NOVEL PROTEIN COMPOSITION OF A BACTERIAL MEMBRANE

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Summary: The ghosts of a Bacillus sp. (Bacillus PP) isolated from a culture of Bacillus megaterium KM were dissociated by treatment with urea-sodium deoxycholate and fractionated by gel filtration. Examination of the protein composition of the unfractionated and the fractionated membrane components by disc gel electrophoresis under dissociating conditions revealed the presence of a protein band of MW 32,000 that comprised at least 90% of the total protein of the ghosts. An electrophoretically identical protein was found to be a relatively minor component of the ghosts of Bacillus megaterium KM.

Introduction: Recent studies in this laboratory have been concerned with the nature of the interactions of the enzymes of phospholipid synthesis with the other components of the cell membrane of a species of Bacillus. Earlier it was reported that the three enzymes that catalyze the conversion of phosphatidic acid to phosphatidylethanolamine, as well as their lipid products, were exclusively associated with the cytoplasmic membrane (1). A similar association has been demonstrated for the enzymes that catalyze the synthesis of phosphatidylglycerol (2).

In connection with this investigation, we have had occasion to study the fractionation of the components of ghosts of this organism by means of gel filtration and disc gel electrophoresis. The results of these studies are the subject of this communication.

Materials and Methods: Bacillus PP was isolated as a single clone without selection from a culture of B. megaterium KM. Whether or not it is a spontaneous mutant of B. megaterium KM remains to be established (see "Discussion") The organism was grown in the medium of Fildes (3) to the onset of stationary

phase (about 17 hrs), harvested, and washed in 50 mM Na phosphate buffer, pH 7.0 To prepare ghosts, the cells (10 g wet weight per liter of medium) were suspended in 40 vol. of sucrose solution (250 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$ in Na phosphate buffer, 50 mM, pH 7.0). Lysozyme (Worthington) was added to a final concentration of 6.25 mg/ml. The cell suspension was gently stirred for 60 min at 23-26° and then centrifuged at 10,000 x g for 15 min at 0°. The pellets were washed twice with 40 vol. of 5 mM $MgCl_2$, then suspended in 20 vol. of 50 mM Na phosphate buffer, pH 7.0, containing 5 mM $MgCl_2$ (PO_4-Mg^{++} buffer) and treated with 10 μg each of RNase and DNase per ml of ghost suspension at 0° for 30 min. Ghosts were collected by centrifugation and washed twice with PO_4-Mg^{++} buffer. The final pellet was suspended in 6 ml of PO_4-Mg^{++} buffer per 10 g of cells, and stored at 0°. Ghosts prepared by this procedure contained less than 1% of two cytoplasmic enzymes and two cell wall components found in intact cells (2). Ghosts of Micrococcus lysodeikticus (ATCC 13632), Bacillus subtilis 168, and B. megaterium KM (ATCC 13632) were prepared in an identical manner.

In some cases ghosts were further purified by centrifugation for 3 hrs at 20,000 rpm (Spinco SW25 Rotor) through a gradient consisting of 8 ml of 80%, 8 ml of 60%, and 8 ml of 20% sucrose (w/v) in 25 mM Tris, pH 9.0. The ghosts were found at the 20-60% interface (1). The ghosts were dialyzed against PO_4-Mg^{++} buffer, concentrated by placing the dialysis bags in Aquacide II (Calbiochem), and again dialyzed against PO_4-Mg^{++} buffer.

Gel filtration was performed at 4° with a column of Sepharose 2B (Pharmacia) (3.7 x 25 cm) equilibrated in urea-sodium deoxycholate (DOC) solvent (7 M urea, 0.15% DOC in PO_4-Mg^{++} buffer). To 2 ml of the ghost suspension (50 mg protein in PO_4-Mg^{++} buffer) 1.25 g urea and 4.5 mg DOC were added. The mixture was stirred at 0° for 90 min, and then applied to the Sepharose column and eluted with the urea-DOC solvent at a flow rate of 10-12 ml per hr. Fractions of 3 ml were collected and analyzed for protein by the method of Lowry (4) and for lipid phosphate after extraction with $CHCl_3$-CH_3OH (5) by the

method of Bartlett (6). The fractions were pooled as described in "Results".

The preparation of SDS gels and samples for the gels followed the method described by Weber and Osborn (7). For the urea-acetic acid system, the procedure of Takayama, et al. (8) was followed. Samples applied to the gels contained 10-200 μg protein. The gels were scanned at 550 mμ with a Gilford gel scanner at a speed of 1 cm/min.

Results: Upon elution of urea-DOC treated ghosts from the Sepharose column (Fig. 1), two protein peaks were obtained, a small one at the void volume (fraction A) and a large, broad peak of proteins included in the column (fractions B-E). It is noteworthy that the membrane phospholipids are largely localized in the excluded peak A, although there is some overlap into the succeeding fractions.

As a matter of convenience, the ghost preparations utilized in this experiment were not purified by sucrose gradient centrifugation. Sucrose gradient analysis of these ghosts showed that these preparations contain a significant amount of non-membranous protein (1), which subsequently has been shown to be lysozyme by disc gel electrophoresis (see below). Sepharose fractionation of ghosts purified by sucrose gradient centrifugation revealed a similar protein profile except the broad included peak was reduced to the extent that it represented only two-thirds of the total protein in the profile.

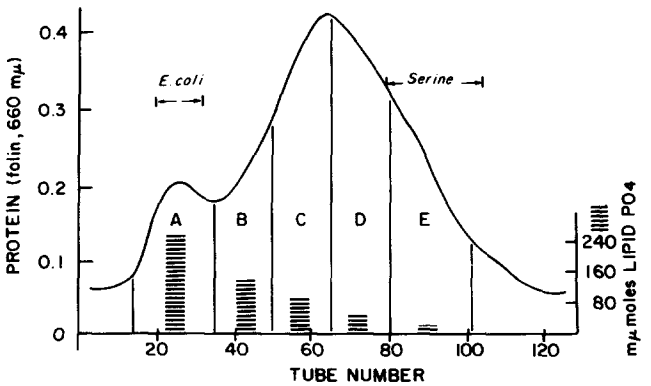

Fig. 1. Gel filtration of urea-DOC treated ghosts of Bacillus PP. The elution positions for V_o and V_i were determined with E. coli and serine, respectively.

Membrane preparations were analyzed for their protein composition by use of the two disc gel electrophoresis systems commonly employed for this purpose with membranous materials. The protein profile of the ghosts of Bacillus PP is shown in Fig. 2. An unexpected result was obtained using both types of gels; virtually all the protein of the ghosts migrates as a single band. Quantitation of the scans shows that this band, excluding contaminating lysozyme, comprises 90-95% of the protein in the gels. When the sensitivity of the scanner was increased five-fold, (cf. Fig. 3a) minor, yet reproducible amounts of other protein species were apparent. Identification of the non-membranous protein contaminant present in the ghost preparations as lysozyme

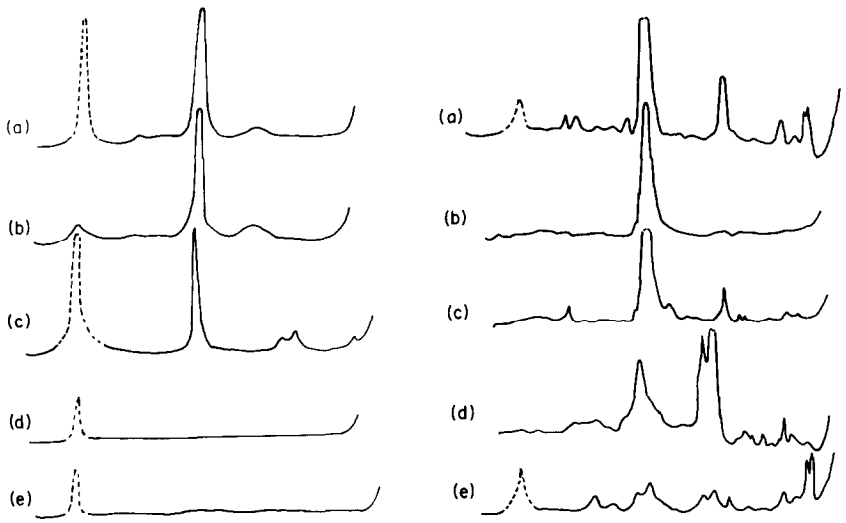

Fig. 2 (left). Disc gel electrophoresis of Bacillus PP ghosts. Ghosts are shown in (a) and (c), sucrose-gradient purified ghosts in (b), and lysozyme in (d) and (e). Electrophoreses (a), (b), and (d) were performed in acetic acid-urea gels and (c) and (e) in SDS gels. In all figures, the top of the gel is to the right and the dashed line refers to lysozyme. With SDS gels the anode is to the left and with urea-acetic acid gels the anode is to the right.
Fig. 3 (right). Disc gel electrophoresis of Sepharose column fractions of sucrose gradient purified Bacillus PP ghosts. The unfractionated ghosts are shown in (a) with the sensitivity of the gel scanner increased 5-fold, and column fractions A, B, C, and E are shown in (b), (c), (d), and (e), respectively. All gels were of the acetic acid-urea type.

is shown in Figs. 2d and e. The results of disc gel electrophoretic analysis of the column fractions A through E obtained after Sepharose chromatography of purified ghosts are shown in Fig. 3. It is seen that the major band of the unfractionated ghosts is predominant in all of the column fractions. A molecular weight for the major protein component (presumably in its monomeric form) was determined by the SDS-gel technique and was found to be 32,000.

The protein patterns obtained with Bacillus PP are quite different from those reported by other investigators examining other types of membranes with these electrophoretic systems. To confirm the efficacy of these disc-gel methods for use in analysis of membrane proteins, the membranes of several other bacterial species were examined. The gel scans obtained with ghosts of B. subtilis, M. lysodeikticus, and B. megaterium KM are shown in Fig. 4a-c. A multitude of bands were obtained in each case. Most pertinent, however, was the comparison between Bacillus PP and Bacillus megaterium KM, from which the former strain presumably is derived. The band profile of all the proteins in the two strains of Bacillus are virtually identical (Fig. 4c and d), the only apparent difference being a quantitative one in the single major band in strain PP. Fig. 4e is a scan of Bacillus PP membranes in which one-tenth as much protein was applied to the gel, demonstrating that virtually all of the membrane protein can move into the gel. Fig. 4f shows that the protein pattern of Bacillus PP is unaffected by prior removal of lipid by extraction.

Since the only apparent difference in the protein composition of the ghosts of Bacillus PP and B. megaterium KM was in the major band of the former, it was of interest to determine the ghost protein distribution of B. megaterium KM upon urea-DOC treatment and Sepharose column chromatography. The protein profile observed was similar to that found with Bacillus PP except that the excluded protein peak was reduced with respect to the broad included peak. Scans of the disc gel electrophoretograms of the column fractions are shown in Fig. 5. Interestingly, even though the major ghost protein band in Bacillus PP is only a minor one in B. megaterium KM, it is the only protein band in the

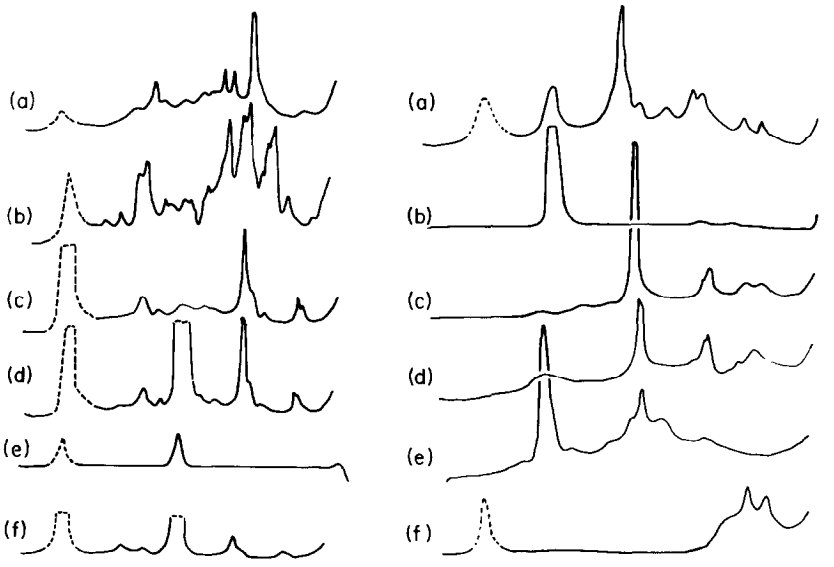

Fig. 4 (left). Urea-acetic acid disc gel electrophoresis of bacterial ghosts. Ghosts of B. subtilis, M. lysodeikticus, B. megaterium KM, and Bacillus PP are shown in (a)-(d), respectively. Scans of gel with one-tenth as much Bacillus PP membrane protein applied, and Bacillus PP ghosts which were delipidated prior to electrophoresis are shown in (e) and (f), respectively.
Fig. 5 (right). Urea-acetic acid disc gel patterns of column fractions of sucrose gradient purified B. megaterium KM. Untreated ghosts are shown in (a), and column fractions A-E are shown in (b-f).

excluded protein peak (Fig. 5b), just as it was with Bacillus PP. The other fractions (Fig. 5c-f) contain a variety of included proteins.

Discussion: The employment of SDS or urea as solvents for gel electrophoresis has enabled many investigators to demonstrate heterogeneity in the protein composition of bacterial membranes (9-14). The results reported here with B. megaterium KM, M. lysodeikticus, and B. subtilis are in accordance with such findings. In view of these studies, the demonstration of a single, low molecular weight protein band comprising approximately 90% of the ghost protein of Bacillus PP is of considerable interest. Chemical studies concerning the homogeneity of this protein are in progress.

The specificity of the membrane dissolution and gel filtration procedures employed in this study for fractionation of membrane components is indicated

by: (a) the asymmetric distribution of the phospholipid in the column fractions, and (b) the multiplicity of different proteins in the various column fractions, and (c) the finding that several of the phospholipid biosynthetic enzyme activities recovered in high yield (>80%) are found primarily in fraction A (presumably as minor protein components) (2). This last finding is of interest since fraction A also contains most of the products of these enzymes, namely the phospholipids.

Also of significance is the protein composition of the column fractions obtained with B. megaterium KM ghosts (Fig. 5). Here, just as with Bacillus PP, fraction A is composed of only one major protein band even though this band is only a relatively minor component of the total ghost protein. The protein band of fraction A is electrophoretically identical in these two bacteria (2). It should also be noted that with B. megaterium KM, the band in fraction A also appears in fraction D. The latter fraction is the approximate location in the elution profile for a protein of 25-35,000 molecular weight. The appearance of this protein band in the void volume (fraction A) may be a result of aggregation of 32,000 molecular weight monomers with themselves and/or with the membrane lipid. This tendency toward aggregation is seen even more clearly in the case of Bacillus PP (Fig. 3) where all the column fractions were found to contain the 32,000 MW protein, presumably in various degrees of aggregation.

Our hypothesis at present is that Bacillus PP is a mutant strain of B. megaterium KM that overproduces this protein. The following observations support this hypothesis: (I) Bacillus PP has the typical morphology and large size of B. megaterium KM. (II) The protein composition of the ghosts of the two organisms appear to be identical except for a quantitative difference in the amount of the fraction A protein band. (III) The cytoplasmic protein band profiles obtained by disc gel electrophoresis appear to be identical for the two organisms (2). Experiments to test this hypothesis and to determine the nature of the interaction of the 32,000 MW protein with the membranes of the ghost preparation are in progress.

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