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CHARACTERIZATION OF THE MYCOPLASMA MEMBRANE PROTEINS

II. SOLUBILIZATION AND ENZYMIC ACTIVITIES OF *ACHOLEPLASMA LAIDLAWII* MEMBRANE PROTEINS

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SUMMARY

Treatment of *Acholeplasma laidlawii* membranes with EDTA in low-ionic strength media released about 11 % of the total membrane protein in a water-soluble form. The released protein fraction had no NADH oxidase, ATPase and *p*-nitrophenylphosphatase activities. The strongly ionic detergents sodium dodecyl sulfate and cetyltrimethylammonium bromide were more effective in the solubilization of *A. laidlawii* membranes than the nonionic detergents Triton X-100, Lubrol W or Brij 58. Sodium deoxycholate occupied an intermediate position. The solubilization of the membranes by detergents affected their NADH oxidase, ATPase and *p*-nitrophenylphosphatase activities in two antagonistic ways: activation and inactivation. The balance of these processes depended on the type and concentration of the detergent used and on the enzymic activity tested. The activation effect was most pronounced with low concentrations of the nonionic detergents and with *p*-nitrophenylphosphatase activity. Inactivation of the enzymes was most pronounced with sodium dodecyl sulfate and cetyltrimethylammonium bromide. The results of the present study favor the use of nonionic detergents for the solubilization and further fractionation of mycoplasma membrane proteins.

INTRODUCTION

Solubilization is a pre-requisite for the fractionation and characterization of membrane proteins. Only a minor fraction of the proteins can be solubilized under mild nondenaturing conditions. These are the ones that are held to the membrane mostly by ionic bonds broken by changes in the ionic strength or pH of the suspending medium, and whose solubilization is usually improved by the addition of a chelating agent, such as EDTA[1–3]. Most membrane proteins, however, are more tightly bound to the membrane lipids, apparently by a combination of hydrophobic and ionic bonds. To break up these composite bonds, more drastic procedures have to be applied. Detergents[4–10] and aqueous organic solvents[11,12] are the most common agents employed for this purpose. Yet these agents are liable to denature the solubilized

protein molecules. The solubilized membrane proteins also tend to aggregate in aqueous media because of their inherent hydrophobic properties and because of the partial denaturation caused by the detergent or organic solvent used[4].

As a preliminary step for the fractionation of the mycoplasma membrane proteins by column chromatography (NE'EMAN *et al.*, in preparation) various methods of solubilization were examined, starting with mild EDTA treatment in media of low ionic strength. Since only a minor part of the mycoplasma membrane proteins was solubilized in this way, detergents had to be employed to effect a more complete solubilization.

MATERIALS AND METHODS

Membrane preparations and EDTA treatment

Acholeplasma laidlawii (oral strain, formerly *Mycoplasma laidlawii* was grown, harvested and lysed and the isolated membranes were washed as described in the accompanying communication[13]. The washed membranes were resuspended in dilute NaCl–Tris–β-mercaptoethanol buffer[13]. and kept at $-20°$ until used. Release of proteins from the washed membranes by prolonged EDTA treatment was carried out according to MARCHESI *et al.*[3]. The soluble fractions collected during the various steps of this procedure were pooled and concentrated by ultrafiltration through a Diaflo PM-10 filter (Amicon N.V. Oosterhout, Holland).

Solubilization of membranes by detergents

Sodium dodecyl sulfate and cetyltrimethylammonium bromide were the products of British Drug Houses, Poole, England. Sodium deoxycholate was purchased from L. Light and Co., Colnbrook, England, and Trition X-100 was obtained from Packard Co., Downers Grove, Ill., U.S.A. Lubrol W was the gift of Imperial Chemical Industries, Manchester, England and Brij 58 was the gift of Atlas Chemical Industries, Wilmington, Del., U.S.A. Membrane suspensions (3.6 mg protein/ml) were treated for 15 min at 37° with the various detergents at concentrations ranging from 0.5 to 16 mg detergent per ml. The solubilized membrane material was separated from the nonsoluble residue by centrifugation at 100000 $\times$ *g* for 1 h at 4°. Protein in the soluble and nonsoluble membrane fractions was determined according to LOWRY *et al.*[15]. Standard curves of bovine serum albumin were prepared in the presence of the detergent concentrations used for membrane solubilization in order to correct for deviations in the results caused by some of the detergents (Triton X-100, Lubrol W, Brij 58 and cetyltrimethylammonium bromide). Electrophoretic analysis of the proteins was carried out by a modification of the Takayama technique[16]. The polyacrylamide gels were stained with 1% Amido Black 10B. Radioactivity of lipids in the membrane fractions was determined in a Packard Tri-Carb liquid scintillation spectrometer using the scintillation mixture described previously[17].

Assay of enzymic activities

NADH oxidase (EC 1.6.99.3) activity was measured spectrophotometrically by determining the decrease in absorbance at 340 nm on addition of NADH to the reaction mixture[18]. Data were expressed as decrease in absorbance at 340 nm per min per mg of protein. Adenosine triphosphatase (EC 3.6.1.3) activity was measured by

the release of inorganic phosphate from ATP[18]. Data were expressed as micromoles 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. The reaction mixture (1.0 ml) consisted of 100 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 10 mM *p*-nitrophenylphosphate and 400 μg of membrane protein. The reaction was stopped after 30 and 60 min of incubation at 37° by adding 2 ml of 0.5 M Tris (not neutralized) in 5 % trichloroacetic acid. The precipitate was removed by centrifugation and the intensity of the yellow color in the supernatant fluid was determined at 420 nm. Data were expressed as increase in absorbance at 420 nm per mg protein in 1 h.

RESULTS

Release of proteins from membranes by EDTA

The proportion of total membrane protein solubilized by the complete Marchesi procedure varied between 8 to 14%, with a mean of 11% in 7 experiments using different batches of membranes. Essentially the same amount of protein was released from membranes pre-washed two, five or ten times alternatively with de-ionized water and with 0.05 M NaCl in 0.01 M phosphate buffer, pH 7.5. Hence the EDTA treatment did not release cytoplasmic proteins adsorbed to the membranes. Only small amounts of membrane lipids were released; the pooled solubilized proteins contained only 0.5% of the total radioactive lipids of the membrane. The electrophoretic pattern of the released proteins was predominated by a heavy band at the center of the gel and was very different from that of the untreated membranes and of the nonsoluble membrane residue. The released protein fraction had no ATPase, *p*-nitrophenylphosphatase and NADH oxidase activities, which were shown to be retained in the nonsoluble membrane residue.

Solubilization of membranes by detergents

Fig. 1 shows the solubilization of *A. laidlawii* membrane proteins and lipids by the various ionic and nonionic detergents. At low detergent concentrations the

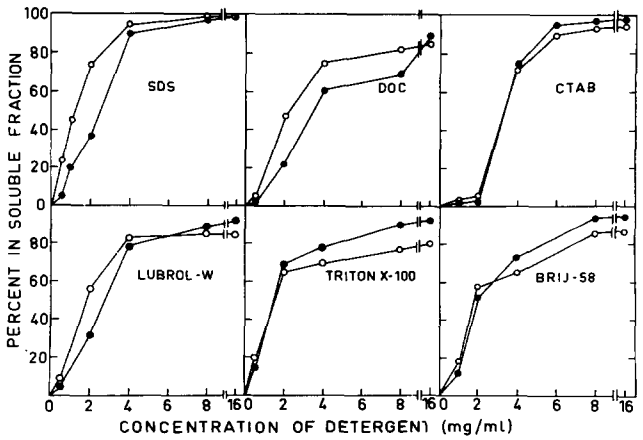

Fig. 1. Solubilization of *A. laidlawii* membrane protein and lipid as a function of the detergent concentration. ○—○, membrane protein; ●— ●, labeled membrane lipid. SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; CTAB, cetyltrimethylammonium bromide.

percentage of membrane proteins solubilized was usually higher than that of membrane lipids. This was reversed at higher detergent concentrations where more lipid than protein was found in the soluble fraction. Fig. 1 also shows that the anionic detergent sodium dodecyl sulfate and the cationic detergent cetyltrimethylammonium bromide solubilized the membranes more effectively than the nonionic detergents Lubrol W, Triton X-100 and Brij 58. The third ionic detergent, sodium deoxycholate, did not solubilize more than 85% of the membrane protein even at 32 mg of detergent per ml, a concentration which solubilized 92% of the lipid (data not shown in figure). As distinct from the solubilization curves of the other detergents the cetyltrimethylammonium bromide curve showed very little solubilization of either protein or lipid at detergent concentrations below 2 mg/ml, followed by a steep increase at higher concentrations. RAZIN AND BARASH[19] have shown that the solubilization of *A. laidlawii* membrane proteins by Triton X-100 is selective. It seemed of interest to test other detergents as well for selectivity. Fig. 2 shows that the degree of selectivity of the membrane protein solubilization depended on the type of detergent used. It was higher with the strongly ionic detergents sodium dodecyl sulfate and cetyltrimethylammonium bromide, where only a few protein bands of the untreated membranes could be detected in the electrophoretic pattern of the nonsoluble residue. The solubility by the other detergents was selective in that certain protein bands were distributed differently in the soluble and the nonsoluble membrane fractions but did not completely disappear from either (Fig. 2).

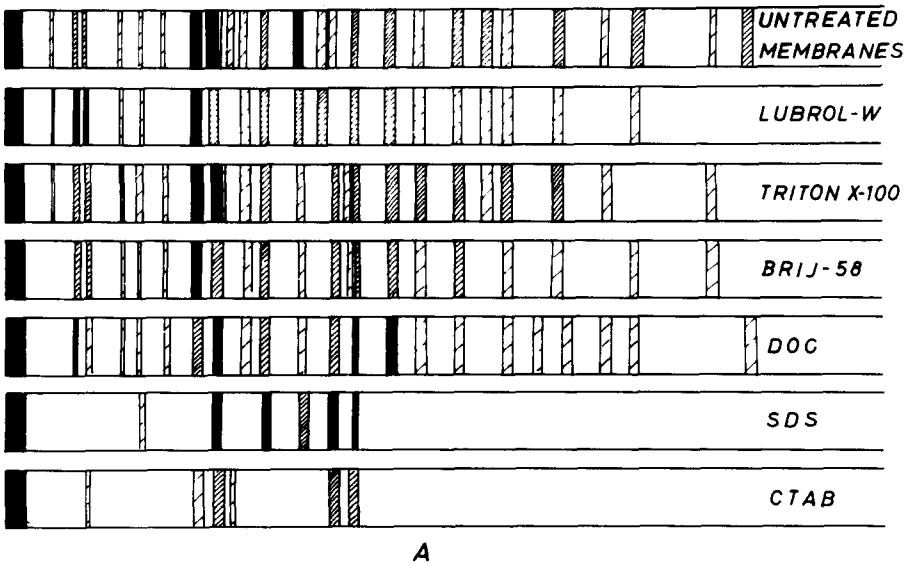

Fig. 2. Schematic representation of the electrophoretic patterns of proteins in the nonsoluble membrane fractions obtained after treatment of *A. laidlawii* membranes with various detergents. The detergents were used at a concentration of 2 mg/ml apart from cetyltrimethylammonium bromide which was used at 4 mg/ml. SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; CTAB, cetyltrimethylammonium bromide.

Solubilization of membrane enzymes by detergents

Fig. 3 shows the distribution of NADH oxidase activity in the soluble and nonsoluble membrane fractions obtained on treatment of the *A. laidlawii* membranes

with different concentrations of ionic and nonionic detergents. Low concentrations of the ionic detergents sodium dodecyl sulfate and cetyltrimethylammonium bromide inactivated the NADH oxidase. Hence, very little of this enzymic activity could be detected in the soluble membrane fraction. The three nonionic detergents and sodium deoxycholate were less harmful; almost 100% of the initial enzymic activity of the membranes could be found in the soluble fraction obtained at 16 mg/ml of Triton X-100, sodium deoxycholate or Brij 58. An activation effect resulting in an increase in total enzyme units was noted with several of the detergents, especially at low concentrations (Fig. 3). For *p*-nitrophenylphosphatase activity alone it was still more pronounced (Fig. 4). However, though this enzymic activity was enhanced

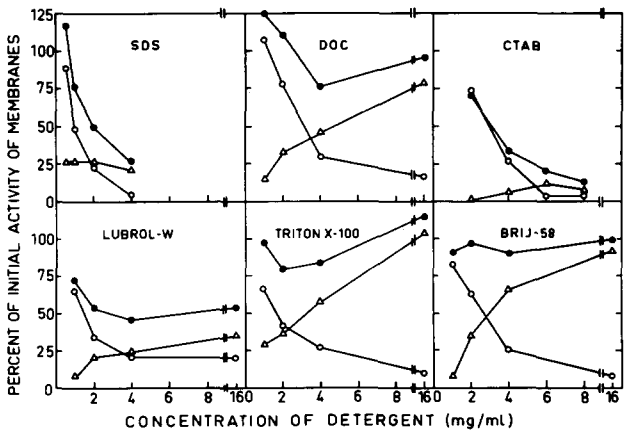

Fig. 3. Solubilization of NADH oxidase activity of *A. laidlawii* membranes by detergents. ●—●, total activity in lysed membranes; ○—○, activity in the nonsoluble fraction sedimented by centrifugation at 100000 × *g* for 1 h; △—△, activity in the soluble fraction. SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; CTAB, cetyltrimethylammonium bromide.

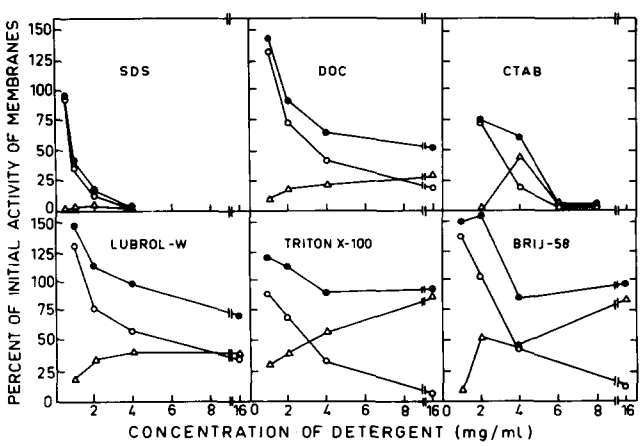

Fig. 4. Solubilization of *p*-nitrophenylphosphatase activity of *A. laidlawii* membranes by detergents. ●—●, total activity in lysed membranes; ○—○, activity in the nonsoluble fraction sedimented by centrifugation at 100000 × *g* for 1 h; △—△, activity in the soluble fraction. SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; CTAB, cetyltrimethylammonium bromide.

at low concentrations of sodium deoxycholate, Lubrol W and Brij 58, it was more susceptible than the NADH oxidase to inactivation by the higher detergent concentrations needed for a substantial solubilization of membrane material. Thus, only up to 80% of the initial *p*-nitrophenylphosphatase activity could be found in the soluble membrane fraction obtained with 16 mg/ml of Triton X-100 or Brij 58 (Fig. 4). ATPase activity of the membranes was generally more susceptible to inactivation by detergents than *p*-nitrophenylphosphatase activity. Thus, sodium dodecyl sulfate and cetyltrimethylammonium bromide inactivated the ATPase at very low concentrations sufficient to solubilize only a small fraction of the membranes. Some ATPase activity remained and could be found in the soluble membrane fraction when Brij 58 was used for solubilization (Fig. 5).

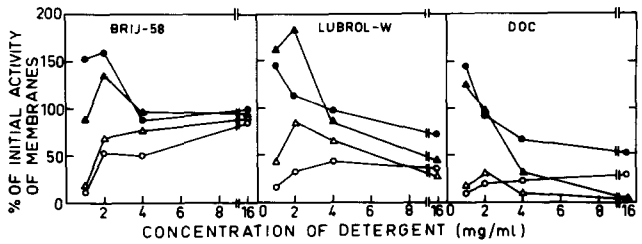

Fig. 5. Comparison of the effect of detergents on the *p*-nitrophenylphosphatase and ATPase activities of *A. laidlawii* membranes. ●—●, total *p*-nitrophenylphosphatase activity in lysed membranes; ○—○, *p*-nitrophenylphosphatase activity in soluble fraction; ▲—▲, total ATPase activity in lysed membranes; △—△, ATPase activity in soluble fraction. The soluble fractions were obtained by centrifugation of the lysed membranes at 100000 × *g* for 1 h. DOC, sodium deoxycholate.

DISCUSSION

The prolonged treatment of *A. laidlawii* membranes with EDTA in low ionic-strength media caused the release of about 11% of the total membrane protein in a water-soluble, almost lipid free form. Erythrocyte membranes treated in a similar manner released spectrin, a protein which accounts for about 20% of the total membrane protein[3]. The electrophoretic profile of the water-soluble protein fraction released from *A. laidlawii* membranes is also predominated by a particular protein, but whether it is related to spectrin remains to be determined. The water-soluble membrane proteins did not contain the Mg^{2+}-activated ATPase present in the native *A. laidlawii* membranes. Thus, the *A. laidlawii* ATPase appears to differ from the ATPases of *Streptococcus faecalis* and *Micrococcus lysodeikticus* which can be solubilized by treatment of the membranes in media of low ionic strength[1,2].

Since preliminary experiments in our laboratory using organic solvents such as *n*-butanol[4,12] or aqueous pyridine[11] for the solubilization of the mycoplasma membrane proteins had shown little promise, our efforts were concentrated on detergents. Not unexpectedly, the ionic detergents were found to be more effective in solubilizing mycoplasma membrane components than the nonionic detergents, but also caused more intensive denaturation of the membrane proteins, as indicated by enzyme inactivation. Sodium deoxycholate appears to occupy an intermediate position, in these respects, between the strongly ionic and the nonionic detergents.

Not only were the membrane lipids and proteins solubilized at different rates, but the different membrane proteins themselves varied in their solubilization rates, so that the solubilization process does not consist of a random disaggregation of the membrane. Evidence for the selective solubilization of membrane enzymes by detergents is available for many types of biological membranes. Thus, Triton X-100 was found to solubilize the Ca^{2+}-dependent ATPase of the sarcoplasmic reticulum membranes, leaving the basic ATPase activity in the nonsoluble residue[6]. Through the solubilization of rat liver plasma membranes by 1% sodium deoxycholate a fraction rich in ATPase activity could be separated[7]. Differences in the solubilization by sodium dodecyl sulfate of the NADH dehydrogenase and succinic dehydrogenase activities of *Bacillus subtilis* membranes were demonstrated by BISHOP *et al.*[20]. Triton X-100, Nonidet P-40 and sodium deoxycholate were also found to dissociate chloroplast membranes from various sources in a non-random fashion, allowing the partial separation of several photochemical systems[8–10].

It appears that during membrane solubilization by detergents two antagonistic processes are taking place simultaneously: the activation or unmasking of some enzymic activities and the denaturation and consequent inactivation of the solublized enzymes. In the outcome, as measured by the total enzymic activity or "enzyme units" in the solubilized material, one effect or the other is the stronger according to the type and concentration of the detergent used. Thus, with the nonionic detergents at low concentrations the activation effect is more pronounced. The strongly ionic detergents sodium dodecyl sulfate and cetyltrimethylammonium bromide exert their denaturing effect at a concentration so low that the balance is in favor of inactivation. The activation of enzymic activities during membrane solubilization has been described for a variety of membranes and detergents[18,21–23]. This effect may be explained by the sterically hindered catalytic sites of the enzyme being exposed to the substrate during membrane solubilization[21]. Inactivation may be due to the disruption of the quarternary structure of the enzyme protein when it is built of several subunits such as several membrane ATPases[1,2]. The detergent might also inactivate the enzymes by causing conformational changes in the tertiary or even the secondary structure of the enzyme protein, by the separation of the enzyme protein from membrane lipids found to be essential for the activity of several membrane enzymes[24], or simply by binding to the active sites of the enzymes.

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