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MEMBRANE ASSOCIATED PHOSPHOLIPOPROTEINS OF *BACILLUS LICHENIFORMIS* 749

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SUMMARY

The membrane-bound penicillinase of *Bacillus licheniformis* 749/C is a phospholipoprotein that differs from the hydrophilic exoenzyme in that its polypeptide chain carries an additional 25 residues (mostly hydrophilic) with phosphatidylserine as the NH_2-terminus. To determine if other phospholipoproteins are present in the plasma membrane, the penicillinase-inducible strain 749 was grown without inducer in the presence of [2-^{3}H] glycerol. Electrophoretic separation of the membrane proteins (after removel of free lipids) showed an association of ^{3}H-activity with certain of the proteins which could not be broken by lipid solvents and strongly denaturing conditions. Pronase digestion of the membrane proteins (after solvent extraction) released phosphatidylserine, thus indicating the covalent linkage of protein and phospholipid. Treatment of the isolated membranes with trypsin solubilized the protein portion of some of the phospholipoproteins (as with penicillinase), but not the ^{3}H-labelled fragment. Penicillinase should be considered as the first observed example of a group of phosphatidylserine-containing proteins present in the plasma membrane of *B. licheniformis* 749 and 749/C.

INTRODUCTION

In the course of studies on the mechanism of penicillinase secretion in *Bacillus licheniformis* 749/C, the lipophilic character of the membrane-bound penicillinase has been documented [1–3]. The enzyme is a phospholipoprotein [4] which differs from the exoenzyme in that its polypeptide chain carries an additional 25 residues (only aspartic acid or asparagine, glutamic acid or glutamine, Gly and Ser) with phosphatidylserine at the NH_2-terminus [4]. This polypeptide segment has a conformation susceptible to proteolytic cleavage, which liberates the exo-form of the penicillinase. The properties of the membrane-bound penicillinase and its possible involvement in formation of the exopenicillinase have been described [4–8].

We have undertaken to determine whether other phospholipoproteins with penicillinase-like lipophilic properties are present in the cell membrane. The penicil-

linase-inducible strain, *B. licheniformis* 749, was grown without the inducer in order to avoid interference by the large amount of penicillinase phospholipoprotein produced by the constitutive mutant 749/C. The occurrence of proteins containing covalently-linked phosphatidylserine would justify an investigation of their potential role in protein secretion in *B. licheniformis*, since the phospholipoprotein form of penicillinase is an intermediate in the formation and secretion of that exoenzyme [5].

MATERIALS AND METHODS

Organism and growth conditions

B. licheniformis 749, a penicillinase-inducible strain, was maintained on slants of Andrade's agar [9, 10]. Inocula were grown overnight in pH 6.5 casein hydrolysate-salts medium (CH/S) [10] with 0.2% glucose. 10 ml of inoculum was added to 200 ml of the same medium in 1 l shake flasks and incubated at 32 °C. After 1 h (cell dry wt. equivalent 0.2 to 0.3 mg/ml), 150 μCi of [^{14}C]amino acids or 1.5 mCi of [2-^{3}H] glycerol, or both, were added. The organisms were harvested when growth had reached early stationary phase (dry w. equivalent 0.8 to 0.9 mg/ml). Cell density was measured turbidometrically with a Klett Summerson colorimeter and No. 54 filter.

Plasma membrane preparation. The organisms were harvested by centrifugation and washed with sodium phosphate buffer (0.05 M, pH 6.5). Cells resuspended in the same buffer with 5 mM $MgCl_2$ (PO_4/Mg buffer) were treated with 100 μg lysozyme and 5 μg pancreatic deoxyribonuclease per ml (Sigma Chem. Co, St. Louis, Mo.) for 30 min at 30 °C. At this time cell lysis was complete, as judged by phase contrast microscopy. The membrane fragments were collected by centrifugation at 39 000 $\times g$ for 2 h and were washed repeatedly with PO_4/Mg buffer.

Membrane solubilization and removal of lipids

The membrane fragments in PO_4/Mg buffer were completely solubilized by the addition of sodium dodecyl sulfate to 2% and incubation at 37 °C for 30 min with constant swirling. The free lipids were separated by chloroform/methanol buffer treatment (monophasic extraction and biphasic separation) according to the method of Bligh and Dyer [11]. The monophasic chloroform/methanol buffer suspension was incubated in a closed flask for 1 h at 30 °C with constant swirling before the biphasic separation step. The organic and aqueous phases were separated, and the protein precipitate at the interface was collected by centrifugation. The interfacial protein precipitate was re-extracted with the solvents to insure complete removal of the free lipids.

Identification of phospholipids

Free phospholipids in the various fractions were identified by thin-layer chromatography using Silica Gel G plates (Brinkman Instruments, Westbury, N.Y.) developed with chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, by vol.) [12]. The plates were exposed to I_2 vapors for the detection of unsaturated fatty acids, or sprayed with 0.2% ninhydrin in acetone/lutidine (9 : 1, v/v) and heated to detect free amino groups. The identity of the phospholipid released by the Pronase digestion of the membrane proteins was also confirmed by the selective

alkaline hydrolysis of the fatty acyl esters [13, 14]. The deacylated, water-soluble phosphate ester was identified by descending paper chromatography using Whatman No. 1 paper developed with a phenol saturated solution of water/acetic acid/ethanol (100 : 10 : 12, by vol.). The paper was dried and sprayed with 0.2 % ninhydrin in acetone for the detection of free amino groups or with ammonium molybdate solution according to the method of Hanes and Isherwood [15] for the detection of phosphate esters.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Electrophoresis of the membrane proteins was conducted in 7 % polyacrylamide gels with 0.1 % dodecyl sulfate, using sodium phosphate buffer (0.2 M, pH 7.2) as the electrolyte. The running buffer was 0.01 M phosphate, pH 7.2 with bromophenol blue as the tracking dye. Protein samples were prepared by solubilization in 2 % dodecyl sulfate, 10 % 2-mercaptoethanol and 4 M urea, and heating for 5 min in boiling water. The samples (20 μg protein in 20 μl) were layered on the gels, and electrophoresis carried out at a constant current of 8 mA/tube for about 4 h at which time the tracking dye had reached the bottom of the gel. The gels were then soaked in 10 % trichloroacetic acid for 2 h and stained overnight for protein with 0.5 % Coomassie blue in methanol/acetic acid (5 : 1, v/v). The gels were destained electrophoretically, and the protein bands were scanned at 570 nm using a Schoeffel S-D 3000 spectrodensitometer.

Radiochemicals and assay of radioactivity

Uniformly labelled ^{14}C-amino acids mixture (200 Ci/mol) and [2-^{3}H]glycerol (200 Ci/mol) were purchased from New England Nuclear Corp., Boston, Mass. ^{3}H- and ^{14}C-activities in various membrane fractions were counted in Aquasol scintillation cocktail (New England Nuclear Corp.). Samples from the chloroform phase were taken to complete dryness under nitrogen before adding the scintillant.

For determination of the radioactivity in the protein bands from electrophoresis, the gels were cut into 1 mm sections using a lateral gel slicer. The slices were dissolved in 0.3 ml of 0.3 N NCS tissue solubilizer (Amersham/Searle, Arlington Hts. Ill) and 10 μl water overnight. The radioactivity was counted in 5 ml of Omnifluor toluene cocktail (New England Nuclear Corp.).

Protein determination. The protein content of the samples was determined by the alkaline ninhydrin procedure of Hirs [16].

RESULTS

Membrane fragments from *B. licheniformis* 749 cells grown in the presence of ^{14}C-labelled amino acids and [2-^{3}H]glycerol were solubilized in 2 % dodecyl sulfate and subjected to chloroform/methanol buffer extraction [11]. Aqueous and organic phases and the protein precipitate at the interface were separated. Thin-layer chromatography of the three fractions showed the presence of both I_2- and ninhydrin-positive lipids in the organic and aqueous phases; these migrated close to the solvent front. No free phosphatidylserine could be detected in any of the fractions. (The R_F of authentic ox brain phosphatidylserine (Koch-Light Labs., Ltd.) was 0.58.)

Table I summarizes the distribution of ^{3}H- and ^{14}C-activity in the fractions

TABLE I

DISTRIBUTION OF ^{14}C- AND ^{3}H-ACTIVITIES IN THREE FRACTIONS FROM LIPID EXTRACTION OF MEMBRANES

Membranes from cells grown in the presence of ^{14}C-labelled amino acids and [2-^{3}H]glycerol were solubilized in 2 % dodecyl sulfate and extracted with chloroform/methanol buffer as described in the text. (Figures in parentheses represent percentage of total radioactivity.)

	^{3}H-activity cpm · 10^{6}	^{14}C-activity cpm · 10^{6}
Membranes	18 (100)	19 (100)
Organic phase	7.7 (42)	2.1 (11)
Aqueous phase	5.3 (30)	1.7 (9)
Interfacial protein precipitate	0.9 (5)	13 (68)

from lipid extraction of the dodecyl sulfate-solubilized membranes. About 5 % of the total ^{3}H-activity incorporated into the membrane from [2-^{3}H]glycerol was associated with the precipitated proteins at the interface. In a similar experiment, in which the membranes were labelled only with [2-^{3}H]glycerol, 3.8 % of the ^{3}H-activity was in the protein fraction.

The insoluble protein precipitate from the interface was resuspended in Tris · HCl buffer (0.05 M, pH 8.0) with 5 mM $CaCl_2$ to a protein concentration of 1.8 mg/ml and digested with Pronase (E.C.3.4.21.4) (300 μg/ml; Calbiochem., La Jolla, Calif.) for 40 h at 37 °C. The digest was then subjected to chloroform/methanol buffer extraction by the procedure of Bligh and Dyer [11].

About 70 % of the ^{3}H-activity and 91 % of the ^{14}C-activity associated with the protein precipitate appeared in the aqueous (methanol buffer) phase after the Pronase

TABLE II

SHIFT IN DISTRIBUTION OF ^{3}H- AND ^{14}C-ACTIVITIES IN THE FRACTIONS FROM LIPID EXTRACTION AFTER PRONASE DIGESTION OF THE MEMBRANE PROTEINS

The labelled interfacial protein fraction from the membranes after the removal of free lipids was hydrolyzed with Pronase (protein/Pronase, 6 : 1, by wt.) for 40 h and again subjected to lipid extraction as described in the text. The control sample (without the Pronase) was similarly treated.

	Percentage of total radioactivity			
	^{3}H	^{14}C	^{3}H	^{14}C
Membrane protein fraction	100*	100**		
	Pronase hydrolyzed		Control	
Organic phase	1.0	0.53	0.23	0.3
Aqueous phase	70	91	4.8	4.6
Interfacial protein precipitate	19	14	85	83

* $3.5 \cdot 10^{5}$ cpm
** $1.4 \cdot 10^{6}$ cpm

TABLE III

THIN-LAYER CHROMATOGRAPHY OF THE FRACTIONS FROM LIPID EXTRACTION OF THE PRONASE-DIGESTED MEMBRANE PROTEINS

Pronase digestion, lipid extraction and thin-layer chromatography for phospholipids were conducted as described in the text. In a similarly prepared control without Pronase, no ninhydrin- or I_2-positive spots were produced. Probable identity of spots: 1, phosphatidylserine; 2, phospholipopeptides; 3, 4, amino acids and peptides.

Fraction	Spots detected		
	Ninhydrin (R_F)	I_2 (R_F)	^{3}H-cpm
Organic phase	0.55	0.56	300
Aqueous phase	(1) 0.56	0.57	737
	(2) 0.31	0.32	344
	(3) 0.21		
	(4) 0.11		
Interfacial protein precipitate	0	0	
Phosphatidylserine (standard)	0.58	0.58	

treatment (Table II), whereas in a control sample similarly incubated but without the Pronase, almost all of the radioactivity was retained in the interfacial precipitate. A small increase in the ^{3}H-activity of the organic phase following the Pronase digestion was also noted. Thin-layer chromatography of the three fractions from the Pronase-treated sample provided a tentative identification of ^{3}H-labelled phosphatidyl-serine in the organic and aqueous phases by comparison with the R_F of authentic phosphatidylserine (Table III). To confirm this identification, the phospholipid fraction was subjected to selective alkaline hydrolysis of the fatty acyl esters [13, 14]. The water soluble phosphate ester released upon deacylation was identified as glycerophosphorylserine (with ^{3}H-activity) by comparison with the R_F (0.32) of authentic glycerophosphorylserine (Supelco Inc., Bellefonte, Pa.) and positive tests for free amino groups and for phosphate esters. The other ^{3}H-labelled product released as a result of Pronase treatment could well be incompletely digested phospholipopeptides, but this material has not been characterized in detail. No free phosphatidylserine was detected in the three fractions from the sample not treated with Pronase. The preferential distribution of the phosphatidylserine into the methanol-water phase was unexpected, but control experiments with ox brain phosphatidylserine provided an explanation. Phosphatidylserine in PO_4-Mg^{2+} buffer (as used for Pronase digestion) was found predominantly in the aqueous phase following chloroform/methanol buffer extraction [11]. Phosphatidylserine will form a chelate complex with Mg^{2+} and this is probably sufficiently hydrophilic to be retained in the aqueous phase.

Electrophoresis of the membrane proteins. The precipitated proteins at the interface (after the chloroform/methanol extraction of the membranes for the removal of free lipids) were heated with dodecyl sulfate, 2-mercaptoethanol and urea to ensure complete denaturation, and then subjected to dodecyl sulfate-polyacrylamide gel electrophoresis. At least fifteen discrete protein bands with a wide range of

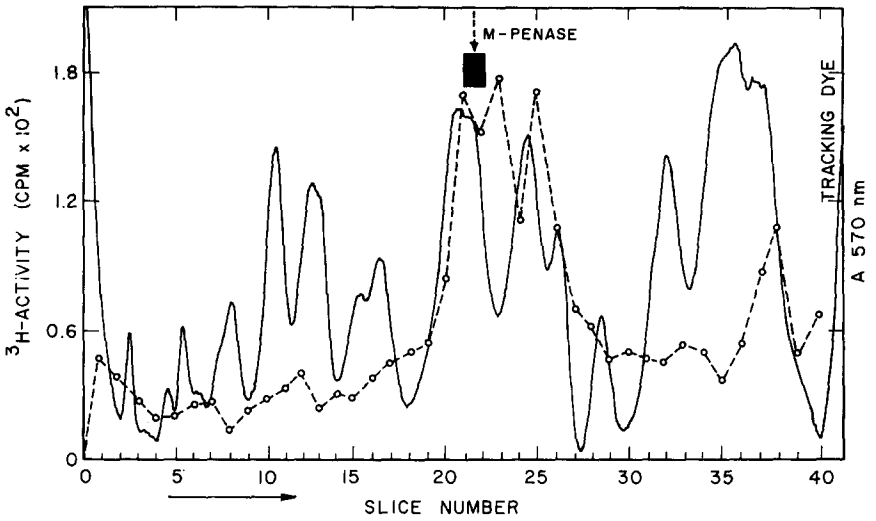

Fig. 1. Dodecyl sulfate polyacrylamide gel electrophoresis of the [2-^{3}H]glycerol-labelled membrane proteins. Membrane proteins were isolated after the removal of free lipids from the membrane and were electrophoresed in 7 % polyacrylamide gels with 0.1 % dodecyl sulfate as described in the text. Proteins were located on the gel (—) by staining with Coomassie blue and scanning for absorbance at 570 nm. ^{3}H-activity (○—○) in 1 mm slices from a replicate gel was measured in an Omnifluor-toluene cocktail. The solid arrow indicates the direction of electrophoresis, and the dotted arrow (M-Penase) the position of membrane penicillinase (M_r = 33 000).

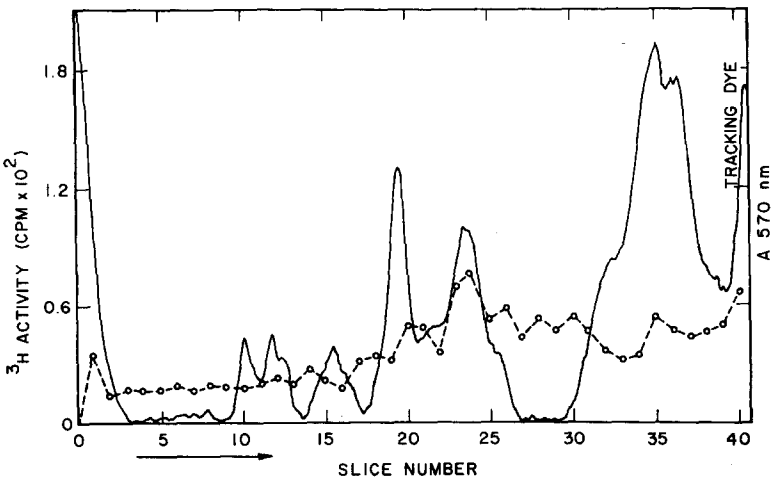

Fig. 2. Dodecyl sulfate polyacrylamide gel electrophoresis of the proteins isolated from membranes treated with trypsin. Electrophoresis and radioactivity measurements as described in Fig. 1. —, Coomassie blue stained proteins; ○- - -○, ^{3}H-activity.

molecular weights were separated. Several major and several minor bands of ^{3}H-labelled protein were detected (Fig. 1). The major bands were in the molecular weight range of 25 000—35 000 (by comparison with membrane penicillinase (M_r = 33 000 [3])).

Effect of trypsin treatment on the composition and properties of membrane proteins. Membranes prepared from *B. licheniformis* 749 cells grown in the presence of [2-^{3}H]glycerol were suspended in Tris · HCl buffer (0.01 M, pH 7.9) with 5 mM $MgCl_2$ and 20 mM $CaCl_2$ and incubated with trypsin [E.C.3.4.4.3] (50 μg/ml; Sigma Chem. Co.) for 2 h at 30 °C. Phenylmethylsulfonylfluoride (500 μg/ml; Sigma Chem. Co.) was then added to inactivate the trypsin. The solubilized proteins were removed by centrifugation at 39 000 × *g* for 2 h, and the pellets were washed thoroughly to remove the trypsin.

The trypsin-treated membrane pellet and a sample similarly incubated but without trypsin were dissolved in 2 % dodecyl sulfate and subjected to lipid extraction [11]. The interfacial precipitates were separated and their protein content and associated ^{3}H-activity were determined. The amount of protein obtained from the treated membranes was 300 μg/ml, whereas the control membranes yielded 800 μg/ml. Nevertheless, the total ^{3}H-activity of the two interfacial fractions was identical (151 000 cpm/ml). The proteins from the trypsin- treated membranes showed only about 7 discrete bands on electrophoresis instead of the 15 or more characteristic of the untreated membranes (Fig. 1), and ^{3}H-activity now appeared to be associated with only a few bands (Fig. 2). Since the recovery of ^{3}H-activity from the gels was low, the two interfacial fractions were subjected to electrophoresis for a shorter time (tracking dye migrated only two-thirds the gel length). Much of the ^{3}H-labelled material removed from the protein band by trypsin now migrated near to or ahead of

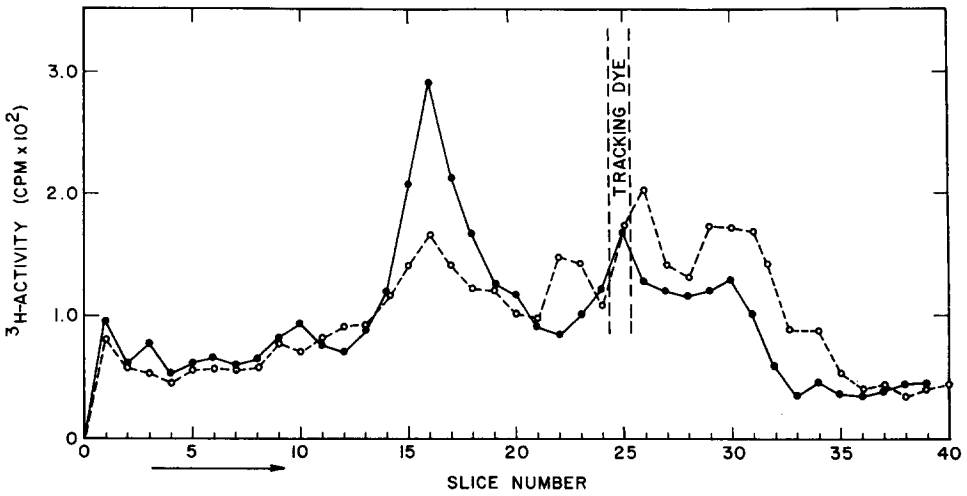

Fig. 3. Migration profile of ^{3}H-activity associated with the interfacial (protein) fraction from chloroform/methanol buffer extraction of membranes incubated without (control) and with trypsin Electrophoresis was stopped when the tracking dye had migrated about 2/3 of the gel length. Other experimental details as described in Fig. 1. ○—○, ^{3}H-activity from trypsin-treated membranes. ●—●, ^{3}H-activity from control membranes.

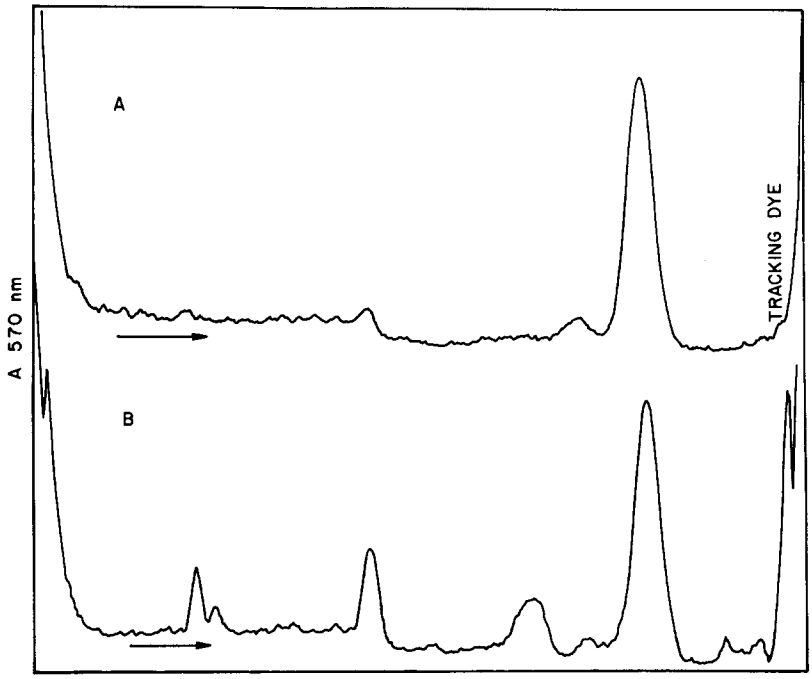

Fig. 4. Dodecyl sulfate polyacrylamide gel electrophoresis of the trypsin-solubilized membrane proteins. Electrophoresis and location of proteins in the gels as described in Fig. 1. (A) buffer solubilized proteins (control); (B) trypsin solubilized proteins.

the tracking dye (Fig. 3). A considerable amount of such low molecular weight, ^{3}H-labelled material was also present in the interfacial protein fraction before treatment with trypsin.

As already mentioned, trypsin solubilized more than 50 % of the protein from the membrane fragments. Electrophoresis of the released material showed at least 4 major protein bands (Fig. 4). None of these corresponded to trypsin which was present in a concentration too low to be detected. We have not attempted to relate the solubilized proteins to individual bands in the original membrane preparations.

DISCUSSION

Membrane proteins are usually associated with lipids through their exposed hydrophobic regions [17], an interaction which can be broken by treatment with lipid solvents. In contrast, some of the membrane proteins of *B. licheniformis* 749 grown in the presence of [2-^{3}H]glycerol, retained ^{3}H-activity even after chloroform/methanol extraction and severe denaturation procedures. This suggests a lipid-protein interaction stronger than the general hydrophobic type. After exhaustive digestion of the proteins by Pronase, the ^{3}H-activity could be separated from the membrane proteins and solubilized in chloroform/methanol buffer. Some of the solubilized ^{3}H-activity was associated with the phosphatidylserine which was released during this digestion of the membrane proteins.

The membrane lipids of *B. licheniformis* are mainly phospholipids. Morman and White [18] in their studies on phospholipid metabolism during penicillinase production in *B. licheniformis*, reported the active turnover of phosphatidylglycerol and cardiolipin in both uninduced and induced cells of strain 749 and in the constitutive penicillinase-forming mutant 749/C. The free membrane phospholipids were mainly phosphatidylethanolamine and phosphatidylglycerol with smaller amounts of cardiolipin, phosphatidic acid and lysolipids. No phosphatidylserine was detected (confirmed in our experiments). Consequently, the release of phosphatidylserine from the membrane proteins by exhaustive proteolytic hydrolysis, strongly indicates a covalent bonding of membrane protein and phospholipid through a phosphodiester linkage.

The ^{3}H-activity could not be separated from the proteins by heating in 4 M urea, 2 % dodecyl sulfate, and 10 % 2-mercaptoethanol at 100 °C for 5 min. Dodecyl sulfate polyacrylamide gel electrophoresis of these solubilized proteins showed about 15 discrete protein bands and ^{3}H-activity associated with several different protein species.

The membrane proteins precipitated after lipid extraction could also be solubilized in Tris · HCl buffer (0.05 M, pH 7.5) containing 2 % Triton X-100 and 2 M urea. However, under these milder denaturation conditions, individual protein species could not be separated by polyacrylamide gel electrophoresis [19]. The proteins thus solubilized remained on the top of the gel as a diffuse band as a result of the formation of large protein-Triton complexes (Aiyappa, P. S. and Lampen, J. O. unpublished data). This is similar to the behavior of the membrane penicillinase of strain 749/C which gives an apparent molecular weight of approximately 100 000 in 0.1 % Triton X-100 and 48 000 in taurodeoxycholate [20] compared with its actual 33 000 molecular weight [4]. The aggregation of the membrane-bound penicillinase is the result of complex formation between the detergents and the covalently linked phospholipid, as the hydrophilic exopenicillinase does not form an aggregate in the presence of taurodeoxycholate [1] or of Triton X-100 (Yamamoto, S. and Lampen, J. O., personal communication).

Trypsin solubilized at least 50 % of the membrane protein but essentially none of the ^{3}H-activity. Dodecyl sulfate polyacrylamide gel electrophoresis of the protein fraction from the trypsin-treated membranes showed fewer protein bands and ^{3}H-activity in only a few bands. Most of the ^{3}H-activity retained in the membrane migrated more rapidly than the tracking dye (Fig. 3) and appears to be associated with relatively small molecules. Such molecules were also present in a considerable amount in the membrane sample incubated in the absence of trypsin.

The phospholipoprotein form of penicillinase is located in the membrane in such an orientation that trypsin can cleave the peptide chain and release a hydrophilic enzyme differing from the native exopenicillinase only in that it lacks the NH_2-terminal lysine residue [3, 4, 20]. This exoform is not susceptible to further trypsin hydrolysis [1]. Trypsin digestion of membranes from strain 749 released four proteins which, like the released penicillinase, were resistant to complete hydrolysis (Fig. 4). Thus some of the four trypsin resistant proteins may be derivatives of the membrane phospholipoproteins.

The phospholipopeptide produced by trypsin cleavage of membrane penicillinase consists of 25 amino acid residues with phosphatidylserine as the NH_2-ter-

minus [4]. The smaller molecules with ^{3}H-activity remaining in treated membranes of strain 749 were not identified directly as phospholipopeptides. Nevertheless, the precipitation of the ^{3}H-activity at the interface after dodecyl sulfate-solubilization and lipid extraction of the trypsin-treated membranes, suggests the presence of a polypeptide attached to the phospholipid (phosphatidylserine).

This study demonstrates that several species of proteins in the membranes of *B. licheniformis* contain covalently-bound phosphatidylserine; consequently, membrane-bound penicillinase should be considered as the first example of a novel group of membrane macromolecules, the phospholipoproteins.

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