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Association of Penicillin-Binding Proteins and Other Enzymes with the Ribosome-Free Membrane Fraction of *Bacillus subtilis*

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We had previously separated the ribosome-complexed and -free membrane fractions of *Bacillus subtilis* by sedimentation in a biphasic sucrose gradient. We now have found that the complexed fraction is contaminated with ribosome-free vesicles and that these can be removed by equilibrium density centrifugation. With this improved preparation, it could be shown that the penicillin-binding proteins are present almost exclusively in the ribosome-free membrane fraction. It thus appears that the fragmentation of the membrane in the lysing protoplast yields separate vesicles for the domains involved in protein translocation and for those involved in the synthesis and reshaping of the peptidoglycan. An enzyme of lipid synthesis (phosphatidylserine synthase) and also H^+-ATPase were similarly found to be concentrated, but less exclusively, in the ribosome-free membrane fraction.

Studies in this laboratory (5, 10) have shown that the membrane of *Bacillus subtilis* can be separated into a fraction complexed with ribosomes (CM) and a fraction free of ribosomes (FM). Since the membranes of the rough and the smooth endoplasmic reticulum in animal cells differ in only two of their major bands (8), we had originally expected that the two bacterial fractions might differ only in a few proteins involved in protein secretion. However, a large number of proteins are unique to CM (six major bands [10], and also several additional differences, as shown by two-dimensional electrophoresis); and, so far, we have been able to link only one of these CM proteins, the 64-kilodalton (kd) protein, to protein secretion, since it is closely covered by ribosomes (6). Moreover, FM also contains unique proteins, including two major bands (10). It therefore appears that the membrane domains separated by our fractionation differ in additional functions besides secretion. It seemed important to verify this conclusion, since the separation of the membrane fractions with different functions should be useful in identifying the functional units and also in determining the topographical relations between these units.

As a first test for the separation of additional functions, we observed the content of various penicillin-binding proteins (PBPs) in the ribosome-complexed and -free membrane fractions. Five PBPs, two of which can be further resolved, have been reported for *B. subtilis* Porton (7). These enzymes catalyze the cross-linking reaction in the formation and reshaping of the peptidoglycan, and they can be conveniently identified by their formation of stable covalent derivatives with [^{14}C]penicillin. We also assayed the membrane fractions for two other functions, phosphatidylserine synthase and H^+-ATPase.

MATERIALS AND METHODS

Bacterial strains and growth media. We used a *B. subtilis* strain, ATCC 6051a, whose membrane has been under investigation in this laboratory. Cells were grown at 37°C with shaking in minimal medium A (4) supplemented with 0.4% glucose and 0.2% Casamino Acids.

Membrane preparations. Membranes were prepared by a modification of the procedure previously described (10). Protoplasts in 20% sucrose in DTKM (1 mM dithiothreitol, 10 mM Tris-hydrochloride [pH 7.6], 50 mM KCl, 10 mM magnesium acetate), containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mM *o*-phenanthroline, were lysed by dilution with 2 volumes of a hypotonic buffer (as above, but without sucrose), followed by passage through a French press at 12,000 lb/in^2. Cell debris was removed by a 5-min spin at 10,000 rpm, and to concentrate the membrane, the supernatant was centrifuged over a 1-ml cushion of 2 M sucrose in TKM (37,000 rpm for 60 min; Beckman SW41 rotor). The material at and below the interface was collected and diluted with 1 volume of hypotonic buffer, overlaid on a triphasic sucrose gradient of 1.35, 1.6, and 2.0 M sucrose in TKM, and spun for 14 to 16 hr (37,000 rpm; SW41 rotor). Material banding below the sample layer, containing no RNA, was denoted as FM, material at 1.6 M was named CM-I, and material

at the 2.0 M interface was named CM-II. For further purification, these three bands were sometimes centrifuged in a second triphasic gradient, and the major component, banding at the same position as in the first gradient, was collected. Each sample was diluted with DTKM and pelleted by centrifugation (50,000 rpm for 60 min; Beckman 60 Ti rotor). The pellets were resuspended in a small volume of DTKM and stored at −76°C.

Equilibrium density gradient centrifugation. The three membrane fractions from a single centrifugation in a triphasic gradient were further fractionated by equilibrium density centrifugation. Samples were layered over a 1.3 to 2.2 M sucrose block gradient in TKM and centrifuged (37,000 rpm for >60 h; SW41 rotor). Membrane fractions of similar density were pooled from parallel tubes.

Penicillin-binding assay. Because ribosomes are present in CM-I and CM-II, but not in FM, the amounts of membrane that would contain equal amounts of nonribosomal proteins (i.e., those above 30 kd) were estimated by scanning gels stained with Coomassie blue. The total protein used in each assay (in units of absorbancy at 280 nm [A_{280}]; measured in 2% sodium dodecyl sulfate) was: FM, 0.1; CM-I, 0.35; and CM-II, 0.25. The samples, in 50 μl of TKM (with 1 mM dithiothreitol where noted), were incubated with 2 μCi of benzyl[^{14}C]penicillin (54 mCi/mmol; Amersham Corp.) for 15 min at 30°C. Gel sample buffer, containing 1% beta-mercaptoethanol, was then added, and the samples were boiled for 5 min and electrophoresed on an 8% polyacrylamide gel (9). The gel was treated with Autofluor (National Diagnostics), dried, and exposed to Kodak X-Omat AR film at −76°C.

Enzyme assays. Phosphatidylserine synthase was assayed by the method of Carman and Weiczorek (3), using CDP-dipalmitin (kindly provided by Eugene P. Kennedy) as the substrate and measuring the conversion of L-[3-^{3}H]serine (28 Ci/mmol; Amersham) to a chloroform-methanol-soluble product. One unit of activity catalyzes the formation of 1 fmol of product per min (assuming a counting efficiency of 40% for ^{3}H).

H^+-ATPase was assayed by the method of Nelson et al. (11), which follows the release of $^{32}P_i$ from [γ-^{32}P]ATP (2 mCi/mmol; New England Nuclear Corp.). One unit hydrolyzes 1 nmol of ATP per min at 30°C.

Reagents. All reagents were of reagent grade, obtained from commercial sources.

RESULTS

Distribution of PBPs. We initially prepared CM and FM from protoplasts of *B. subtilis* 6051a, after lysis in the French press, by centrifugation in a biphasic discontinuous sucrose gradient (1.35 and 1.8 M), as previously described (10). For assay of PBPs, an approximately equal quantity of each membrane fraction, in terms of membrane protein (see above), was incubated with an excess of benzyl[^{14}C]penicillin, and the product was analyzed by electrophoresis, followed by autofluorography. The five known PBPs of *B. subtilis* were all present in FM, in considerably higher concentrations than in CM (data not shown).

It seemed possible that CM was contaminated with FM, since earlier work had shown that when some CMs were sonicated they released material that could be separated as FM in the usual biphasic gradient (10). To try to eliminate such contamination, we modified the fractionation by providing an additional layer of 1.6 M sucrose in the gradient, thus yielding three fractions: FM banding beneath the sample layer, CM-I at 1.6 M, and CM-II at 2.0 M. When analyzed by electrophoresis, followed by staining with Coomassie blue, CM-I could be seen to be intermediate in protein composition between FM and CM-II (data not shown). CM-II, the band farthest from FM, proved to have very little PBP, and after further purification through a second triphasic gradient, it contained essentially none (Fig. 1A). However, CM-I, after purification in a second triphasic gradient, still contained PBPs (Fig. 1A).

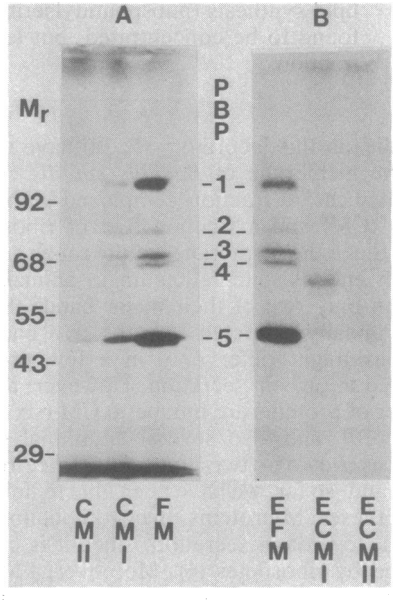

FIG. 1. PBPs of *B. subtilis* 6051a. Assay conditions were as outlined in the text. Approximately equivalent amounts of membrane protein from each fraction were reacted with an excess of benzyl[^{14}C]penicillin. (A) Membrane fractions recovered after two triphasic gradients; (1 mM) dithiothreitol was included in the incubation. (B) Membrane fractions recovered after one triphasic and one equilibrium density gradient; dithiothreitol was not present. Protein concentrations used (in A_{280} units) were: FM, 0.13; ECM-I, 0.54; and ECM-II, 0.56. After being boiled in sample buffer, samples were run on an 8% gel (9) and treated as described in the text. Molecular weights (in thousands) were taken from a straight line through the positions of the standards: phosphorylase *b*, 92; bovine serum albumin, 68; heavy chain of gamma globulins, 55; ovalbumin, 43; and carbonic anhydrase, 29.

Equilibrium density centrifugation. The persistence of PBPs in CM-I, after two fractionations, suggested that it contained hybrid vesicles, with both a ribosome-bearing and a PBP-containing domain. However, since our 16-h sedimentation in a discontinuous gradient separates on the basis of both size and density and since the vesicles differed in both, it seemed possible that this method of fractionation might not be separating free from complexed vesicles completely. We therefore further fractionated CM-I by equilibrium density centrifugation for 60 h on a 1.3 to 2.2 M sucrose gradient (see above). CM-I separated into an EFM (fraction free of ribosomes as judged by its A_{280}/A_{260} ratio) peak and several peaks of membrane complexed with ribosomes; the latter peaks were collected as ECM-I (the lighter fraction) and ECM-II (the denser fraction) (Fig. 2.). EFM contained 43%, ECM-I contained 27%, and ECM-II contained 30% of the total membrane protein, as estimated by Coomassie blue staining of proteins with molecular weights above 30,000.

PBPs were present, as expected, in EFM but were virtually absent from ECM-I and ECM-II (Fig. 1B). It thus appears that in the lysate the domains containing PBPs and those carrying ribosomes form distinct vesicles; there is not a significant number of hybrid vesicles.

To test the possibility that a CM might fail to react because its PBPs are inaccessible, we solubilized the proteins of each fraction of Fig. 1 without denaturation by adding 1% Triton X-100 before exposure to penicillin. The results were indistinguishable from those obtained without solubilization.

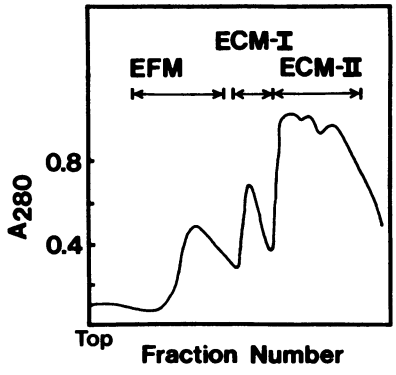

FIG. 2. Equilibrium density centrifugation of CM-I of *B. subtilis* 6051a. Fractions from the triphasic gradients were pooled, concentrated, and centrifuged for 60 h, as described in the text, and A_{280} was monitored on an ISCO fractionator. Fractions (16 drops) were collected, and those in the ranges designated EFM, ECM-I, and ECM-II were pooled and concentrated.

Further characterization of PBPs. The apparent molecular weights observed for PBPs of *B. subtilis* 6051a (102,000, 82,000, 73,000, 70,000, and 50,000) differed substantially from those reported for *B. subtilis* Porton by Kleppe and Strominger (7), using a 7.5% gel (122,000, 96,500, 88,000, 78,000, and 50,000). Moreover, Sowell and Buchanan (12) obtained intermediate values, which can be estimated from their Fig. 2. However, the differences are due to the analytical procedures and not to the strains; we obtained identical values for the two strains in our 8% gel. Moreover, with *B. subtilis* Porton, we also found PBPs only in FM (data not shown).

Since PBPs are unique to FM, it was of interest to determine whether they might account for the two major protein bands, with M_rs of 102,000 and 42,000, found earlier to be unique to that fraction (10). Staining of the gels of Fig. 1A with Coomassie blue identified PBP 1 with the band with an M_r of 102,000 (data not shown). PBP 5 also corresponds to, and may well account for, a heavy band in FM, but that band is not unique to FM (10); evidently a protein of similar M_r is also present in CM. The 42-kd unique major band of FM does not correspond to a PBP; its function is unknown.

Initially, CM-I and CM-II exhibited several additional PBP bands, mostly of lower M_r than those previously observed but also one at 60,000 (ECM-I; Fig. 1B). These varied in intensity in different experiments, and they were never as pronounced as PBP 1 and PBP 5. They appeared at first to be novel, minor PBPs associated with complexed membranes, but it now seems clear that they are due to interactions of labeled penicillin with ribosomal or non-PBP membrane proteins, in the absence of a sulfhydryl reagent. When dithiothreitol was present during the incubation, the additional bands were much fainter, whereas the PBPs in FM were not significantly reduced (Fig. 1A). Moreover, the addition of an excess of unlabeled benzylpenicillin before boiling in electrophoresis sample buffer further reduced these faint bands, suggesting the possibility of a nonenzymatic reaction.

One of the sulfhydryl-sensitive bands, of 60 kd, was quite pronounced (ECM-I; Fig 1B). It appears to be a ribosomal protein (presumably S1), since it was also obtained from free ribosomes incubated with radioactive penicillin in the absence of a sulfhydryl reagent (data not shown).

Distribution of other enzymes. We also examined the distribution of two other enzymatic activities of the membrane: phosphatidylserine synthase, as a component of lipid synthesis, and the H^+-ATPase, involved in oxidative phosphorylation. These activities also were found predominantly in FM (>50%; Table 1). Howev-

TABLE 1. Distribution of membrane functions between FM and CM

Membrane fraction[a]	PBPs[b]	Enzyme activity (U)[c]	
		Phosphatidyl-serine synthase	ATPase
FM	100	27.3	2.5
CM-I	18	16.0	1.5
CM-II	2	4.7	1.0

[a] Samples of the three fractions containing equal amounts of membrane protein (see text) were assayed.

[b] For quantitation of PBPs, the areas of the peaks obtained by scanning with the fluorogram were measured and expressed as values relative to FM (100%).

[c] Enzyme assays were as described in the text. Similar results were obtained with other membrane preparations.

er, unlike the PBPs, the concentrations of these enzymes in CM-II were significant. After equilibrium density centrifugation, phosphatidylserine synthase fractionated predominantly with FM, which contained >80% of the activity; H^+-ATPase was not assayed.

DISCUSSION

Earlier studies showed that in minicells of *B. subtilis* (1) and *Escherichia coli* (2) some PBPs were reduced in concentration as compared with normal cells, but none was absent. The present work reports the first sharp separation of PBPs between fractions of the bacterial cell membrane. PBPs of *B. subtilis* were found in FM but were absent from a complexed fraction (CM-II) which had been well separated from FM in a triphasic sucrose gradient.

The membrane fraction isolated at an interface intermediate in density (CM-I) was intermediate in protein composition between FM and CM-II, and it also contained PBPs. However, prolonged centrifugation to density equilibrium (60 h) separated CM-I into free and complexed vesicles, and these vesicles also exhibited PBPs almost exclusively in the free fraction (Fig. 1B). It thus appears that in the usual 16-h centrifugation in a discontinuous gradient, the differences in size of the vesicles prevent complete separation on the basis of density. The cleaner separation by equilibrium density centrifugation showed that essentially all of the vesicles carrying ribosomes are free of the PBP-bearing domains.

Two other membrane enzymes, phosphatidylserine synthase and H^+-ATPase, were also concentrated in FM, but they were less sharply separated from CM (Table 1). It is not surprising that in the fragmentation of the membrane during lysis, along presumably arbitrary lines, the ribosome-bearing domains should be separated more completely from some than from other nonribosomal domains.

It is striking, and certainly could not have been predicted, that the fragmentation of the membrane in the lysing protoplasts sharply separates the domains involved in protein translocation from those involved in peptidoglycan metabolism. One possible explanation is that these two major sets of domains are distributed as a mosaic in much or all of the cell membrane but that the vesicles formed on lysis are small enough so that they rarely overlap the domains. An alternative possibility is that the domains carrying the PBPs are located in the zones of septum formation and reshaping and that protein secretion might be restricted to regions away from these areas. However, the advantage of such a distribution would not be obvious for proteins being translocated into, rather than through, the membrane. It is interesting that PBP 5 is synthesized predominantly on membrane-bound ribosomes (S. Ahlers, M. P. Caulfield, P. C. Tai, and B. D. Davis, manuscript in preparation); hence, it is inserted in CM and must subsequently migrate to its destination in FM.

The presence of two major protein bands unique to FM had already strongly suggested that FM and CM differ in additional functions besides protein translocation (5, 10). The present findings demonstrate this conclusion directly. One of the unique FM bands, of 102 kd, appears to correspond to PBP 1, but the other, of 42 kd, is not a PBP. Evidently, unknown major functions are separated sharply between CM and FM.

The effectiveness of fractionation based on complexing with ribosomes suggests that other methods may be able to separate vesicles carrying various functions even more cleanly, perhaps by the use of antibodies or other ligands interacting with specific surface components.

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