteins A, B, and C all have lower molecular weights and radically different amino acid compositions from all but a few of the ribosomal proteins which have been described in \textit{Escherichia coli} or \textit{Bacillus stearothermophilus} (27, 28, 31-33), as yet I cannot rule out the possibility that one or more of these proteins is identical with a ribosomal protein in \textit{B. megaterium}. It should also be noted that several workers
have recently noted changes in DNA-binding proteins late in sporulation of *Bacillus subtilis* (6, 34), and one of these studies reported the accumulation of a significant amount of a low molecular weight DNA-binding protein(s) very late in sporulation (34). It is possible that this protein(s) is either protein A, B, or C or a mixture of all three.

Clearly, knowledge of the internal location of the massive amount of these unique spore proteins A, B, and C will be of great importance in assessing their possible functions in the dormant spore, if any. It is, of course, possible that these proteins are not bound to spore DNA in *vivo* but are bound weakly to some other spore component such as tRNA, dipeptidyl aminopeptidase etc. However, certainly one of the most suggestive findings made in this study is the observation that proteins A, B, and C all bind to DNA. To this observation should be added the finding that the new group of spore proteins degraded during germination also binds to DNA, possibly even more strongly than proteins A, B, and C, since many of these new proteins require 0.3 M NaCl for elution from DNA-cellulose. Thus, dormant bacterial spores appear to contain a family of DNA-binding proteins comprising 13 to 19% of the total protein of the spore, and it may possibly be significant that the levels of some of these proteins in spores, when calculated as protein to DNA ratios, are only slightly below the values giving maximum change in the T_m of *B. megaterium* DNA or a nuclear fraction. This observation, coupled with the finding that proteins A and B (and probably C) are all synthesized at the time that the developing spore becomes resistant to ultraviolet light, lends support to the suggestion made previously that these basic proteins may be involved in altering the conformation of spore DNA in *vivo* (2). It should be noted, however, that the affinity between proteins A, B, and C and DNA is not extremely high, since they can be removed from DNA by low salt. This finding may explain the lack of association of protein A, B, or C with DNA in spore lysates where the salt concentration is approximately 0.15 M (2). However, DNA and all three proteins are present in the spore itself at extremely high concentrations (DNA, 3 mg/ml; protein A, 6 to 9 mg/ml; protein B, 5 to 7 mg/ml; protein C, 1.3 to 2 mg/ml) which may compensate for this low affinity.²

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² Calculated assuming a spore water content of 70%.
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