Biochimica et Biophysica Acta, 298 (1973) 876–886

BBA 76274

BINDING OF PROTEINS TO MYCOPLASMA MEMBRANES

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(Received October 19th, 1972)

SUMMARY

1. Isolated *Acholeplasma laidwalii* membranes were capable of binding large quantities of the basic proteins cytochrome *c* and lysozyme (up to 0.5 mg per mg membrane protein), usually smaller amounts of *n*-butanol-solubilized *A. laidlawii* membrane proteins and still less of bovine serum albumin.

2. Removal of about 70% of *A. laidlawii* membrane proteins by pronase digestion increased and the removal of lipids decreased the binding capacity of the membrane, so that the lipids appear to provide most of the binding sites for the soluble proteins.

3. Isolated membranes bound about twice as much cytochrome *c* and lysozyme as membranes of intact cells, implying that about half the binding sites for these proteins are exposed on the outer membrane surface.

4. The binding of the proteins to the membranes depended on temperature, pH and ionic strength of the medium. The binding of albumin and butanol-solubilized membrane proteins increased considerably at acid pH values while that of cytochrome *c* and lysozyme was only slightly affected. The membrane-bound cytochrome *c* and lysozyme, but neither the albumin nor the butanol-solubilized proteins, could be almost quantitatively released by 1 M NaCl, showing electrostatic bonds to be responsible for the binding of the basic proteins.

5. Butanol-solubilized *A. laidlawii* membrane proteins could bind to *Mycoplasma mycoides* var. *mycoides* membranes so as to produce "hybrid" membranes having antigenic properties of both membrane types. The possibility of binding proteins from the growth medium to the mycoplasma membrane and its implications regarding immunological characterization are discussed.

INTRODUCTION

A variety of model systems has been employed to investigate the bonds by which proteins and lipids in biomembranes are held together. Most popular are the models based on monolayers[1], bilayers[2], liposomes[3–5] or phospholipid dispersions[6]. A somewhat different approach has been to study the recombination or reaggregation of solubilized membrane proteins and lipids to membranous structures[7–9] where the findings generally coincided with those of the phospholipid model systems[10]. Another less common approach, based on the binding of water-soluble or solubilized membrane proteins to the plasma membrane of intact cells[11] or to isolated ghosts or membrane

fragments[12] has obvious advantages in investigating the binding of hormones or foreign protein antigens to cell surfaces[11–13]. This was the approach used by us to study the binding of several soluble and membranous proteins to mycoplasma membranes in order to elucidate the type of bonds formed, the chemical nature and location of the binding sites and the immunogenic properties of the bound proteins.

MATERIALS AND METHODS

Membrane preparations

Acholeplasma laidlawii (oral strain) and *Mycoplasma mycoides* var. *mycoides* (strain V5) were grown in a modified Edward medium[14]. To label the membrane lipids, 50 μCi of [9, 10-^{3}H]oleic acid or 2 μCi of [1-^{14}C]oleic acid were added to each l of the growth medium. The organisms were harvested after 18–20 h of incubation at 37 °C and were washed and osmotically lysed as described before[15]. To label the membrane proteins, *A. laidlawii* cells grown for 16 h in 1 l of Edward medium were transferred to 100 ml of the partially defined medium of Razin and Cohen[16] containing 100 μCi of L-[^{3}H]phenylalanine. After 6 h of further incubation with shaking at 37 °C, the organisms were harvested, washed and lysed. The isolated membranes were washed three times alternately with deionized water and 0.05 M NaCl in 0.01 M phosphate buffer, pH 7.5. The washed membranes were suspended in NaCl–Tris–β-mercaptoethanol buffer (β-buffer, ref. 15) diluted 1:20 in deionized water (dilute β-buffer) and kept at —20 °C until used.

Digestion of membranes by pronase

Digestion of *A. laidlawii* membranes (2 mg membrane protein per ml) by pronase (200 μg/ml) was carried out at 45 °C for various periods of time ranging from 2 to 24 h. To stop digestion, the samples were chilled rapidly to 0 °C and centrifuged at 100000 × *g* for 30 min. The supernatant was discarded and the pellet was resuspended in deionized water.

Solubilization of membrane proteins by n-butanol

Maddy's procedure[17] for the solubilization of erythrocyte membrane proteins was applied with some modifications. 2 ml of ice-cold *n*-butanol were added to 4 ml of a membrane suspension in deionized water containing 4 mg membrane protein per ml. The mixture was vigorously shaken for 30 s and transferred to an ice-bath for 15 min. Centrifugation of the mixture at 34000 × *g* for 15 min in the cold resulted in its separation into three phases: upper butanol-rich yellow phase, a small creamy interphase and a lower colorless aqueous phase containing most of the membrane protein. The aqueous phase was carefully separated, dialyzed overnight against 1000 vol. of cold β-buffer diluted 1:100 with deionized water, and extracted twice more with *n*-butanol as described above. The solution of membrane proteins so obtained was kept at 4 °C and used within 3 days of its preparation after centrifugation at 34000 × *g* for 1 h for the removal of aggregated proteins.

Binding of soluble proteins to membranes

Equal volumes of membrane suspension (0.5–2 mg membrane protein per ml) and soluble protein solution (0.25–8 mg/ml) in 0.025 M Tris–maleate–citrate buffer,

pH 7.5 or 4.5 were mixed and incubated at 37 °C for 30 min. The resulting membrane-soluble protein complex was collected by centrifugation at 100000 × *g* for 30 min and washed once in deionized water. To release proteins bound electrostatically to the membrane, the sedimented membrane-soluble protein complex was washed twice in 5 ml of a cold solution of 1 M NaCl–0.01 M sodium citrate–0.02 M $MgCl_2$ (pH 8.5). The amount of soluble protein bound to the membranes was obtained by determination of the protein content of the complexes by the Folin–phenol method of Lowry *et al.*[18]. In cases where the solubilized membrane proteins were prepared from *A. laidlawii* grown with L-[^{3}H]phenylalanine, an estimate of the amount of solubilized proteins bound to the membranes was obtained by radioactivity measurements.

Analytical procedures and reagents

Electrophoretic analysis of membrane proteins was carried out in polyacrylamide gels containing 0.1% sodium dodecyl sulfate[19]. Densitometer tracings of the stained gels were made in a Kipp and Zonen Densitometer model DD2. Density gradient analysis of membranes and membrane-soluble protein complexes was performed as described before[15], using a linear sucrose gradient of 30–60%. Lipids were extracted from membranes by five successive extractions with acetone–water–ammonia (90:10:0.03, by vol.) at 4 °C for 30 min. Protein was determined by the Folin–phenol method of Lowry *et al.*[18], using bovine serum albumin as standard. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer using dioxane–toluene scintillation liquor[15]. The soluble proteins used were crystallised bovine serum albumin (Armor, Kankakee, Ill., U.S.A.), cytochrome *c* (99% pure, Sigma, St. Louis, Mo., U.S.A.) and crystalline lysozyme (Nutritional Biochemical Co., Cleveland, Ohio, U.S.A.).

Immunological techniques

Antisera to membranes and to membrane-soluble protein complexes were prepared in rabbits as described by Kahane and Razin[20]. Each rabbit received a total of 4 mg protein. The antisera were tested for metabolism-inhibiting antibodies to *A. laidlawii* and *M. mycoides* var. *mycoides* in a medium containing glucose, as described by Taylor-Robinson *et al.*[21] and for agglutination of the mycoplasma cells by the method of Bailey *et al.*[22].

RESULTS

Properties of the butanol-solubilized membrane proteins

Treatment of *A. laidlawii* membranes with cold *n*-butanol according to Maddy[17] resulted in the separation of 80–90% of the membrane proteins in the aqueous phase together with 5–10% of the membrane lipid. The bulk of the lipid was found in the butanol-rich phase. The small interfacial layer contained the rest of the protein (10–20% of the total) and some lipid. Electrophoretic analysis of the aqueous phase showed it to contain essentially all the major protein bands characterizing the native membrane (Fig. 1). Chromatography of the aqueous phase on a Bio-Gel P200 column (40 cm × 2.5 cm) equilibrated with dilute β-buffer resulted in the exclusion of over 85% of the protein in the void volume, suggesting the presence of aggregated protein in the aqueous phase (see also Maddy[17]). The precipitation of the solubilized mem-

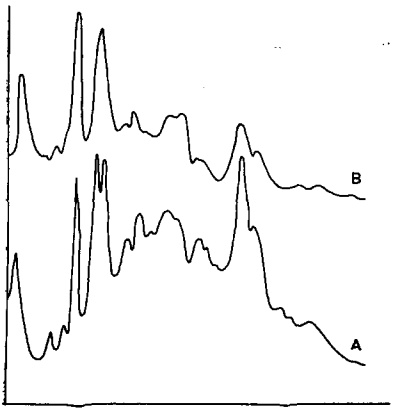

Fig. 1. Electrophoretic patterns (densitometer tracings) of *A. laidlawii* membrane proteins. A, native membranes; B, *n*-butanol-solubilized membrane proteins.

brane proteins on freezing and thawing the aqueous phase was also indicative of their tendency to aggregate. Even when the aqueous phase was stored at 4 °C, after 7 days about 40% of the protein sedimented by centrifugation at $34000 \times g$ for 1 h. When the pH of the solution was reduced to 4.0 or when NaCl was added to a final concentration of 1 M, 20–30% of the protein sedimented upon centrifugation at $34000 \times g$ for 1 h. No significant differences in protein patterns was, however, found by the electrophoretic analysis of the sedimentable and non-sedimentable protein fractions, so that no selective aggregation of protein species seems to occur in the aqueous phase.

Binding of soluble proteins to A. laidlawii membranes

A. laidlawii membranes were capable of binding large quantities of cytochrome *c* and lysozyme, usually smaller amounts of the butanol-solubilized membrane proteins and still less bovine serum albumin (Table I). Removal of about 70% of membrane proteins by pronase digestion increased the binding capacity of the membranes (Table I), while the removal of over 90% of membrane lipids by aqueous acetone extraction brought it down to about 40% that of the native membranes. Hence membrane lipids seem to provide the majority of binding sites for the soluble proteins so that it is more appropriate to express the results as mg soluble protein bound per labeled membrane lipid than as mg soluble protein bound per mg membrane protein (Table I). However, because of the difference in the intensity of label of the various batches of membranes the binding values obtained in different experiments varied and cannot be compared.

The binding of the soluble proteins caused membrane density to increase (Table II) and thus speaks against the soluble proteins being merely co-precipitated with the membrane rather than bound to it. More evidence for the lack of co-precipitation was provided by the absence of any band additional to that of the membranes in all of the density gradients examined. Furthermore, protein determination gave negative results with all the fractions separated from the gradients apart from those containing the membrane band. When labeled *n*-butanol-solubilized membrane proteins were used for binding, the radioactivity was found to be associated with the

TABLE I

BINDING OF SOLUBLE PROTEINS TO NATIVE AND PRONASE-DIGESTED *A. LAIDLAWII* MEMBRANES

Native membranes (1 mg protein per ml) or pronase-treated membranes from which 68% of the protein was removed, were incubated with the soluble protein (1 mg/ml) at 37 °C for 30 min in 0.025 M Tris-maleate-acetate buffer, pH 7.5.

Soluble protein added	*Soluble protein bound*			
	Native membranes		*Pronase-digested membranes*	
	*mg/10⁶ cpm of labeled membrane lipid**	*mg/mg membrane protein*	*mg/10⁶ cpm of labeled membrane lipid*	*mg/mg membrane protein*
Lysozyme	5.78	0.51	7.07	1.62
Cytochrome *c*	5.23	0.47	6.50	1.35
Bovine serum albumin	0.23	0.04	0.33	0.15
Butanol-solubilized membrane proteins	2.32	0.21	3.98	0.83

* The membranes contained labeled lipid; 1 mg of membrane lipid being equivalent to $1.2 \cdot 10^5$ cpm in this batch of membranes.

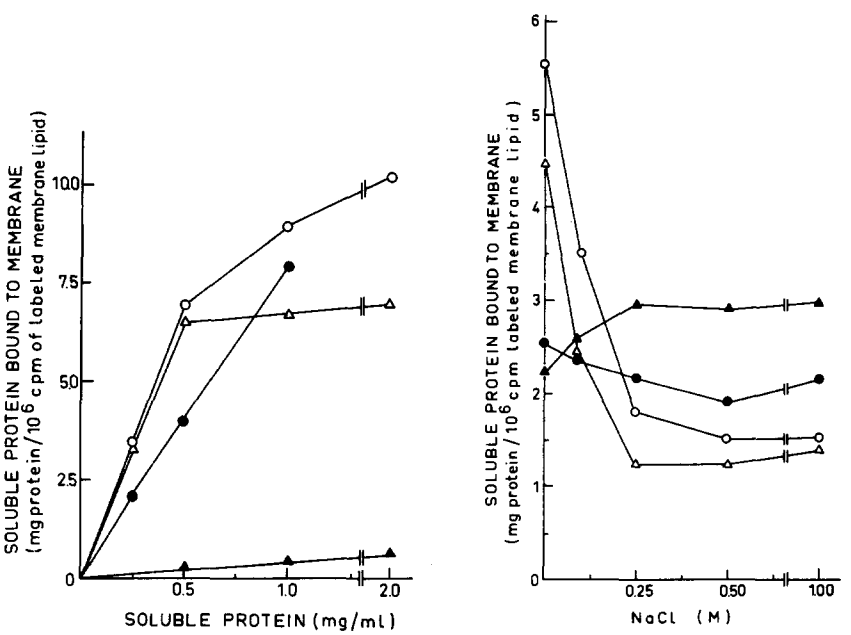

Fig. 2. Binding of soluble proteins to pronase-digested *A. laidlawii* membranes at pH 7.5. ○—○, lysozyme; △—△, cytochrome *c*; ●—●, butanol-solubilized *A. laidlawii* membrane proteins; ▲—▲, bovine serum albumin. Pronase digestion was as described in Table I.

Fig. 3. Effect of NaCl on the binding of soluble proteins to *A. laidlawii* membranes. ○—○, lysozyme; △—△, cytochrome *c*; ●—●, butanol-solubilized *A. laidlawii* membrane proteins; ▲—▲, bovine serum albumin. The binding was tested at pH 7.5, except for albumin where the pH was 4.5.

membrane band only, again speaking against co-precipitation of the soluble proteins. The possibility that part of the soluble protein was trapped within the membranous vesicles seems unlikely since sonication of the membrane-soluble protein complex at 20 kcycles for 1 min failed to release any soluble protein.

Fig. 2 shows the dependence of the amount of soluble protein bound on its concentration in the binding medium. Under the test conditions, the binding sites on the membranes were saturated at a concentration of 0.5 mg cytochrome *c* per ml. With the butanol-solubilized membrane protein no saturation was achieved at 1 mg/ml, and solutions of higher concentrations were not available.

The addition of NaCl to the binding medium strongly inhibited the binding of cytochrome *c* and lysozyme to *A. laidlawii* membranes, but had little effect on the binding of albumin or of the butanol-solubilized membrane proteins (Fig. 3). Moreover, the washing of the membrane-soluble protein complexes with 1 M NaCl in 0.05 M citrate buffer, pH 8.5, released over 95% of the bound cytochrome *c* or lysozyme but none of the bound albumin or butanol-solubilized proteins. The effect of Mg^{2+}, similar to that of NaCl, could be demonstrated at much lower concentrations, either by its addition to the binding medium or by overnight dialysis of the membrane-soluble protein complex against 20 mM $MgCl_2$. The binding of albumin and the butanol-solubilized membrane proteins was somewhat enhanced in the presence of $MgCl_2$ (Fig. 4).

The effect of pH on binding is shown in Fig. 5 and Table II. While the binding of albumin and the butanol-solubilized membrane proteins increased considerably at acid pH values, the binding of cytochrome *c* or lysozyme was affected only slightly.

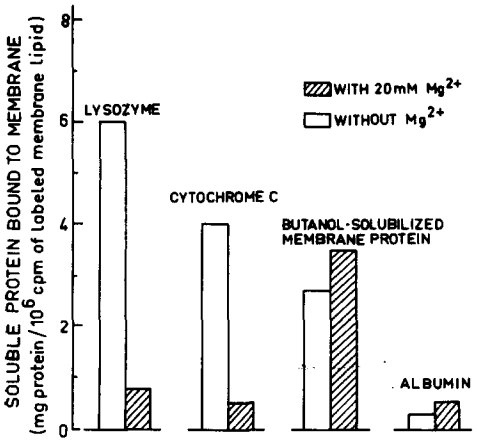

Fig. 4. Effect of Mg^{2+} on the binding of soluble proteins to *A. laidlawii* membranes at pH 7.5.

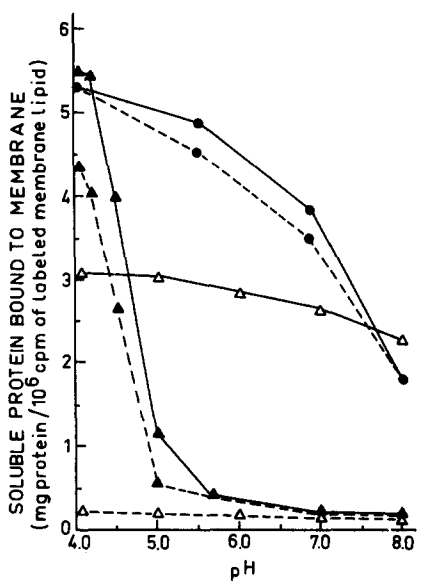

Fig. 5. Effect of pH on the binding of soluble proteins to *A. laidlawii* membranes. △, cytochrome *c*; ●, butanol-solubilized *A. laidlawii* membrane proteins; ▲, bovine serum albumin. Broken lines, the membrane-soluble protein complex was treated with 1 M NaCl in 0.01 M sodium citrate at pH 8.5; solid lines, untreated membrane-soluble protein complex.

TABLE II

CHANGES IN THE DENSITY OF *A. LAIDLAWII* MEMBRANES ON BINDING OF SOLUBLE PROTEINS AT DIFFERENT pH VALUES

Equal volumes of membrane suspension (2 mg protein per ml) and soluble protein solution (2 mg/ml) in 0.025 M Tris–maleate–citrate buffer, pH 7.5 or 4.0, were mixed and incubated at 37 °C for 30 min. The resulting membrane-soluble protein complex was collected by centrifugation, washed once in deionized water and subjected to density-gradient analysis. The density of the untreated membranes was 1.154 g/cm^3.

Soluble protein bound	*Membrane density (g/cm^3)*	
	pH 4.0	*pH 7.5*
Cytochrome *c*	1.171	1.169
Lysozyme	Not tested	1.179
Bovine serum albumin	1.179	1.154
Butanol-solubilized membrane proteins	1.176	1.165

The binding of all the four proteins to the membranes was very rapid and maximum binding values were obtained after 5 min of incubation. As the experimental technique used was based on centrifugation it does not allow of kinetic measurements at less than 5 min incubation.

The effect of temperature on binding is shown in Fig. 6. With all the four proteins tested maximum binding took place at about 45 °C but the dependence on temperature was more pronounced with the butanol-solubilized membrane proteins and with albumin of which almost twice as much was bound when the temperature was increased from 10 to 45 °C as against only 20% more of cytochrome *c* or lysozyme.

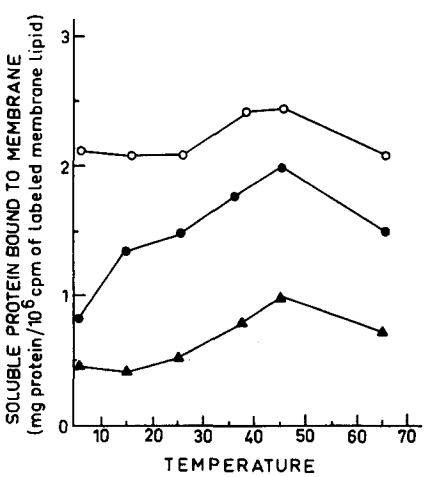

Fig. 6. Effect of temperature on binding of soluble proteins to *A. laidlawii* membranes. ○—○, lysozyme; ●—●, butanol-solubilized *A. laidlawii* membrane proteins; ▲—▲, bovine serum albumin. The binding was tested at pH 7.5, except for albumin where the pH was 4.5.

Isolated *A. laidlawii* membranes bound about twice as much lysozyme and cytochrome *c* as membranes which still formed an integral part of the cell. With butanol-solubilized membrane proteins the difference was less pronounced (Table III).

TABLE III

BINDING OF SOLUBLE PROTEINS TO CELLS AND ISOLATED MEMBRANES OF *A. LAIDLAWII*

Cells (3 mg cell protein/ml) and membranes (1 mg membrane protein/ml) were incubated for 30 min at 0 °C with the soluble protein (1 mg/ml) in the binding medium supplemented with 0.15 M NaCl. The addition of NaCl and the low binding temperature were necessary to protect the cells from lysis during the binding period. After binding the cells were osmotically lysed to isolate the membranes. The results are thus expressed in both cases as the amount of protein bound per membrane lipid

Soluble protein	*Soluble protein bound per labeled membrane lipid (mg/10^6 cpm)*	
	Whole cells	*Isolated membranes*
Lysozyme	4.18	7.66
Cytochrome *c*	3.50	6.18
Butanol-solubilized *A. laidlawii* membrane proteins	1.67	2.14

Binding of solubilized A. laidlawii membrane proteins to M. mycoides membranes

Incubation of *M. mycoides* membranes with butanol-solubilized membrane proteins of *A. laidlawii* resulted in the binding of about 0.2 mg of the solubilized proteins per mg of *M. mycoides* membrane protein, about the same amount as was bound by *A. laidlawii* membranes (see Table I). After binding of the solubilized *A. laidlawii* membrane proteins the density of the *M. mycoides* membranes increased from 1.182 g/cm^3 to 1.195 g/cm^3.

Table IV shows that *M. mycoides* membranes complexed with membrane proteins of *A. laidlawii* elicited the production of antibodies agglutinating both organisms. The agglutination titers were comparable to those produced by injecting each of the membrane types or the solubilized membrane proteins separately. The ability of *A. laidlawii* membrane proteins to elicit the production of the metabolism-inhibiting antibodies was, however, seriously impaired by the butanol treatment, although their ability to elicit agglutinin production remained unaffected. Nevertheless, the results presented in Table IV show that binding affected neither the immunogenic properties of the solubilized membrane proteins nor the immunogenicity of the membranes to which they were bound. The possibility that the binding of soluble proteins to the cell surface may obstruct the attachment of specific antibodies to the membrane was investigated. The results obtained so far with cytochrome *c* showed that its binding to *A. laidlawii* (up to 0.3 mg/mg cell protein) did not reduce the agglutination titer of the cells by an antiserum to the membrane.

TABLE IV

IMMUNOGENICITY OF MEMBRANES, SOLUBILIZED MEMBRANE PROTEINS AND COMPLEXES OF SOLUBILIZED MEMBRANE PROTEINS WITH MEMBRANES OF A DIFFERENT MYCOPLASMA

Materials used for immunization	*Agglutination of cells (reciprocal of titer)*		*Metabolism inhibition (reciprocal of titer)*	
	A. laidlawii	*M. mycoides*	*A. laidlawii*	*M. mycoides*
A. laidlawii membranes	64	< 2	2560	< 10
n-Butanol-solubilized *A. laidlawii* membrane proteins	128	< 2	80	< 10
M. mycoides membranes	< 2	64	< 10	2560
Complex of *M. mycoides* membranes with butanol-solubilized *A. laidlawii* membrane proteins	128	64	160	2560

DISCUSSION

The basic proteins, cytochrome *c* and lysozyme appear to bind to the mycoplasma membrane almost exclusively by electrostatic bonds since they could be almost quantitatively released by washing with 1 M NaCl. The serum albumin and butanol-solubilized membrane proteins could not be removed by 1 M NaCl and were apparently bound to the membrane mostly by apolar hydrophobic bonds or by a combination of both apolar and polar bonds. The marked increase in the binding of albumin and butanol-solubilized membrane proteins at acid pH values may be explained by their bearing a net negative charge at neutral pH[8]. Under the usual binding conditions, at neutral pH, the protein molecules are accordingly electrostatically repelled by the acidic phosphate groups on the membrane surface. A decrease in the pH of the binding mixture reduces the electrostatic repulsion and thus enables the proteins to come into closer contact with the membrane components so that hydrophobic bonds can be formed. Similar findings have been reported on the binding of soluble and membranous proteins to model phospholipid systems[2–5].

Although the marked increase in protein binding with rising temperatures (Fig. 6) may simply be ascribed to the higher collision frequency between the protein molecules and the membrane surface[11], the fact that it was much more pronounced with butanol-solubilized membrane proteins and with albumin suggests that the temperature effect may be partly mediated through changes in the physical state of the membrane lipids. The formation of hydrophobic bonds, which involves the intercalation of the polypeptide chains into lipid regions, may well be affected by the physical state of these regions, so as to be considerably reduced at temperatures near or below the phase-transition point of the lipids.

Although both serum albumin and butanol-solubilized membrane proteins were apparently bound by the same type of bonds the amount of albumin bound was

less than 15% of the amount of butanol-solubilized proteins. Hence membrane proteins seem to be better fitted for hydrophobic bonding to membrane lipids than soluble plasma protein, as has recently also been shown in experiments using phospholipid dispersions[6].

There is little doubt that the majority of binding sites for the basic lysozyme and cytochrome *c* consist of the acidic phosphate groups of membrane phospholipids as was also shown with respect to the binding of divalent cations to the mycoplasma membrane[23]. The actually increased binding capacity of the membrane on the removal of membrane proteins by pronase may be due to the exposure of new binding sites previously occupied by membrane proteins. Since our experiments indicate that about half the sites responsible for cytochrome *c* and lysozyme binding are exposed on the outer membrane surface, a significant part of the phosphate groups of the membrane phospholipids appears to be located there.

Proteins bound to the membrane were found to retain their immunogenicity, as was shown by the binding of butanol-solubilized *A. laidlawii* membrane proteins to *M. mycoides* membranes (Table IV). As has already been suggested[13], foreign proteins bound to the cell membrane during growth may interfere with the immunological characterization of the organism. Our results indicate that the soluble plasma proteins which have isoelectric points at the acid pH range bind only in small quantities at alkaline or neutral pH values. Much larger quantities, however, may be bound to the cell surfaces at the acid pH values prevailing during the late growth phases of fermentative mycoplasmas grown in the presence of serum and glucose. Hence organisms harvested when the pH of the growth medium becomes highly acidic (it may drop to pH 5.5, ref. 24) are liable to have significant quantities of serum proteins bound to their surface, a factor to be borne in mind in immunological studies of mycoplasmas.

ACKNOWLEDGMENT

This work was supported by grant FG-Is-286 from the U.S. Department of Agriculture under Public Law 480.

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