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CHARACTERIZATION OF THE MYCOPLASMA MEMBRANE PROTEINS V. RELEASE AND LOCALIZATION OF MEMBRANE-BOUND ENZYMES IN *ACHOLEPLASMA LAIDLAWII*

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SUMMARY

The peripheral membrane protein fraction released by washing *Acholeplasma laidlawii* membranes with low-ionic strength buffers contained about 50 % of the total membrane-bound ribonuclease and deoxyribonuclease activities. The ATPase, NADH oxidase and *p*-nitrophenylphosphatase activities remained bound to the membrane even when EDTA was added to the wash fluids, and thus appear to belong to the integral membrane protein group.

Serving as a marker for peripheral membrane proteins, the membrane-bound ribonuclease activity was solubilized by bile salts much more effectively than the integral membrane-bound enzymes. On the other hand, the solubilized ribonuclease showed a much lower capacity to reaggregate with other solubilized membrane components to membranous structures. Yet, most of the ribonuclease molecules which were bound to the reaggregated membranes could not be released by low-ionic strength buffer. The reaggregated membranes differed from the native membranes in the absence of particles on their fracture faces obtained by freeze cleaving, and by their much higher labeling by the [^{125}I]lactoperoxidase iodination system. These results suggest that most of the proteins are exposed on the reaggregated membrane surfaces, with very little, if any, protein embedded in its lipid bilayer core.

Enzyme disposition in the *A. laidlawii* membrane was studied by comparing the activity of isolated membranes with that of membranes of intact cells after treatment with pronase or with an antiserum to membranes. The data indicate the asymmetrical disposition of these activities, the ATPase and NADH oxidase being localized on the inner membrane surface, while the nucleases are exposed on the external membrane surface.

INTRODUCTION

Previous communications in this series [1, 2] dealt mainly with the solubilization and fractionation, in the presence of detergents, of the integral membrane

proteins of *Acholeplasma laidlawii*. Special emphasis was put on those proteins exhibiting catalytic and immunogenic activities. In the present investigation an attempt was made to characterize some of the peripheral membrane proteins of *A. laidlawii*, which possess catalytic activity. Peripheral membrane proteins, as defined by Singer and Nicolson [3], are those proteins releasable in a water-soluble and lipid-free form by mild agents, including low-ionic strength buffers or EDTA, as against the integral membrane proteins which require detergents or organic solvents for their solubilization. The ability of the released peripheral membrane proteins to reassociate with solubilized membrane components to membranous structures by the reaggregation procedure [4, 5] was compared with that of the integral membrane proteins, enzymic activities serving as convenient markers. The results obtained reveal significant differences between the peripheral and integral membrane proteins in this respect and, in addition, shed more light on the molecular organization of reaggregated mycoplasma membranes. Finally, the disposition of the various enzymic activities in the *A. laidlawii* membrane was investigated. The data presented in this communication supplement the information on the asymmetrical disposition of proteins in mycoplasma membranes reported recently by Amar et al. [6] and by Kahane and Marchesi [7].

MATERIALS AND METHODS

Cells and membrane preparations. A. laidlawii was grown in a modified Edward medium [8]. To label membrane lipids, 50 μCi of [9, 10-$^3H_2$]oleic acid were added to each liter of the growth medium. The organisms were grown, harvested and washed once in a 0.25 M NaCl solution. To obtain membrane preparations, the cells were osmotically lysed as described before [4]. The isolated membranes were washed once with deionized water and then with 0.05 M NaCl in 0.01 M phosphate buffer, pH 7.5. The washed membranes were resuspended in NaCl–Tris–β-mercaptoethanol buffer (β-buffer, ref. 4) diluted 1:20 in deionized water (dilute β-buffer) and stored at −20 °C.

Release of proteins from the membranes. The membranes were subjected to a series of washings in decreasing concentrations of Tris–HCl buffer following the procedure of Muñoz et al. [9] with modifications. After osmotic lysis, the membranes were suspended in 100 mM Tris–HCl buffer, pH 7.4, to a final concentration of 2.5–3.0 mg of membrane protein per ml. One additional wash was made in the same buffer. The membranes were then subjected to two washes with the same volume of 30 mM of the Tris–HCl buffer. The pellet was suspended in 3 mM Tris–HCl buffer and kept overnight at 4 °C. On the following day the suspension was centrifuged and washed again in 3 mM Tris–HCl. Membranes were sedimented after each washing by centrifugation at 37 000 × g for 30 min. The wash fluids were collected and concentrated by ultrafiltration using a Diaflo PM-10 filter (Amicon, Oosterhout, Holland). All procedures were carried out at 4 °C.

Membrane solubilization and reaggregation. Sodium deoxycholate was the product of British Drug Houses (Poole, England). The sodium salts of cholic acid and taurodeoxycholic acid were obtained from Sigma (St Louis, Mo., U.S.A.) and those of taurocholic acid from Calbiochem (San Diego, Calif., U.S.A.). The washed membranes (3.6 mg protein per ml) were treated for 20 min at 37 °C with the various bile salts. The solubilized membrane material was separated from the nonsoluble residue by centrifugation at 100 000 × g for 1 h [1, 5]. For reaggregation, the solubilized mem-

brane material was dialyzed for 4 days in the cold against 1000 vol. of dilute β-buffer containing 20 mM $MgCl_2$. The reaggregated material was collected by centrifugation at 37 000 × *g* for 1 h.

Digestion by pronase. Digestion of isolated membranes or intact cells suspended in β-buffer was carried out by 100 μg/ml pronase (A grade, Calbiochem., Los Angeles, Calif.) at 37 °C. Digestion was stopped by adding an equal volume of ice-cold suspending buffer and immediate centrifugation at 100 000 × *g* for 1 h. The sediment was washed twice, the cells were lysed and enzymic activities were determined on the washed membrane preparations.

Iodination of cells and membrane preparations. Iodination was carried out by the method of Phillips and Morrison [10] as described in detail by Amar et al. [6].

Treatment with antisera. Antiserum to isolated membranes was prepared in rabbits as described by Kahane and Razin [11]. The inhibitory effect of the antiserum on enzymic activities of the membranes was tested in a reaction mixture containing either isolated membranes or intact cells (containing the equivalent of 1 mg membrane protein/ml) and antiserum diluted 1:10 in 0.25 M NaCl. After incubation at 37 °C for 1 h, the tubes were shaken vigorously to break up the agglutinates, the cells were lysed and the enzymic activities were determined on the isolated membrane preparations.

Assay procedures. Protein was determined according to Lowry et al. [12]. Radioactivity in membrane lipids was determined in a Packard Tri-Carb liquid scintillation spectrometer using the scintillation mixture described before [13]. NADH oxidase (EC 1.6.99.3) activity was measured spectrophotometrically [14]. Results were expressed as decrease in absorbance at 340 nm /min per mg of protein. Adenosine triphosphatase (EC 3.6.1.3) activity was measured by release of inorganic phosphate from ATP [14]. Results were expressed as μmoles of inorganic phosphate released per mg of protein in 30 min. *p*-Nitrophenylphosphatase activity was measured spectrophotometrically by determining the yellow color of the liberated *p*-nitrophenol, using the reaction mixture described previously [1]. Results were expressed as increase in absorbance at 420 nm per mg protein in 1 h. Ribonuclease activity was determined by measuring the absorbance at 260 nm of soluble nucleotides released from RNA according to the method of Eaves and Jefferies [15]. The reaction mixture (1 ml) contained 80 mM Tris–HCl buffer, pH 8.8, 8 mM $MgCl_2 \cdot 6H_2O$, 400 μg of ribonucleic acid (from yeast Type XI, Sigma Co.) and 80–200 μg protein of the tested preparation. The reaction was stopped after 10, 30 and 60 min of incubation at 37 °C by adding 0.25 ml of a 0.75 % (w/v) solution of uranyl acetate in 25 % perchloric acid. The tubes were centrifuged and the absorbance of the supernatant fluids was measured at 260 nm. Deoxyribonuclease activity was determined by the same procedure using 200 μg of highly polymerized DNA (Calbiochem.) as substrate. Results were expressed as increase in absorbance at 260 nm/mg protein per 6 min.

Column chromatography. The wash fluids obtained by washing the membranes with 3 mM Tris–HCl buffer were fractionated on a Sepharose 4B column (2.5 cm × 45 cm). The column was equilibrated and eluted with dilute β-buffer. The void volume was determined by Blue Dextran 2000. Flow rate was about 20 ml/h and fractions of 3.5 ml were collected and analyzed for protein, radioactive lipid, ribonuclease and deoxyribonuclease activities. The chromatographic procedure was carried out at 4 °C.

Gel electrophoresis. Gel electrophoresis was performed in 7 % polyacrylamide

gels containing 0.1 % sodium dodecylsulfate according to Weber and Osborn [16] as described in detail by Amar et al. [6]. Densitometer tracings of the stained gels were made in a Kipp and Zonen Densitometer Model DD2.

Electron microscopy. Thin sections were prepared from pellets of the reaggregated membrane material fixed with 4 % glutaraldehyde and 2 % OsO_4 as described before [17]. The material was embedded in Epon according to Luft [18] and sectioned in an LKB Ultrome -II microtome. The sections were placed on uncoated 400-mesh grids and stained with uranyl acetate and lead citrate [19]. Freeze fracturing of reaggregated membranes was performed by the method of Moor and Mühlethaler [20] in a Balzers' freeze-etching apparatus (Balzers, Fürstentum Liechtenstein) as described in detail by Rottem et al. [21].

RESULTS

Release of membrane-bound enzymes by low-ionic strength buffers

The first two washes of *A. laidlawii* membranes in 100 mM Tris, according to the procedure of Muñoz et al. [9], released about 10 % of the total membrane-associated protein. However, electrophoretic analysis (Fig. 1) indicated that the proteins released in 100 mM Tris were mostly of cytoplasmic origin. Table 1 shows that the two additional washes of membranes in 30 mM Tris released 12 % of the total protein while the two washes in 3 mM Tris released an additional 17 % of the membrane-associated proteins. Of the five enzymic activities tested, only the ribonuclease and deoxyribonuclease were released in considerable quantities in the 3 mM Tris wash fluids. The small amounts of the ATPase, *p*-nitrophenylphosphatase and NADH oxidase activities found in the wash fluids may well represent enzymic activities bound to

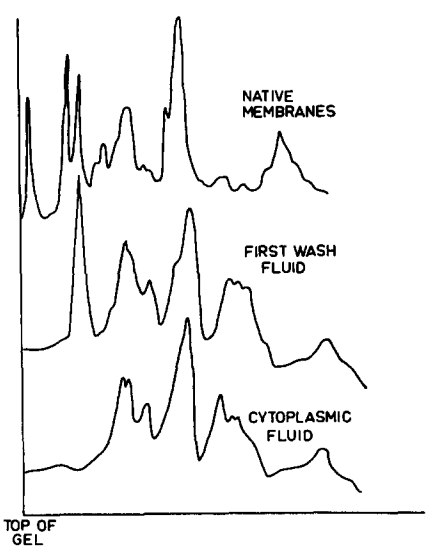

Fig. 1. Densitometer tracings of electrophoretic patterns in polyacrylamide gels of proteins derived from the cytoplasmic fluid obtained after osmotic lysis of *A. laidlawii* cells; first wash fluid of the isolated membranes in 100 mM Tris, and the washed membranes. The pattern of the wash fluid resembles that of the cytoplasmic fluid in most parts.

TABLE I

RELEASE OF MEMBRANE COMPONENTS AND ENZYMES BY LOW-IONIC STRENGTH BUFFERS

For details of the washing procedure see Materials and Methods.

Fraction	Percent of total						
	Protein	Lipid	ATPase	*p*-Nitrophenyl-phosphatase	NADH oxidase	Ribo-nuclease	Deoxy-ribonu-clease
30 mM Tris wash fluids	12.0	3.0	4.5	2.6	3.6	1.6	8.0
3 mM Tris wash fluids	17.0	15.5	6.3	5.3	5.7	43.1	51.0
Washed membranes	71.0	81.5	89.2	92.1	90.7	55.3	41.0

minute nonsedimentable membrane fragments produced during the washing procedure, as evidenced by the decrease in the amount of sedimentable membrane lipid (Table I).

Fig. 2 compares the release of ribonuclease and ATPase activities in each of the steps of the washing procedure. This figure shows clearly that the most crucial step in the release of the ribonuclease activity is that in which the ionic strength of the

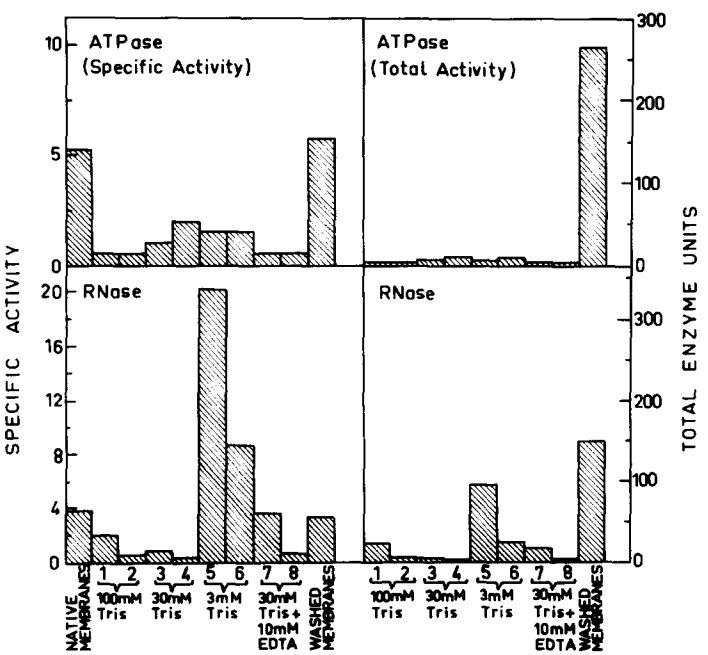

Fig. 2. Distribution of the ATPase and ribonuclease activities in the various wash fluids of *A. laidlawii* membranes treated by a modification of the washing procedure of Muñoz et al. [9].

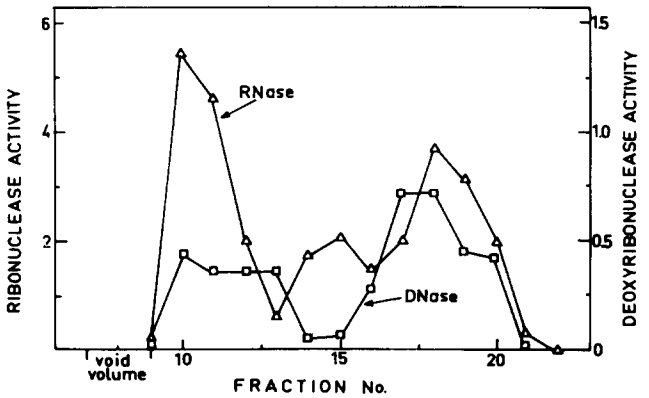

Fig. 3. Ribonuclease and deoxyribonuclease activities in the fractions obtained by filtration of the nuclease-enriched 3 mM Tris wash fluids through a Sepharose 4B column.

washing solution is decreased 10-fold, from 30 mM Tris to 3 mM Tris. The last two washes with buffer containing 10 mM EDTA failed to release any significant quantities of the nucleolytic enzymes (Fig. 2).

Filtration of the nuclease-enriched 3 mM Tris -wash fluids through a Sepharose 4B column showed a different distribution of the ribonuclease and deoxyribonuclease activities in the fractions collected (Fig. 3), indicating that the two activities do not represent the activity of a single nonspecific phosphodiesterase. Moreover, the two nucleolytic activities are also distributed differently between the cytoplasmic and membrane fractions obtained by osmotic lysis of *A. laidlawii* cells. While approximately one-half of the total ribonuclease activity of the cell is membrane bound, only one-tenth of the cell deoxyribonuclease activity is associated with the membrane, the rest being found in the cytoplasm (see also ref. 14).

TABLE II

SOLUBILIZATION OF MEMBRANE COMPONENTS AND ENZYMES BY BILE SALTS

Enzymic activity, expressed as percentage of the enzymic activity of native membrane; A, total activity in lysed membranes; B, activity in soluble fraction.

Bile salt	Percent in soluble fraction		Enzymic activity							
	Protein	Lipid	ATPase		NADH oxidase		*p*-Nitrophenyl-phosphatase		Ribonuclease	
			A	B	A	B	A	B	A	B
Deoxycholate (50 mM)	83	92	0	0	92	61	28	6	123	117
Taurodeoxycholate (50 mM)	72	85	29	0	56	16	34	16	224	206
Cholate (100 mM)	47	70	29	1	40	1	69	1	133	121
Taurocholate (100 mM)	45	61	40	1	63	2	91	4	203	181

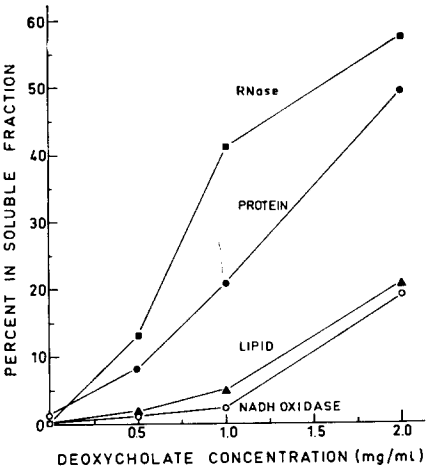

Fig. 4. Solubilization of membrane components and enzymes by low concentrations of deoxycholate. For experimental details see Materials and Methods.

It could have been argued that the ribonuclease activity detected in the cytoplasmic fraction originates from the release of membrane-bound ribonuclease during cell lysis in deionized water. However, cell lysis carried out in 10 mM Tris or in 0.25 mM NaCl containing 50 μg digitonin/ml [22] did not reduce the percentage of cell ribonuclease activity found in the cytoplasm, speaking against the possibility that cytoplasmic ribonuclease originates from membrane-bound ribonuclease released during osmotic lysis in low-ionic strength media.

Solubilization of membrane-bound enzymes by bile salts

Table II shows that the various bile salts differ in their capacity to solubilize the *A. laidlawii* membrane components. Of the bile salts tested, deoxycholate appears to

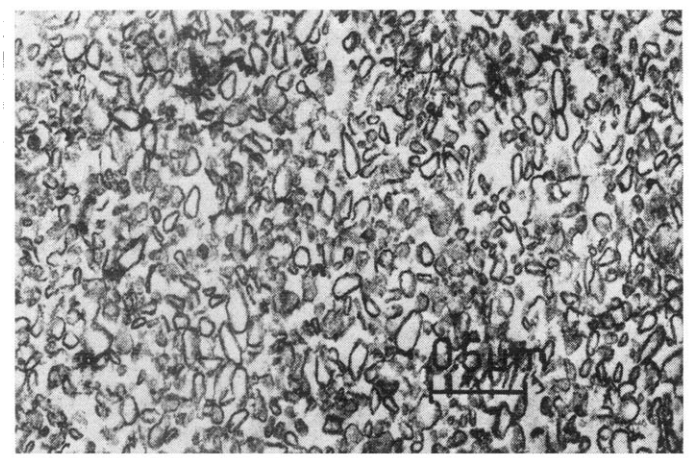

Fig. 5. Thin section of reaggregated material from *A. laidlawii* membranes solubilized by taurocholate. The vesicular nature of the reaggregated material is clearly seen.

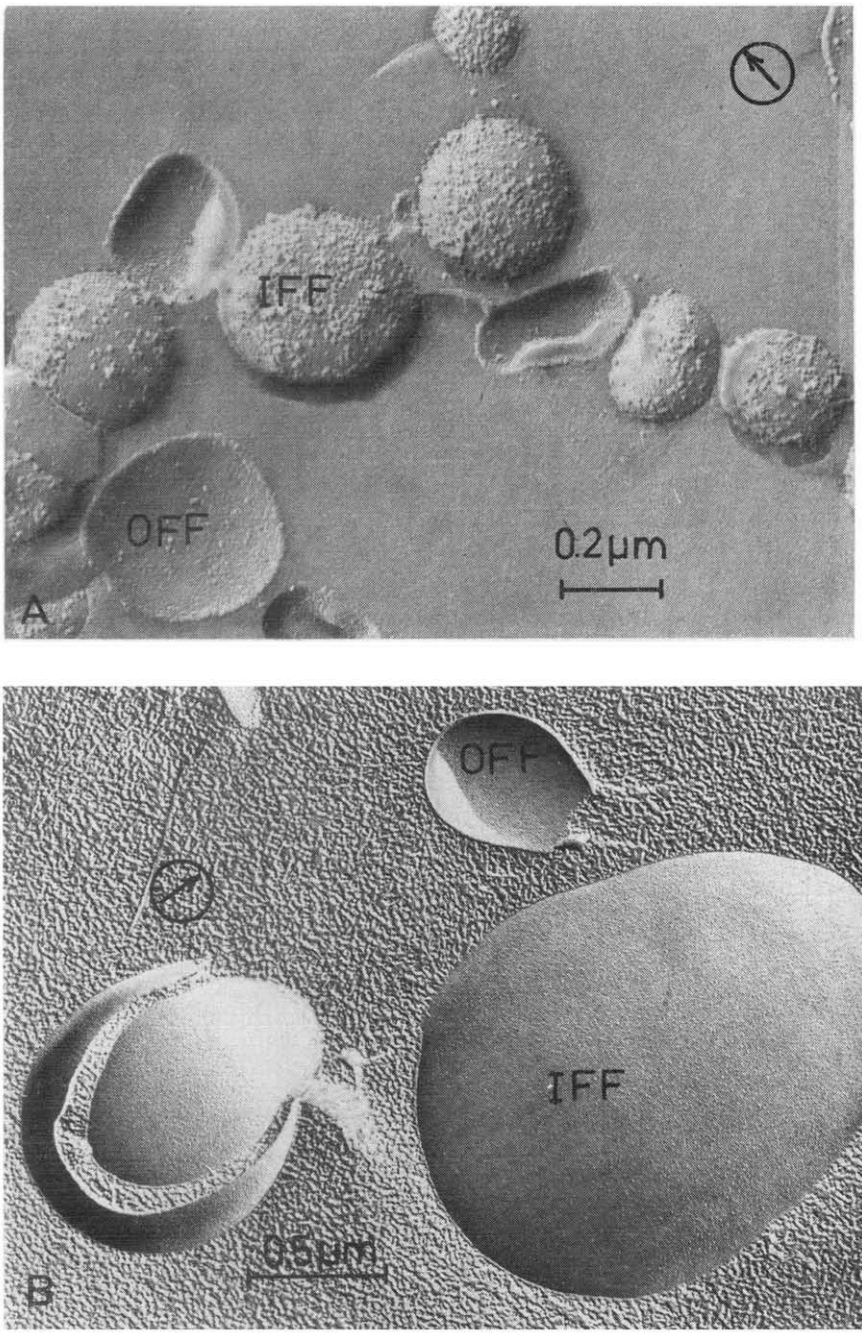

Fig. 6. Replicas of freeze-cleaved native (A) and reaggregated (B) membranes of *A. laidlawii*. The reaggregated membranes were obtained from cholate-solubilized material. The relatively smooth fracture faces of the reaggregated membranes are contrasted with the particle-studded fracture faces of the native membranes. IFF, inner (convex) fracture face; OFF, outer (concave) fracture face. The arrow indicates the direction of shadowing.

be the best solubilizing agent. ATPase activity was essentially absent from any of the soluble fractions shown in Table II. It should be pointed out that the membrane-bound ribonuclease activity was solubilized much more effectively than any of the other enzymic activities tested and was actually stimulated by the solubilization process. Essentially, it was completely solubilized even under conditions when more than half of the membrane protein was still found in the nonsoluble residue (Table II). This point could be demonstrated even more clearly when solubilization was carried out at low deoxycholate concentrations (Fig. 4).

Reaggregation of solubilized membrane-bound enzymes

The bile-salt solubilized membrane material reaggregated to membranous structures on dialysis against dilute β-buffer containing 20 mM $MgCl_2$. In thin sections the reaggregated membranes resembled the native membranes in thickness and trilaminar profile, and most often formed vesicular structures (Fig. 5). However, the fracture faces of the reaggregated membranes differed from those of the native membranes in the absence of the characteristic particles (Fig. 6).

With the exception of the material solubilized by taurodeoxycholate about 85 % of the solubilized membrane protein and over 90 % of the solubilized lipid was incorporated into the reaggregated membranes (Table III). As to the enzymic activities, the incorporation of ribonuclease into the reaggregated membranes was much lower than that of the NADH oxidase and *p*-nitrophenylphosphatase. The portion of the solubilized ribonuclease which was incorporated into the reaggregated membranes showed a higher degree of resistance to release by washing in 3 mM Tris, when compared to the ribonuclease bound to the native membranes (Table IV).

It seemed of interest to find out whether the reaggregated membranes formed sealed vesicles. Properly sealed vesicles may enclose soluble components which are not membrane bound. Moreover, enzymes located on the inner membrane surface may fail to act if their substrate cannot penetrate into the vesicle. Several lines of evidence indicate that the reaggregated membrane vesicles obtained in our study are not sealed.

TABLE III

REAGGREGATION OF SOLUBILIZED MEMBRANE COMPONENTS AND ENZYMIC ACTIVITIES

Bile salt	Percent reaggregated*				
	Protein	Lipid	NADH oxidase	*p*-Nitrophenyl phosphatase	Ribonuclease
Deoxycholate (50 mM)	89	91	84	91	56
Taurodeoxycholate (50 mM)	67	42	16	32	11
Cholate (100 mM)	85	92	100	86	39
Taurocholate (100 mM)	84	92	80	40	41

* Percent of the total dialysis bag content.

TABLE IV

RELEASE OF PROTEINS AND ENZYMES FROM NATIVE AND REAGGREGATED *A. LAIDLAWII* MEMBRANES BY LOW-IONIC STRENGTH BUFFERS

Reaggregated membranes, obtained from membrane material solubilized by deoxycholate.

Fraction	Percent of total					
	Native membranes			Reaggregated membranes		
	Protein	NADH oxidase	Ribo-nuclease	Protein	NADH oxidase	Ribo-nuclease
30 mM Tris wash fluids	12	3.6	1.6	11.8	5.4	9.1
3 mM Tris wash fluids	17	5.7	43.1	13.3	7.6	10.3
Washed membranes	71	90.7	55.3	74.9	87.0	80.6

Thus, the disruption of the vesicles by sonication did not increase the NADH oxidase and ribonuclease activities, and even decreased the *p*-nitrophenylphosphatase activity of reaggregated membranes. Our results also indicate that the macromolecule lactoperoxidase has access to proteins on both sides of the reaggregated membrane vesicles since the lactoperoxidase-mediated iodination values of reaggregated membranes were four to five times greater than those of membranes of intact cells (Table V). Nevertheless, the high iodination values of the reaggregated membranes may also be due to factors other than imperfect vesicularization, such as the unfolding and different organization of the polypeptide chains in the reaggregated membranes (see Discussion). More direct evidence for imperfect vesicularization was obtained on examination of thin sections of reaggregated membrane vesicles. Small discontinuities in the membranes could often be observed at high magnification (Fig. 7).

Localization of the membrane-bound enzymic activities

Enzymic activities of intact *A. laidlawii* cells and isolated membranes are compared in Table VI. The nucleases and *p*-nitrophenylphosphatase exhibited about the same activities when tested with intact cells or isolated membranes, whereas the NADH

TABLE V

LABELING OF MEMBRANES OF INTACT CELLS, ISOLATED MEMBRANES AND REAGGREGATED MEMBRANES BY THE LACTOPEROXIDASE-MEDIATED ^{125}I IODINATION

Preparation	Radioactivity (cpm/mg membrane protein)	Comparison of labeling*
Membranes of intact cells	519 000	1.0
Isolated membranes	1 607 000	3.1
Reaggregated membranes from:		
Sodium dodecylsulfate	2 243 000	4.3
Taurocholate	2 514 000	4.8
Deoxycholate	2 629 000	5.1

* Compared to that of membranes of intact cells.

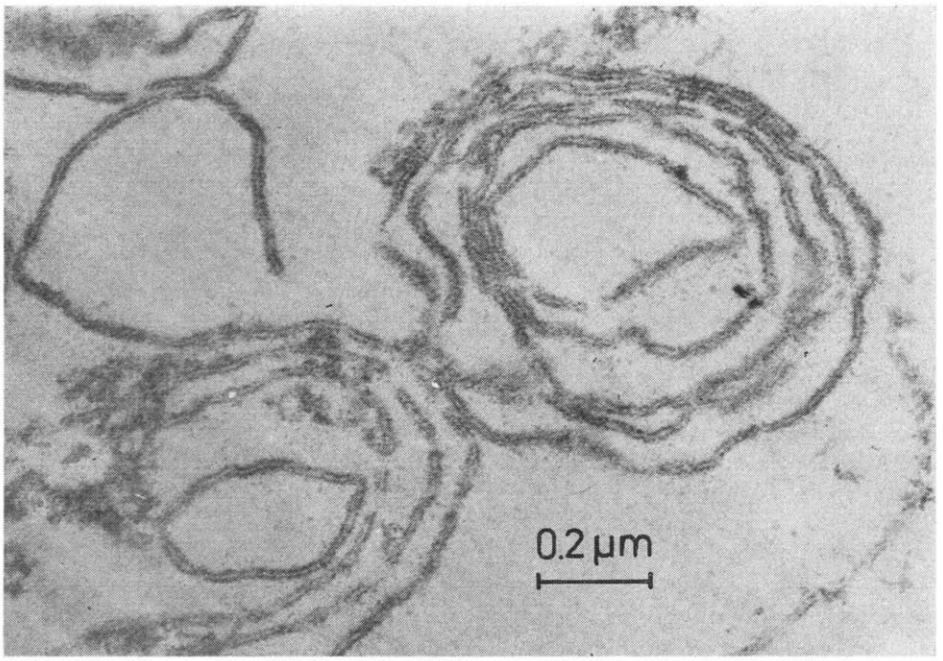

Fig. 7. Thin section of reaggregated material from *A. laidlawii* membranes solubilized by cholate. The discontinuities in the reaggregated membranes indicate that the "onion-like" vesicles are not sealed.

oxidase and ATPase activities were much higher when tested with isolated membranes, where the inner membrane surface is also exposed to the substrate.

Although the ATPase and NADH oxidase activities of isolated membranes were very sensitive to inactivation by pronase, they resisted inactivation when treatment was carried out on intact cells, indicating that these enzymic activities are not exposed on the outer membrane surface (Table VII). The degree of ribonuclease inactivation by pronase was about the same with both intact cells and isolated membranes, while *p*-nitrophenylphosphatase was essentially unaffected in both cases. It must be stressed that pronase acted only suboptimally under the conditions of the experiment. Less than 20 % of the total membrane protein was digested on incubation of isolated mem-

TABLE VI

ENZYMIC ACTIVITIES OF *A. LAIDLAWII* CELLS AND ISOLATED MEMBRANES

Specific activity, calculated per mg of membrane protein.

Preparation	Specific activity				
	ATPase	*p*-Nitrophenyl-phosphatase	NADH oxidase	Ribonuclease	Deoxyribo-nuclease
Intact cells	1.3	5.9	0.3	8.3	1.3
Isolated membranes	9.8	4.9	3.1	7.7	0.7

TABLE VII

EFFECT OF PRONASE TREATMENT ON ENZYMIC ACTIVITIES OF *A. LAIDLAWII* MEMBRANES

Preparation, for experimental details see Materials and Methods; specific activity calculated per mg of membrane protein.

Preparation	Specific activity			
	ATPase	*p*-Nitrophenyl-phosphatase	NADH oxidase	Ribonuclease
Isolated membranes	9.8	5.35	4.3	5.4
Isolated membranes treated with pronase	0	5.22	1.0	2.4
Membranes of cells treated with pronase	8.8	5.28	3.8	1.5

branes with 100 μg pronase/ml for 15 min at 37 °C. The high-ionic strength β-buffer included in the reaction mixture to protect the cells from osmotic lysis was found to be responsible for the repression of pronase activity. When the buffer was diluted 1:20 in deionized water, pronase digested over 55 % of the total membrane protein under the same conditions of test.

Further support for the localization of the ATPase and NADH oxidase on the inner membrane surface was provided by experiments in which intact cells and isolated membranes were treated with an antiserum to *A. laidlawii* membranes. Table VIII shows that the antiserum caused partial inhibition of the ATPase and NADH oxidase activities when isolated membranes were treated, but had no effect when treatment was carried out on intact cells.

TABLE VIII

EFFECT OF ANTISERUM ON ENZYMIC ACTIVITIES OF *A. LAIDLAWII* MEMBRANES

Preparation, isolated membranes or intact cells were treated with immune or normal serum as described in Materials and Methods. The cells were then lysed and the enzymic activities were determined on the isolated membrane preparations. Specific activity, calculated per mg of membrane protein.

Serum	Preparation	Specific activity	
		ATPase	NADH oxidase
Antiserum to *A. laidlawii* membranes	Isolated membranes	4.2	1.4
	Membranes of intact cells	11.2	2.2
Normal serum (control)	Isolated membranes	11.6	2.2
	Membranes of intact cells	11.7	2.0

DISCUSSION

A definitive answer to the question of how much of the *A. laidlawii* membrane protein consists of peripheral proteins cannot be given because the amount seems to depend on the handling of the membrane during its isolation. The isolation of *A. laidlawii* membranes by osmotic lysis of the organisms in deionized water or in very dilute buffer, and subsequent washings of the membranes probably cause the loss of some of the peripheral membrane proteins. The addition of Mg^{2+} to the lysis mixture, a practice successfully utilized to minimize peripheral protein loss from bacterial protoplast membranes [9], could not be applied in this case because divalent cations, even at extremely low concentrations, protect mycoplasma cells from osmotic lysis [23].

Our finding that the *A. laidlawii* ATPase cannot be released from the membrane by low-ionic strength buffers and EDTA is of great phylogenetic interest as all the microbial ATPases characterized so far can be released in this way in a lipid-free and water-soluble form and may thus be regarded as peripheral membrane proteins [24–30]. The inclusion of the mycoplasma ATPases in the integral membrane protein group, as are the ATPases of the plasma membranes of eukaryotes [31, 32], gains further support from the recent demonstration of the dependence of mycoplasmal ATPase activity on the physical state of membrane lipids [33, 34].

Of the enzymic activities of *A. laidlawii* membranes tested by us only the ribonuclease and deoxyribonuclease activities were released in significant quantities by washing in low-ionic strength buffer. Yet, a significant percentage (up to 50 %) of the nucleolytic activity remained membrane bound. Does this result reflect the presence of different membrane-bound enzymes acting on the same substrate, or is this due to small conformational changes in only some of the enzyme molecules rendering them more hydrophobic than the others? Our data are insufficient to decide this point. Similar findings are available for other microbiol membranes. Thus, the *Escherichia coli* ATPase could not be completely released from the membrane by washings with low-ionic strength buffer; the amount released varied from about 30 % [28] to about 90 % [30] of the total membrane ATPase activity, depending, it seems, on variations in the washing procedure.

Since, by definition, the peripheral membrane proteins are located on the membrane surfaces, it might be expected that these will be the first membrane components to be detached by detergents, as was in fact shown for the ribonuclease activity in our study (Table II, Fig. 4) and for the *E. coli* ATPase, which was released from the membrane by a low concentration (0.04 %) of sodium dodecylsulfate [35]. The high susceptibility of the peripheral membrane proteins to detachment by low detergent concentrations may explain our previous finding [1] that more membrane protein than lipid is solubilized at low detergent concentrations, the reverse being true at high detergent concentrations.

Our study provides more information on the ultrastructure and organization of reaggregated *A. laidlawii* membranes. Bile salts, which are milder detergents than the sodium dodecylsulfate used so far in studies on reaggregation of mycoplasma membranes [36, 37], were employed to solubilize the membranes. We had hoped that the bile salts would cause minimal conformational changes in membrane proteins, enabling the formation of reaggregated membranes which would resemble the native

membranes in molecular organization. However, the data obtained do not seem to justify these hopes. The fracture faces of the reaggregated membranes obtained from material solubilized by bile salts, like those of reaggregated membranes produced from sodium dodecylsulfate solubilized membrane material [38], lacked the particles appearing on the fracture faces of the native membranes. This suggests that the reaggregated membrane core is built of a lipid bilayer with very little, if any, protein embedded in it [36, 37]. Accordingly, most of the proteins are exposed on the surface of the reaggregated membranes. The higher labeling by the [^{125}I]lactoperoxidase system of the reaggregated membrane proteins as compared to the labeling of the native membrane proteins (Table V) supports this suggestion.

The asymmetrical distribution of proteins on the inner and outer membrane surfaces of *A. laidlawii* and *Mycoplasma hominis* has already been reported in a previous communication of this series [6]. The present communication extends these observations to the localization of several of the membrane-bound enzymic activities in *A. laidlawii*. Our results clearly indicate that the NADH oxidase and ATPase activities are located on the inner or cytoplasmic membrane surface while the membrane-bound nucleases appear to be exposed on the outer membrane surface. The relative resistance of the *p*-nitrophenylphosphatase to inactivation by pronase (Table VII) suggests that the enzyme is embedded within the membrane and in this way is protected from proteolysis. Detergents, which should expose this enzyme, inactivate it at low concentrations [1].

ATPase has also been localized histochemically on the inner side of the cell membrane of *Mycoplasma gallisepticum* [39], as has been found for the ATPase of *Micrococcus lysodeikticus* [40]. Considering that the ATPase and NADH oxidase activities fulfill a key role in respiration and energy coupling, their localization on the cytoplasmic surface of the plasma membrane was to be expected. The membrane-bound nucleases of *A. laidlawii*, which are at least partially exposed on the external membrane surface, may function in the provision of transportable nucleotides and nucleosides from macromolecular nucleic acids present in the growth medium [41].

ACKNOWLEDGEMENT

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