Identification of iron- and phosphorus-containing antigens of the *Acholeplasma laidlawii* cell membrane

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Received 9 June 1982

Acholeplasma laidlawii *cell membrane* *Autoradiography* *Crossed immunoelectrophoresis* *Iron-containing proteins* *Membrane proteins* *Phosphorus-containing proteins*

1. INTRODUCTION

Mycoplasmas are prokaryotes, without a cell wall. This property makes them useful for membrane studies, as the only permeability barrier surrounding the cell, is the plasma membrane [1]. Some mycoplasmas have membrane-bound respiratory-associated components [2], but, compared to other prokaryotes, remarkably little is known about how they transport electrons. Most mycoplasmas lack cytochromes as well as ubiquinones [3] and their respiratory system has, accordingly, been characterized as 'flavin-terminated' [4]. However, iron and acid-labile sulphur have been found in both the membrane and the cytoplasmic fraction of the cells [4]. The NADH oxidase of *Acholeplasma laidlawii*, which is membrane-bound in this mycoplasma (as well as in the other *Acholeplasma* species), has been purified after extraction with the neutral detergent Triton X-100 [5]. The enzyme was shown to be a copper-containing iron sulphur flavoprotein composed of 3 peptide subunits. To relate the structure with the function of this enzyme, it is important to establish to which of its subunits iron, copper, and FMN are bound.

Incorporation of ^{59}Fe, analysis of membranes by crossed immunoelectrophoresis (CIE), and autoradiography have been used to identify 7 iron-containing membrane protein antigens from *Escherichia coli* [6]. One of these antigens was identified as the NADH dehydrogenase by a zymogram-staining technique [6]. Here, ^{59}Fe was incorporated in vivo in *A. laidlawii* and the membranes were analysed by CIE, and SDS pore gradient gel electrophoresis, followed by autoradiography. Two iron-containing proteins were found, one of which is of membrane origin. By a similar technique it was shown that two membrane proteins contain phosphorus.

Abbreviations: CIE, crossed immunoelectrophoresis; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Materials*

Agarose A and M_r markers were obtained from Pharmacia Fine Chemicals (Uppsala); Tween 20 (polyoxyethylene sorbitan monolaurate) from Atlas Chemie GmbH (Essen); sodium dodecyl sulphate (SDS), from Kebo AB (Stockholm); Kodak X-Omat AR film from Eastman Kodak Co. (Rochester NY); $^{59}Fe\ Cl_3$ (8.3 mCi/mg Fe) and [^{32}P]orthophosphate (carrier-free) from the Radiochemical Centre (Amersham); tryptose and PPLO serum fraction from Difco Lab. (Detroit MI).

2.2. *Incorporation of ^{59}Fe and ^{32}P in vivo and preparation of membranes*

Acholeplasma laidlawii, strain B(ju), was grown statically at 37°C in a tryptose medium, supplemented with 1% (v/v) of PPLO serum fraction [7]. $^{59}Fe\ Cl_3$ in 1 ml 0.1 M HCl was added to 100 ml the medium (final conc. 9 mCi/l) after inoculation with 10 ml of an overnight culture and incubation for 90 min. After 24 h, the cells were chilled on ice, and harvested by centrifugation at 8700 × *g* for 30 min at 5°C. The cells were washed once with 22 ml β-buffer [0.05 M Tris–HCl buffer (pH 7.4), 0.156

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M NaCl and 0.01 M β-mercaptoethanol] and centrifuged as above.

The cells were disrupted by osmotic lysis [7] in 40 ml total vol. and the membranes were collected by centrifugation at 34 000 × *g* for 30 min at 5°C. The supernatant fraction of the whole cell lysate, which will be referred to as the cytoplasmic fraction, was concentrated to 0.4 ml in a Minicon® B 15 and stored at −20°C until use. The membrane pellet was washed by repeated centrifugations in diluted β-buffer [7] until the absorbance of the supernatant at 280 nm was ≤0.02. The pellet was finally stored at 4°C overnight.

In a separate but similar experiment, the cells were labelled with ^{32}P by growing them in 50 ml medium containing [^{32}P]orthophosphate (final conc. 40 mCi/l).

2.3. *Preparation of detergent extracts*

The ^{59}Fe-labelled membranes were equally distributed into 2 centrifuge tubes. One portion was dissolved in 50 μl 0.2 M SDS in 0.1 M Tris–HCl buffer (pH 8.0), and the other was extracted with 50 μl 5% Tween 20 in the above buffer as in [8] and stored at −20°C until use. The ^{32}P-labelled membranes were also extracted with Tween 20, but not dissolved in SDS.

2.4. *Crossed immunoelectrophoresis (CIE) and autoradiography*

Poly- and mono-specific antisera were produced in rabbits as in [10]. Cie [9] was performed in the presence of 1% Tween 20 in 80 mM Tris–acetic acid buffer (pH 8.6) at a field strength of 15 V/cm for 40 min during first-dimensional electrophoresis and at 2 V/cm for 15 h during second-dimensional electrophoresis. The CIE plates were then soaked in 0.2 M NaCl for 2 days prior to pressing. Autoradiograms were obtained by exposing the dried CIE plates to the X-ray film. The plates were stained with Coomassie brilliant blue after exposure [6].

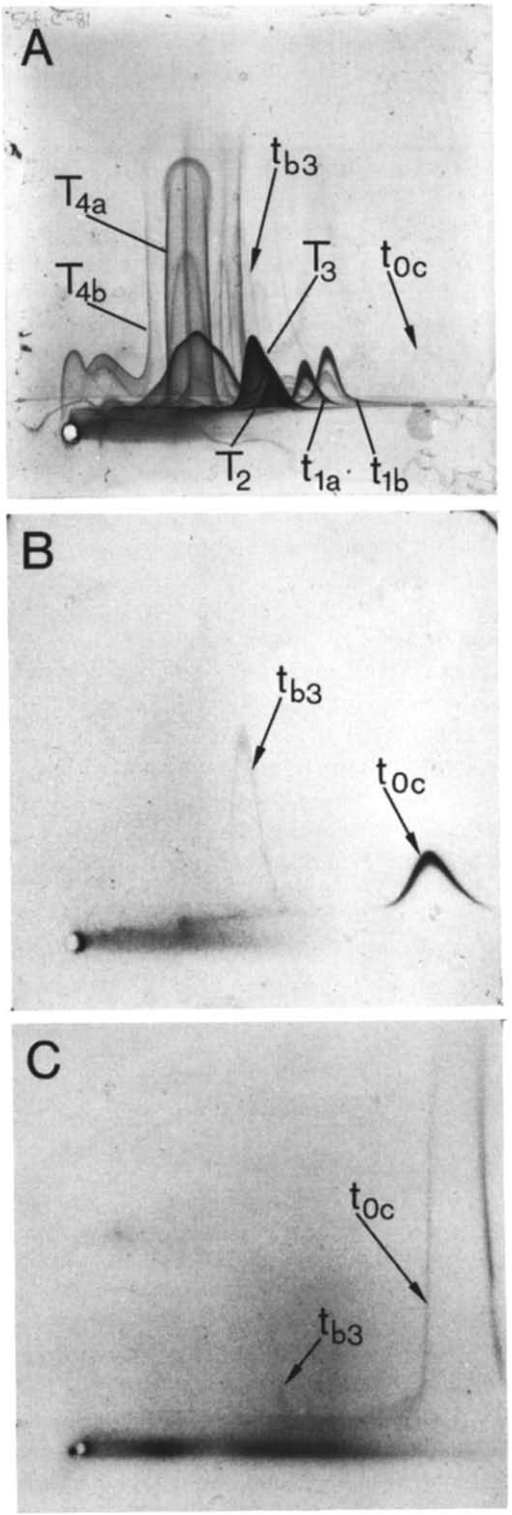

Fig.1. Crossed immunoelectrophoresis (CIE) of ^{59}Fe-labelled cell fractions of *A. laidlawii* with antiserum against membranes: (A) analysis of the Tween 20 extract of the membrane by protein staining of the CIE plate; (B) autoradiogram of the CIE plate shown in (A) exposed for 7 days; (C) autoradiogram of a CIE of the cytoplasmic fraction exposed for 32 days.

2.5. *Polyacrylamide gel electrophoresis*

The ^{59}Fe-labelled cell fractions were analysed by SDS pore gradient gel electrophoresis. A linear gradient of polyacrylamide (6–30%) was first cast by photopolymerization in the gel cassette (15 × 15× 0.15 cm). A stacking gel (5%) was then chemically polymerized on top of the separation gel. Electrophoresis was carried out for 48 h at 50 V in 40 mM Tris–acetic acid buffer (pH 7.4) containing 2 mM EDTA and 0.1% SDS. Samples were run in duplicate and after termination of the electrophoresis, the gel was cut longitudinally in slices. One slice of each sample was soaked in a mixture of methanol, acetic acid, and water (4:1:5) and subsequently stained in 0.05% of Coomassie brilliant blue. The other set of slices was fixed in 25% of isopropanol to avoid losses of nonheme iron, dried in a Slab Gel Dryer (Hoofer Scientific Instruments, model SE 540), and exposed to the X-ray film for 31 days.

3. RESULTS

3.1. *Identification of iron-containing antigens*

Fig.1A shows the result of a CIE of the ^{59}Fe-labelled membranes with some of the immunoprecipitates denoted according to the reference immunoprecipitation pattern [10]. The autoradiogram

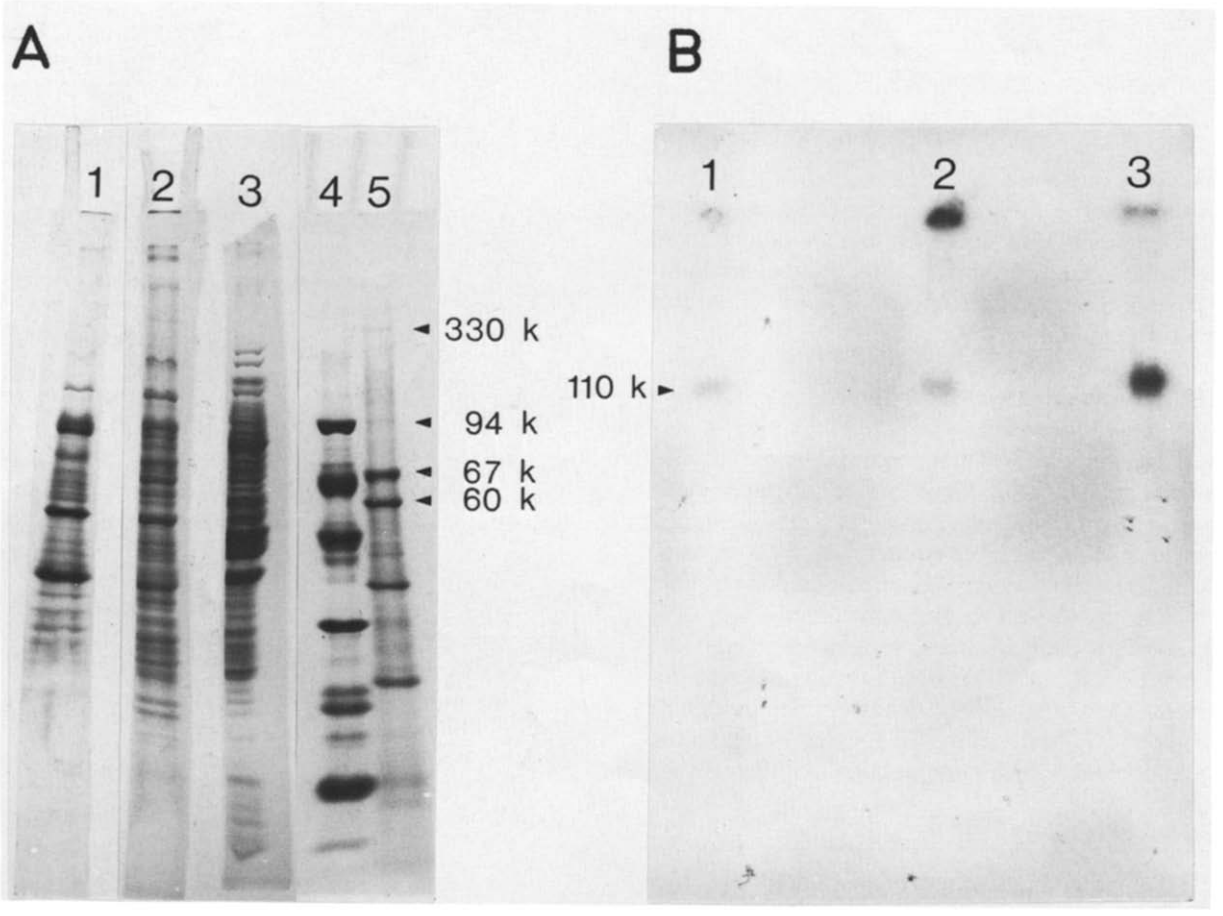

Fig.2. Polyacrylamide gel electrophoresis of ^{59}Fe-labelled cell fractions of *A. laidlawii*. (A) Samples from the left: (1) Tween 20 extract of membranes; (2) SDS-solubilized membranes; (3) cytoplasmic fraction; (4) low M_r markers; (5) high M_r markers. (B) The corresponding autoradiogram of the labelled samples shown in (A), exposure time 31 days.

(fig.1B) reveals two major iron-containing antigens, denoted t_{b3} and t_{0c}. Antigen t_{0c} is more heavily labelled than t_{b3}. Both antigens are minor components of the Tween extract and t_{0c} is particularly difficult to visualize by protein staining (fig.1A) but not by autoradiography. Some iron-containing material was retained close to the application well and some material seemed to be adsorbed to the agarose and migrated as a very diffuse zone (cf. [11]) during first-dimensional electrophoresis (fig.1B).

In fig.1C is shown an autoradiogram of a CIE of the cytoplasmic fraction of the cells. Several minor-containing antigens can be seen and there is one major iron-containing antigen, which is probably identical with t_{0c}. Thus, t_{0c} seems to be a cytoplasmic protein (or possibly a peripheral membrane protein), which contaminated the membrane preparation. There are only small amounts of antigen t_{b3} present in the cytoplasmic fraction, indicating that this antigen is of membrane origin.

3.2. *Polyacrylamide gel electrophoresis*

An SDS pore gradient gel electrophoresis and the corresponding autoradiogram of ^{59}Fe-labelled cell fractions are shown in fig.2. Two iron-containing bands of different relative intensities were found in the fractions. The upper band coincides with the material retained at the starting position (cf. the CIE experiments). The lower band corresponds to ~110 000 M_r and the cytoplasmic fraction of the cells contained more of this material than did the membrane fractions. The radioactivity of the gels was lost if, prior to autoradiography, they were stained in a solution containing acetic acid indicating that the iron is of non-heme origin [6].

3.3. *Identification of phosphorus-containing antigens*

Fig.3 illustrates the result of a CIE of a Tween 20 extract of ^{32}P-labelled membranes. Two ^{32}P-labelled immunoprecipitates were found after autoradiography (fig.3B). Judging from the electrophoretic migration rate and the shape of the immunoprecipitates, one of them was identified as the membrane protein T_3. The other immunoprecipitate appeared in the region (T_4) where several precipitates are found [10]. A monospecific antiserum against one of the antigens (T_{4a}) in this region is available and the sample was, therefore, also analysed by CIE with this antiserum. Fig.3C shows the

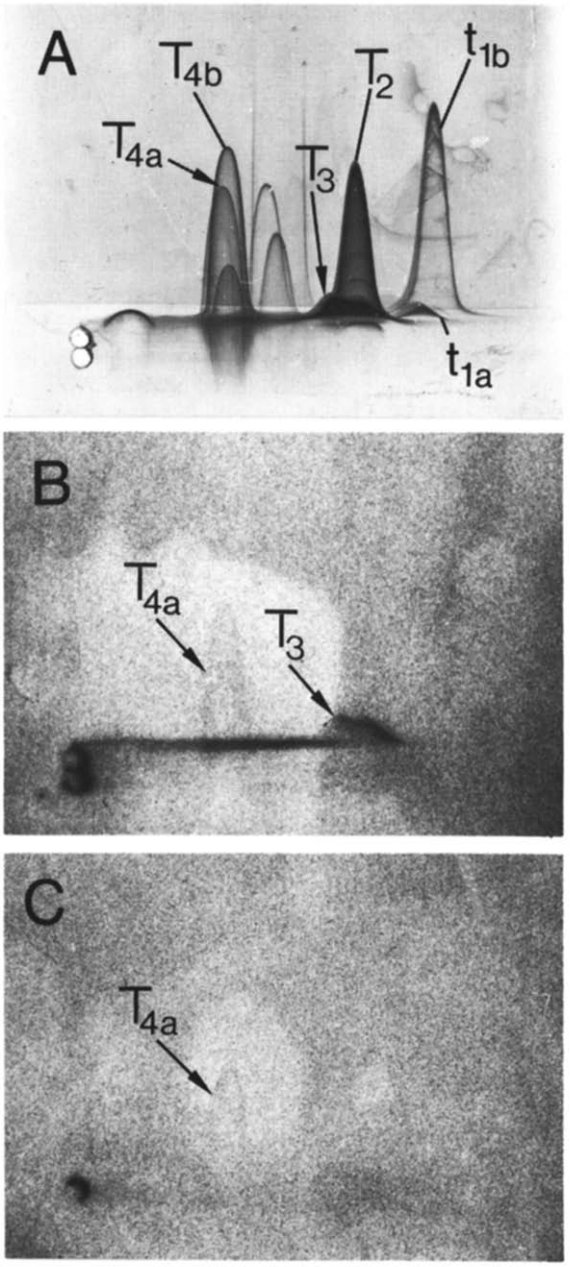

Fig.3. CIE of a Tween 20 extract of ^{32}P-labelled *A. laidlawii* membranes: (A) with antiserum against membranes; (B) autoradiogram of the CIE plate shown in (A), exposure time 35 days; (C) autoradiogram of a CIE experiment performed with a mono-specific antiserum against the flavoprotein T_{4a}, exposure time 35 days.

corresponding autoradiogram and, as can be seen, ^{32}P was incorporated in the flavoprotein antigen T_{4a}.

4. DISCUSSION

Two iron-containing antigens of *A. laidlawii* were identified in this work. One of them, t_{0c}, seemed to be of cytoplasmic origin (fig.1C) and had M_r 110 000 (fig.2B). Interestingly, the antigen t_{b3} is not present in large amounts in the cytoplasmic fraction of the membrane (fig.1C), which suggests that it is a true membrane protein. However, this iron-containing antigen seemed to contain too little of ^{59}Fe to be visualized by autoradiography of the polyacrylamide gel under the conditions used. Non-heme iron might also have been partly lost during the SDS electrophoresis [12]. The M_r of t_{b3} could, therefore, not be determined.

The nature of the phosphorus-containing material of the membrane protein antigen T_3 has not been determined. Phosphorus might be associated to this protein in the form of a phosphorylated amino acid, nucleic acid or phospholipids. The flavoprotein T_{4a}, has been isolated from the *A. laidlawii* membrane after extraction with the neutral detergent Tween 20 [13] and it has also been partially characterized [14]. Thus, T_{4a} was expected to contain phosphorus, since phosphate is a building block in both FMN and FAD. The flavoprotein T_{4a} is probably identical with protein 7 from *A. laidlawii* [15], which also could be labelled *in vivo* with ^{32}P.

It is very likely that the flavoprotein T_{4a} isolated in [13,14] is identical with one of the subunits of the NADH oxidase characterized in [5] for the following reasons. The app. M_r of T_{4a} in a continuous electrophoretic system is 34 000 [14]. However, when T_{4a} was analysed by discontinuous SDS polyacrylamide gel electrophoresis, $M_r \sim 40\,000$ was obtained (see fig.1 in [8]). The NADH oxidase contains FMN and one of its subunits has M_r 40 000 [5]. This subunit should accordingly be the one to which the FMN molecule is bound.

T_{4a} does not contain iron as judged from the autoradiogram. Whether the iron-containing antigen t_{b3} is identical to one of the other two subunits (the 19 000 or 65 000 M_r peptide) of the NADH oxidase [5] remains to be established.

ACKNOWLEDGEMENTS

We thank Dr J.D. Pollack, who encouraged us to search for iron-containing antigens. This work was supported by the Foundations of Carl Trygger, O.E. and Edla Johansson, Magn. Bergvall and the Swedish Natural Science Research Council.

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