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Isolation and Properties of Membranes from *Bacillus megaterium* Spores

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Membranes from dormant and heat-activated spores of *Bacillus megaterium* QM B1551 were isolated and purified by gentle lysis procedures followed by differential and sucrose density gradient centrifugations. The purified membranes were enriched for inner membranes and were characterized by their density and content of proteins, phospholipids, enzymes, cytochromes, and carotenoids. These purified spore membranes could be used to investigate their role in the triggering of germination.

Recent evidence has suggested that the triggering of spore germination may be a membrane-associated event (20, 27). Unfortunately, little is known about spore membranes, and there are no data on membranes from *Bacillus megaterium* QM B1551 spores. In another *B. megaterium* strain, membranes have been prepared by mechanical disruption of spores and partially characterized (13, 36). In addition, it was reported that inner and outer membranes could be isolated by differential centrifugation, and several enzyme marker activities were measured (10, 22).

We report here a relatively gentle method of disrupting spores of *B. megaterium* QM B1551 which allows the isolation of inner membranes. The membranes from dormant or heat-activated spores were purified, and they could be studied by biophysical techniques (20) as well as by the binding of proline to a possible trigger site (27).

MATERIALS AND METHODS

Membrane isolation. The methods for the preparation of spores of *B. megaterium* QM B1551 and their extraction with sodium dodecyl sulfate-dithiothreitol (SDS-DTT) were previously described (31, 32). All references to spore weights are on a dry-weight basis. Spores extracted with SDS-DTT had the same requirements for triggering germination as nonextracted spores, yet they were lysozyme sensitive (32). Spore membranes were prepared by several methods. In method A, lysates were prepared by suspending 2 g of extracted spores in 20 ml of 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5) containing lysozyme (0.5 mg/ml), RNase (2.5 μg/ml), and DNase I (2.5 μg/ml) at 30°C. After 12 min, the suspension was cooled on ice, 8 g of glass beads was added, and the suspension was sonicated eight times for 30 s each as previously described (32) with 90 s of cooling at 0°C each time. This procedure gave >95% spore disruption as judged microscopically. Debris was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant fraction was made to 5% (vol/vol) sucrose and centrifuged at 304,000 × *g* (maximum) for 105 min to sediment membrane fractions. Further purification was done with discontinuous sucrose gradients as described by Schnaitman (30) by using 4 ml of 2.12 M sucrose, 13 ml of 1.44 M sucrose, and 12 ml of 0.77 M sucrose, all in 10 mM HEPES buffer (pH 7.5). On each gradient, 5 ml of suspended membrane pellet was layered, and the gradients were centrifuged at 20,000 rpm in a Beckman SW27 rotor for 16 h at 2°C. The gradients were fractionated with a gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebr.) by pumping 2.12 M sucrose into the bottom of the tube and collecting fractions from the top. The absorption at 280 nm of each fraction was measured, and the peak fractions, located just above 1.44 M sucrose, were pooled. Membranes were recovered by centrifugation at 304,000 × *g* for 105 min, and the pellets were washed with 25 ml of 10 mM HEPES buffer (pH 7.5) by centrifugation under the same conditions. The washed pellets were resuspended overnight in 10 mM HEPES buffer (pH 7.5) at approximately 20 to 40 mg of membrane protein per ml.

Method B was similar to method A except that EDTA at pH 7.5 was added after sonication to a final concentration of 10 mM. Also, the gradients were made with 8 ml of 1.44 M sucrose and 20 ml of 0.77 M sucrose, both in 10 mM HEPES–10 mM EDTA (pH 7.5). Centrifugation was the same as in method A, and pooled fractions were washed in 10 mM HEPES buffer without EDTA as above. Method C was the same as method A except that the spore lysate was passed four times through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 10,000 lb/in^2 instead of being sonicated.

Assays. Previously published methods were used to assay for protein (24), phosphate (7), RNA (5), and DNA (6), and peptidoglycan was measured after hydrolysis and determination of hexosamine (19). Phospholipids were extracted by the method of Bligh and Dyer (4) and analyzed by thin-layer chromatography as described by Bertsch et al. (solvent system i in reference 3). Carotenoid was extracted from the membranes (3.5 mg of membrane protein) by the method of Bligh and Dyer (4) and dried under N_2. Spectra were obtained in $CHCl_3$, and thin-layer chromatogra-

phy was done on silica gel plates (EM Laboratories, Elmsford, N.Y.) by using methanol-benzene (25:75) or ethanol as the solvent. Proteins were separated by polyacrylamide gel electrophoresis (23) with 15% acrylamide except that a 0.98% cross-linker was used. Gels were stained with Coomassie brilliant blue and destained as described previously (32).

Previously published procedures (35, 36) were used to assay NADH oxidase, succinate dehydrogenase (EC 1.3.99.1), malate oxidase (EC 1.1.3.3), DL-glycerol-3-phosphate oxidase, NADH dehydrogenase (EC 1.6.99.3), and malate dehydrogenase (EC 1.1.1.37). Other methods were used to assay ATPase (EC 3.6.1.3) (12) and dehydrogenases of the following: glucose (EC 1.1.1.47) (28), leucine (EC 1.4.1.9) (29), glutamate (EC 1.4.1.2) (8), lactate (EC 1.1.1.27) (11), glycerol 3-phosphate (EC 1.1.1.8) (2), proline (EC 1.4.3.2) (9), 6-phosphogluconate (EC 1.1.1.44) (16), and glucose 6-phosphate (EC 1.1.1.49) (16).

RESULTS

Membrane isolation and composition. Spores extracted with SDS-DTT were disrupted, the debris was removed by centrifugation at 10,000 × *g*, and a membrane fraction was sedimented at 304,000 × *g* for 105 min. In the sucrose density gradient, most of the material sedimented to the interface between 0.77 and 1.44 M sucrose (Fig. 1), and those fractions were pooled and washed as described above. A similar technique was recently reported for *B. sphaericus* spore membranes (17) in which "cytoplasmic membrane" sedimented to the same place as described above with the same gradient procedure (30), whereas spore integuments sedimented through the 1.44 M sucrose to the 2.12 M cushion. By analogy, our pooled fractions would correspond to cytoplasmic or inner spore membranes (see below).

The composition of membranes from dormant and heat-activated spores is shown in Table 1. The ratios of phospholipid to protein were from 0.25 to 0.29, a range which was lower than that reported for the cytoplasmic membranes of two gram-negative organisms (26, 30). However, the above values were similar to those reported for vegetative cell membranes of *B. subtilis* (15, 21) and *B. megaterium* QM B1551 (Racine, unpublished data). When the density of spore membranes was measured by equilibrium density centrifugation (26), the densities ranged from 1.160 to 1.168 g/ml. These values are similar to those of other membrane preparations (26, 30) and to that found for *B. megaterium* QM B1551 vegetative cells (1.168 g/ml; unpublished data). The recoveries of protein and phospholipid in the membrane fraction were 3 to 4% and 30 to 40%, respectively, compared with total spore protein and phospholipid content.

The membranes contained no DNA or peptidoglycan, but both preparations contained RNA (Table 1) in amounts similar to those reported for vegetative cell membranes of other *Bacillus* species (15). Unfortunately, a comparison with published values for spore membranes cannot be made because the nucleic acid content of membranes from spores has never been reported. For peptidoglycan, it may be noted that the limit of detection was about 0.5 mg of hexosamine per g of spores, which is 0.3% of the total spore peptidoglycan. We would not have detected the glucosamine from glucosaminylphosphatidylglycerol, which was present at about 90 μg/g of spore.

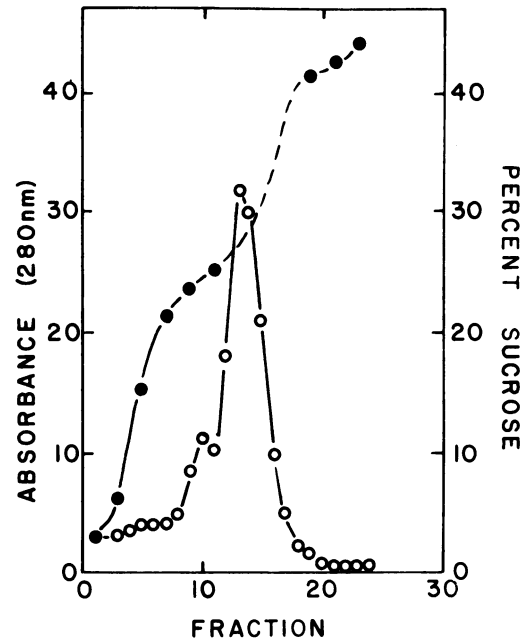

FIG. 1. *Sucrose density gradient. Spore membranes (5 ml) prepared by method A were fractionated on discontinuous sucrose gradients as described in the text. From each fraction (32 drops), 20 μl was used to determine sucrose concentration (●) by refractive index; 20 μl was diluted 50-fold in water, and the absorbance (○) at 280 nm was measured. Fractions 12 to 17 were pooled.*

The patterns of membrane proteins from dormant and heat-activated spores on SDS-polyacrylamide gels were the same, and one is shown in Fig. 2. There were 18 major and minor bands ranging in molecular weight from slightly less than 10,000 to about 130,000, a range which is similar to that of other membrane preparations (10, 26). The number of proteins shown in Fig. 2 is considerably less than the number presented in a preliminary report for *B. megaterium* KM spore membranes (10), which is indicative of the greater purity of membranes prepared by the

TABLE 1. *Chemical composition of spore membranes*

Type of spores	Component content (mg/g of spores)[a]					Phospholipid/protein ratio	Density (g/ml)[c]
	Protein	Phospholipids[b]	DNA	Hexosamine	RNA		
Dormant	9.9	2.9	0	<0.5	7.7	0.29	1.160
Heat-activated	11.6	2.9	0	<0.5	7.2	0.25	1.168

[a] Based on the weight of SDS-DTT-extracted spores. Spore membranes were isolated and purified from dormant or heat-activated spores by method A.

[b] Values are calculated from millimoles of phospholipid phosphate by using a conversion factor of 894 mg/mmol. This factor is the weighted average of the molecular weights of the phospholipid species in spores of this organism as determined according to their relative abundance by using the data in Table 2.

[c] Density was determined by equilibrium sucrose density gradients (26).

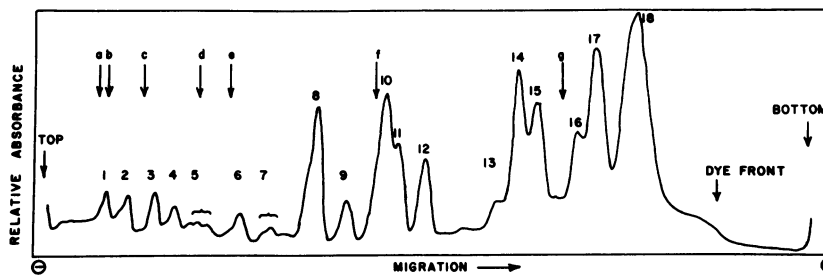

FIG. 2. *Electrophoresis of membrane proteins. The purified membrane proteins (150 μg) from heat-activated spores (method A) were separated by SDS-polyacrylamide gel electrophoresis at 3 mA and 25°C and stained as described in the text. The gel was scanned with a Zeineh soft laser scanning densitometer, and the peaks were numbered. The arrows indicate the positions of the following standards with the indicated molecular weights: (a) β-galactosidase (130,000); (b) phosphorylase B (94,000); (c) bovine serum albumin (68,000); (d) ovalbumin (45,000); (e) aldolase (40,000); (f) chymotrypsinogen (25,000); (g) RNase (13,700).*

procedures reported here. The same protein pattern was found repeatedly for membranes from either dormant or heat-activated spores. Also, membranes that had been stored for 7 to 10 days at 4°C had the identical pattern, suggesting that significant proteolysis did not occur with purified membranes. A rigorous exclusion of proteolytic activities by the use of protease inhibitors such as EDTA cannot be done because EDTA destroys much of the biological activity of these membranes (see below).

The membrane preparations were examined by electron microscopy of thin sections by the method of Schnaitman (30). The preparations contained membrane fragments but were primarily (>60%) composed of vesicles with diameters ranging from 0.06 to 0.2 μm (data not shown). We have not determined whether these vesicles are inside out or not, but preliminary data suggest a mixture of both types.

Phospholipids. The phospholipids of spore membranes were extracted and analyzed by thin-layer chromatography (Table 2). Four species of phospholipids were found: diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and glucosaminylphosphatidylglycerol. The predominant species was phosphatidylglycerol (44%) as it is in vegetative organisms (3). Diphosphatidylglycerol constituted 29% of the total, whereas phosphatidylethanolamine and glycosaminylphosphatidylglycerol constituted 13 and 14%, respectively. There were no significant differences between membranes from dormant and heat-activated spores.

To test whether the above phospholipid distribution in membranes was a representative fraction of the total spore phospholipid content, the phospholipid composition of dormant and heat-activated SDS-DTT-treated spores was determined. The distributions of phospholipids were similar from both dormant and heat-activated spores, and these distributions were also quite similar to those from spore membranes (Table 2). Therefore, the membrane fraction isolated as described above contained a representative distribution of the total phospholipid content from intact spores.

The phospholipid distributions from SDS-DTT-extracted spores were close, but not identical, to those calculated from data of whole, non-SDS-DTT-extracted spores of this same strain by Bertsch et al. (3). They reported that diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and glucosaminyl-

phosphatidylglycerol constituted approximately 17, 50, 14, and 19%, respectively, of the total spore phospholipid content. However, Bertsch et al. extracted only 3.4 μmol of phospholipids per g of spores, whereas our yield was 4.5 μmol of phospholipids per g of spores extracted after lysis and sonication. By applying the exact method of Bertsch et al. (3) to nonextracted spores, we confirmed that only 3.6 μmol of phospholipids per g of spores was extracted. However, an additional 1 μmol/g was extracted from these spores by boiling the residue for 20 min. The major component (60 to 70%) of the phospholipids extracted by this last step was diphosphatidylglycerol (data not shown). This agrees with the conclusions of Bertsch et al. (3) that diphosphatidylglycerol is more difficult to extract than other phospholipid species. We conclude that the major difference between our results and those of Bertsch et al. is that our lysis and sonication procedure extracted more phospholipids, mainly diphosphatidylglycerol.

Ellar and Postgate (13) have also analyzed the phospholipids of *B. megaterium* KM spores. Compared with our results, they found a higher proportion of diphosphatidylglycerol (35%), much lower phosphatidylglycerol (only 24%), lysylphosphatidylglycerol (7%) in place of glucosaminylphosphatidylglycerol, and two unknown phospholipids constituting 21% of the phospholipids of that strain. Analysis of the differences between their results and ours is difficult because they did not report the total amount of phospholipids extracted in their experiments.

Enzymes. The activities of several enzymes were determined (Table 3) as a means of judging the functional integrity of the spore membranes. The membranes isolated by method A had

TABLE 2. *Phospholipid content of spores and spore membranes*

Phospholipid	Total phospholipid content (%)			
	Membranes[a]		Spores[b]	
	Dormant	Heat-activated	Dormant	Heat-activated
Diphosphatidylglycerol	29.6	28.4	27.8	27.8
Phosphatidylglycerol	43.5	44.1	48.6	45.4
Phosphatidylethanolamine	12.2	13.5	16.6	13.4
Glucosaminylphosphatidylglycerol	14.8	14.0	12.1	13.4

[a] Purified membranes isolated by method A were extracted, and the phospholipid distribution was determined as described in the text.

[b] Spores extracted with SDS-DTT were disrupted by lysis and sonication, and the entire suspension was extracted to obtain the phospholipids (>90% recovery).

TABLE 3. *Membrane-associated enzyme activities*[a]

Enzyme	Sp act (μmol/min per mg of protein)	
	Dormant	Heat-activated
NADH oxidase	0.68	0.43
NADH dehydrogenase	1.20	1.39
Succinate dehydrogenase	0.020	0.015
Malate dehydrogenase	0.006	0.007

[a] Membranes were isolated by method A from dormant and heat-activated spores. The enzymes were assayed immediately as described in the text.

NADH oxidase, succinate dehydrogenase, malate dehydrogenase, and NADH dehydrogenase activities. Membranes did not contain dehydrogenases of glucose, glucose 6-phosphate, 6-phosphogluconate, leucine, lactate, proline, glutamate, or glycerol 3-phosphate. Also, there was no activity for glycerol 3-phosphate oxidase or ATPase.

The NADH oxidase of membranes was completely inhibited by 10 mM KCN and was not stimulated by flavin adenine dinucleotide; there was no activity with NADPH in the presence or absence of flavin adenine dinucleotide. In contrast to the membrane-bound NADH oxidase, there was a soluble NADH oxidase activity in the 304,000 × *g* supernatant fraction that was >90% resistant to 10 mM KCN and was stimulated twofold by flavin adenine dinucleotide. This supernatant fraction also contained a soluble NADPH oxidase activity at about the same level as the soluble NADH oxidase, but it was stimulated 20-fold by flavin adenine dinucleotide. These results for NADH oxidase are similar to those reported by Wilkinson and Ellar for *B. megaterium* KM (36), except that our overall specific activities were almost 10 times higher, and they reported no succinate or malate dehydrogenase activities (10, 36). Since NADH oxidase activity has been shown to be quite sensitive to the harshness of the method of preparing lysates (36), these results suggest that our method of preparing membranes may be more gentle than those previously used (36). In addition to the high level of recovered enzyme activities, the lack of marker-soluble enzyme activities in the membrane indicated that this method achieved a high level of purification.

Other components. The final membrane preparation was reddish-orange. This color was due in part to cytochromes *a*, a_3, *b*, and *c*, which were identified by difference spectra of reduced-minus-oxidized membranes by the method of Weber and Broadbent (34). These same cytochromes have been reported in spores of *B.*

subtilis (34) and in *B. megaterium* KM inner spore membranes (36).

In addition to cytochromes, our spore membranes contained a carotenoid with an absorption maximum at 484 nm and shoulders at ~460 and 512 nm (Fig. 3). By extraction in chloroform-methanol and chromatography on silica gel plates with two solvent systems (see above), there appeared to be only one species of carotenoid. Although the carotenoid has not been further characterized, it could be similar to the carotenoid previously reported in *B. megaterium* KM inner membranes (22) and may be a useful marker for that membrane. There was no yellow pigment which has been reported for spore integument fractions (22).

Alternate procedures. Other methods of spore disruption were tested, and the properties of the membranes were compared with membranes prepared by method A. First, method B employed the addition of 10 mM EDTA after spore lysis. These membranes contained no RNA, DNA, or peptidoglycan and had phospholipid-to-protein ratios ranging from 0.43 to 0.47. The higher ratios (compared with method A) were due to a threefold loss in membrane protein, whereas phospholipid content was reduced by about one-half. The reduced protein content was also reflected in the 80 to 90% reduction in both NADH oxidase and dehydrogenase activities which could not be restored with Ca^{2+}, Mg^{2+}, or Mn^{2+}. Also, there was an alteration and loss in several high-molecular-weight protein peaks as determined by electrophoresis. However, membranes made by either method A or method B were similar in density, phospholipid distribution, cytochromes, and carotenoid content. Concentrations of EDTA lower than 10 mM were ineffective in reducing the RNA content of membranes.

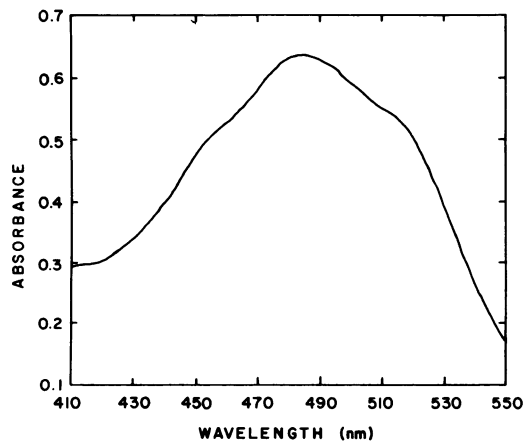

FIG. 3. *Spectrum of spore membrane carotenoid. The carotenoid was extracted from membranes of heat-activated spores prepared by method A as described in the text. A visible absorption spectrum was recorded by using $CHCl_3$ as the solvent in the sample and reference compartments.*

A second procedure was method C, which employed the French pressure cell rather than sonication. These membranes were similar to those prepared by method A in protein recovery, density, and protein profile by electrophoresis. However, all the enzyme specific activities were 50 to 90% less than those in membranes made by method A.

Finally, we also disrupted nonextracted spores with direct sonication (32), with a colloid minimill (33), or with a Wig-L-Bug (19), and in each case, no membranes could be recovered by centrifugation.

DISCUSSION

The isolation of spore membranes presents special problems because often the harsh treatments that are necessary to disrupt spores also damage membranes. The method that we devised appeared to be gentle and resulted in a membrane preparation with the expected membrane-associated enzyme activities, no soluble enzymes, a phospholipid content that appears representative of the total spore phospholipid content, a unique carotenoid, and the expected cytochromes. This method was used for either dormant or heat-activated spores, and the results were essentially the same. In our hands, most other mechanical methods of spore disruption resulted in recoveries of membranes and enzyme activities that were below those found in membranes prepared by method A.

Membranes prepared by method A were free of peptidoglycan and DNA but not RNA. In the few cases where membrane purity has been examined, RNA has been found as a common contaminant (15). Treatment with 10 mM EDTA (method B) completely eliminated the RNA but also caused other alterations leading to losses in protein and enzyme activities. Therefore, this second method may not be optimal for biologically active membranes. Preliminary experiments with membranes prepared by method B with fluorescence depolarization and electron spin resonance probes suggest that those membranes do not have the same properties as membranes prepared by method A.

In this work, it should be noted that we compared the total phospholipid content from intact spores with that of SDS-DTT-extracted spores and found them to be similar. This might at first seem surprising because the SDS-DTT treatment disrupts spore coats and would presumably

remove the outer membrane, which one would expect to contain phospholipids. However, there appears to be some confusion as to whether the outer membrane, which is morphologically distinct during sporulation, is, in fact, an intact membrane in mature spores. From electron micrographs of mature spores, several species of spores have been reported to have an indistinguishable or ill-defined membrane (1, 14, 18, 25; our unpublished data). In addition, electron micrographs of SDS-DTT-extracted spores of *B. megaterium* QM B1551 show no discernible outer membrane (P. S. Vary and J. C. Vary, manuscript in preparation). We also analyzed the material extracted by the SDS-DTT treatment and found a large amount of neutral lipid and a yellow pigment, but essentially no phospholipids (unpublished data). Thus, the morphologically distinct outer membrane seen during sporulation may not be a typical membrane in the mature spore and could be composed of remnants of that membrane needed for sporulation.

It has been reported that inner and outer membranes of mature *B. megaterium* KM spores could be isolated by differential centrifugation (10; P. C. Fitz-James, personal communication). The two fractions were not identical in their difference spectra or in patterns on polyacrylamide gels of the proteins (10), and 80% of the phospholipids were in the membrane fraction, whereas the integument fraction contained 20% of the phospholipids and most of the neutral lipid (22). By our methods, little integument fraction was detected as defined by Guinand et al. (Fig. 4 in reference 17). This result is not surprising since the spores were first extracted with SDS-DTT, which disrupts the spore coats and should remove most remnants of outer membrane. Therefore, the methods described in this report are most useful for isolating spore inner membranes.

There are several reasons why it is important to isolate spore inner membranes. Spores treated with SDS-DTT, which have little or no outer membrane, still can be triggered for germination by the same compounds that are effective on nonextracted spores (32). Therefore, it is unlikely that components extracted by the SDS-DTT treatment are critical components of the trigger mechanism. Also, recent studies have shown that spore membranes isolated by method A can respond in a stereospecific manner to L-proline when studied with an electron spin resonance probe, 5-doxyl stearate (20). Finally, an investigation of the L-proline trigger site suggests that it may be located in the inner membranes (27). Further studies are under way to probe the function of spore membranes and their possible role in triggering germination.

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