SOLUBILISATION OF A TEICHOIC ACID-SYNTHESISING SYSTEM FROM THE MEMBRANE OF *BACILLUS LICHENIFORMIS* BY FREEZING AND THAWING

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1. Introduction

Both electrostatic and non-polar interactions help to maintain the structure of biological membranes, and techniques designed to disrupt one or both of these interactions have been used to solubilise membrane-bound enzymes from animal and bacterial sources [1]. Repeated washings of bacterial membranes with buffers of low osmolarity in the absence of bivalent cations releaae a significant proportion of the membrane protein, notably ATPase [2], which is presumed to be electrostatically bound. The residual membrane is still vesicular and retains the respiratory complexes and the enzymes that catalyse the biosynthesis of cell-wall polymers. We report here that repeated freezing and thawing of this residual membrane prepared from *B. licheniformis* releases a non-sedimentable lipoprotein which is active in the biosynthesis of the wall teichoic acids poly(glycerol phosphate) (polyGP) and poly(glycerol phosphate glucose) (poly GPGlc), and contains the undecaprenol phosphate intermediate for the synthesis of the glucose-containing polymer.

2. Results

B. licheniformis A.T.C.C. 9945 was grown in batches to mid-logarithmic phase in complex medium containing 0.1% glucose [3]. Membranes were prepared by lysis of the cells with lysozyme in the absence of Mg^{2+} or osmotic stabiliser [4], and were washed once with 2 M KCl, twice with 0.025 M Tris–HCl, pH 8.0, twice with 0.15 M sodium pyrophosphate, pH 7.5, and twice more with 0.025 M Tris–HCl, pH 8.0. The final product was resuspended in 0.025 M Tris–HCl, pH 8.0, at a concentration of 20–25 mg of protein/ml and the suspension was rapidly frozen in an acetone–solid CO_2 bath. For optimal solubilisation of the teichoic acid synthesising system the frozen membrane suspension was held at −20° for 10 days before the next stage of the procedure.

Solubilisation was carried out as follows: the frozen membrane suspension was allowed to thaw slowly at room temperature and then transferred to a beaker the bottom of which it just covered. The suspension was frozen for 45 sec in acetone–solid CO_2 (−70°C), then thawed to 0°C in a water bath at 30°C. This process was repeated three more times and after the final thawing the suspension was centrifuged at 38 000 *g* for 30 min at 0°C. The pellet was resuspended in 0.025 M Tris–HCl, pH 8.0, at the original concentration and kept at −20°C (membrane residue, MR). The supernatant was treated with 1.5% (w/v) protamine sulphate (0.1 ml of protamine/ml of supernatant) at 0°C for 30 min and the mixture was then centrifuged at 120 000 *g* for 2 hr at 0°C. The supernatant contained the solubilised enzymes (crude soluble material, CS). From a membrane suspension at 25 mg of protein/ml, MR and CS contained 20.6 mg of protein/ml and 2.8 mg protein/ml respectively, measured by the Lowry method after solubilisation in 0.1 M NaOH, using bovine serum albumin as a standard [5].

Some of the enzymic activities of the original membrane, MR and CS are compared in table 1. The very low succinic dehydrogenase and NADH dehydrogenase levels in CS suggested that the respiratory enzyme

Table 1
Enzyme activities* of soluble fraction.

Enzyme	MR	CS	Assay Method
Alkaline phosphatase	80	8	[6]
Acid phosphatase	89	1.5	[6]
Succinate dehydrogenase	97.5	0	[7]
NADH dehydrogenase	81.5	17.5	[8]
poly GP synthetase	55	409	[4]
poly GPGlc synthetase	80	196	[4]

* Relative specific activities; activity in original membrane = 100.

complex was not solubilised by the freezing and thawing process. This conclusion was supported by the fact that no differences between the spectra of CS, before and after treatment with dithionite, could be detected, indicating the absence of cytochromes.

The crude soluble preparation (CS) could be fractionated on a column of Sephadex G-200 as shown in fig. 1. The elution profile of poly GPGlc synthesising activity was identical with that of poly GP synthesising activity, both being excluded from the column. Material (purified soluble, PS) corresponding to the void volume peak 1 was freeze-dried, redissolved in water and applied to a DEAE–cellulose column, from which it was eluted as a single peak in about 0.7 M NaCl on application of a linear gradient of 0–1.0 M NaCl. After chromatography on Sephadex G-200 the activity of the material (PS) could be approximately doubled by dialysis against 1 mM sodium EDTA in 0.05 M Tris, pH 8.0, for 16 hr at 4°C. The overall purification of poly GP synthetase at the end of this process was approximately 8-fold.

The purified material (PS) was lipoprotein in nature. Extraction of lipids by chloroform–methanol [9] removed 40% (w/w) of the material, while butanol extraction [3] indicated a lipid content of 48% (w/w). These compare with a figure of 45% for the original membrane, estimated by chloroform–methanol extrac-

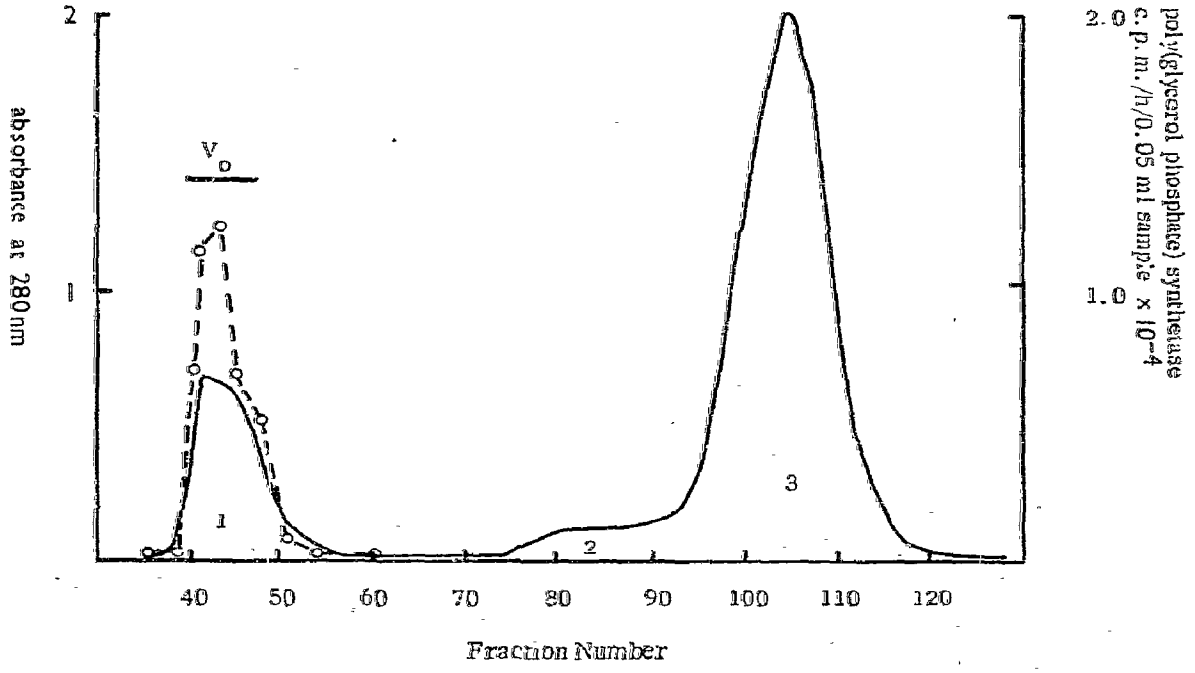

Fig. 1. Chromatography of CS on Sephadex G-200. CS (5 ml) was applied to a column (90 × 2.5 cm) of Sephadex G-200 (Pharmacia Ltd., Uppsala, Sweden) and was eluted in 0.025 M Tris–HCl, pH 8.0, containing 0.02% (w/v) sodium azide, by upward flow at 4°C; 4 ml fractions were collected and the absorbance at 280 nm was measured. Poly GP synthetase and poly GPGlc synthetase were measured [4] in 0.05 ml portions of the samples. The void volume of the column was measured using blue dextran. (•——•) Absorbance at 280 nm; (o——o) poly GP synthetase activity.

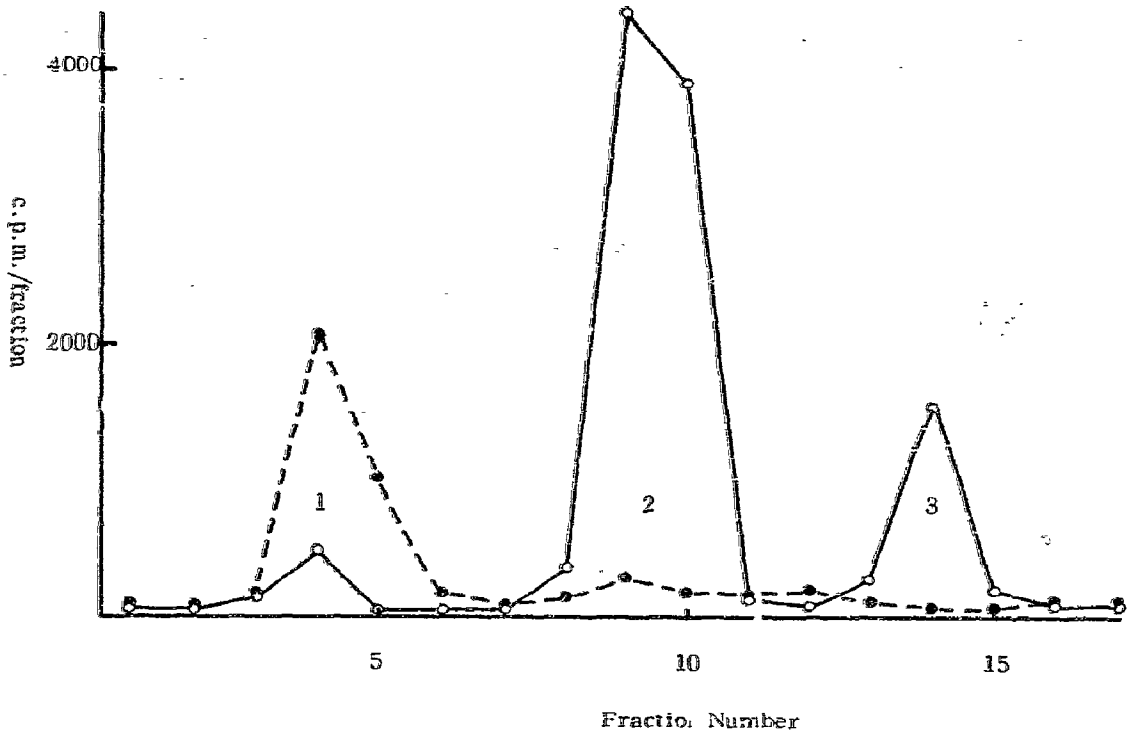

Fig. 2. TLC of labelled lipids synthesised from UDP-[U-^{14}C]glucose by membranes and CS. 0.5 ml of membrane suspension (30 mg dry wt/ml) or 3 ml of CS was incubated with 2 mM UDP-[U-^{14}C]glucose (1.5×10^6 cpm/μmol) and 10 mM $MgCl_2$ at 37°C for 30 min. The reaction mixtures were freeze-dried and lipids were extracted into 4 ml of butan-1-ol containing 0.5 ml of water for 8 hr at 4°C. The butanol phase was washed three times with 2 ml of water at 4°C, evaporated to dryness *in vacuo*, and the lipids were redissolved in a small volume of chloroform. The lipids were separated by chromatography on thin layers (0.4 mm) of silica C-1 H in chloroform–anhydrous methanol 80:25 (v/v). 1 cm Bands of gel were scraped off for scintillation counting [3]. (o—o—o) Whole membrane; (•—•—•) CS.

tion. These values may include proteins extracted as apolar proteolipid complexes. A preliminary comparison of the polar lipids of MR and PS by butanol extraction, 2-dimensional t.l.c. and densitometry [10] indicated that the soluble material contained significantly less acidic phospholipid (phosphatidyl glycerol and cardiolipin) and more amphoteric lipid (phosphatidyl ethanolamine) than the MR. Intact membranes of *B. licheniformis* synthesise three glucose-containing lipids from UDP-glucose and endogenous acceptor. Two of these are diglucosyl diglyceride (DG) and its precursor monoglucosyl diglyceride (MG); the third is a polyprenol phosphate glucose intermediate in the biosynthesis of poly GPGlc [3]. The identification of these lipids was described previously [3]. A comparison by thin-layer chromatography of the glucose lipids synthesised by membrane and CS is shown in fig. 2. CS only yielded lipid intermediate (component 1) while the membrane also gave DG (component 2) and MG (component 3).

3. Discussion

It is well known that freezing and thawing damages biological membranes, either through a mechanical effect of ice crystals or by the production of extremely high solute concentrations during freezing. Whatever may be the basic cause of disruption, repeated freezing and thawing appears to provide a new and selective tool for the dissection of the bacterial membranes. Of particular interest is the observation that the solubilised complex retains the integrity of the enzyme–lipid intermediate association which is essen-

tial for the biosynthesis of poly GPGlc. Methods of detergent solubilisation which have been used to study lipid-mediated processes have invariably disrupted such complexes [11, 12]. Our isolation of a multienzyme complex that retains its polyprenol phosphate in a functional association with the enzymes is consistent with the model of wall synthesis proposed by Anderson et al. [13] in which the enzyme systems that catalyse the synthesis of the different wall polymers are closely associated with a shared pool of undecaprenol phosphate in the membrane.

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