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IDENTIFICATION OF SURFACE PROTEINS OF A BACTERIAL MEMBRANE USING THIOLACTONE-ACTIVATED POLYACRYLAMIDE BEADS

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Summary

A new method for the determination of protein accessibility in membranes and membrane fractions using a resin, 'Enzacryl' polythiolactone, is described. Enzacryl polythiolactone is a hydrophilic polymer of acrylamide and acrylamide derivatives with thiolactone ring substituents. The binding of enzymes and proteins to this resin is accomplished very simply by mixing them together in a simple aqueous buffer. Groups which react with the polymer in the pH range 5—9 include aliphatic and phenolic hydroxyls and aliphatic amino groups. Surface proteins of *Bacillus licheniformis* membrane and solubilised membrane fractions are bound irreversibly to this resin. Inaccessible proteins remaining in the fractions are solubilised with sodium dodecyl sulphate and examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Introduction

The asymmetric nature of biological membranes has been studied for many years and several techniques have been developed to label membrane proteins possessing exposed reactive groups [1—5]. Most of these techniques use chemical labelling compounds and depend on the inability of the chemical reactants to diffuse through the membrane. Many of the chemical reagents are only impermeant to membranes within limits of pH and temperature. The reaction conditions have, therefore, to be carefully controlled if accurate results are to be obtained.

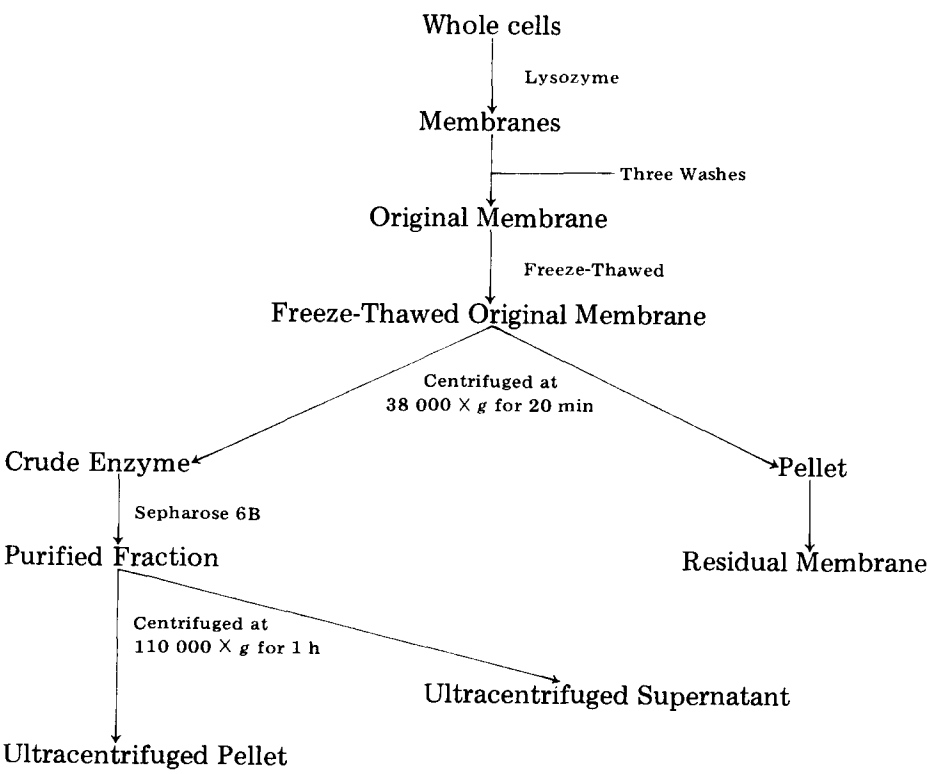
There is, presumably, less risk of labelling reagents penetrating the mem-

brane if the reactive groups are attached to a large polymer particle. Recently, surface and integral proteins have been studied by the use of activated resins and dextran which are too large to penetrate the membrane. Dextran can be activated with CNBr [6,7] and will then react with amino groups in proteins to form covalent links. Kamio and Nikaido [8] have used this method to compare surface proteins in the outer and inner membranes of *Escherichia coli*. They were able to correlate the proteins bound in whole cells with those known to be present in the outer membrane. The resin, Enzacryl polythiolactone, a reactive polyacrylamide derivative (Koch-Light Chemicals, Ltd.) was therefore investigated with a view to its possible use in the determination of membrane structure. Membrane and solubilised membrane fractions were prepared from *Bacillus licheniformis* and the accessibility of the proteins in each of the fractions was determined.

Methods

Membrane preparation and fractionation

B. licheniformis A.T.C.C. 9945 was grown in batches to mid-logarithmic phase in complex medium containing 0.1% glucose [9]. Membranes were prepared by lysis of the cells with lysozyme [10] and were washed three times with 0.05 M Tris-HCl, pH 8.0. The final product was resuspended in the same



Scheme I. Membrane preparation and fractionation procedure.

Tris buffer to a concentration of 20—25 mg protein/ml. This final membrane preparation (original membrane) was rapidly frozen and stored at −20°C. Membrane fractions were obtained by freeze-thawing this membrane suspension after it had been stored for 10 days. The method used was slightly modified from that of Hancock and Baddiley [11]. After the final thawing, some of the membrane was retained uncentrifuged (freeze-thawed original membrane), the rest of the suspension was centrifuged at 38 000 × *g* for 20 min at 4°C. The pellet was resuspended in the above Tris-HCl buffer to the original concentration and kept at −20°C (residual membrane). The supernatant contained the 'solubilised' enzymes and was decanted off (crude extract). The crude extract was further fractionated in a column of Sepharose 6B (Pharmacia, Ltd.) and a purified teichoic acid-synthesising fraction (purified fraction) was obtained in a peak corresponding to the void volume of the column. The purified fraction was further fractionated by centrifugation at 110 000 × *g* for 1 h in a Beckman L.2. ultracentrifuge. The supernatant was decanted off and the small residual pellet which contained most of the teichoic acid-synthesising activity was resuspended to its original concentration (ultracentrifuged pellet). From an original membrane suspension at 25 mg protein/ml, the residual membrane, crude extract, purified and resuspended pellet fractions contained 21, 5, 0.5 and 0.35 mg protein/ml, respectively, measured by using the method of Lowry et al. [12] after solubilisation in 0.1 M NaOH using bovine serum albumin as standard. The membrane preparation and fractionation are summarised in Scheme I. All of the fractions contained lipid, and purified and resuspended pellet fractions appeared to be completely composed of lipoprotein complexes [11,13].

The irreversible binding of exposed membrane proteins to Enzacryl polythiolactone

Enzacryl polythiolactone resin (Koch-Light) was reacted with exposed membrane proteins by mixing the membrane fractions with the resin in 0.1 M phosphate buffer. The optimum conditions for the binding of the resin to exposed proteins were elucidated as follows. 0.05 ml of an original membrane suspension containing 1 mg of protein was incubated with each of 0, 20, 40 and 75 mg of the resin in 0.2 ml of 0.1 M phosphate buffer at pH values of 6, 7 and 8. Incubations were carried out for 15 min at 37°C (continually shaken), then any remaining resin was inactivated by the addition of 1 M hydroxylamine (10 μl per 20 mg resin). After a further 5 min, 0.1 ml of a concentrated polyacrylamide gel electrophoresis sample buffer was added and the mixture was boiled for 5 min. The incubation tubes were then spun for 10 min at 2000 × *g* to sediment the resin polymer and 20-μl samples of the clear supernatants containing unbound proteins were decanted for analysis by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. The phosphate buffer used was 0.1 M KH_2PO_4 which was adjusted to the required pH with KOH. SDS-polyacrylamide gel electrophoresis was carried out by using the method of Laemmli [14].

Using the optimum conditions for the binding of exposed proteins to the resin (20 mg resin/mg of protein at pH 6.0, discussed in Results), the original membrane, freeze-thawed original membrane, crude extract, purified and resus-

pended pellet fractions were treated as above. However, the very low protein concentration of the crude extract, purified and resuspended pellet samples led to the use of larger volumes of sample and phosphate buffer and as a result the experimental procedure had to be modified slightly. After the inactivation of excess resin with hydroxylamine, the samples were heated for 5 min in a boiling-water bath to inactivate any proteases before being dialysed overnight (0°C) against distilled water to remove the hydroxylamine, phosphate and sample buffers. The samples were then freeze-dried, polyacrylamide gel electrophoresis sample buffer was added to them and they were then analysed as before. If the samples were freeze-dried immediately after neutralisation of the excess resin, an unusable gel was obtained on the addition of the polyacrylamide gel electrophoresis sample buffer. Protein was assayed by using the method of Lowry et al. [12], corrections being made for the presence of Tris. Lipid phosphorus was measured by using the method of Chen et al. [15] after extraction of the lipids by the method of Bligh and Dyer [16].

Results

The reaction of the resin with exposed proteins

The results of reacting the resin with membranes under different conditions are shown in Fig. 1. At each of the three pH values tested, some polypeptides

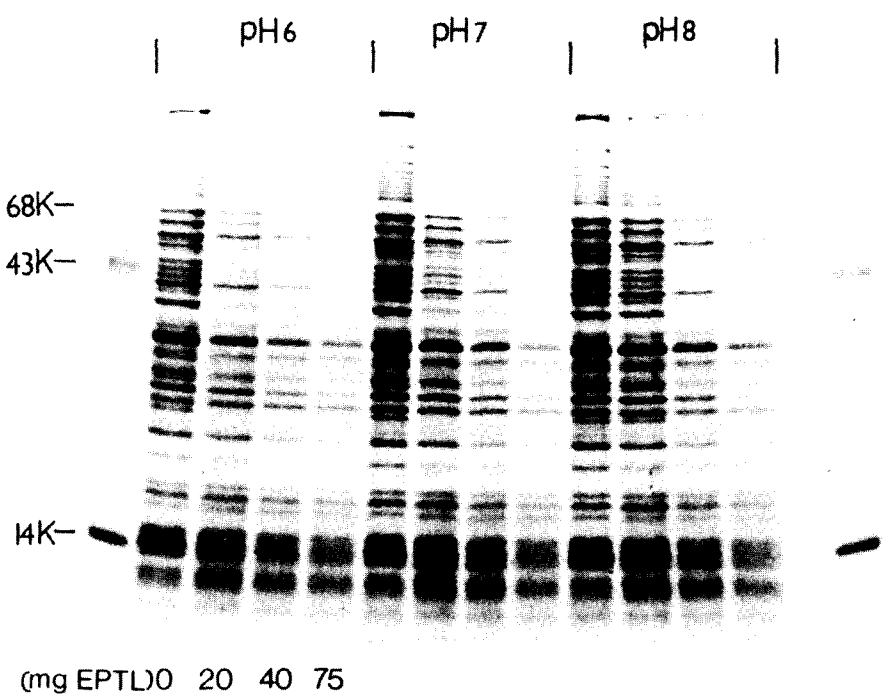


Fig. 1. SDS-polyacrylamide gel electrophoresis of membrane proteins remaining unbound to Enzacryl polythiolactone resin after incubation of the membrane proteins with the resin under different conditions, for 15 min. Molecular weight markers were: 68K, bovine serum albumin; 43K, ovalbumin; 14K, Lysozyme. EPTL, Enzacryl polythiolactone resin.

rapidly became covalently linked to the resin beads while others did not react to a detectable extent. The differences in extent of reaction were most readily detectable in incubation with 20 mg resin/mg protein at pH 6.0. At other pH values, more resin was required to give the same rate of removal of polypeptides from the membrane, and at pH 6 larger amounts of resin led to detectable loss of even the most slowly reacting polypeptides.

The reactivity of the resin after incubation with membrane was measured by its ability to covalently bind [^{3}H]lysine. After 15 min it had lost 10% of its binding capacity and this was due to reaction with membrane; incubation of the resin in buffer alone led to no loss of reactivity over a period of 1 h. Longer periods of incubation with the membrane or the use of more resin, led to greater protein bindings, but in both cases all of the proteins of the membrane began to react, presumably as the membrane was disrupted by reaction of the phospholipids as well as protein with the resin. Thus, all the proteins have the potential to react with the resin. Selectivity in the binding of proteins required a moderate excess of resin together with a short incubation period. Since a large amount of reactive resin remained after 15-min incubations, it was essential that the resin be inactivated before disruption of the remaining membrane with SDS. Hydroxylamine was found to give complete inactivation within 1 min. The hydroxylamine probably disrupted the membrane by deacylating the lipids, but the reaction of the resin with hydroxylamine was so much faster than with proteins (100% of resin inactivated in 1 min by NH_2OH compared with 10% inactivated in 15 min by protein) that this disruption was unlikely to lead to a significant amount of non-specific reaction of the proteins with EPTL.

The exposed and inaccessible proteins of bacterial membrane and its subfractions

The technique was used to examine protein exposure in fractions of bacterial membrane released by freeze-thawing [11]. Samples of the fractions, obtained as shown in Scheme I, were incubated with the resin under the optimum conditions. The controls were incubated without resin. The polypeptides of the proteins remaining unbound after incubation were separated by SDS-polyacrylamide gel electrophoresis in a 12% gel (Fig. 2). At least 11 polypeptides were removed completely from the original preparation in 15 min, and the polypeptide patterns of the inaccessible proteins of the original membrane before and after freeze-thawing were found to be very similar. The freeze-thaw process did not, therefore, appear to have altered the lipid-protein associations in the membrane to such an extent that any protein became more or less accessible to the resin. The crude extract also appeared to have almost the same accessible and inaccessible proteins as the original membrane and freeze-thawed original membrane fractions. Many of the polypeptides in these fractions, especially those with molecular weights above 68 000, were exposed and irreversibly bound to the resin. On the other hand, in the purified and resuspended pellet fractions most of the proteins were inaccessible to the resin as the polypeptide patterns from the samples incubated with the resin were almost the same as the polypeptide patterns of the controls. Most of the bands that reacted with the resin in the original membrane, freeze-thawed original membrane and crude extract fractions were, however, absent from the purified and resuspended pellet frac-

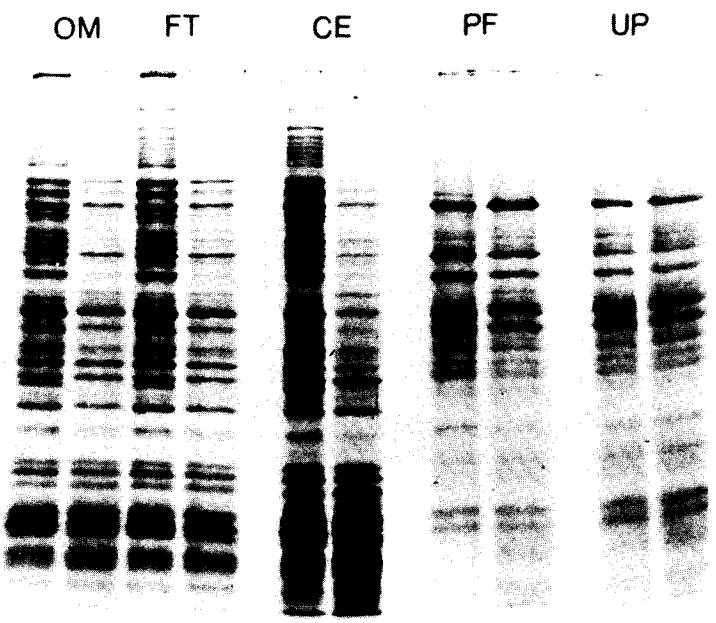


Fig. 2. SDS-polyacrylamide gel electrophoresis of proteins in membrane fractions after reaction with the resin. The left-hand sample of each pair is the control that had not been exposed to the resin. OM, original membrane; FT, freeze-thawed original membrane; CE, crude extract; PF, purified fraction; UP, resuspended pellet.

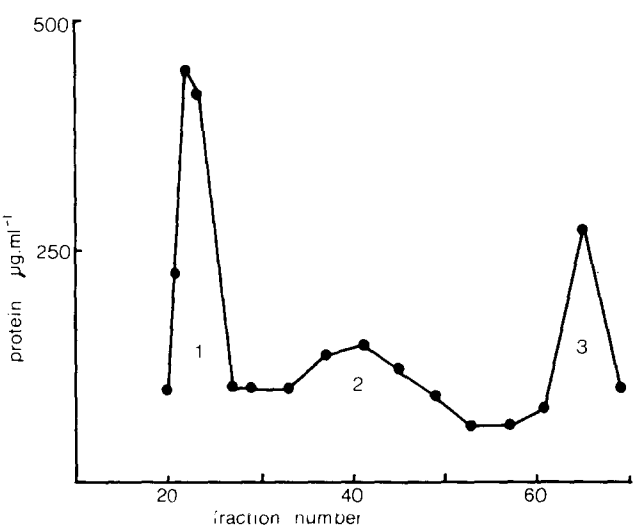


Fig. 3. Chromatography of crude extract from freeze-thawed membranes on Sepharose 6B. Crude extract (4 ml) was applied to a Sepharose 6B column (40 × 1.5 cm) and was eluted in 0.025 M Tris-HCl, pH 8.0, by upward flow at 4°C; 3-ml fractions were collected and assayed for protein ceontent, and for lipid phosphorus. Peak 1 was purified fraction; peak 2 was not analysed; peak 3 contained only soluble proteins (no lipid).

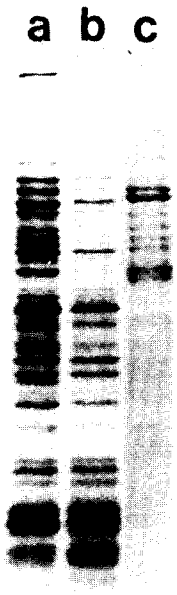


Fig. 4. A comparison of the peak 3 soluble proteins with the proteins which react rapidly with the resin in the original membrane. a, original membrane; b, original membrane plus resin; c, peak 3.

tions before their reaction with the resin. The reactive polypeptides separated from the major lipoprotein peak during chromatography of crude extract on Sepharose 6B and most of them eluted in peak 3 (Fig. 3), which contained no lipid. Fig. 4 compares these polypeptides (c) with the original membrane before (a) and after (b) reaction with the resin. Peak 3 contained most, but not all, of the proteins of the original membrane that reacted rapidly with the resin.

Discussion

Treatment of membranes with the resin distinguished a particular class of polypeptides that reacted rapidly and irreversibly with the activated resin. These polypeptides were released from the membrane by freeze-thawing but, unlike the bulk of the protein so released, they were not associated with lipids after this treatment.

Apparently, the majority of the proteins that were released as lipoprotein complexes by freeze-thawing (peaks 1 and 2, Fig. 3) were protected from the resin, both in the membrane and after extraction, by their association with phospholipids, possibly due to restrictions imposed by the very short 'spacer arm' between the resin bead and the thiolactone group. If this was the reason for the failure of the lipoprotein polypeptides to react, then the reactive proteins must be largely exposed to the aqueous environment of the membrane. They could be extrinsic membrane proteins absorbed by electrostatic or weak hydrophobic interactions, or integral proteins where a sufficient proportion of

the polypeptides protruded from the membrane surface.

A similar selectivity for exposed membrane-surface proteins has been demonstrated for several dicarboxylic acid anhydrides [17]. This hypothesis requires testing by an examination of the fate of known extrinsic and externally accessible membrane proteins during reaction of membranes with the resin. In preliminary investigations with human erythrocyte ghosts, it has been found that the only proteins accessible to the resin are band 3 and approx. 20% of bands 1 and 2 (Thompson, S. unpublished results). Band 3, a transmembrane glycoprotein, is reported to be the only accessible surface polypeptide of intact erythrocytes [2,18] while bands 1 and 2 comprise spectrin, which is known to be an extrinsic protein on the inner surface of intact cells [4,19]. Enzacryl polythiolactone, therefore, appears to provide a very useful means for studying protein exposure on membrane surfaces.

References

1 Phillips, D.R. and Morrison, M. (1971) Biochemistry 10, 1766–1771
2 Bretscher, M.S. (1971) J. Mol. Biol. 58, 775–781
3 Cabantchik, I.Z., Balshin, M., Brever, W. and Rothstein, A. (1975) J. Biol. Chem. 250, 5130–5136
4 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
5 Juliano, R.L. and Behar-Bannelier, M. (1975) Biochim. Biophys. Acta 375, 249–267
6 Axen, R., Porath, J. and Ernback, S. (1969) Nature 214, 1302–1304
7 Ahrgren, L., Kagedal, L. and Akserstrom,S. (1972) Acta. Chem. Scand. 26, 285–288
8 Kamio, Y. and Nikaido, H. (1977) Biochim. Biophys. Acta 464, 589–601
9 Hancock, I.C. and Baddiley, J. (1972) Biochem. J. 127, 27–37
10 Hughes, A.H., Hancock, I.C. and Baddiley, J. (1973) Biochem. J. 132, 83–93
11 Hancock, I.C. and Baddiley, J. (1973) FEBS Lett. 34, 15–18
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, J. (1951) J. Biol. Chem. 193, 265–275
13 Thompson, S. (1977) Ph.D. Thesis, University of Newcastle upon Tyne
14 Laemmli, U.K. (1970) Nature 227, 690–685
15 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–58
16 Bligh, E.G. and Dyer, W.J. (1951) Can. J. Biochem. Physiol. 37, 911–917
17 Howlett, G.J. and Wardrop, A.J. (1978) Arch. Biophys. Biochem. 188, 429–437
18 Phillips, D.R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284–289
19 Kirkpatrick, F.H. (1976) Life Sci. 19, 1–18