Purification and Characterization of Cytoplasmic Proteins Synthesized in the Developing Forespores of *Bacillus subtilis* during Sporulation

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Forespores of *Bacillus subtilis* 60015 were isolated from sporulating cells at t_5 and were incubated with an amino acid mixture containing [^{14}C]phenylalanine. Three species of [^{14}C]-labelled cytoplasmic proteins synthesized in the forespores were purified to homogeneity by gel filtration in the presence of detergents and by ion-exchange chromatography. The molecular weights of the purified proteins, called A, B and C, were estimated by SDS-PAGE as 24200, 11500 and 12700, respectively. Protein B contained remarkably high amounts of tyrosine (23.8%) and alanine (22.8%), and proteins A and C contained relatively high amounts of glutamate + glutamine, glycine, and alanine (10.4 to 13.3%). The synthesis of these proteins can provide markers for the control events in the forespore compartment during sporulation.

INTRODUCTION

Nutritional deprivation in the medium accompanying an alteration of metabolic balance causes an initiation of bacterial sporulation (Schaeffer, 1969; Freese, 1976), where a forespore compartment is formed by the asymmetric partition of the stationary phase cell into two compartments following the rapid growth of a folded mother-cell double-membrane septum. The sporulating cell consists of two discrete sections, and the separation of the sporangium into forespore and mother-cell compartments is useful for investigating the major biochemical changes which take place in the developing forespore during sporulation.

Procedures for isolation of intact forespores have been developed in many species of *Bacillus* (Andreoli *et al.*, 1973; Ellar & Posgate, 1974; Fujita *et al.*, 1977; Watabe *et al.*, 1980; Nakayama *et al.*, 1980) and some of the biochemical properties closely related to the developing forespores have been investigated (Eaton & Ellar, 1974; Ellar & Posgate, 1974; Andreoli *et al.*, 1975, 1978; Ellar *et al.*, 1975; Fujita *et al.*, 1977; Nakayama *et al.*, 1980; Watabe *et al.*, 1980, 1981; Setlow, 1981). We have developed a [^{14}C]phenylalanine-incorporation system using isolated forespores of *Bacillus subtilis* 60015, and found continuous protein synthesis in the young forespore throughout sporulation. About 75% of the radioactive amino acid incorporated into the forespore at t_5 was located in the cytoplasmic fraction, and a major protein labelled with [^{14}C]phenylalanine was found to have a molecular weight of about 20000 and lower molecular weight polypeptides also contained radioactivity (Watabe *et al.*, 1981). In the present paper, [^{14}C]-labelled proteins in the forespore cytoplasmic fraction were purified to homogeneity, and their amino acid composition and molecular weights were analysed.

METHODS

Organism, cultural conditions and media. The transformable Marburg strain of *B. subtilis* 60015 (*metC tryC*), originally obtained from Ernst Freese (National Institute of Health, Bethesda, Md., U.S.A.), was used. Bacteria were grown in the nutrient sporulation medium containing nutrient broth, metal mixture, methionine, tryptophan and glucose as described previously (Watabe *et al.*, 1981). The growth stage was identified by t_n , where n is the number of hours after the end of exponential growth.

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Preparation of forespores and labelling experiment. Preparation of forespores from sporulating cells at t_5 and confirmation of their purity were carried out as described previously (Watabe *et al.*, 1981). The labelling experiment with the isolated forespores was performed by incubation with $0.1 \mu\text{Ci L-[U-}^{14}\text{C]phenylalanine ml}^{-1}$ (3.7 kBq ml^{-1}) and $10 \mu\text{M}$ of each of 20 unlabelled amino acids (Watabe *et al.*, 1981).

Purification of ^{14}C -labelled cytoplasmic proteins. (i) *Preparation of cell extract.* A cell extract of ^{14}C -labelled forespores was prepared, as described previously, in the presence of 2 mM-EDTA , $2 \text{ mM-phenylmethylsulphonyl fluoride}$ and haemoglobin-Sepharose (Watabe *et al.*, 1981). The haemoglobin-Sepharose was prepared as described by Nakayama *et al.* (1977).

All column chromatographies were performed at 25°C . Protein content of the eluate from Sepharose CL-6B was measured by the absorbance at 450 nm following the addition of $0.8 \text{ ml } 4\% \text{ (w/v) trichloroacetic acid}$ to 0.2 ml eluate. Protein in eluates from Sephadex G-150 and DEAE Bio-Gel A was estimated from the absorption at 280 nm . Radioactivity was measured as described previously (Watabe *et al.*, 1981).

(ii) *Sepharose CL-6B gel filtration.* After dialysing overnight at 25°C against $50 \text{ mM-carbonate buffer (pH } 9.8)$ containing 8 M-urea , $0.1 \text{ M-}\beta\text{-mercaptoethanol}$ and $0.4\% \text{ (w/v) SDS (UMS buffer)}$, the cell extract was applied to a column ($2.0 \text{ cm diam.} \times 90 \text{ cm}$) of Sepharose CL-6B equilibrated with UMS buffer and eluted with the same buffer.

(iii) *Sephadex G-150 gel filtration.* Gel filtration by Sephadex G-150 was performed using columns of two different lengths; short ($1.5 \text{ cm diam.} \times 100 \text{ cm}$) and long ($1.5 \text{ cm diam.} \times 150 \text{ cm}$). Both columns were equilibrated and eluted with $50 \text{ mM-carbonate buffer (pH } 9.8)$ containing $0.1\% \text{ (w/v) SDS}$.

(iv) *DEAE Bio-Gel A chromatography.* The sample was dialysed overnight against $50 \text{ mM-Tris/HCl buffer (pH } 8.0)$ and was then applied to a column ($0.9 \text{ cm diam.} \times 13 \text{ cm}$) of DEAE Bio-Gel A (Bio-Rad) equilibrated with the same buffer. The column was washed with 20 ml of the same buffer and eluted stepwise with 15 ml portions of the same buffer containing 0.2 M , 0.4 M and 0.6 M-NaCl , successively.

SDS-PAGE. Proteins were fractionated by electrophoresis either in tubes or in a slab apparatus. For tube gel electrophoresis, proteins were treated with detergents and were run in gels containing $7.5\% \text{ (w/v)}$ of polyacrylamide at 6 mA per tube for about 5 h as described by Fairbanks *et al.* (1971). The gels were then stained with Coomassie brilliant blue-R and destained. For slab gel electrophoresis, samples were solubilized in Laemmli solubilization buffer (Laemmli, 1970), and were separated with the SDS-PAGE system of Ames (1974). The slab gels, which were 2.0 mm thick and consisted of $4\% \text{ (w/v)}$ stacking gel and a $15\% \text{ (w/v)}$ separating gel, were run at 28 mA until the tracking dye flowed out from the bottom (approximately 4 h). The gels were stained with Coomassie blue, destained and dried.

The tube gels containing radioactive samples were cut into 1.5 mm pieces with a gel slicer, and the radioactivity was measured after solubilization of the gels by ammoniacal H_2O_2 solution as described previously (Watabe *et al.*, 1981).

Amino acid analysis. The purified proteins were hydrolysed in 6 M-HCl in evacuated sealed tubes at 110°C for 14 h . The solutions were evaporated to dryness *in vacuo*, redissolved in $0.2 \text{ M-citrate buffer (pH } 2.2)$, and analysed on a JEOL amino acid analyser (model JLC-6H).

Chemicals. Tris, EDTA, phenylmethylsulphonyl fluoride and SDS were obtained from Sigma. L-[U- ^{14}C]phenylalanine (specific activity $513 \text{ mCi mmol}^{-1}$; 18.9 GBq) was purchased from Amersham. All other materials were of analytical grade quality.

RESULTS

Purification of ^{14}C -labelled cytoplasmic proteins

Forespores were isolated from sporulating cells at t_5 and were incubated with an amino acid mixture containing [U- ^{14}C]phenylalanine for 4 h . The cell lysate of ^{14}C -labelled forespores was fractionated on Sephadex G-200 eluted with $0.1 \text{ M-Tris/HCl buffer (pH } 8.0)$. Two peaks of radioactive fractions were obtained and the main peak was eluted at a position corresponding to a molecular weight of more than 200000 (data not shown). SDS-PAGE of the ^{14}C -labelled cytoplasmic protein resulted in one sharp peak with a molecular weight of about 20000 and broad peaks of about 10000 and 50000 (Watabe *et al.*, 1981). The molecular size of the radioactive proteins implied from the elution pattern of Sephadex G-200 with a non-denaturing buffer was quite different from that estimated by SDS-PAGE, which suggested an aggregation of these proteins. Therefore, the cell lysate was dialysed against $50 \text{ mM-carbonate buffer (pH } 9.8)$ containing 8 M-urea , $0.1 \text{ M-}\beta\text{-mercaptoethanol}$ and $0.4\% \text{ (w/v) SDS}$ to disaggregate the proteins, and was applied to a Sepharose CL-6B column. As expected, more than 85% of the radioactivity was eluted at fractions 34 to 54 which correspond to molecular weights of less than 50000 (Fig. 1a). These fractions were pooled and then applied to the short column of Sephadex

Fig. 1. Gel filtration of ^{14}C -labelled cytoplasmic fraction in isolated forespores of *B. subtilis* 60015. The cell lysate was applied to a Sepharose CL-6B column (a); radioactive fractions (indicated by the bar) were pooled and then applied to Sephadex G-150 column (b). Protein in the eluates was estimated from the absorbance at 450 nm or at 280 nm. Protein (---); radioactivity (●).

G-150 in the presence of 0.1% (w/v) SDS (Fig. 1b). Radioactivity eluted in a high-molecular-weight peak (fractions 53 to 70) containing 78.7% of the applied radioactivity and a low-molecular-weight peak (fractions 70 to 82). The pool of fractions 60 to 68 was called Fra.I and that of 70 to 82, Fra.II.

Fra.I was re-chromatographed on the long Sephadex G-150 column, and one major peak of protein with radioactivity was obtained. After pooling the main fraction, this gave only a single protein peak on SDS-PAGE analysis, which coincided with the radioactive peak in the tube gel (data not shown); this homogeneous protein was called protein A.

The low-molecular-weight peak of Fra.II was applied to the long Sephadex G-150 column in the presence of 0.1% (w/v) SDS (Fig. 2). One major radioactive peak, called Fra.III, was obtained by this step, but SDS-PAGE analysis of the main peak fraction of Fra.III yielded at least four protein bands with molecular weights of 11 300 to 14 000 (data not shown). Further purification of Fra.III was then accomplished by chromatography on DEAE Bio-Gel A; proteins B and C were obtained from this process by stepwise elution with 0.2 M and 0.4 M NaCl, respectively (Fig. 3). The homogeneity of these proteins was confirmed by SDS-PAGE; when they were run on slab gel, both showed only a single protein band and radioactivity comigrated with each of them (data not shown). Further purification of the non-absorbed fraction obtained from the ion-exchange chromatography was unsuccessful because of the small amount of the fraction.

Overall recoveries of radioactivity from the cell lysate in the proteins A, B and C were calculated as 43.5%, 8.1% and 4.1%, respectively.

Molecular weight

The molecular weights of the purified proteins were estimated by coelectrophoresis with standard proteins in SDS-PAGE. The molecular weights of the proteins A, B and C were estimated as 24 200, 11 500 and 12 700, respectively.

Amino acid composition

Amino acid analysis of an acid hydrolysate of the purified proteins revealed all the common amino acids to be present (tryptophan was not examined; Table 1). Proteins A and C contained

Fig. 2. Gel filtration of Fra.II on Sephadex G-150. ^{14}C -labelled protein of Fra.II was re-chromatographed on the long column of Sephadex G-150. Protein (---); radioactivity (●).

Fig. 3. DEAE Bio-Gel A ion-exchange chromatography of Fra.III. Fra.III, prepared from Sephadex G-150 gel filtration, was applied to a DEAE Bio-Gel A column. The column was equilibrated with 50 mM-Tris/HCl buffer (pH 8.0) and eluted stepwise with the same buffer containing 0.2 to 0.6 M-NaCl. Protein (---); radioactivity (●).

a high amount of glutamate + glutamine, glycine, and alanine (10.4 to 13.3%). It is notable that protein B contained a very high amount of tyrosine (23.8%) and alanine (22.8%), and relatively high amounts of serine and methionine. Each protein contained one unusual peak in the acid hydrolysates, which was expressed as a leucine equivalent. The unknown in protein A was identified as glucosamine based on its elution time on an amino acid analyser, but those in proteins B and C were not identified.

Table 1. Amino acid composition of proteins A, B, and C

Amino acid	Mol % of protein		
	A	B	C
Lys	4.8	1.5	4.4
His	3.2	0.8	1.2
Arg	3.6	1.3	3.8
Asx*	9.1	3.7	8.8
Thr	4.8	1.8	4.9
Ser	7.9	13.5	8.0
Glx†	13.2	9.5	11.1
Pro	3.7	1.5	3.8
Gly	11.1	3.1	10.4
Ala	10.6	22.8	12.9
Cys‡	trace	trace	0.1
Val	5.7	1.8	7.7
Met	1.8	10.4	3.0
Ile	5.2	1.0	3.3
Leu	7.4	1.9	6.0
Tyr	1.9	23.8	1.2
Phe	3.6	1.0	2.6
Unknown§	2.4	0.9	6.9

* Aspartic acid + asparagine.

† Glutamic acid + glutamine.

‡ Calculated as cysteic acid.

§ Calculated as leucine equivalent.

DISCUSSION

The developing forespores of *B. subtilis* 60015 show changes in the specific activity of several enzymes and in the protein profile of the cytoplasm (Watabe *et al.*, 1980), and also show continuous protein synthesis throughout forespore maturation (Watabe *et al.*, 1981). Among the forespores isolated at t_5 , t_9 and t_{13} (corresponding to early stage V, late stage V and stage VI of sporulation, respectively), t_5 forespores showed the highest activity of protein synthesis (Watabe *et al.*, 1981). Low-molecular-weight cytoplasmic proteins (molecular weight of about 20000 and polypeptides of lower molecular weight) were highly labelled with [^{14}C]phenylalanine when the isolated forespores at t_5 were incubated with amino acid mixture, and the polypeptides corresponding to them in their molecular sizes were found in developing forespores after late stage V and in mature spores, but not in vegetative cells or mother-cell compartments (Watabe *et al.*, 1980, 1981). The results strongly suggest that these proteins are synthesized in the developing forespores during the middle stages of sporulation. The purification of these proteins was first tried by conventional gel filtration with Tris/HCl buffer. The elution pattern of radioactive proteins, however, was quite different from that expected from the electrophoretic profile in SDS gels (Watabe *et al.*, 1981), which suggested an aggregation of the cytoplasmic protein. Changes of molecular size and activity of spore cytoplasmic enzymes have been shown in many species of *Bacillus*, for example, glucose dehydrogenase in heated spores of *B. subtilis* is present in an inactive form with a molecular weight of about 40000 and treatment of the inactive enzyme with EDTA or dipicolinic acid converts it into an active form with a molecular weight of about 100000 (Hachisuka & Tochikubo, 1971). Analysis of cytoplasmic proteins in forespores or dormant spores of *B. subtilis* 60015 by PAGE in the absence of SDS often gives an unresolved profile (our preliminary data). These results, therefore, suggest that cytoplasmic proteins in forespores and mature spores tend to aggregate. Thus, purification of the ^{14}C -labelled cytoplasmic proteins was carried out in the presence of detergents.

The molecular weights of the purified proteins with radioactivity, called A, B and C, were estimated as 24200, 11500 and 12700, respectively. There is little possibility that these polypeptides are proteolytic products produced by forespore proteases during preparation of the cell lysate because ^{14}C -labelled forespores were ruptured in the presence of phenylmethylsul-

phenyl fluoride, haemoglobin-Sepharose and EDTA, which are specific inhibitors for serine and metal proteases (Nakayama *et al.*, 1977). Some unique low-molecular-weight basic proteins are found in *Bacillus megaterium* (Setlow & Primus, 1975; Setlow, 1975*a, b*; 1978), *B. subtilis* (Johnson & Tipper, 1981; Tipper *et al.*, 1981), and *Bacillus cereus* (Yuan *et al.*, 1981), and some of these are closely related immunologically (Yuan *et al.*, 1981). In *B. megaterium*, seven spore-specific low-molecular-weight proteins are degraded by spore proteases to produce free amino acids, which support much of the protein synthesis early in spore germination (Setlow & Primus, 1975). These proteins are found in the forespore; consequently they may be synthesized within the forespore (Dignam & Setlow, 1980), and some of them (Setlow's proteins A and B) are associated with spore DNA *in vivo* and are involved in the high resistance of spore DNA to UV light (Setlow, 1975*a*; Setlow & Setlow, 1979). In the present study, protein B was found to contain a high level of tyrosine and alanine, comprising about half of the total amino acids present (Table 1). When amino acid compositions of the low-molecular-weight proteins from dormant spores of several *Bacillus* species are compared, it can be seen that proteins B and E of *B. megaterium* and γ of *B. subtilis* contain a high amount of glutamate (25.6%, 22.2% and 28%, respectively) (Setlow, 1975*b*; Setlow, 1978; Johnson & Tipper, 1981); protein B of *B. subtilis* 60015 was the only polypeptide which contained a similar amount of tyrosine. Since proteins A, B and C of *B. subtilis* 60015 were purified in the presence of detergents, biological activity of these polypeptides could not be measured, but the high content of tyrosine in protein B suggest that this protein acts as a reserve of that amino acid in the forespore core during sporulation.

As mentioned above, the existence of these polypeptides is presumed to be closely associated with one of the biologically specific events taking place within the forespore at t_5 . Gene expression in the forespore and mother-cell compartments is regulated in a manner different from that suggested for *B. megaterium* (Eaton & Ellar, 1974), *B. subtilis* (Linn & Losick, 1976) and *B. cereus* (Andreoli *et al.*, 1978), therefore it is expected that the synthesis of proteins A, B and C would be a good marker for the study of the control mechanism of gene expression in the forespore compartment during development of sporulation.

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