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Membrane Protein Alterations During The Early Stages of Sporulation of Bacillus Subtilis

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Membrane protein alterations during the early stages of sporulation were examined by polyacrylamide gel electrophoresis. Solubilized samples of the vegetative cell membrane (VCM), sporulation membrane fraction (SMF), and inner forespore membranes (IFM) were compared with respect to their protein compositions. The VCM contained 39 protein components, distinguishable as separate bands on gel electrophoresis, and these ranged in molecular weight from 16,000 to greater than 100,000. During the first 5 hr of sporulation, 6 of these 39 protein bands disappeared, 8 increased and 12 decreased in concentration, and 13 showed no discernible change. In addition, 15 new protein components were identified in the SMF during the first 5 hr. The new components consisted of 7 protein bands that were transiently associated with the SMF, and 8 proteins that persisted in the SMF from their time of appearance until at least T5 of sporulation. Comparison of the protein composition of the IFM with those of the VCM and SMF revealed that membrane protein alterations occur during sporulation.

The turnover of H^3-tryptophan-labeled membrane protein was followed during growth and sporulation. During the 30 min of growth following a simple chase with excess unlabeled tryptophan, membrane protein appeared stable, whereas 5–10% of the nonmembrane protein turned over to acid-soluble material. However, manipulation of the cells by dilution into fresh medium, or centrifugation, as part of the chase procedure, resulted in elution of membrane protein to the cytoplasm. In contrast, proteins labeled during vegetative growth were always eluted to the cytoplasm during the first 2 hr of sporulation, and this was followed by a period of reassociation with the membrane fraction. The results are discussed with respect to membrane differentiation as it relates to spore development.

Key words: sporulation, membranes, Bacillus subtilis

INTRODUCTION

The bacterial endospore is the end result of a complex series of cellular events. These include a sequential series of alterations in the physiological state of the cell, which lead to morphogenesis of a mature spore. The morphological events of spore development have been elucidated by thin section electron microscopy (1, 2) and traditionally consist of the following seven stages: 1) axial filament formation, 2) septation, 3) forespore engulfment, 4) cortex formation, 5) coat formation, 6) maturation, and 7) release of the mature spore. However, these stages are arbitrary representation, and do not fully illustrate the complexity of morphogenesis.

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Stages 2 and 3 involve the extension of membrane material, which results in a forespore surrounded by two unit membranes of apparently opposite polarity (orientation of inner and outer surfaces). The inner forespore membrane (IFM) is formed as the result of an asymmetric septation and thus retains a polarity similar to the vegetative cell membrane (VCM). Formation of the outer forespore membrane (OFM) occurs during the engulfment process, suggesting (from a morphological standpoint) a reversed polarity with respect to the spore body it surrounds. A previous investigation (3) showed that the protein composition of the IFM, isolated from a mutant designated SB-23, differed from that of the VCM. Mutant SB3-23 is classified as an Spo IV mutation. Several proteins found in the VCM were not found in IFM preparations, and there appeared to be at least one new protein unique to the IFM. These data suggested that membrane differentiation occurs during spore formation. The one protein that was unique to the IFM preparation was considered a sporulation-specific IFM protein. In addition, Felix and Lundgren (4) reported that succinate cytochrome C reductase and NADH cytochrome C reductase, which were associated with the VCM, were not detectable in IFM preparations from B. cereus forespores, again suggesting specific membrane differentiation.

At present, neither the structure nor mechanism of synthesis of the OFM is known. One surface of the OFM faces the mother cell cytoplasm and the other faces the IFM of the forespore. The OFM is thus a barrier between the mother cell cytoplasm and the spore core, rather than a barrier between intra- and extracellular environments. In addition, the OFM probably functions both in the synthesis of spore cortex peptidoglycan, which is structurally different from that of vegetative cell wall peptidoglycan (5), and as the site for initiating coat protein assembly. It seems unlikely that the OFM is merely synthesized by expansion of the vegetative cell membrane, and thus identical with it. This raises interesting questions as to the true polarity of this membrane and its mechanisms of assembly.

The IFM is derived from a polar septation during stage 2 of sporulation, and serves as the cell membrane upon germination of the spore. One may consider the IFM as the unaltered polar end of the cell membrane, or as the polar end of the cell membrane with subsequent sporulation-specific modifications occurring after septation. Previous results (3) indicated that the IFM has a different protein composition from that of the cell membrane, suggesting the latter possibility. However, one further complication exists in that the cell membrane itself may be altered during the early stages of sporulation. Indirect evidence suggesting that such modification does occur has been reported (6, 7).

The purpose of the present investigation was to examine membrane protein alterations during the early stages of sporulation and to reexamine the protein composition of the IFM in view of these new data.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacillus subtilis 168 was used as the wild type sporulating strain. Mutant SB-23 was used for isolating the IFM as previously described (3). This mutant is classified as an Spo IV mutant, and is characterized by overproduction of three sporulation-associated protease activities, production of less than 1% of the dipicolinic acid produced by the parent strain, and release of immature spores at the end of sporulation (3).

Cells grown overnight in brain heart infusion (Difco) at 30°C were used as an inoculum (1:20 dilution) into the sporulation medium described by Leighton (8). Cells were grown by the active culture technique of Santo et al. (9). Growth and sporulation

were monitored by following optical density with a Klett Summerson colorimeter (#66 filter), determining viable cell numbers on nutrient agar plates (Difco) both before and after heating at 80°C for 10 min, and by examining cultures by phase contrast microscopy. T0 is defined as the time cells cease logarithmic growth, and sporulation is complete in 9 hr (T9). T0 occurs at 200 Klett units under the conditions described.

Forespore Isolation

Forespores were isolated from strain SB-23 as previously described (3), with the following modifications: 1) concentrated cultures were treated sonically for 5 min, at 0°C, using a Biosonik III sonifer (Bronwill Scientific, N.Y., yellow probe, power setting 40); and 2) samples were then treated with 100 μg/ml DNAse for 15 min at 37°C.

Preparation of Membrane Fractions

The VCM was isolated from logarithmically growing cells by the following procedure. Growth was stopped by the addition of chloramphenical to 100 μg/ml and sodium azide to 0.001 M. Cells were collected by centrifugation, washed in citrate-phosphate buffer (trisodium citrate 0.01 M and pH 7.4 phosphate buffer 0.1 M), resuspended in the same buffer, and lysed by the addition of 200 μg/ml of lysozyme, 20 μg/ml of DNAase and 20 μg/ml of RNAase. After incubation at 35°C for 15 min, membranes were isolated by centrifugation as described in detail by Sargent (10).

The SMF was isolated from sporulating cells by the same procedure. These preparations appeared to contain all of the membrane material from sporulating cells isolated as late as T5, since the expected increase in total protein in this fraction, due to membrane synthesis during septation and engulfment, was observed (see Fig. 2).

The IFM was obtained from forespores isolated from mutant SB-23 as previously described (3). Protein in the membrane fraction was assayed by the method of Lowry (11) using bovine serum albumin as a standard.

Membrane Protein Stability and Effect of Cell Extracts

Tritium-labeled membranes were prepared from cells grown to 150 Klett units in medium containing 5 μCi/ml of (H^3)-L-tryptophan. Membranes isolated in citrate-phosphate buffer as described by Sargent (10) were washed by centrifugation and resuspended in 0.05 M tris (hydroxymethyl) aminomethane (Tris) pH 7.4

The stability of protein in isolated membranes was examined by incubating membranes in Tris buffer (0.05 M, pH 7.4) at 0 (ice bath), 37, and 47°C. Samples were withdrawn at time 0, 15, 30, 45 and 60 min and centrifuged at 20,000 g for 20 min. The pellets were dissolved in 0.1 M NaOH, and after the addition of bovine serum albumin (100 μg/ml), they were precipitated with cold trichloroacetic acid (7.5% final concentration). Precipitates were collected on Whatman GF/C filters and counted in toluene scintillation fluid.

The stability of protein in isolated membranes was also examined in the presence of crude cell extracts. Soluble extracts were prepared from vegetative and sporulating cells by a similar lysis procedure used in preparing membranes. The only differences were the use of Tris buffer (0.05 M, pH 7.4) and the addition of 100 μg/ml of DNAase and RNAase. After lysis and centrifugation at 20,000 g for 20 min, supernatants were removed and assayed for protein. Labeled membranes were incubated in the presence of these extracts, and the amount of label present in the membrane pellets, after incubation and centrifugation, was the criterion for assessing membrane protein stability. Membrane pellets were

processed as described above to assay for radioactive material with the following modification. After collecting the trichloroacetic acid precipitate on GF/C filters, these samples were solubilized off the filters with NCS solubilizer (Amersham Searle) before counting. This procedure was necessary owing to quenching by protein, which precipitated from the crude extracts during incubation at 37°C.

Gel Electrophoresis

Purified membranes were suspended in electrophoresis sample buffer (5 ml of 2-mercaptoethanol, 30 ml of 10% sodium dodecylsulfate, 12.5 ml of 1.5 M Tris, pH 6.8, plus deionized water to 100 ml) at a protein concentration of 1 mg/ml. The suspension was immediately heated in a boiling water bath for 5 min. Samples were electrophoresed in 1-mm-thick slab gels of 5, 10, and 20% acrylamide. Gels were prepared by the basic methods of Reid and Bieleski (12). Proteins were stacked at 10 mA in the spacer gel, and electrophoresed through the running gel at 15 mA. Gels were stained for 1.5 hr in a mixture of 25% isopropanol, 10% acetic acid, and 0.04% Coomassie blue, followed by overnight destaining in 10% acetic acid. Gels were gently agitated at room temperature during staining and destaining. The following proteins were used as molecular weight standards: hemoglobin, trypsin, pepsin, bovine serum albumin, and conalbumin (molecular weights 16×10^3, 23×10^3, 32×10^3, 68×10^3, and 86×10^3 respectively).

Turnover of Growth-Labeled Proteins

Protein turnover during sporulation was determined by the following procedure. Cellular proteins were labeled during the last 40 min of logarithmic growth by the addition of 5 μCi/ml of (H^3)-L-tryptophan. When the culture density reached 200 Klett units (T0), 1,000 μg/ml of unlabeled L-tryptophan was added as a chase. Unlabeled L-tryptophan was dissolved in 0.2 N HCl to a final concentration of 4.0% immediately prior to use. The addition of excess unlabeled tryptophan to sporulating cultures did not alter the time of appearance of heat resistant spores (data not shown). Turnover of total label incorporated was followed by precipitating 0.5 ml samples of culture with 0.5 ml cold 10% trichloroacetic acid (TCA) containing 500 μg/ml unlabeled L-tryptophan. Samples (2 ml) for determining turnover of protein in the soluble and membrane fractions were transferred to 2 ml of an ice cold solution containing 200 μg/ml chloramphenicol, 0.002 M sodium azide, and 200 μg/ml L-tryptophan in deionized water. Membrane and soluble fractions were prepared as described by Sargent (10).

Protein turnover during logarithmic growth was examined by two procedures. The first consisted of labeling cells during the penultimate cell growth period with 5 μCi/ml of labeled tryptophan. During late logarithmic growth, these cells were transferred into fresh medium (1:20 dilution) containing 1,000 μg/ml of unlabeled tryptophan as a chase. Turnover of total cell, soluble, and membrane protein was followed immediately after the chase. The second procedure consisted of labeling during the last growth period with 5 μCi/ml labeled tryptophan. Labeling began when the culture density reached 50 Klett units, and at a culture density of 100 Klett units 1,000 μg/ml of unlabeled tryptophan was added as a chase. Turnover of total cell, soluble, and membrane protein was followed by sampling for 30 min (approximately 1 generation).

Synthesis and Turnover of Membrane Protein During Sporulation

The synthesis and turnover of membrane protein during sporulation was examined by labeling cells with 5 μCi/ml (H^3)-L-tryptophan at T0, and sampling for incorporation into

the membrane fraction. Turnover of membrane proteins synthesized during sporulation was followed by chasing portions of the labeled culture at T1, T2, and T3 with 1,000 μg/ml of unlabeled tryptophan.

Radioactive Methods

Labeled samples were collected on GF/C glass fiber filters (Whatman, 2.4 cm), presoaked in 5% TCA containing 500 μg/ml L-tryptophan, dried under a heat lamp, and transferred to scintillation vials. Samples were counted in scintillation fluid consisting of toluene plus Spectrafluor (Amerst/Searle). (H^3)-L-tryptophan with a specific activity of 4.6 Ci/mM was obtained from Schwarz-Mann.

The effective dilution of labeled tryptophan by the addition of 1,000 μg/ml of unlabeled tryptophan was computed on the basis of the medium containing 0.29% tryptophan (Difco Manual, ninth edition). This could contribute 29 μg/ml of tryptophan by proteolysis. The effective dilution of labeled tryptophan (0.22 μg/ml) would be 34-fold under these conditions, giving rise to a 2.9% error in subsequent analysis of protein turnover. In addition, the effective dilution of label by the above chase procedure was determined by following incorporation of label added to cultures with and without 1,000 μg/ml of unlabeled tryptophan. The results showed a 48-fold effective dilution of label, which would give rise to a 2.1% error in the subsequent analysis of protein turnover during growth. Although not determined experimentally, the error in determining protein turnover during sporulation would be less than that for growing cells, since the rate of protein synthesis decreases during sporulation (unpublished data).

RESULTS

Growth and Sporulation

The final cell transfer yields a culture that grows exponentially to a cell density of 200 Klett units (about 5.0×10^8 cells/ml). At this time, T0, the cells enter the development period, and sporulation is complete at T9. Figure 1 shows growth and sporulation of strain 168 under the conditions used. Strain SB-23 behaves identically to its parent 168 strain during exponential growth; however, it was shown previously (3) that most of the cells are blocked before stage 5 of sporulation.

Figure 2 shows the results of analysis of total cell protein, and protein present in the membrane fraction during growth and sporulation of strain 168. The membrane fraction accounts for approximately 15% of the total cell protein during exponential growth. This value increases to 25% in late exponential cells and remains at this value until about T2 of sporulation. During the transition from T2 to T3, the net amount of membrane protein increases by 40% as a result of membrane synthesis during the engulfment of the prespore. Total cell protein and membrane protein increase slightly from T0 to T1. This is due to a slight increase in cell mass, as determined by analysis of dry weight, and results in an increase in cell size (unpublished observations). Total cell protein remains relatively constant after T1. Similar results were obtained for strain SB-23 (data not shown).

Gel Electrophoresis

Gel electrophoresis was performed on samples of membranes prepared from vegetative, T1, T2, T3, T4, and T5 cells. Figure 3 shows the results of electrophoresis of membrane fractions from strain SB-23. The results were identical for strain 168. Membrane fractions from vegetative cells consist of 39 protein components discernible by

Coomassie blue staining. The molecular weight range is from 16,000 to greater than 100,000. These proteins were numbered 1 to 39, starting with the highest-molecular-weight species, and comparison was then made between vegetative cell membrane proteins and those proteins found in the membrane fraction during the early stages of sporulation. New protein bands were observed and given alphabetical subscripts following

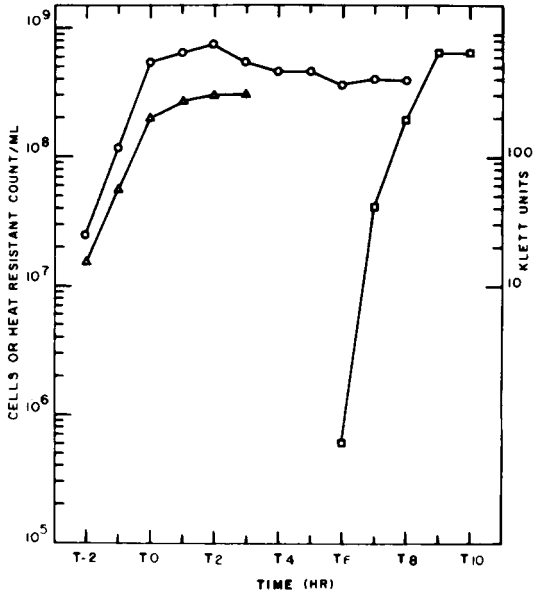

Fig. 1. Growth and sporulation of strain 168 in nutrient sporulation medium. Klett units, △; viable count, ○; and heat resistant count, □.

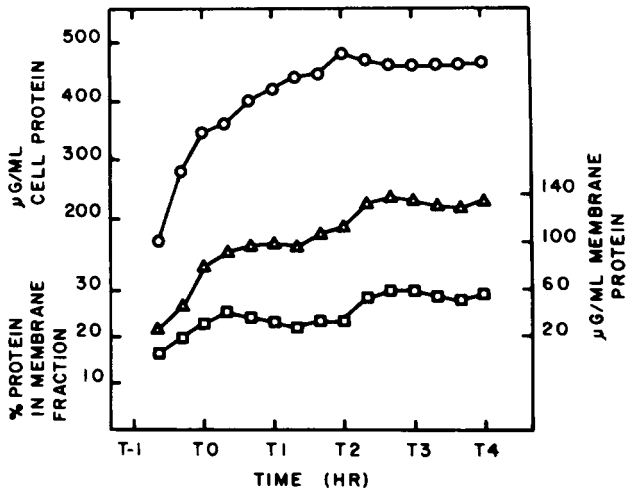

Fig. 2. Analysis of cell and membrane protein during growth and sporulation. Cell protein, ○; membrane protein, △; and % membrane protein, □.

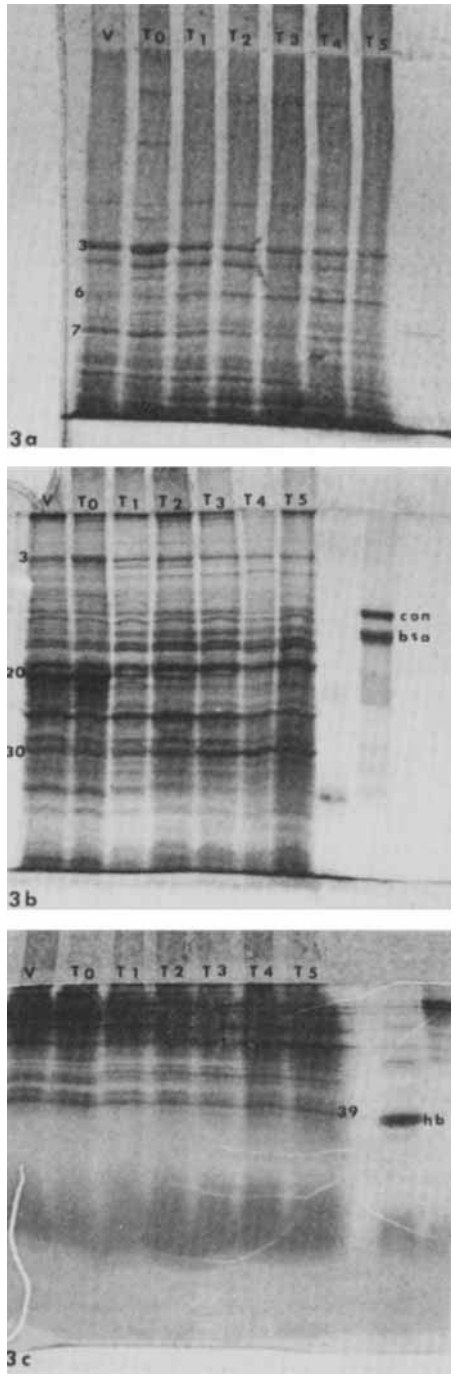

Fig. 3. Gel electrophoresis patterns of the VCM and SMF isolated at T0 and at hourly intervals to T5. Bands 3, 6, 7, 20, 30, and 39 are labeled for reference. (a) 5% gel, (b) 10% gel, and (c) 20% gel (Con, conalbumin; bsa, bovine serum albumin; and hb, hemoglobin.)

the number of the next-higher-molecular-weight vegetative cell membrane protein (except for sporulation membrane protein a, which was the highest-molecular-weight protein observed). Visual analysis of the gels indicated the following six classes of proteins: 1) those that disappear, 2) those that increase, 3) those that decrease, 4) those that are new and persistent to T5, 5) those that are new and transient, and 6) those that show no change. Results of analysis of the gels, with respect to specific protein compositions, are given in Fig. 5, since all changes observed did not reproduce adequately in Figs. 3 and 4.

The protein composition of purified IFM material was compared to those of the VCM and SMF. Figure 4 shows sample gels. The differences observed indicate the following nine classes of proteins: 1) those unique to the IFM, 2) those unique to the SMF and present in the IFM, 3) those unique to the SMF and not present in the IFM, 4) those present in the VCM fraction and missing from the IFM, 5) those VCM proteins that disappear from the SMF and are not found in the IFM, 6) those VCM proteins that disappear from the SMF but are found in the IFM, 7) those in common with the VCM and IFM, but at reduced levels in the IFM, 8) those in common with the VCM and IFM, but at increased levels in the IFM, 9) those VCM proteins not found in the IFM, but persistent in the SMF. These results indicate a complex series of alterations in the protein composition of the membrane fraction from sporulating cells. These data are summarized in Fig. 5.

Turnover of Growth-Labeled Proteins

In view of the fact that compositional changes take place with respect to membrane proteins during sporulation, turnover of growth-labeled membrane proteins was examined. During logarithmic growth, the pattern of turnover observed depended upon the chase procedure used. Figure 6 shows the results obtained by a dilution and cold tryptophan chase. Turnover of total and soluble protein appears biphasic, characterized by more rapid rates during the first 10–15 min. In addition, 15% of membrane-associated label turns over in the 10 min following chase. Following this initial burst of turnover, a slower rate characteristic of growing cells is observed. When centrifugation is used as part of the chase procedure, the initial burst of turnover is stimulated approximately two-fold (data not shown).

In contrast, when cells were labeled and then chased without dilution into fresh medium or centrifugation, a different pattern of turnover was observed (Fig. 6). During the first 30 min (about 1 generation) turnover of 5–10% of total and soluble protein was observed. Very little, if any, turnover was observed in membrane protein.

Turnover of growth-labeled proteins during sporulation was examined by chasing cells at T0. The results are shown in Fig. 7. Total protein begins to turn over at T1, and proceeds at a rate of about 20%/hr. During the first 2 hr of sporulation, 25% of the growth-labeled membrane proteins were eluted to the cytoplasm in the absence of a net decrease in membrane protein content (as determined by Lowry assay, Fig. 2). This represents about 19 μg/ml of membrane protein. The corresponding increase in soluble protein label accounts (in cpm/ml) for the eluted membrane protein. During the period from T2 to T2.5, about 80% of the previously eluted membrane proteins reassociated with the membrane fraction. This represents about 15 μg/ml of protein. The net increase in membrane protein during engulfment is about 45 μg/ml of culture. Thus, synthesis of an additional 30 μg/ml of membrane protein must occur during this period (see below). Turnover of soluble and membrane proteins proceeds at a rate of 16 to 20%/hr from T3 to T5.

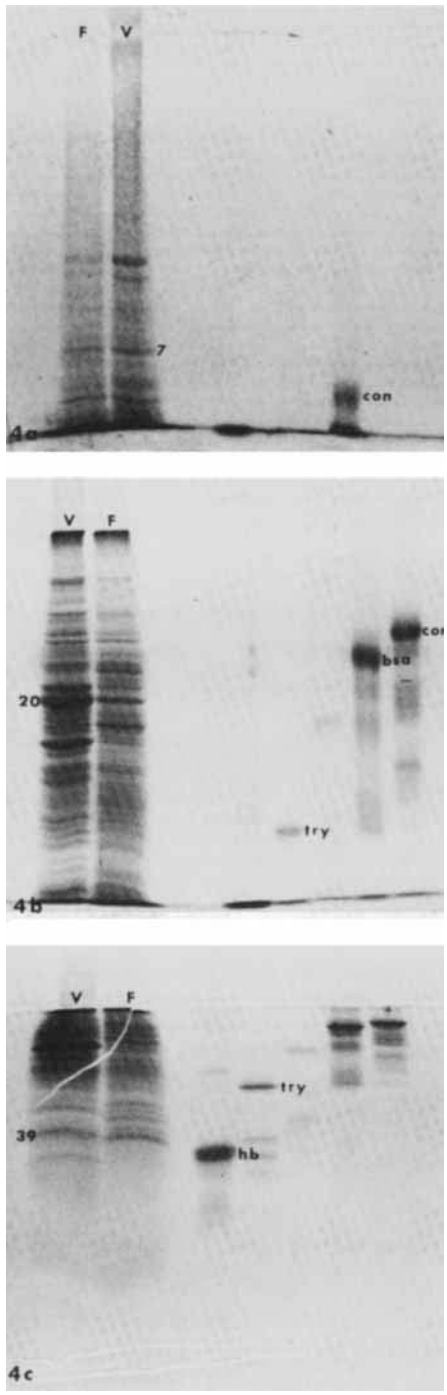

Fig. 4. Gel electrophoresis patterns of the VCM (V) and IFM (F). Bands 7, 20, and 39 are labeled for reference. (a) 5% gel; (b) 10% gel, and (c) 20% gel. (Con, conalbumin; bsa, bovine serum albumin; try, trypsin; and hb, hemoglobin.)

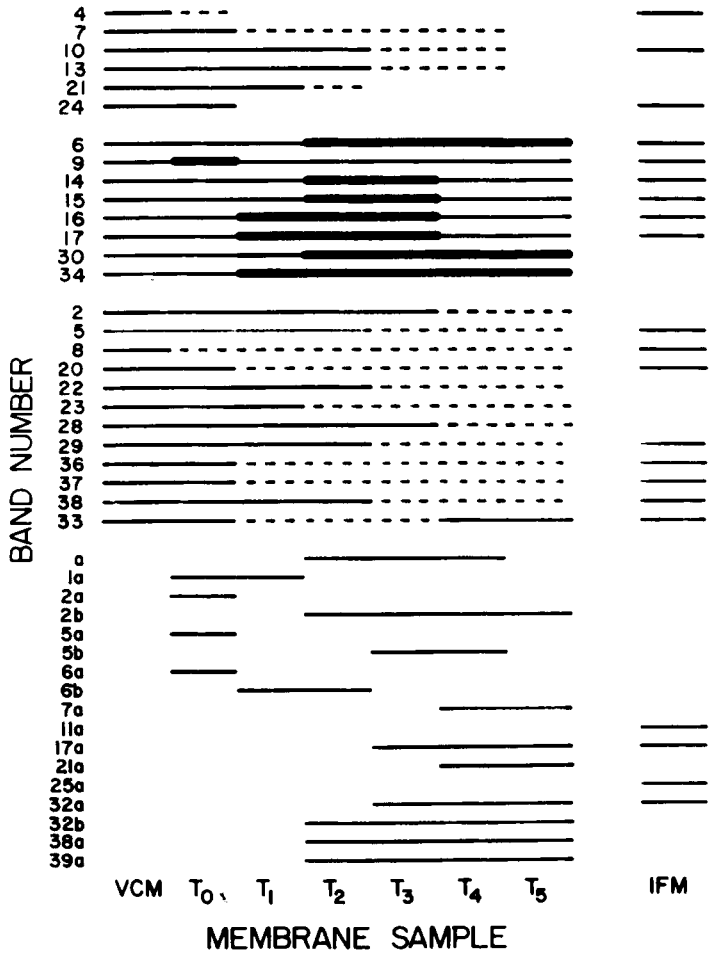

Fig. 5. Schematic summary of membrane protein alterations during sporulation. Numbers on the left side of the diagram correspond to polypeptides observed after gel electrophoresis of membrane samples (see Figs. 3 and 4 for reference bands). This summary is based on the visual inspection of several slab gels. A solid line indicates the presence of a polypeptide band at a concentration characteristic of the VCM; a thickened solid line indicates a polypeptide present in a greater concentration that that of the VCM; a dashed line indicates reduced amounts; and the absence of a line indicates a polypeptide that was absent, or present in an amount that precluded detection.

Synthesis and Turnover of Membrane Protein During Sporulation

Figure 8 shows the net incorporation of label into the membrane fraction during sporulation. The results parallel those obtained by assaying the total protein in the membrane fraction, showing an initial increase followed by a plateau and a second increase. During the time period prior to engulfment, net membrane protein increases about 15 μg/ml of culture. An additional 19 μg/ml of membrane protein must have been synthesized to balance turnover to the cytoplasm. This represents 34 μg/ml of membrane protein, and corresponds to the accumulation of 40,000 cpm/ml of culture. During engulfment, membrane protein increases about 45 μg/ml of culture, 15 μg being accounted for by reassociation of

previously eluted membrane proteins. This leaves 30 μg/ml of culture as membrane protein required by new synthesis during engulfment. Labeling during sporulation shows an increase of about 40,000 cpm/ml of culture during engulfment, which would be equivalent to about 34 μg/ml of culture. During a chase of 1,000 μg/ml of unlabeled tryptophan,

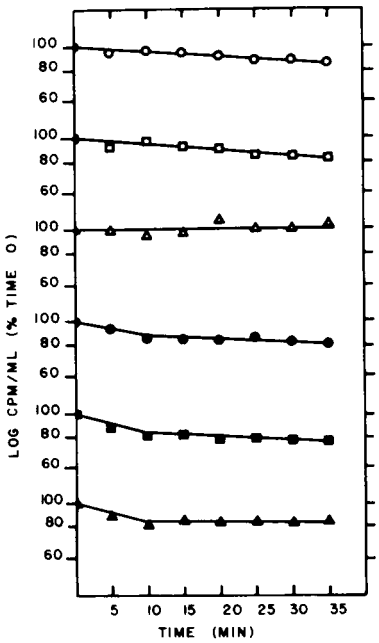

Fig. 6. Protein turnover during logarithmic growth. Turnover in the absence of cell manipulation; total protein, ○; soluble protein, □; and membrane protein, △. Turnover after dilution into fresh medium: total protein, ●; soluble protein, ■; and membrane protein, ▲. Results expressed as log % cpm/ml remaining after chasing.

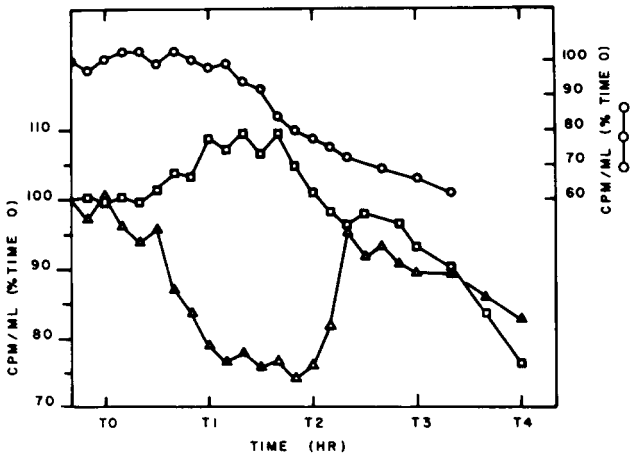

Fig. 7. Protein turnover during sporulation. Total protein, ○; soluble protein, □; and membrane protein, △. Results expressed as % cpm/ml remaining after chasing.

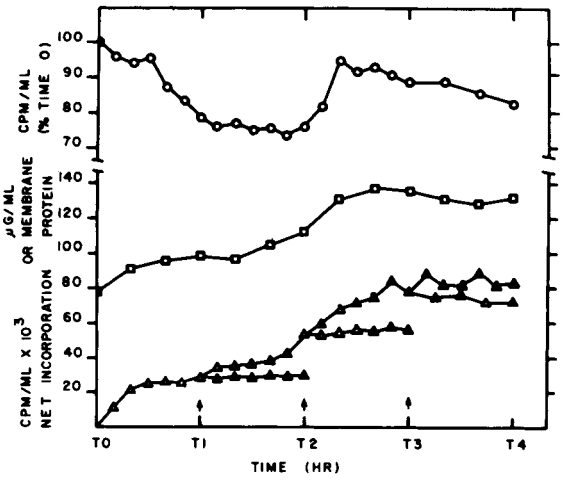

Fig. 8. Net incorporation to the membrane fraction during sporulation. Cpm/ml incorporated in the membrane fraction, △; μg/ml membrane protein, □; and % cpm/ml remaining in the membrane fraction after chasing prelabeled cells at T0 (data from Fig. 7), ○. Arrows indicate time of chase for cells labeled during sporulation.

membrane protein synthesized during sporulation were relatively stable until T3; however, this does not preclude turnover of stage-specific proteins that constitute a minor fraction of the total membrane protein. After T3 there appears to be some turnover of membrane protein synthesized during sporulation. Rates of turnover for total and soluble protein labeled during sporulation were similar to those reported by Spudich and Kornberg (13); however, initial rates of greater than 16–20%/hr were usually observed in the first 10 min following chase (unpublished observation).

Membrane Protein Stability and Effect of Cell Extracts

Two alternatives were considered to explain the observed elution of membrane proteins during sporulation. First of all, elution could be effected by modification of membrane structure, resulting in decreased stability of membrane proteins. This is reminiscent of Schimke's proposal for turnover of eukaryote membrane proteins (14) and suggests that isolated membranes would exhibit a loss of labeled protein when incubated in vitro. However, data in Table I shows that VCM and T1 membranes are relatively stable at low temperatures, and at 37°C for 1 hr. The results of incubation at 47°C also fail to support the hypothesis that membranes during sporulation are less stable with respect to their proteins.

On the other hand, continual action of cytoplasmic factors on the membrane could be responsible for membrane protein elution. This prompted examination of the effect of cytoplasmic proteins from crude cell extracts on the VCM. The results in Table II indicate that such an event does not take place under the in vitro conditions used.

DISCUSSION

The conditions used for growth of B. subtilis in this study lead to a large and fairly synchronous burst of sporulation. During the developmental period, numerous alterations occur in the physiological state of the cells, resulting in production of a mature spore. The

results of the present investigation indicate that extensive alterations in the protein composition of the membrane fraction from sporulating cells occur.

Vegetative cell membranes were shown to consist of 39 proteins discernible by Coomassie blue staining of slab gels. The molecular weight range is from 16,000 to greater than 100,000. The membrane fraction isolated during the first 5 hr of sporulation will contain the cell membrane and newly synthesized spore membranes. Unfortunately, procedures are not yet available for the separate isolation and analysis of the VCM, OFM, and IFM during the early stages of sporulation. However, results from analysis of the complete membrane fraction from sporulating cells can be used in drawing some conclusions about membrane protein alterations as they pertain to sporulation.

First of all, certain proteins found in the VCM are lost from the membrane fraction during sporulation. These most likely reflect proteins whose functions are not required for, or which are incompatible with, spore development. It has been reported previously (15) that the membrane-associated glucose phosphotransferase system, of the class initially reported by Kundig et al. (16), declines rapidly at the end of logarithmic growth. The loss of this transport system may be required if cells are to become committed to sporulation, as glucose strongly suppresses sporulation.

In addition, certain VCM proteins were found to increase or decrease in concentration in the sporulation membrane fraction. These may reflect adjustments in membrane functions, which are required in response to the cell's altered physiological state, and may

TABLE I. Stability of Membranes in 0.05 M Tris Buffer

Time	0°C	37°C		47°C	
(min)	VCM	VCM	T1	VCM	T1
0	100	100	100	100	100
15	98	96	103	85	97
30	97	92	109	86	95
45	101	97	107	57	91
60	112	105	108	39	66

Results expressed as % of cpm/ml present at the start of incubation. Membranes stored in Tris buffer (0.05 M, pH 7.4) at 4°C for 1 week lost about 5% of their initial cpm/ml determined immediately after isolation. VCM and T1 indicate membranes isolated from vegetative cells and cells 1 hr after growth ceases.

TABLE II. Effect of Crude Cell Extracts on the VCM[a]

Time	Crude extract[b]			
(min)	VC	T0	T1	T2
0	100	100	100	100
10	94	93	101	97
20	95	97	101	105
30	97	101	104	99
40	95	107	96	103

[a]Results are expressed as % of cpm/ml present at the start of incubation.

[b]Final protein concentrations used during the incubations were 3.3 mg/ml, 2.4 mg/ml, 3.4 mg/ml, and 3.7 mg/ml for the VC, T0, T1, and T2 extracts respectively.

or may not be directly related to sporulation. Previously reported alterations that may relate to the above-mentioned changes in membrane protein composition include Tochikubo's study of concentration changes in the particulate cytochrome system during sporulation (17) and Szulmajster's observation of lower NADH and NADPH oxidation activity in the particulate fraction during sporulation (18). It should be mentioned that in the latter example much of the lost membrane oxidation activity was found in the cytoplasm.

The 15 new protein components found in the membrane fraction during sporulation fall into two classes. The first consists of 7 proteins (a, 1a, 2a, 5a, 5b, 6a, and 6b) that are transient in their association with the membrane fraction. At the present time it is difficult to say with certainty in which membrane these new transient proteins are found. Those that appear and disappear early (1a, 2a, 5a, and 6a) are likely candidates for a VCM cell membrane location and may function in some sporulation-specific capacity. Some of these new transient proteins could be cleavage products of proteins that disappear from the membrane fraction, suggesting removal of specific proteins by proteolytic cleavage followed by extrusion from the membrane. Those proteins that appear and disappear after T1 may be associated with the cell membrane; however, it is also possible that they are transient proteins associated with newly synthesized spore membranes. The second class of new proteins consists of 8 proteins (2b, 7a, 17a, 21a, 32a, 32b, 38a, and 39a) that persist in the membrane fraction through T5 of sporulation. Although assignment cannot be made to a specific membrane site for all of these proteins, it is likely that some of these are unique to spore membranes. Two of these proteins (17a and 32a, Fig. 5) are found in the IFM, and may be unique to this membrane structure. Those that are not found in the IFM may well be OFM proteins; however, isolation of this membrane is necessary for conclusive proof. At present there is no direct evidence for specific functions for these new proteins. They may be related to probable membrane-associated sporulation events such as Ca^{++} transport into the cell and forespore (19), DPA transport into the forespore (20), cortex peptidoglycan synthesis, or coat protein assembly (3).

The membrane protein alterations reported in this investigation are the same for strain 168 and SB-23. Advantage was taken of the fact that pure IFM could be obtained from incomplete spores isolated from strain SB-23. The results of protein analysis, by gel electrophoresis of IFM proteins, reinforce the hypothesis that membrane alterations are extensive during sporulation. The IFM was found to lack 11 of the 39 proteins found in the VCM, and of these 11, 3 proteins (7, 13, and 21) correspond to proteins that disappear from the membrane fraction during sporulation. Those proteins that are missing from the IFM, but remain in the membrane fraction isolated from sporulating cells, suggest specific differentiation of the IFM after its formation. There were also concentration differences in some of the proteins in common with the IFM and VCM. The IFM also contained 2 proteins (11a, 25a) that were unique to the IFM. These results show that the IFM is not identical to the VCM. Although the IFM arises from the VCM on a morphological basis, it must be subject to sporulation-specific modifications in protein composition.

Protein turnover is known to occur during bacterial growth and sporulation (21, 13). The results reported in this paper suggest that during growth 5–10% of the cytoplasmic proteins are degraded per cell generation. On the contrary, membrane proteins appear stable during growth. However, these observations are dependent upon the experimental procedures used. Manipulation of cells by centrifugation, or dilution into fresh medium, was found to induce some turnover of membrane proteins. The induction of altered metabolic processes by experimental manipulation of cells has been discussed by Sargent

(10) in his study of membrane synthesis during synchronous growth of B. subtilis, and by Pine in his report of protein turnover in Escherichia coli (21, 22).

During the first 2 hr of sporulation, growth-labeled membrane proteins were eluted to the cytoplasm in an acid precipitable form. Since this occurred without a decrease in net membrane protein, a process of turnover was indicated. Since the protein composition of the sporulation membrane fraction is altered during development, the turnover phenomenon is indicated as a mechanism for restructuring the membranes. The facts that can be used in constructing a model to explain the observed membrane modifications are as follows: 1) net protein in the membrane fraction remains relatively constant during the first 2 hr of sporulation, followed by a 40% increase between T2 and T3 due to engulfment of the prespore by newly assembled membrane; 2) membrane proteins, which turn over to the cytoplasm, remain in an acid precipitable form until at least T1, when protein turnover to acid soluble material begins (this does not rule out limited proteolysis to acid precipitable material prior to T1); 3) the protein composition of the sporulation membrane fraction changes during development; 4) some of the eluted membrane proteins reassociate with the membrane fraction at a time when the prespore is engulfed; 5) membrane protein turnover continues until at least T5 of sporulation; 6) membrane proteins synthesized during the developmental period appear relatively stable to T3 of sporulation; and 7) incorporation of labeled membrane proteins to the membrane fraction decreases at the end of growth, and then resumes between T2 and T3.

When proposing a model to explain the above facts, a fundamental consideration is the mechanism by which proteins are eluted from the membrane. The following two possibilities have been considered: 1) elution as a result of an alteration in membrane structure leading to a decreased stability of proteins in the membrane, and 2) elution as a result of a continual metabolic process acting from the cytoplasm. Results reported in this study suggest that the first alternative is not the case. Attempts to demonstrate the second alternative were also negative; however, this may be the result of the in vitro conditions employed.

A second question of major importance concerns the mechanism by which specificity of membrane protein turnover is effected. This must be considered both before and after initiation of protein turnover to acid soluble material, and with respect to rapidly and slowly disappearing membrane proteins. Specificity most likely involves selective synthesis coupled with 1) selective inactivation of rapidly disappearing proteins; and 2) entrance of slowly disappearing proteins into the cytoplasmic protein pool, which is subject to degradation to acid-soluble material. It is not known whether rapidly disappearing proteins are inactivated before or after elution from the membrane (perhaps by proteolysis); however, the end result is their rapid removal from the membrane fraction. Unfortunately, very little is known about the regulation of protein turnover during sporulation. A number of proteases have been identified (23); however, specificity of proteases for different classes of proteins (i.e., membrane vs cytoplasmic) has not been examined. In addition, proteases associated with the membrane fractions of bacteria have been reported (24). MacGregor (25) has shown the release of membrane-attached nitrate reductase by a membrane protease. Such observations suggest a physiological role for membrane-associated proteases in the regulation of membrane metabolism.

The fact that proteins can be removed from the membrane by a process of turnover to the cytoplasm is an important consideration in determining the mechanism of membrane differentiation during sporulation. Since the IFM is modified and the OFM is likely

modified with respect to the protein composition of the VCM, the observed turnover phenomenon is considered a possible mechanism of membrane differentiation during development. The fact that 80% of the eluted membrane label reassociates with the membrane fraction is considered, for the purpose of this discussion, to indicate a reassociation of membrane proteins that are not specifically inactivated. Since reassociation occurs during engulfment, the eluted proteins form a putative pool of membrane proteins that, along with newly synthesized protein, are assembled into spore membranes. This indicates that elution of membrane proteins is not specifically limited to those that will be inactivated. A second alternative, that of reutilization of labeled tryptophan by way of acid soluble residues generated during proteolysis, was considered unlikely for the following reasons. Unlabeled tryptophan, added as a chase, was always effective in competing with previously added labeled tryptophan, and pulse labeling during sporulation always resulted in the expected incorporation, based on consideration of rates of protein synthesis and turnover in vegetative and sporulating cells (unpublished observations). Thus exogenously added tryptophan always had access to the internal pool. Since the generation of free amino acid by proteolysis also occurred by way of a pool, exchangeable with exogenously added chase amino acids, there is little probability of selectively reutilizing label that was previously incorporated, and then released during turnover.

Figure 9 shows a diagram relating the above-mentioned events to membrane differentiation during sporulation. It should be noted that Sargent (10) has reported the elution of membrane proteins to the cytoplasm at a certain stage of the cell cycle. However, the observation that centrifugation, and even dilution into fresh medium, cause membrane protein elution to the cytoplasm suggests that this event can be induced by experimental manipulation of cells, perhaps those used in producing cell synchrony.

In conclusion, the system of bacterial sporulation offers the membrane biologist a chance to study membrane differentiation on the molecular level. The separation of the

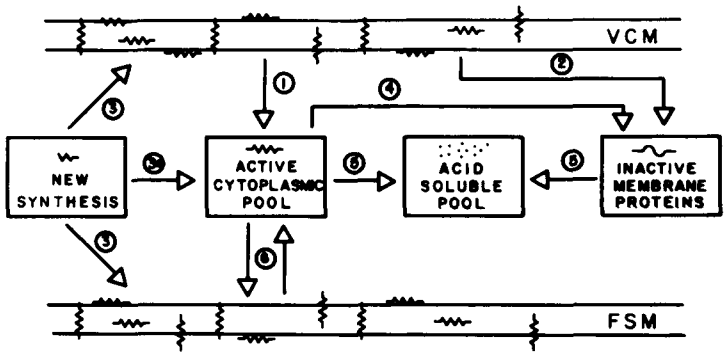

Fig. 9. Diagram of membrane differentiation during sporulation. 1) Elution of membrane protein from the VCM to the active cytoplasmic pool, 2) rapid inactivation of those membrane proteins that disappear rapidly from the membrane, 3) and 3a) new synthesis of membrane protein (3a indicates newly synthesized membrane protein that may enter the cytoplasmic pool prior to insertion to the membrane), 4) inactivation of membrane proteins from the active cytoplasmic pool, 5) degradation to acid soluble material, and 6) reutilization of membrane protein from the active cytoplasmic pool during engulfment of the prespore. Certain reactions may be localized in the sporangium or developing forespore, depending upon which membrane is being differentiated, i.e., the VCM, IFM, or OFM. In this figure, VCM, vegetative cell membrane; FSM, forespore membrane. See text for detailed description.

membrane fraction of sporulating cells into VCM, OFM, and IFM is of utmost importance to further investigation of this developmental system. In addition, the lipid portion of the membranes must be examined. Hopefully further research will describe membrane differentiation in sporulating bacilli in precise molecular terms.

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