Characterization, purification and synthesis of spore-coat protein in *Bacillus megaterium* KM

Gordon S. A. B. STEWART and David J. ELLAR

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(Received 16 September 1981/Accepted 14 October 1981)

The spore-coat fraction from *Bacillus megaterium* KM, when prepared by extraction of lysozyme-digested integuments with SDS (sodium dodecyl sulphate) and urea, contains three *N*-terminal residues and a major component of apparent mol.wt. 17 500. Electron microscopy of this fraction shows it to consist of an ordered multilamellar structure similar to that which forms the coat region of intact spores. The 17 500-dalton protein, which has been purified to homogeneity, has an *N*-terminal methionine residue, has high contents of glycine, proline, cysteine and acidic amino acids and readily polymerized even in the presence of thiol-reducing agents. It is first synthesized between late Stage IV and early Stage V, which correlates with the morphological appearance of spore coat. Before Stage VI the 17 500-dalton protein is extractable from sporangia by SDS in the absence of thiol-reducing reagents. Between Stage VI and release of mature spores the protein becomes resistant to extraction by SDS unless it is supplemented by a thiol-reducing reagent. In addition to that of the spore-coat protein, the timing of synthesis of all the integument proteins was analysed by SDS/polyacrylamide-gel electrophoresis and non-equilibrium pH-gradient electrophoresis. Several integument proteins are conservatively synthesized from as early as 1 h after the end of exponential growth (t_1), which may reflect protein incorporation into the spore outer membrane.

When the growth of vegetative cells of endospore-forming bacteria becomes limited by the depletion of an essential metabolite, sporulation is initiated. The sporulation process is characterized by a well-defined sequence of morphological changes which can be divided into seven distinct stages (Ryter *et al.*, 1961; Schaeffer *et al.*, 1963).

State III is characterized by the formation of a new intracellular compartment (forespore). Cortex synthesis continues throughout Stage IV and early Stage V, at which time the deposition of multiple proteinaceous layers around the periphery of the forespore is first observed. Spore-coat morphology as revealed by electron microscopy is apparently species-specific (Aronson & Fitz-James, 1976). *Bacillus cereus* spores show three distinct coat layers in thin section, and in freeze-fracture one of these layers appears to be composed of a repeating pattern of stacked parallel bars or rods, which has been termed 'cross-patching' (Aronson & Fitz-

James, 1976). Below this layer a pitted layer has been detected (Holt & Leadbetter, 1969). The spores of *Bacillus coagulans* and *Bacillus subtilis* generally appear to have more complex coats containing additional layers. *Bacillus megaterium* KM has a single multilayered coat in which pitted and cross-patched layers have been seen by thin section (Aronson & Fitz-James, 1976).

The structure and function of the various layers of proteinaceous coat material have been the subject of intensive study. The insecticidal activity of several strains of *Bacillus thuringiensis* attributable to parasporal crystals produced during sporulation may be linked with the uncontrolled production of one or more components of the coat (Delafield *et al.*, 1968; Bulla *et al.*, 1980).

A study of several mutants, either with defective spore coats or lacking coats entirely, has indicated a possible role for the coat in spore germination and resistance. For example, a mutant of *Clostridium perfringens* has been found to be germinable by lysozyme but not by amino acids (Cassier & Ryter, 1971; Cassier & Sebald, 1969). The sensitivity of spores which are coat-deficient (Suzuki & Rode,

Abbreviations used: SDS, sodium dodecyl sulphate; Dns or dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

1969), or whose coats have been rendered permeable to several lytic enzymes by chemical treatment (Gould & Dring, 1972), indicate a role for the coat layers in maintaining the spore's tolerance to bacteriocidal agents. In addition, it has also been suggested that the coats may play a structural role in maintaining core dehydration (Gould & Dring, 1974, 1975; Ellar, 1978).

These characteristic morphological properties of the bacterial spore coat have prompted its detailed examination as a putative spore-specific gene product. Composed largely, if not exclusively, of polypeptides, it can provide selective protein markers to facilitate a study of the control of gene expression in a relatively simple differentiating system (Munoz *et al.*, 1978; Nakayama *et al.*, 1978; Linn & Losick, 1976). In parallel with these objectives a biochemical analysis of spore-coat structure, synthesis and maturation may answer the following questions about the biochemistry of the sporulation process. (i) Does the coat consist of a heterogeneous population of polypeptides or an ordered assembly of one or at most a few monomeric units? (ii) Are the coat proteins synthesized as precursors which are subsequently modified and is such a scheme reflected in their sequence of synthesis? (iii) How are the biochemical and biophysical features of individual coat proteins correlated with their structure and function in the intact coat?

The spore coat accounts for between 30 and 60% of the spore dry weight (Murrell, 1969; Tipper & Gauthier, 1972). In addition to species heterogeneity, the apparent discrepancy in these values arises from different definitions of the coat, ranging from the complete integument structure to enzymically digested and denaturant-extracted basal layers. In the present paper the spore coat is defined (see also the Discussion section) as those integument proteins that resist extraction with SDS in the absence of thiol-reducing agents. Contamination of coat material by the spore outer membrane, which forms part of the integument fraction (Crafts-Lighty & Ellar, 1980), is therefore virtually eliminated. (The term 'integuments' is used to describe the particulate fraction obtained after centrifugation of disrupted spores for 10 min at 15000g.) A major component of the resulting coat fraction is a protein of apparent mol.wt. 17500. The isolation, purification and sequence of synthesis of this protein are described. The results suggest that the major coat components in *Bacillus megaterium* KM are derived by polymerization of a low-molecular-weight monomeric unit whose synthesis is concomitant with the first morphological appearance of coat material. These results are discussed in relation to those recently published for *Bacillus subtilis* (Jenkinson *et al.*, 1981) and *Bacillus cereus* (Aronson, 1981).

Materials and methods

Bacterial strain and growth

The organism used in these studies was a sporogenic strain of *Bacillus megaterium* KM. The conditions for growth and sporulation were as previously described (Ellar & Posgate, 1974), with the exception of a modified sporulation medium (Stewart *et al.*, 1981). Spores were harvested by centrifugation (10000g for 3 min at 4°C) and re-centrifuged at least six times from deionized water (4°C), each time removing and discarding the uppermost layer of spores until the pellet was free from contamination by cells or debris.

Chemicals

Acrylamide and *NN'*-methylenebisacrylamide were obtained from BDH. L-[³⁵S]Methionine and [U-¹⁴C]protein hydrolysate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A., unless otherwise specified.

Purification of spore coat

Dormant spores were stored frozen at -20°C and either used directly or refluxed for 10 min in 80% propan-2-ol in order to inactivate autolytic enzymes. Spores or sporangial pellets were resuspended to 200 mg dry wt./ml in either distilled water or 30 mM-EDTA/1 mM-1,10-phenanthroline/1 mM-*p*-aminobenzamidine dihydrochloride/1 mM-phenylmethanesulphonyl fluoride/5 mM-*N*-ethylmaleimide, before breakage in a Braun cell homogenizer (model MSK) with 40 g of glass beads (0.10–0.11 mm diameter) for 6 × 30 s with a liquid-CO₂ cooling system operating. Subsequent fractionation and extraction was as shown in Scheme 1. All centrifugation steps were for 10 min at 15000g and all pelleted fractions were resuspended by mild sonication in a Kerry KS 200 ultrasonic bath.

Purification of a 17500-dalton protein by preparative electrophoresis

Spore-coat material from step [6b] in Scheme 1 was resuspended to 8 mg dry wt./ml in 50 mM-Tris/HCl (pH 7.5)/3% (w/v) SDS/25 mM-dithiothreitol/2 mM-phenylmethanesulphonyl fluoride/1 mM-EDTA/10% (w/v) glycerol and solubilized by incubation at 100°C for 10 min. Proteins were fractionated by electrophoresis in a 2.1 cm-bore Canalco preparative tube-gel apparatus (Fisons Scientific Apparatus, Loughborough, Leics., U.K.). SDS (0.1%)/polyacrylamide gels were prepared essentially as described by Laemmli & Favre (1973). A 5 cm separating gel contained 15% (w/v) acrylamide and 0.15% (w/v) *NN'*-methylenebisacrylamide, and a 3 cm stacking gel contained the corresponding concentrations of 6 and 0.06%. A

Scheme 1. Coat extraction and purification

total of 80 mg of protein was loaded under electrode buffer (Laemmli & Favre, 1973) and electrophoresis was performed at 12 mA constant current. Proteins were recovered by elution of the slit disc with electrode buffer (18 ml/h) and collected as 3 ml fractions. Portions were analysed by electrophoresis in a slab apparatus as described below. Those fractions containing the 17 500-dalton protein were pooled, dialysed against several changes of distilled water and freeze-dried.

SDS/polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of SDS was conducted as described by Laemmli & Favre (1973), an acrylamide/*NN'*-methylenebisacrylamide ratio of 100:1 being used. Protein samples were suspended to between 1 and 10 mg/ml in SDS/sample buffer [50 mM-Tris/HCl (pH 7.5)/1% (w/v) SDS/25 mM-dithiothreitol/2 mM-phenylmethanesulphonyl fluoride/1 mM-EDTA/10% (w/v) glycerol/0.0025% (w/v) Bromophenol Blue] and solubilized by incubation for 5 min at 100°C. Whole spores and spore integuments (step [2],

Scheme 1) were extracted at 100 mg dry wt./ml and 15 mg dry wt./ml respectively in SDS/sample buffer by heating for 5 min at 100°C. The resulting insoluble residue was removed by centrifugation at 15 000 g for 10 min. Samples were applied to wells, formed in the stacking gel, under electrode buffer and run at 20 mA constant current until the dye marker had entered the separating gel, at which time the current was increased to 30 mA until the marker was 0.5 cm from the end of the gel. The gel was removed from the apparatus, stained for 30 min at 60°C with 0.1% (w/v) Coomassie Brilliant Blue R in 50% (v/v) methanol/10% (v/v) acetic acid and destained with several changes of 5% methanol/10% acetic acid. Gels were prepared for fluorography with sodium salicylate as described by Chamberlain (1979). Stained gels were scanned with a double-beam recording microdensitometer (Joyce Loebel and Co., Gateshead, Tyne and Wear, U.K.). Molecular weights were estimated by the relation of log (molecular weight) to the R_F of Sigma SDS/polyacrylamide-gel-electrophoresis marker proteins or Amersham [^{14}C]methylated protein mixture. For radioactivity quantification, gel tracks were cut into 1 mm slices and prepared for scintillation counting by the method of Di Rienzo & Inouye (1979).

Extraction of the 17 500-dalton protein from gel slices

Protein recovered from preparative electrophoresis was further purified by SDS/polyacrylamide-slab-gel electrophoresis and excision of the 17 500-dalton band made visible by staining for 2 min. Gel slices were cut into small cubes and extracted with 0.1% (w/v) SDS/20 mM-Tris/HCl (pH 7.5)/25 mM-dithiothreitol/1 mM-EDTA/2 mM-phenylmethanesulphonyl fluoride for 4 h at 60°C. The gel cubes were removed by filtration with a 0.45 µm-pore-size membrane filter (Millipore Corp.) and the filtrate dialysed for 18 h against continuous-flow 0.05 M- NH_4HCO_3 and subsequently freeze-dried.

Two-dimensional electrophoresis

Samples were solubilized at 10 mg/ml in SDS/sample buffer as described for SDS/polyacrylamide-gel electrophoresis. Before electrophoresis, the solubilized samples were diluted 2:1 with sample dilution buffer comprising 9.5 M-urea/2% Ampholines (pH range 3.5–10; LKB Produkter)/25 mM-dithiothreitol and 8% (w/v) Nonidet P40 (Sigma). Two-dimensional electrophoresis was as described by O'Farrell (1975) and non-equilibrium pH gradient electrophoresis was as described by O'Farrell *et al.* (1977). Non-equilibrium pH-gradient electrophoresis was for 1200 V-h (400 V for 3 h) with sample application at the anode, and all second-

dimension gels were prepared essentially as described for SDS/polyacrylamide-gel electrophoresis.

Analytical methods

Samples were assayed for phosphorus by the method of Ames & Dubin (1960), carbohydrate by the method of Devor (1950) and protein by the method of Lowry *et al.* (1951). *N*-Terminal analysis was by a modification of the dansylation procedure of Gray (1972). A sample (145 µg) of protein was dissolved in 50 µl of 1% (w/v) SDS by heating to 100°C for 5 min. Redistilled *N*-ethylmorpholine (50 µl) and dansyl chloride (75 µl; 25 mg/ml in dimethylformamide) were added and after thorough mixing the suspension was left at room temperature for 3 h. Water-saturated diethyl ether (4 vol.) was then added. The mixture was shaken and then centrifuged for 1 min at 2000 *g* to separate phases. The ether was removed and the extraction repeated four times. Traces of diethyl ether were removed in a stream of air and sufficient 50% (w/v) trichloroacetic acid was added to give a one-phase system. After cooling on ice for 30 min, the precipitate was recovered by centrifugation, washed twice with diethyl ether and hydrolysed with 6 *M*-HCl containing 2-mercaptoethanol (0.5 ml/l) for 12 h at 105°C. The Dns-amino acid(s) were identified by chromatography on polyamide-coated sheets (Perham, 1978). Protein samples for amino acid analysis were reduced and *S*-carboxymethylated by the procedure described by Perham (1978). Samples were analysed on an LKB 4400 amino acid analyser after hydrolysis at 105°C for 24 or 48 h in 6 *M*-HCl containing 0.5 ml of 2-mercaptoethanol/litre.

Radioactive labelling

Spores were radioactively labelled by the aseptic addition of either [U-¹⁴C]protein hydrolysate (10 µCi/litre) or L-[³⁵S]methionine (200 µCi/litre) [1 Ci = 37 kBq]. For pulse-labelling experiments, 20 µCi of L-[³⁵S]methionine was added aseptically to 100 ml of sporulating culture taken from a 1-litre

master flask every hour from t_0 to t_8 . After incubation for 1 h at 30°C, 1 ml was removed for the determination of total radioactivity and radioactivity incorporated into trichloroacetic acid-precipitable material. The culture was subsequently harvested by centrifugation and resuspended to its original volume in the supernatant from a parallel culture incubated without radioactive label and containing 50 µg of L-methionine/ml. All cultures were incubated to $t_{11.5}$, at which time lysozyme (50 µg/ml) was added to each culture, and after a 30 min incubation the spores were harvested as described above. Determination of radioactivity was as described by Stewart *et al.* (1981).

Results

Purification of spore coat

The extraction and purification procedures for the preparation of spore coat from *Bacillus megaterium* KM are shown in Scheme 1. Table 1 presents the percentage composition of the fractions obtained.

Extraction with SDS/sample buffer of spore integuments (step [2], Scheme 1) prepared from spores steady-state-labelled with L-[³⁵S]methionine solubilized 95% of the radioactivity. Amino acid analysis of the remaining insoluble residue (cortex peptidoglycan) of unlabelled integuments has previously shown less than 1% contamination with non-cortical amino acids (Johnstone & Ellar, 1981). These results indicate that all of the coat proteins are solubilized by the extraction methods used.

The SDS/polyacrylamide-gel-electrophoretic pattern of proteins extracted from whole spores and spore integuments are compared in tracks 1 and 2 of Fig. 1. Although differences in minor protein components and a major component of apparent mol.wt. 62 500 can be identified, the patterns are extremely similar. This suggests that extraction of whole spores does not solubilize inner membrane or core proteins to any significant extent. Extraction of

Table 1. Percentage composition of fractions obtained during purification of the spore coat

Fraction	Dry wt.	[U- ¹⁴ C]Protein hydrolysate	L-[³⁵ S]Methionine
Spores	100	100	100
Integuments [2]*	26	21.4	40.4
Lysozyme extract	ND†	ND	4.2
2% SDS extract	ND	ND	7.9
Coat [6b]	6.3	4.6	22.3
Urea-extracted coat [8]	5	ND	19.4
Glutathione-extracted coat [12]	ND	ND	12.9
17 500-Dalton protein	ND	0.72	ND

* Values in square brackets represent the appropriate step in Scheme 1.

† ND, Not determined.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of spore proteins

All tracks were loaded with 100 μ g of protein unless otherwise stated. Track (1), spores extracted at 100 mg/ml with SDS/sample buffer (100°C/5 min); track (2), integuments extracted as for track (1), but at 15 mg/ml (Step [2], Scheme 1); track (3), 2% SDS extract of lysozyme-digested integuments (Step [5], Scheme 1); track (4), residual material from 2% SDS extract solubilized in SDS/sample buffer (100°C for 5 min) (Step [6b], Scheme 1); track (5), residue after coat extraction with 9 M-urea (Step [8], Scheme 1); track (6), 50 μ g of 17 500-dalton protein purified by preparative electrophoresis; track (7), 70 μ l of the soluble fraction from coats extracted with 25 mM-glutathione at 5 mg/ml (Step [9], Scheme 1); track (8), 25 μ g of the freeze-dried material obtained after Sephadex G-25 filtration of the glutathione extract (Step [9b], Scheme 1); track (9), as for track (8), but 250 μ g of protein applied; track (10), residue after glutathione extraction of the spore coat solubilized in SDS/sample buffer (100°C/5 min) (Step [12], Scheme 1); track (11), 50 μ g of Sigma molecular-weight standards: albumin (mol.wt. 66 000), ovalbumin (45 000), trypsinogen (24 000), β -lactoglobulin (17 500) and lysozyme (14 400).

lysozyme-digested integuments with 2% SDS at 50°C provides a soluble and insoluble fraction. The soluble fraction (track 3 of Fig. 1) contains the majority of the integument proteins. The major band corresponding to mol.wt. 14 400 is lysozyme. The residual material (step [6b], Scheme 1), which comprises 6.3% of the spore dry weight and is rich in sulphur-containing amino acids (Table 1), can be completely solubilized in SDS/sample buffer containing 25 mM-dithiothreitol as a thiol-reducing agent. Track 4 (Fig. 1) shows the SDS/polyacrylamide-gel-electrophoretic profile of this fraction, and Fig. 2 shows a microdensitometer scan and

U-¹⁴C-labelling pattern of a similar preparation from spores labelled with [U-¹⁴C]protein hydrolysate. These data show that this fraction contains relatively few proteins by comparison with the original integument fraction and a major component of apparent mol.wt. 17 500. The latter was estimated as 16% of the coat protein by densitometry and 15.7% by liquid-scintillation counting of ¹⁴C radioactivity.

Munoz *et al.* (1978) have reported that preparations of *Bacillus subtilis* spore coat are highly susceptible to proteinase activity. Steps were taken therefore to determine the effect of various proteolytic inhibitors in the preparation of material characterized by tracks 2–4 of Fig. 1. Neither refluxing in 80% propan-2-ol before spore breakage nor a combination of the proteinase inhibitors 1,10-phenanthroline, *p*-aminobenzamidine dihydrochloride, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide and EDTA resulted in any observable change in the electrophoretic pattern. Nevertheless, phenylmethanesulphonyl fluoride and EDTA were routinely used in all preparations.

Track 5 of Fig. 1 shows the material remaining after urea extraction of spore coat (step [8], Scheme 1). Although this fraction constitutes 5% of the total spore dry weight, it has a very simple polypeptide profile. *N*-Terminal analysis gave only three Dns-amino acids (excepting Dns- ϵ -lysine), even on heavily loaded chromatograms, and these were represented by methionine as the major component, with additional spots of glycine and serine. Despite this simplicity in composition, the material consists of a highly ordered lamellar structure as revealed by thin-section electron microscopy (Fig. 3). This fine structure is consistent with the material representing a highly purified spore-coat preparation that may be compared with the coat region of intact spores (Fig. 3 inset).

Extractability of coat proteins during sporulation

Sporangial cultures at Stage IV, V and VI were harvested and disrupted by homogenization as described in the Materials and methods section. The pelleted material was fractionated by the procedure used for dormant spores up to, and including, the extraction with 2% SDS at 50°C (step [6], Scheme 1). In marked contrast with the situation in mature spores, the integuments from these sporulation stages were almost completely soluble in 2% SDS. Moreover, before Stage VI no coat proteins could be identified when the small amounts of insoluble residue remaining were dissolved in SDS/sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis. These proteins were first detectable in the 2%-SDS-insoluble residue at Stage VI in amounts corresponding to 10% of those present in the equivalent residue obtained from dormant spores. Several high-molecular-weight proteins ob-

Fig. 2. Microdensitometer scan and distribution of ^{14}C radioactivity in coat protein (Step [6b], Scheme 1) prepared from spores grown in medium containing $10\mu\text{Ci}$ of $[\text{U-}^{14}\text{C}]\text{protein hydrolysate/litre}$
 -----, Radioactivity (c.p.m.); —, densitometer trace.

served in the residue throughout the three stages were identified as mother-cell-membrane components.

Purification and analysis of the 17500-dalton coat protein

The major 17500-dalton component of the spore coat was purified by preparative disc gel electrophoresis of material from step [6b] of Scheme 1 (track 4, Fig. 1). The material obtained was purified further by excision of the appropriate band from SDS/polyacrylamide-slab-gel electrophoresis. About 2.4 mg of material were prepared by this procedure, and track 6 of Fig. 1 shows the degree of homogeneity obtained. Of particular significance is the presence of a band corresponding to an apparent mol.wt. of 35000. By the nature of the purification

method, this band can only represent dimerization of the 17500-dalton protein and this dimerization must be stable to reduction by dithiothreitol.

In a series of studies on the effect of extraction of the urea-purified coat with thiol-reducing reagents of various reduction potentials, it was observed that reduced glutathione could specifically solubilize the 17500-dalton protein. The supernatant from such an extraction is shown in track 7 of Fig. 1, and track 8 shows the freeze-dried material after desalting on Sephadex G-25 (step [9b], Scheme 1). Track 9 of Fig. 1 shows $250\mu\text{g}$ of this material to indicate the degree of homogeneity. Apart from the 17500-dalton band, bands corresponding to 35000 and 53000 daltons can be detected. *N*-Terminal analysis of this material gave a single Dns-amino acid corresponding to methionine. The presence of a

Fig. 3. Thin-section electron micrograph ($\times 174\,000$) of urea-extracted coat as prepared in Step [8], Scheme 1
The inset shows a segment of an intact spore.

single *N*-terminal residue and the relationship of the high-molecular-weight bands to the major protein, identifies these bands as dithiothreitol-resistant polymers of the 17 500-dalton monomer.

No carbohydrate or phosphorus was detected in the pure 17 500-dalton protein, and the amino acid analysis is shown in Table 2. The presence of both cysteine and carboxymethylcysteine in the hydrolysate indicates that complete denaturation of the protein was not obtained under the standard alkylation conditions (Perham, 1978). A remarkable feature of this analysis is that 30% of the total protein is composed of the small hydrophobic residues glycine and proline. The acidic residues aspartate and glutamate comprise a further 20%; these are, however, almost certainly amidated, since analysis by non-equilibrium pH-gradient electrophoresis shows the protein running very close to lysozyme, which has a *pI* of 11.0–11.2. The protein could not be retained on gels during isoelectric focusing to equilibrium. The polymers of the 17 500-dalton protein show more acidic *pI* values than does the monomer, which may reflect charge shielding or some as-yet-undetermined structural modification during polymerization.

Table 2. Amino acid analysis of the 17 500-dalton protein (step [9b], Scheme 1)

Amino acid	Content (mol %)	No. of residues relative to Ile (1)
Asx*	4.6	10
Thr	5.1	11
Ser	4.2	9
Glx*	15.5	33
Pro	11.7	25
Gly	18.2	38
Ala	5.2	11
Cys	1.4	3
Cm-Cys†	5.7	12
Val	6.1	13
Met	3.5	7
Ile	1.0	2
Leu	0.2	0
Tyr	5.2	11
Phe	2.6	6
His	5.9	12
Lys	2.3	5
Arg	1.5	3
		Total 211
		Nominal mol.wt. 21910

* Determined as aspartic acid and glutamic acid after hydrolysis.

† abbreviation: Cm-Cys, carboxymethylcysteine.

Timing of synthesis of coat and integument proteins

Cultures of sporulating cells were pulse-labelled with L-[35 S]methionine every hour from t_0 – t_8 . After allowing sporulation to continue until release of free spores, the contribution of proteins synthesized at each time point to the final integument profile was examined. The distribution of radioactivity from whole-spore extracts, which accurately reflects integument proteins (see tracks 1 and 2 in Fig. 1), was analysed by both SDS/polyacrylamide-gel electrophoresis and non-equilibrium pH-gradient electrophoresis. The percentage of total radioactivity incorporated at each time point is given in Fig. 4. The fluorograph from SDS/polyacrylamide-gel electrophoresis (Fig. 5) shows that protein components of the integuments are conservatively

Fig. 4. Profile of incorporation of L-[35 S]methionine into sporulating cultures pulse labelled every hour from t_0 to t_8 as described in the text

●, Radioactivity incorporated into trichloroacetic acid-precipitable material given as a percentage of the total label. ○, Percentage of the total radioactivity incorporated located in the spore integument fraction.

synthesized from as early as t_1 , more than 1 h before asymmetric membrane septation. Two major proteins synthesized at this time have apparent mol.wts. of 22 100 and 46 000. They can be observed until t_4 , after which time their synthesis is markedly decreased. The first detectable synthesis of coat proteins is at t_4 , which correlates with the morphological appearance of Stage IV. A major increase in the synthesis of these proteins occurs during t_5 and t_6 and continues to t_8 at which time approx. 50% of the spores are released. One other major component synthesized between t_5 and t_8 is a 62 500-dalton protein that is a particular characteristic of whole-spore extracts and may be easily removed from integuments by repeated washing.

Although considerable information can be obtained by analysis with SDS/polyacrylamide-gel electrophoresis, a more quantitative and detailed examination is possible by using the technique of two-dimensional electrophoresis. As the coat protein monomer is extremely basic, equilibrium isoelectric focusing was unsuitable for protein separation and the alternative technique of non-equilibrium pH-gradient electrophoresis was utilized. Fig. 6 shows the separation of pulse-labelled extracts by this technique. No protein spots could be detected on a 20-day fluorograph of t_0 extracts, but by t_1 , however, six spots are clearly identifiable, which increase to 19 by t_2 . The profile at t_3 is essentially

Fig. 5. Fluorograph (15-day) of the SDS/13% polyacrylamide-gel electrophoretic profile from spores pulse-labelled with L-[35 S]methionine as described in Fig. 4 and extracted at 100 mg/ml with SDS/sample buffer. Tracks (1–9), cultures pulse-labelled from t_0 to t_8 respectively; track (10), L-[35 S]methionine steady-state-labelled spore extract; track (11), Amersham [14 C]methylated molecular-weight standards: myosin (mol.wt. 200 000), phosphorylase *b* (92 500), albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000) and lysozyme (14 300).

Fig. 6. Fluorographs (20-day) of non-equilibrium pH-gradient-electrophoresis-separated spore integument proteins obtained from spores pulse-labelled with L-[^{35}S]methionine every hour from t_1 to t_8 as described in the text
 ➔ identifies 17 500-dalton protein and its polymers; ➜ identifies 62 500-dalton protein.

identical with that at t_2 , but a major change in protein pattern occurs at t_4 . There are in excess of 34 proteins synthesized conservatively at this time, the most significant of which is the appearance of coat protein dimer and trimer without any detectable monomer. It is noteworthy that because there are two proteins with molecular weights extremely close to that of the coat monomer which are synthesized at this time, the total absence of monomer can only be demonstrated by analysis with a two-dimensional system. Synthesis of coat monomer is clearly

apparent by t_3 and, in addition, the 62 500-dalton protein identified by SDS/polyacrylamide-gel electrophoresis can be seen. This protein has a heterogeneous charge distribution which may reflect glycosylation.

Discussion

The spore coat is the outermost permeability barrier of the dormant endospore (Gerhardt & Black, 1961). An understanding of the interrela-

tionships of coat structure and function, particularly as these may relate to the spore's remarkable resistance to heat, radiation and bacteriocidal reagents, can only be attempted after the detailed analysis of coat formation and composition. In much of the previous literature, the coats have been considered to be the insoluble fraction from which all the cortical material has been removed (Murrell, 1969). This definition includes at least some of the proteins derived from the spore outer membrane which remain associated with the integuments after lysozyme digestion (Crafts-Lighty & Ellar, 1980). By treating *Bacillus subtilis* integuments with a combination of 0.1% SDS, 1M-KCl and lysozyme, Goldman & Tipper (1978) removed a significant proportion (14%) of total spore protein, which they considered to be composed in the main of membrane proteins. In their view the integuments treated in this way still contained residual membrane components, and they estimated that 28% of the protein originally present in their unfractionated coat preparations could be accounted for by membrane proteins. The presence of such proteins in putative coat material may be crucial in the interpretation of studies which relate to the timing of coat-protein synthesis. Aronson & Fitz-James (1968) have reported that spore-coat-protein precursors are synthesized at a constant rate beginning shortly after the start of spore formation. Nakayama *et al.* (1978) reported that sporulating cells from stages t_2 and t_4 make large amounts of spore-coat protein, and Jenkinson *et al.* (1981) report that 9 out of 14 coat proteins are synthesized as early as Stage II or III. The possibility should be considered, however, that at least some of the proteins synthesized at these early times may reflect the conservative synthesis of outer-membrane, rather than coat, components.

Results described in the present paper show that the heterogeneity of the SDS/polyacrylamide-gel-electrophoresis profile of lysozyme-digested integuments can be markedly decreased by extraction with 2% SDS and 9M-urea. The residual material (step [8], Scheme 1) retains the ultrastructure typical of the spore coat and yet contains only three *N*-terminal residues. The major component derives from a low-molecular-weight protein that, through polymerization, may constitute over 35% of the purified material. This degree of homogeneity is consistent with current theories of coat structure which propose only a very few structural coat components (Aronson & Fitz-James, 1976; Aronson & Pandey, 1978). The amino acid composition of the purified coat monomer also supports previous conclusions that the structural layers consist predominantly of polypeptides which interact by hydrophobic bonds with deep-seated cystine residues (Aronson & Fitz-James, 1976); furthermore, the high glycine and proline content may permit regions of a collagen-like

triple helix, as previously suggested for *Bacillus subtilis* (Goldman & Tipper, 1978). No hydroxyproline or hydroxylysine could be detected in the hydrolysates, however.

By contrast with previous reports of coat precursor processing (Aronson, 1981; Stelma *et al.*, 1980; Munoz *et al.*, 1978; Cheng & Aronson, 1977), no evidence has yet been obtained for processing of the coat proteins of *Bacillus megaterium* KM. In addition, since no processing system has yet been identified which results in the production of an *N*-terminal methionine residue, the presence of this residue at the *N*-terminus of the 17500-dalton coat monomer may be presumptive evidence for synthesis *de novo*.

Analysis of the timing of coat-protein synthesis during sporulation was carried out on extracts of whole spores to enable comparison with the results obtained for *Bacillus subtilis* by Jenkinson *et al.* (1981). These analyses show that integument proteins, which may include the outer membrane, are conservatively synthesized from as early as t_1 . Those proteins which constitute the structural elements of the coat, however, are not synthesized before deposition, but precisely when the morphological appearance of the coat can first be detected. Although these results differ from those previously published, this may reflect the relatively simple coat structure of *Bacillus megaterium* compared with that of *Bacillus subtilis*. In this context it is important to note that more than 99% of the integument proteins from *Bacillus megaterium* KM mature spores are soluble in the SDS/sample buffer after 5 min heating at 100°C. By using a similar solubilizing regime with *Bacillus subtilis* integuments treated with 0.1% SDS and lysozyme, Goldman & Tipper (1978) and Jenkinson *et al.* (1981) found respectively 30% and 41% of integument protein to be insoluble.

Although the active synthesis of coat proteins proceeds during Stage V, they cannot be isolated from Stage-V sporangia as proteins that are resistant to SDS extraction in the absence of thiol-reducing reagents. This strongly suggests that a very late maturation of the coat structure renders it resistant to these extraction procedures. A marked change in the ease of extraction between t_9 and t_{30} of a 36000-dalton integument protein from *Bacillus subtilis* has also been noted by Jenkinson *et al.* (1981). Although the nature of the maturation process is unknown, its timing is co-ordinate with dramatic changes occurring in membrane-lipid fluidity. Between Stage VI and release of mature spores, the electron-spin-resonance spectrum of doxylstearic acid spin label changes from fluid to polycrystalline (Stewart *et al.*, 1980). In addition, between Stage V and VI, the developing forespore acquires the heat-resistance properties and lack of

detectable metabolism that are characteristic of the dormant spore (Ellar *et al.*, 1975; Scott & Ellar, 1978). Such major changes in the structure of macromolecular components, which occur very late in sporulation, must have considerable impact on the establishment of cryptobiosis.

The availability of a highly purified spore-coat component and the ability to purify forespores at various stages (Ellar & Posgate, 1974) offer an ideal system in which to study the compartmentalization of coat-protein synthesis.

We are grateful to the Science Research Council (S.R.C.) for financial support. We thank Mr. J. G. Lay for electron microscopy and Miss M. Mather for amino acid analysis (supported by S.R.C. grant GR/B 37288).

References

- Ames, B. N. & Dubin, D. T. (1960) *J. Biol. Chem.* **235**, 769–775
- Aronson, A. I. (1981) *J. Bacteriol.* **145**, 541–547
- Aronson, A. I. & Fitz-James, P. C. (1968) *J. Mol. Biol.* **33**, 199–212
- Aronson, A. I. & Fitz-James, P. C. (1976) *Bacteriol. Rev.* **40**, 360–402
- Aronson, A. I. & Pandey, N. K. (1978) in *Spores VII* (Chamblis, G. & Vary, J. C. ed.), pp. 54–61, American Society for Microbiology, Washington, DC
- Bulla, L. A., Jr., Bechtel, D. B., Kramer, K. J., Shethna, Y. I., Aronson, A. I. & Fitz-James, P. C. (1980) *Crit. Rev. Microbiol.* **7**, 147–204
- Cassier, M. & Ryter, A. (1971) *Ann. Inst. Pasteur Paris* **121**, 717–732
- Cassier, M. & Sebald, M. (1969) *Ann. Inst. Pasteur Paris* **117**, 312–327
- Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132–135
- Cheng, H. M. & Aronson, A. I. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1254–1258
- Crafts-Lighty, A. & Ellar, D. J. (1980) *J. Appl. Bacteriol.* **48**, 135–145
- Delafield, F. P., Somerville, H. J. & Rittenberg, S. C. (1968) *J. Bacteriol.* **96**, 713–720
- Devor, A. W. (1950) *J. Am. Chem. Soc.* **72**, 2008–2012
- DiRienzo, J. M. & Inouye, M. (1979) *Cell* **17**, 155–161
- Ellar, D. J. (1978) in *Symposium of the Society for General Microbiology* (Stanier, R. Y., Rogers, H. J. & Ward, B. J., eds.), pp. 296–235, Cambridge University Press
- Ellar, D. J. & Posgate, J. A. (1974) in *Spore Research 1973* (Barker, A. N., Gould, G. W. & Wolf, J., eds.), pp. 21–40, Academic Press, London
- Ellar, D. J., Eaton, M. W., Hogarth, C., Wilkinson, B. J., Deans, J. & La Nauze, J. (1975) in *Spores VI* (Gerhardt, P., Costilow, R. N. & Sadoff, H. L., eds.), pp. 425–433, American Society for Microbiology, Washington, DC
- Gerhardt, P. & Black, S. H. (1961) *J. Bacteriol.* **82**, 750–760
- Goldman, R. C. & Tipper, D. J. (1978) *J. Bacteriol.* **135**, 1091–1106
- Gould, G. W. & Dring, G. J. (1972) in *Spores V* (Halvorson, H. O., Hanson, R. & Campbell, L. L., eds.), pp. 401–408, American Society for Microbiology, Washington, DC
- Gould, G. W. & Dring, G. J. (1974) *Adv. Microb. Physiol.* **11**, 137–164
- Gould, G. W. & Dring, G. J. (1975) in *Spores VI* (Gerhardt, P., Sadoff, H. L. & Costilow, R. N., eds.), pp. 541–546, American Society for Microbiology, Washington, DC
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Holt, S. C. & Leadbetter, E. R. (1969) *Bacteriol. Rev.* **33**, 346–378
- Jenkinson, H. F., Sawyer, W. D. & Mandelstam, J. (1981) *J. Gen. Microbiol.* **123**, 1–16
- Johnstone, K. & Ellar, D. J. (1982) *Biochim. Biophys. Acta* in the press
- Laemmli, U. K. & Favre, M. (1973) *J. Mol. Biol.* **80**, 575–599
- Linn, T. & Losick, R. (1976) *Cell* **8**, 103–114
- Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Munoz, L. E., Sadie, Y. & Doi, R. H. (1978) *J. Biol. Chem.* **253**, 6694–6701
- Murrell, W. G. (1969) in *The Bacterial Spore* (Gould, G. W. & Hurst, A., eds.), pp. 214–273, Academic Press, London
- Nakayama, T., Munoz, L. E., Sadie, Y. & Doi, R. H. (1978) *J. Bacteriol.* **135**, 952–960
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142
- Perham, R. N. (1978) in *Techniques in Protein and Enzyme Biochemistry, B110* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. & Tipton, K. F., eds.), pp. 1–39, Elsevier/North-Holland Scientific Publishers Ltd., Amsterdam
- Ryter, A., Ionesco, H. & Schaeffer, P. (1961) *C. R. Hebd. Séances Acad. Sci. Ser. D* **252**, 3675–3677
- Schaeffer, P., Ionesco, H., Ryter, A. & Balassa, G. (1963) in *Colloques Internationaux du Centre National de la Recherche Scientifique, Sciences Humaines* (Senez, J., ed.), pp. 529–544, Gordon and Breach, New York
- Scott, I. R. & Ellar, D. J. (1978) *Biochem. J.* **174**, 635–640
- Stelma, G. N., Aronson, A. I. & Fitz-James, P. C. (1980) *J. Gen. Microbiol.* **116**, 173–185
- Stewart, G. S. A. B., Eaton, M. W., Johnstone, K., Barrett, M. D. & Ellar, D. J. (1980) *Biochim. Biophys. Acta* **600**, 270–290
- Stewart, G. S. A. B., Johnstone, K., Hagelberg, E. & Ellar, D. J. (1981) *Biochem. J.* **198**, 101–106
- Suzuki, Y. & Rode, L. J. (1969) *J. Bacteriol.* **98**, 238–245
- Tipper, D. L. & Gauthier, J. J. (1972) in *Spores V* (Halvorson, H. O., Hanson, R. & Campbell, L. L., eds.), pp. 3–12, American Society for Microbiology, Washington, DC