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THE NATURE AND LOCATION OF *ACHOLEPLASMA LAIDLAWII* MEMBRANE PROTEINS INVESTIGATED BY TWO-DIMENSIONAL GEL ELECTROPHORESIS *

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Summary

The high resolution, two-dimensional electrophoresis system for the separation of proteins described by O'Farrell, (O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007—4021) has been modified for the separation of *Acholeplasma laidlawii* proteins.

Reproducible protein patterns have been obtained from *A. laidlawii* cell, membrane and soluble protein preparations. The isoelectric focusing of membrane proteins was greatly improved by removing the bulk of the membrane lipid before solubilizing the protein.

A. laidlawii peripheral membrane proteins were removed from the membrane by low ionic strength washing and by treatment with EDTA. The effect of an exhaustive EDTA treatment and a rapid, warm EDTA treatment were compared. By comparing the protein patterns obtained in these ways it was possible to distinguish two separate groups of peripheral membrane proteins and one integral membrane protein group. The peripheral membrane proteins which were removed from the membrane at low ionic strength (group I) were also insoluble in Triton X-100, whereas additional peripheral membrane proteins extractable by subsequent EDTA treatment (group II) were soluble in Triton X-100.

Exterior-facing membrane proteins were distinguished from the interior-facing ones by lactoperoxidase-catalyzed iodination of intact cells and membranes. Group I peripheral membrane proteins faced the cell interior whereas group II proteins faced the cell exterior. We counted approximately 320

* A preliminary report of part of this work was presented at the eleventh Australian Biochemical Society Meeting (Archer, D.B., Rodwell, A.W. and Rodwell, E.S. (1978) Proc. Aust. Biochem. Soc. 11, 109).

Abbreviations: SDS, sodium dodecyl sulphate; TEMED, *N,N,N′,N′*-tetramethyl-ethylenediamine; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulphonic acid.

individual whole cell proteins. Of these, about 140 were membrane associated and a maximum of 40 proteins were iodinated after iodinating intact cells.

A. laidlawii was also grown in the presence of $NaH_2{}^{32}PO_4$ and whole cell proteins were separated by two-dimensional gel electrophoresis. One membrane protein and two soluble proteins were labelled.

Introduction

Studies on the distribution of proteins in cell membranes have relied extensively upon sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis to separate individual proteins from protein mixtures. This technique has been invaluable with membranes that have only a few proteins, such as the erythrocyte membrane. Its use is limited where more complex mixtures of proteins need to be separated. We have adapted the high resolution two-dimensional gel electrophoretic system originally described by O'Farrell [1] to a study of the distribution of proteins in the *Acholeplasma laidlawii* membrane. *A. laidlawii* is a mycoplasma which has been widely used as an experimental organism in membrane studies [2].

It is accepted that membrane proteins may be classed as peripheral or integral depending upon the closeness of their association with the lipid bilayer [3]. Peripheral membrane proteins are bound to the membrane surfaces and are removed from membranes by mild treatment such as an alteration of the ionic strength or by chelating agents, whereas integral membrane proteins are more intimately associated with the membrane and their removal needs more drastic treatment [3]. In *A. laidlawii* membranes, ATPase, NADH oxidase, *p*-nitrophenyl phosphatase [4–6] and thioesterase [7] enzyme activities belong to the integral membrane protein group whereas significant amounts of ribonuclease and deoxyribonuclease activities are released from *A. laidlawii* membranes at low ionic strength and in the presence of EDTA [5]. Proteins are distributed asymmetrically in the *A. laidlawii* membrane [5,8] but the extent of this asymmetry is unknown. The ATPase and NADH oxidase enzymes are located on the cell interior membrane surface, whereas the nuclease enzymes are on the cell exterior membrane surface [5].

We have been able to separate the individual proteins of the *A. laidlawii* membrane in two dimensions. We have compared the effect of low ionic strength upon *A. laidlawii* membranes with exhaustive EDTA extraction [9] and with rapid EDTA treatment [10,11] and have been able to distinguish peripheral and integral membrane proteins. By using the lactoperoxidase-catalyzed iodination procedure [12,13] we have located a number of membrane proteins on either the cell interior or exterior surfaces of the membrane.

Methods

Growth of cells

The organism used throughout this work was *A. laidlawii* oral strain (originally from Dr. S. Razin, the Hebrew University, Hadassah Medical School, Jerusalem, Israel). The cells were grown in a modified Edward medium [14] at

37°C. The sodium chloride in this medium was replaced by 0.075 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (pH 7.8).

The organism was grown in the presence of either protein hydrolysate uniformly labelled with 10 μCi ^{14}C/ml or 0.1 mCi sodium dihydrogen ortho[^{32}P]-phosphate/ml. Both radioactive materials were obtained from the Radiochemical Centre, Amersham, U.K. For growth in the presence of ^{32}P the potassium phosphate in the growth medium was replaced by potassium chloride.

The cells were harvested at the late logarithmic stage of growth by centrifugation in a Sorvall RC2-B centrifuge for 15 min at 17 300 × *g*. Cells grown in the presence of protein hydrolysate uniformly labelled with ^{14}C were washed once in 0.15 M sodium phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$ and 1% (w/v) casamino acids (buffer I), and once in the same buffer without the casamino acids (buffer II). Cells grown in the presence of ^{32}P were washed twice in 20 mM HEPES buffer (pH 7.2) containing 0.15 M NaCl, 10 mM $MgSO_4$ and 2 mM dithiothreitol to lessen the possibility of phosphate exchange.

Preparation of membranes and soluble proteins

Membranes were prepared by hypotonic lysis of washed cells. The cell pellet from 5 ml culture was suspended in 100 μl 2 M glycerol for 10 min at 22°C. This cell suspension was injected by syringe into 5 ml distilled water at 0°C where the cells lysed. Membranes were harvested by centrifugation in a Sorvall RC2-B centrifuge for 20 min at 39 100 × *g* and washed twice in 20 mM Tris-HCl (pH 7.1). The membrane pellet was suspended in 0.2 ml 20 mM Tris-HCl (pH 7.1)/1 mM $MgSO_4$ with 10 μl stock DNAase solution added and kept at 0°C for 30 min. 10 μl stock RNAase solution was then added for a further 30 min at 0°C. Stock DNAase solution contained 1 mg pancreatic DNAase/ml 10 mM Tris-HCl (pH 7.5)/1 mM $MgSO_4$. Stock RNAase solution contained 1 mg RNAase/ml 0.1 M sodium EDTA (pH 7.5).

For the preparation of soluble (cytoplasmic) proteins, the supernatants after hypotonic lysis of cells and the membrane washes were combined and concentrated about ten-fold by pressure filtration through a PM 10 Diaflo Ultrafilter using a Model 10PA Ultrafiltration Cell (Amicon, Lexington, Mass., U.S.A.). The resulting protein solution was dialysed for 20 h against two changes of distilled water at 0°C and lyophilized. The protein was then redissolved in 10 mM Tris-HCl (pH 7.5)/1 mM $MgSO_4$ and treated with DNAase and RNAase as described above.

EDTA treatment of membranes

Washed membranes were treated either by an exhaustive EDTA procedure [9] or by a rapid EDTA procedure [10,11]. The soluble and membrane fractions prepared by these methods were obtained by centrifugation in a Beckman L5-65 ultracentrifuge in an SW-56 rotor for 1 h at 140 000 × g_{max}.

Solubilization of A. laidlawii membranes by Triton X-100

Membranes were resuspended in 20 mM Tris-HCl (pH 7.5)/1% (w/v) Triton X-100/10 mM KCl/0.2 mM ATP/0.2 mM $MgCl_2$/0.2 mM $CaCl_2$ for 15 min at 22°C. A supernatant (Triton soluble) and precipitate (Triton insoluble) were

obtained by centrifugation in a Beckman L5-65 ultracentrifuge in an SW-56 rotor for 1 h at 140 000 $\times g_{max}$. The protein from either the supernatant or precipitate was collected by ethanol precipitation in 10 vols. ethanol at 0°C, followed by washing in ethanol, ethanol/diethyl ether (1 : 1, v/v) and diethyl ether.

Iodination of cells and membranes

The iodination procedure was similar to that described by Hubbard and Cohn [13]. To the cell suspension (2.5 mg cell protein/ml buffer II: was added 150 μCi carrier-free $Na^{125}I$/ml (The Radiochemical Centre, Amersham, U.K.) and 200 munits lactoperoxidase/ml (EC 1.11.1.7) (Calbiochem B grade). The mixture was incubated at 22°C for 10 min and then the reaction stopped by adding 4 vols. 20 mM cysteine in buffer II. The cells were harvested by centrifugation, washed once with 20 mM $K^{127}I$ in buffer II and then once more in buffer II. The addition of a hydrogen peroxide generating system was not required for the iodination of whole cell proteins. Negligible iodination occurred in the absence of lactoperoxidase.

Membrane proteins (1 mg membrane protein/ml) were iodinated by the addition of 150 μCi $Na^{125}I$/ml, 10 mM glucose, 2.2 munits glucose oxidase/ml (EC 1.1.3.4) (Type II, Sigma) and 200 munits lactoperoxidase/ml to the membranes suspended in 20 mM Tris-HCl (pH 7.1). The mixture was incubated at 22°C for 10 min. The reaction was stopped by the addition of 4 vols. 20 mM cysteine in the same Tris buffer. The membranes were harvested by centrifugation, washed once with 20 mM $K^{127}I$ in the Tris buffer and then once in the Tris buffer. Negligible iodination occurred in the absence of lactoperoxidase.

Estimation of radioactivity

The amount of radioactivity in ^{14}C- and ^{32}P-labelled cells and membranes was estimated by liquid scintillation counting in a Packard Model 2650 Tri-Carb Liquid Scintillation Spectrometer. Liquid samples contained 10% (v/v) water and 90% (v/v) scintillant (4 g PPO and 0.1 g/l POPOP/toluene/Triton X-100, 2 : 1, by vol.).

The radioactivity in iodinated cells and membranes was estimated by a modified disc batch method [15]. 50-μl samples of cell or membrane suspension were pipetted onto 1'' Whatman 3 MM paper discs which were placed in ice-cold 10% (w/v) trichloroacetic acid containing 50 mM $K^{127}I$ (at least 10 ml per disc). After shaking for half an hour the discs were then left for 16 h. They were then washed for 1 h in each of 10% (w/v) trichloroacetic acid containing 50 mM $K^{127}I$ and then twice in 10% (w/v) trichloroacetic acid. Two 15-min washes in ice-cold 90% (v/v) acetone completed the procedure. The discs were dried and the radioactivity estimated in a Packard Model 3002 autogamma spectrometer.

The distribution of radioactivity in the isoelectric focusing gel after running was determined after dissolution of the gel with 2% periodic acid [16].

Two-dimensional gel electrophoresis of proteins

Two dimensional gel electrophoresis was performed by the method of O'Farrell [1] modified to improve the solubilization and focusing of membrane proteins in the first (isoelectric focusing) dimension. The modified method is described in detail.

Buffers and solutions. Solutions which differ in composition from those described by O'Farrell are designated by a prime or double prime.

A′ lysis buffer: 8 M urea/2% (w/v) Triton X-100/2% (v/v) ampholines (LKB-Produkter AB, Bromma, Sweden) (1% pH range 5—7 and 1% pH range 3.5—10)/12% (w/v) sucrose/5% (v/v) β-mercaptoethanol/0.001% (w/v) bromphenol blue. B′, sonication buffer: 10 mM Tris-HCl (pH 7.5)/1 mM $MgSO_4$. C′: 1 mg pancreatic DNAase/ml B′. C″: 1 mg RNAase/ml 0.1 M sodium EDTA (pH 7.5). D′, 30% acrylamide stock for isoelectric focusing gels: 25.5% (w/v) acrylamide/4.5% (w/v) *N,N′* diallyltartardiamide. E′: 10% (w/v) Triton X-100 in water. F′, ampholine mixture: 1/1 (v/v) mixture of ampholines (LKB, Sweden) of pH range 5—7 and 3.5—10 (40%, w/v). K′, sample overlay solution: 8 M urea/2% (w/v) Triton X-100/2% (v/v) ampholine mixture (F′)/5% (v/v) β-mercaptoethanol. V: 60% sucrose. W, agarose gel for sealing bottom slot of sodium dodecyl sulphate (SDS) gel slab: 1 g agarose melted in 100 ml Tris-glycine electrophoresis buffer (O'Farrell's [1] solution Q) and stored in 4-ml aliquots.

Other solutions, G, H, I, J, L, M, N, O, P, Q and S were as described by O'Farrell [1]. Solutions R (running buffer with high SDS), T (staining solution) and U (destaining solution) were not used in this work.

Sample preparation for electrophoresis

The washed cell pellet from 5 ml cell culture containing approximately 1 mg protein was suspended in 100 μl B′ with the aid of a sonic probe (Type USD-25 Sonipen, Technic International, Bergenfield, N.J., U.S.A.). 10 μl stock DNAase (C′) was added and the cells lysed by four cycles of freezing and thawing. 10 μl RNAase (C″) was then added and the suspension kept at 0°C for 30 min. The volume of solution was determined by weighing; solid urea was added to 8 M and an equal volume (equal to the volume of suspension before addition of urea ×1.67) of lysis solution A′ was added. The final protein concentration was about 2 mg/ml.

Soluble proteins in B′ were brought to 8 M urea by the addition of solid urea and an equal volume of solution A′ added to give a final protein concentration of about 2 mg/ml.

Membranes were defatted before solubilization. 20 vols. ethanol at 0°C were added by drop to 1 vol. membrane suspension in 20 mM Tris-HCl (pH 7.1) at 0°C. After 15 min at 0°C the precipitate was collected by centrifugation in a Sorvall RC2-B centrifuge for 15 min at 10 400 × *g*. The precipitate was washed successively in ethanol, ethanol/diethylether (1/1, v/v), and diethyl ether at 0°C. The residue was dried in vacuo before it was dissolved at room temperature in lysis buffer A′ to give a protein concentration of approximately 2 mg/ml.

Samples in A′ were stored frozen at −20°C.

First dimension gel electrophoresis: isoelectric focusing

Gels for isoelectric focusing were cast in plastic tubes (105 mm × 3.0 mm internal diameter) sealed at the bottom with wet dialysis membrane. To make 10 ml gel mixture, 4.8 g urea was dissolved in a mixture of 2 ml Triton X-100 solution E′/2 ml sucrose solution V/1.33 ml *N,N′* diallyltartardiamide/acrylamide solution D′/0.2 ml water/10 μl *N,N,N′,N′*-tetramethyl-ethylenediamine

(TEMED). 0.5 ml ampholine mixture F′ was then added and the solution filtered through a coarse sintered glass filter to remove particles which might facilitate the subsequent crystallization of urea in the gels. The solution was then de-aerated by swirling under vacuum for about 1 min and 25 μl fresh ammonium persulphate solution (10%, w/v) (G) was added. Gels were poured to a height of 100 mm and overlaid with solution H. After 1—2 h for polymerization, solution H was replaced by 20 μl sample overlay solution K′ and this was overlaid with a small amount of water. After 1—2 h, the gels were transferred to the electrofocusing apparatus (Model M137, manufactured by MRA Corporation, Boston, Massachusetts, U.S.A.). Water and solution K′ were replaced with 20 μl fresh solution K′ and the tubes filled with catholyte solution J. Water at 12°C was circulated through the core of the apparatus during prerunning and electrofocusing. The gels were pre-run at 200 V for 15 min, 300 V for 30 min and 400 V for 30 min. Samples were loaded by displacement through the gel overlay solution K′. Electrofocusing was at 400 V for 18 h, 600 V for 3 h and 800 V for 1 h, a total of 9800 Volts hours.

Storage and equilibration of isoelectric focusing gels. Isoelectric focusing gels were equilibrated with shaking for 15 min in 5 ml solution 0 before storing at —70°C. Equilibration was continued for a further 15 min after the gels were thawed before mounting for electrophoresis in the second dimension.

Second dimension. Electrophoresis in the second dimension on SDS gel slabs was essentially as described by O'Farrell [1]. The slabs were 1.5 mm thick and the heights of resolving and stacking gels were 150 mm and 25 mm respectively. Gel slabs were poured 1—3 days before use, the surface above the stacking gel being covered with diluted stacking gel buffer (M) during storage. The resolving gels were made with a uniform 12% (w/v) acrylamide concentration. The equilibrated isoelectric focusing gels were mounted on the SDS gel slabs using sufficient agarose gel P to just cover the cylinders. The slot formed by removing the bottom gasket below the running gel was filled with agarose gel W before clamping the gel slab assembly to the electrophoresis apparatus. Electrophoresis was done in a cold room (0°C) at constant current with periodic adjustments to keep the power at 10 W. The tracker dye (bromphenol blue) reached the bottom of the resolving gel in about 6 h.

Detection of spots

^{14}C-labelled proteins were detected by the fluorographic method of Bonner and Laskey [17] as modified by Laskey and Mills [18]. An exposure period of about 500 000 dpm × days at —70°C was required to detect the maximum number of spots. An exposure of 150 000 dpm × days was sufficient to compare major spots and the fluorograms shown have all been exposed for this period. Proteins labelled with ^{125}I or ^{32}P were detected by exposing the dried gel slabs to Kodak X-Omat film at room temperature after staining the proteins.

To detect unlabelled proteins, isoelectric focusing gels were immersed in a fixing solution (5% (w/v) 5-sulphosalicyclic acid/10% (v/v) methanol/water) at 60°C for 30 min. Proteins were stained at 60°C for 30 min in a 0.12% (w/v) solution of Coomassie Brilliant Blue R250 in destaining solution. The destaining solution contained 25% (v/v) ethanol/8% (v/v) acetic acid in water. Gels

were destained at room temperature by shaking in several changes of this solution. This procedure was taken from LKB Application Note 75 (LKB-Produkter, Bromma, Sweden).

Results

The two-dimensional separation of A. laidlawii proteins

The pH gradient in the isoelectric focusing gel, when measured after elution of gel slices in 0.1 M KCl ranged from 4 to 8, being approximately linear over the pH range of 4 to 6.5 and rising steeply between pH 6.5 and 8. The two-dimensional electrophoretogram of *A. laidlawii* whole cell proteins, together with molecular weight and pH scales is shown in Fig. 1. The linear scales in this and subsequent figures are in mm. The great majority of the cell proteins focused within the pH range of 4—7 and had molecular weights between 25 000 and 100 000. We estimate that less than 5% of the proteins fell outside these limits. All radioactivity applied to the gels was recovered after the isoelectric focusing, showing that no proteins were lost from the system due to having p*I* values outside the range in the focusing gels.

The protein patterns of the soluble and membrane fractions from *A. laidlawii* cells are shown in Figs. 2a and 2b, respectively. The soluble protein sam-

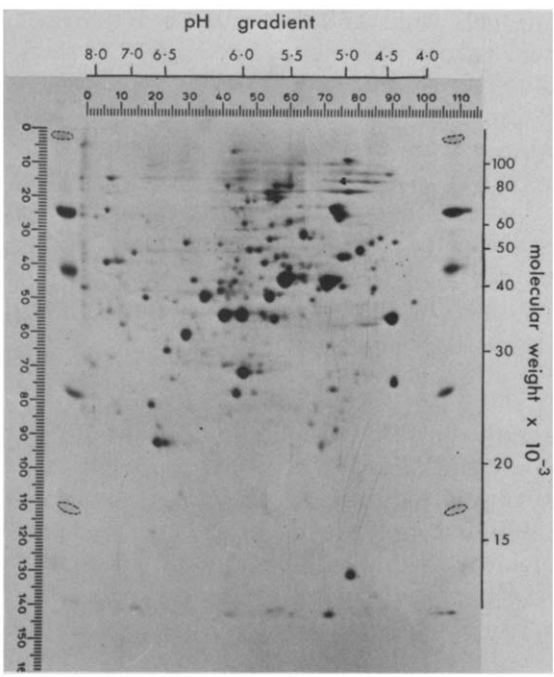


Fig. 1. Separation of proteins from *A. laidlawii* cells. Alongside the sample of ^{14}C-labelled *A. laidlawii* cell proteins on both sides of the gel are samples of molecular weight markers run in the dodecyl sulphate dimension. The protein standards were made radioactive by reaction with dansyl (1-dimethylaminonaphthalene-5-sulphonyl) chloride uniformly labelled with ^{14}C, as described by Gray [19]. The protein standards were: (1) β galactosidase (M_r = 116 000); (2) bovine serum albumin (M_r = 68 000); (3) ovalbumin (M_r = 43 000); (4) chymotrypsinogen (M_r = 25 700); (5) myoglobulin (M_r = 17 200).

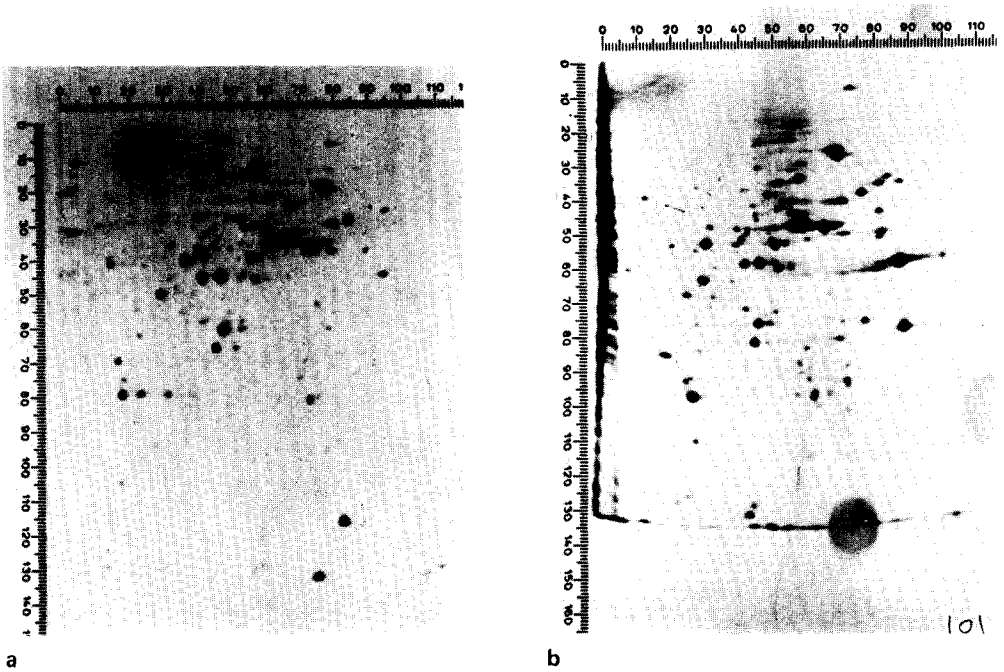


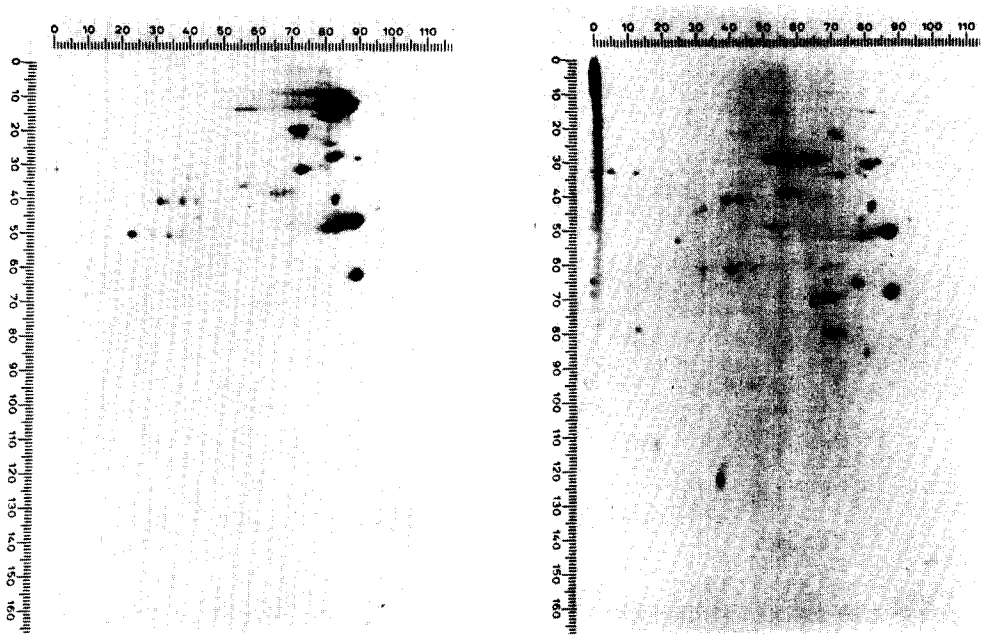
Fig. 2. *A. laidlawii* soluble and membrane proteins. (a) Soluble proteins; (b) membrane proteins.

ple contained the cell lysate supernatant (LS) and the two subsequent membrane washes in 20 mM Tris-HCl (pH 7.1), though the LS and both washes were all identical. This identity of pattern indicates that no further disruption of the membrane occurred after the initial hypotonic lysis of the organism, although the lysis and washing probably all remove some peripheral membrane proteins from the membranes.

Although in practice it is difficult to count more than 300 individual spots, there are approximately 320 individual proteins which can be counted in the whole cell preparation. This represents a significant expression (approx. 30%) of the genome's maximum coding capacity. Of these, about 260 are found in the soluble fraction and about 140 in the membrane fraction. There are many proteins which are common to both the soluble and membrane fractions (i.e. are counted twice) and these are loosely bound peripheral membrane proteins partly removed from the membrane by the cell lysis and washing procedure. Although the LS and the subsequent washes had identical protein patterns, the amount of protein in the fractions was quite different, with only 4.7% of the initial cell protein appearing in the second wash, compared to 57.3% of the cell protein in the LS. The combined soluble fractions contained 78.8% of the cell protein whereas the membrane preparation contained 21.2%.

EDTA treatment of A. laidlawii membranes

It is already apparent that the osmotic lysis and low ionic strength washing of *A. laidlawii* released peripheral proteins from the membranes. Treating the washed membranes with EDTA should release more peripheral proteins. After



a b

Fig. 3. *A. laidlawii* membrane proteins released by EDTA treatment [9]. (a) The proteins released by the second EDTA treatment of this procedure; (b) the proteins remaining after this treatment.

fractionation of cell protein by the exhaustive EDTA treatment of Marchesi et al. [9], the amount of protein in the membranes was halved from the start of the EDTA treatment to the end of the final water wash. This compares with a previous report concerning the use of this procedure with *A. laidlawii* of between 8 and 14% [4] and up to 90% with erythrocyte membranes [20]. Indeed, this exhaustive procedure has been criticised because it may give rise to non-specific aggregation of protein or proteolysis of erythrocyte membrane proteins [10]. The effect of short exposure of the membranes to warm EDTA [10,11], a procedure proposed by the critics of the exhaustive procedure, has also been investigated.

The first EDTA wash of the exhaustive treatment [9] removed proteins from the membrane which are also removed by low-ionic strength alone together with a number of membrane-associated proteins having a p*I* value of about 5. These additional proteins predominated in the second EDTA wash (Fig. 3a). The remaining membrane-associated proteins are shown in Fig. 3b. There were 70—80 remaining proteins compared to the 140 in the initial membrane preparation.

The rapid EDTA treatment [10,11] released many proteins which appeared in the soluble cell proteins (Fig. 2a), but the newly released membrane proteins which were also present in the previous EDTA extracts predominated.

In none of these protein preparations was there any sign of proteolysis as there was no radioactive material at the ion front in the second dimension. There was a small degree of induced charge heterogeneity evident in the water

wash step of the exhaustive EDTA extraction procedure. The question of whether any non-specific protein aggregation has occurred will be discussed below.

Peripheral and integral membrane proteins

By comparing the proteins present in these fractions it is possible to distinguish peripheral from integral membrane proteins. For this purpose we have decided to concentrate on 27 proteins chosen because they are major proteins of the cell, all but 3 of which are membrane proteins (Fig. 4). The group of spots collectively numbered 1 may be protein aggregates. It is a major component of the EDTA extract (Fig. 3a) but is only very rarely apparent in whole cell protein preparations.

The intrinsic membrane proteins are those present in the membrane preparation after the exhaustive EDTA washing procedure, but absent from the LS, the two EDTA extractions of the exhaustive procedure and the rapid warm EDTA treatment. The water wash after the two EDTA extractions of the exhaustive procedure seemed to be very disruptive to the membrane. The small amount of protein released by this step is probably comprised of disrupted membrane fragments as most integral membrane proteins were present in this fraction. The integral membrane proteins are numbered 9—13 and 23—26.

Two groups of peripheral membrane proteins can be distinguished. The first group is comprised of those proteins common to the LS and the resulting membrane proteins after the exhaustive extraction, i.e. they are proteins partially removed from the membrane by the low ionic strength cell lysis and washing procedures but incompletely removed from the membrane even after the exhaustive extraction procedure. These proteins are numbered 14, 15, 19—22 and 27. They are group I peripheral membrane proteins.

The second group of peripheral membrane proteins are those which are absent from the LS and dilute Tris washes but appear in the EDTA washes, i.e. are removed from the membrane by the action of EDTA; whether it be the exhaustive procedure or the rapid procedure makes no difference. The EDTA extraction in low-ionic strength also releases group I proteins but this second group of peripheral membrane proteins are additional proteins released by EDTA. These group II peripheral membrane proteins are numbered 1—3 and 5—8.

Membrane protein 4 appeared in all fractions. Proteins 16—18 are either soluble proteins, or membrane associated but then totally removed from the membrane by the EDTA extraction. Proteins 16 and 18 are included because of the labelling experiment with $^{32}P_i$ to be described below, and protein 17 is included because it is a major protein which helps locate the protein patterns.

Triton X-100 treatment of A. laidlawii membranes

The Triton X-100 soluble and insoluble proteins of *A. laidlawii* membranes produced quite different protein patterns after two-dimensional gel electrophoresis. The Triton X-100 soluble protein pattern was remarkably similar to the pattern of membrane proteins, although many quantitative differences may well exist. The insoluble protein residue (Fig. 5) was enriched with pro-

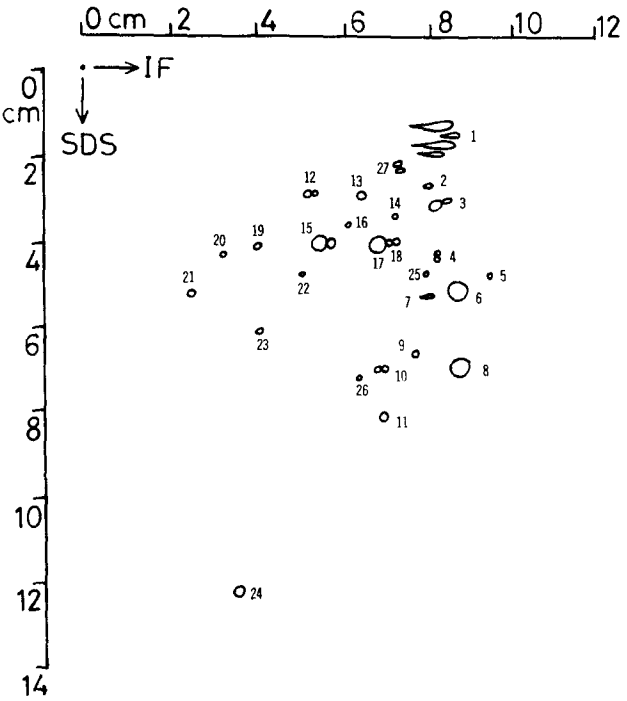


Fig. 4. Numbering of *A. laidlawii* proteins. This is a trace of some major *A. laidlawii* cell proteins which were then numbered so that individual proteins may be referred to in the text.

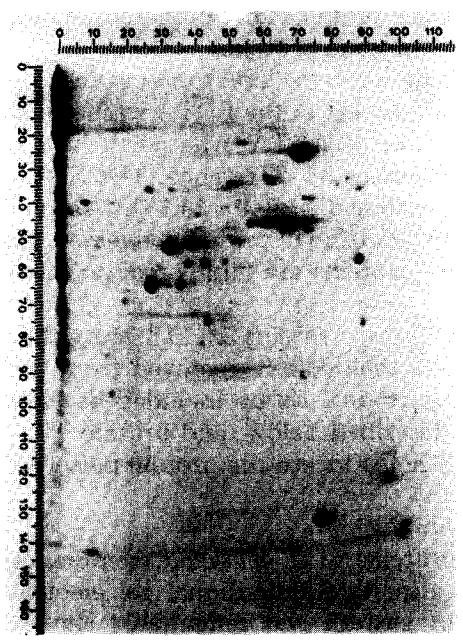
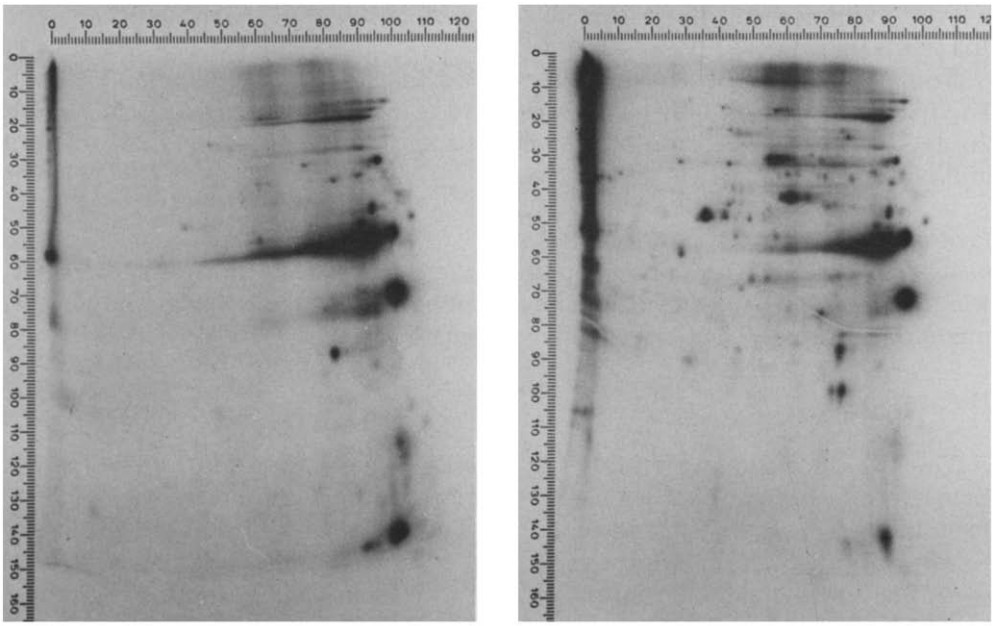


Fig. 5. Triton X-100 extraction of the *A. laidlawii* membrane. Those proteins which were insoluble in Triton X-100 are shown.



a b

Fig. 6. Iodination of *A. laidlawii* membrane proteins. Intact cells (a) and membranes (b) of *A. laidlawii* were iodinated by lactoperoxidase-catalyzed iodination procedure [12,13]. The iodinated proteins were then separated by two-dimensional gel electrophoresis as described. 140 000 dpm were loaded on to the gel in a and 150 000 dpm in b. Both gels were exposed to X-ray film for 35 days before developing.

teins 19—21 and 27. These four proteins belong to the group of peripheral proteins removed from the membranes by low ionic strength, i.e. group I proteins.

The asymmetric distribution of proteins in the A. laidlawii membrane

Iodination of whole cells should only iodinate those membrane proteins having an external face, whereas iodination of membranes will label membrane proteins facing both the interior and exterior of the cell.

Iodinated whole cell proteins and membrane proteins derived from these iodinated cells were separated by two-dimensional electrophoresis. Identical autoradiograms were obtained from both preparations (Fig. 6a). A proportion of iodinated protein was also found in the soluble fraction (7.6—11.8%), but the labelled proteins were shown to be unsedimented membrane proteins, confirming that only membrane proteins were iodinated by this procedure.

The iodination of membranes labelled the proteins shown in Fig. 6b. Of the 140 membrane proteins, 90—100 proteins can be iodinated and of these a maximum of 40 were labelled after iodination of whole cells. It is clear that a number of membrane proteins are not iodinated by this procedure. This indicates that the iodination procedure either has a disruptive effect upon the membrane in that some membrane proteins are removed from the membrane or that many proteins are not available for iodination. Protein 7 was always

heavily iodinated and streaked in the electrofocusing dimension, but the reason for this is unclear.

From Figs. 6a and b it can be concluded that membrane proteins 1—8, 11, 13, 14, 22 and 25 face the cell exterior and membrane proteins 12, 15, 19—21, 26 and 27 face the cell interior. Membrane proteins 9, 10, 23 and 24 were not iodinated.

Proteins labelled by growth with $^{32}P_i$

When whole cell proteins from *A. laidlawii* grown in the presence of $^{32}P_i$ were run in the two-dimensional electrophoresis system, it was shown that membrane protein 7 and the soluble proteins 16 and 18 were labelled.

Discussion

The two-dimensional method of O'Farrell [1] has far greater resolving power than any one-dimensional gel electrophoresis method for the analysis of complex mixtures of proteins in biological materials. Unfortunately, the results were poor when the method was applied to mycoplasma membranes. A large part of the protein failed to enter the isoelectric focusing gels, and many of those which did enter failed to focus properly, resulting in horizontal streaking in the second dimension. The following modifications to the method were made to improve the solubilization and focusing of mycoplasma membrane proteins.

Sucrose (12%, w/v) was added to both gel and sample. This necessitated a reduction in the concentration of urea from 9.5 to 8 M. The purpose of the sucrose addition was to reduce the cathodic drift during prolonged focusing in the polyacrylamide gels [21]. Also, sucrose reduces the aggregation of membrane protein · Triton X-100 complexes [22].

Acrylamide/*N,N'*-methylene bisacrylamide (T = 4%, C = 5%) was replaced by acrylamide/*N,N'* diallyltartardiamide (T = 4%, C = 17%), resulting in less restrictive gels with improved mechanical properties [23]. The use of *N,N'* diallyltartardiamide as a cross-linking agent had the further advantage that the gels were soluble in 2% periodic acid for scintillation counting [16].

It was necessary to extend the pH gradient by altering the proportions of the component ampholine solutions to avoid losing a significant number of proteins at the alkaline end of the gradient.

An increase in the total focusing time and voltage to 9800 Volt hours resulted in a sharpening of some protein bands without any change in the pH gradient in the gel.

Nonidet P-40 in gel and sample was replaced by Triton X-100. This was probably of no consequence as the two detergents are very similar. Cooling the gels by water circulation at 12°C during focusing eliminated band distortion.

With the above modifications, focusing of mycoplasma membrane proteins was improved, but some proteins still failed to focus properly, resulting in horizontal streaking in the second dimension. This was largely overcome by defatting the membranes as described prior to solubilization in lysis buffer A'. More than 99% of the radioactivity in ^{14}C-labelled membranes was recovered in the defatted residue.

A. laidlawii membrane proteins focused very well after defatting but

between 12 and 15% of the loaded radioactivity failed to enter the gel, compared to 10% with non-defatted samples. The small increase in protein remaining at the top of the gel after defatting was fully compensated by the excellent resolution of individual spots. Other methods for focusing membrane proteins have relied upon initial complete solubilization by dodecyl sulphate which is then subsequently removed from its association with the proteins by electrofocusing in the presence of an excess of non-ionic detergent [24] or by acetone precipitation of the proteins [25]. We have tried both methods with *A. laidlawii* membranes but neither are as suitable as the defatting procedure; 32% of the radioactivity applied failed to enter the gel after dodecyl sulphate removal by detergent [24] and 24% failed to enter after acetone precipitation [25]. The method of Ames and Nikaido [24] did not result in an altered protein pattern but there was appreciable streaking of all protein spots in the focusing dimension (unpublished observations).

A further 15% of the protein is lost from isoelectric focusing gels during the 30 min equilibration in O'Farrell's solution O. Shorter equilibration periods resulted in some vertical streaking in the second dimension. This loss could probably be avoided by using the procedure described by O'Farrell for non-equilibrated gels [1]. The method we have described is not universally applicable to the analysis of membrane proteins from all membranes. When applied to the human erythrocyte membrane a large proportion of the protein failed to focus.

A comparison of the two EDTA extraction procedures upon the *A. laidlawii* membrane is possible from the results shown here. Such a comparison would only be possible with a high resolution two-dimensional method for protein analysis. It appears that both the exhaustive extraction procedure [9] and the rapid extraction [10,11] release a heterogeneous mixture of proteins from the membranes. Both procedures release the group of proteins numbered 1 from the membranes and this group, which is comprised of four individual spots, is normally absent from whole cell or membrane protein preparations and is probably therefore composed of protein aggregates. No other aggregates were observed. There was some indication of limited membrane disruption during the water wash stage of the exhaustive procedure but no extensive proteolysis occurred. It is interesting to note that the proteins which were released from the membrane by the EDTA treatment but not by the osmotic lysis or low ionic strength washes have p*I* values of about 5.

The results show that the majority of *A. laidlawii* proteins have p*I* values between 4 and 7. The molecular weight range reported here of 25 000–100 000 for the great majority (95%) of *A. laidlawii* proteins differs from the molecular weight range of 30 000 to over 200 000 reported by Amar et al. [8]. These workers estimate that over 20% of the *A. laidlawii* membrane protein has a molecular weight of over 100 000, due mainly to one protein band in one dimensional SDS polyacrylamide gels containing over 19% of the membrane protein. No major proteins with a molecular weight of 120 000 were evident on our two-dimensional gels although many higher molecular proteins are present in two-dimensional gels of proteins from other mycoplasmas (unpublished observations).

Triton X-100 has been used to solubilise proteins from many different mem-

branes [27] including *A. laidlawii* [4,28]. It binds to hydrophobic regions of proteins [27]. The results show clearly that of the numbered membrane proteins, only proteins 19—21 and 27 are significantly enriched in the Triton-insoluble pellet. These proteins are also peripheral proteins removable by low-ionic strength (group I) and are all located on the interior membrane surface. The EDTA-extractable peripheral proteins (and the integral proteins) were Triton X-100 soluble. Here again the distinction between the two classes of peripheral membrane proteins is clear. For the EDTA-extractable proteins (group II) to be soluble in Triton X-100 they must have hydrophobic regions and are probably, therefore, more intimately associated with the membrane than the low-ionic strength extractable proteins which evidently are membrane bound solely by virtue of ionic interactions of a protein-phospholipid or protein-protein nature. Comparisons may be made with other published data. For example, in *A. laidlawii*, NADH oxidase, ATPase and *p*-nitrophenyl-phosphatase were soluble in Triton X-100 [28] and were later shown to be interior-facing integral membrane proteins [5]. In the human erythrocyte membrane it has been shown that the Triton X-100-soluble proteins are comprised of exterior-facing proteins which have a hydrophobic interaction with the membrane, whereas the Triton X-100 insoluble proteins are those bound to the interior membrane surface solely by ionic interactions [29].

By combining the results from the division of the membrane proteins into peripheral or integral membrane proteins with the results from the iodination experiments, a picture of the *A. laidlawii* membrane can be built up. Integral proteins are found which either face the cell interior or the cell exterior or which are not iodinated. Those integral membrane proteins facing the cell exterior may span the membrane and also face the cell interior; it is impossible to say whether this is so from these results. The EDTA-extractable peripheral proteins (group II) are found exclusively on the cell exterior whereas the low ionic strength extractable membrane proteins, apart from proteins 14 and 22, are located on the interior surface of the membrane. It must be emphasized that the EDTA extraction releases both group I and II peripheral proteins, but the group II proteins are those specifically extracted by EDTA after the low ionic strength washing.

It is known that there is an asymmetrical distribution of proteins in the *A. laidlawii* membrane [8]. Of the 90—100 membrane proteins which were iodinated, a maximum of 40 faced the cell exterior. The proportion of those facing the cell exterior and which traverse the membrane is not known. There are many (about 40) membrane proteins which were not iodinated, or which may have been iodinated but lost from the membrane during the procedure. Proteins 9, 10, 23 and 24 were not iodinated. They are all integral membrane proteins but may well face a membrane surface and expose no tyrosine residues. The inner membrane surface of *Mycoplasma hominis* becomes richer in protein as the cell culture ages [30]. The reorganisation of protein on the inner membrane surface results in a reduction of iodination by the lactoperoxidase-catalyzed procedure as the *M. hominis* culture ages [30] and it is probable that the age of culture of *A. laidlawii* will be an important factor in the extent of iodination of its membrane proteins. Some proteins may be masked from iodination by the hexosamine polymer which is loosely associated with the outer

surface of the *A. laidlawii* membrane [31,32].

As far as we are aware there are no previous reports of mycoplasma proteins labelled with $^{32}P_i$. Protein 7, a $^{32}P_i$-labelled membrane protein, never focused well in the isoelectric focusing dimension and was abnormally heavily labelled by iodine after lactoperoxidase-catalyzed iodination, though the reasons for this are unclear. The other two $^{32}P_i$-labelled proteins in *A. laidlawii* were located in the soluble protein fraction. The type of labelling by $^{32}P_i$ is unknown.

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