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IMMUNOCHEMISTRY AND PEPTIDE MAPPING OF *MICROCOCCUS LYSODEIKTICUS* MEMBRANE PROTEINS

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SUMMARY

Three proteins, ATPase (EC 3.6.1.3), NADH dehydrogenase (EC 1.6.99.3) and a component migrating rapidly on electrophoresis in polyacrylamide gel ('fast-moving component') have been purified from isolated membranes of *Micrococcus lysodeikticus*. Immunochemical analysis of these proteins demonstrated that each protein possessed a unique antigenic specificity when tested against antiserum to *M. lysodeikticus* membranes. Treatment of the proteins with sodium dodecyl sulfate revealed a common antigenic specificity. The common antigen was not detectable in catalase purified from the cytoplasmic fraction of *M. lysodeikticus* and is considered to be peculiar to the membrane proteins. It is heat labile and destroyed by trypsin or by pronase. Trypsin digestion of the purified proteins yielded peptide "fingerprints" showing the presence of common major peptides. Some minor peptides appeared to be unique for each protein. Purified sodium dodecyl sulfate subunit of ATPase and the fast-moving component had strikingly similar peptide fingerprints, although their migration in polyacrylamide gels differed. Common peptides were also obtained from tryptic digests of whole membranes and the deoxycholate-insoluble residue containing electron transport components. In contrast to the membrane fractions the purified cytoplasmic protein catalase, gave an entirely different pattern of peptides on tryptic digestion and fingerprinting.

INTRODUCTION

Membranes are composed primarily of proteins and lipids and there is now considerable information on the overall chemical composition and nature of the phospholipids of isolated membranes[1,2]. In general, membrane proteins have been less well characterized but in recent years the variety of proteins in membranes has been investigated by polyacrylamide-gel electrophoresis and the complexity of protein patterns of the dissociated membranes from diverse sources has thereby

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been demonstrated[3–8]. Little is known, however, about the nature and immunogenic properties of individual proteins of membranes or membrane-associated enzymes. Adenosine triphosphatase (ATPase, EC 3.6.1.3) has been identified as a major antigen of *Micrococcus lysodeikticus* membranes[9] and its interaction with homologous antibody has recently been studied[10].

In our previous investigation[11], it was shown by immunoelectrophoresis that three major antigenic components are released from *M. lysodeikticus* membranes, one of which reacted strongly with antiserum to purified ATPase[10]. Moreover, the evidence from this study suggested the presence of a common antigen[11]. Accordingly, the present immunochemical and peptide-mapping studies were undertaken to further clarify the existence of a common membrane antigen and to determine the antigenic relationships between certain purified components of the membrane. The successful isolation of the enzymes ATPase and NADH dehydrogenase and a 'fast-moving component' of unknown function, by a method of electrophoresis on polyacrylamide gel described in this paper, has enabled us to define the immunochemical characteristics of these membrane antigens more precisely. Although the results reported here give evidence of the presence of a common antigenic component in the three proteins treated with sodium dodecyl sulfate, it should be emphasized this does not constitute proof of the existence of identical antigenic determinants or identical peptide subunits. However, the intriguing possibility that certain membrane proteins may contain common and specific peptide chains requires further exploration and is being actively investigated in this laboratory.

MATERIALS AND METHODS

Preparation of membranes

Membranes were isolated from *M. lysodeikticus* (NCTC 2665), cultivated, washed, and lysed with lysozyme as previously described[3,12]. The membranes were washed with 50 mM Tris–HCl buffer (pH 7.5), as described in an earlier study[9].

Preparation of antisera and immunodiffusion techniques

Rabbits were immunized with membrane fractions dispersed in Freund's incomplete adjuvant as described recently[11].

In some experiments, membrane fractions separated by polyacrylamide-gel electrophoresis were used directly for immunization. Although polyacrylamide gel has been shown to be a good adjuvant[13], we have found that better results were obtained by mixing homogenized gel slices with Freund's incomplete adjuvant. Equal volumes of gel homogenate and Freund's adjuvant were mixed and sonicated for 30–60 sec. The thick emulsion was injected intramuscularly into rabbits, following the immunization schedule and procedures used previously[11].

Immunodiffusion tests were performed in agar by the methods of CAMPBELL *et al.*[14–16].

Analytical polyacrylamide-gel electrophoresis

Electrophoresis in polyacrylamide gel was performed in the Model 12 Canalco equipment (Canal Industrial Corporation, Rockville, Md.) by the methods originally described by DAVIS[17]. For most of the electrophoretic examinations of fractions, the

7 % polyacrylamide gel and standard Canalco buffer and gel formulations previously used in this laboratory[18] were employed. Where the standard procedure was not used, the precise conditions of pH, polyacrylamide gel strength and buffer are indicated in the appropriate sections below.

Partial purification of ATPase, sodium dodecyl sulfate-treated ATPase, NADH dehydrogenase, fast-moving component and catalase

ATPase. Membrane fractions were extracted with *n*-butanol by a method used by SALTON AND SCHOR[19] in this laboratory. The essential features of the method were as follows: 2 vol. of membrane suspension (in 0.05 M Tris–HCl buffer (pH 7.5); initial membrane protein approx. 10 mg/ml) were extracted for 10 min at 0° with 1 vol. of *n*-butanol. The organic solvent phase was removed and discarded after separation from the aqueous phase and interfacial zone by centrifugation for 15 min at 0° at 27000 × *g*. The aqueous phases and interfacial emulsion dispersed in the Tris–HCl buffer were re-extracted with *n*-butanol at a 1:2 by vol. ratio of organic solvent to buffer phase, as described above. The procedure was repeated until virtually all of the carotenoid had been extracted into the organic solvent (usually two to four extractions). Any residual insoluble material was removed by centrifugation and pooled aqueous preparations were dialyzed at 0° against several changes of 0.005 M Tris–HCl buffer (pH 7.5) to remove dissolved butanol. These aqueous-phase fractions from the extracted membranes were then used as the starting material for purification of the ATPase. The precipitate formed on saturation with 50 % $(NH_4)_2SO_4$ contained 85 % of the original ATPase activity. This fraction was purified further by gel filtration on Sephadex G-200 as described by MUNOZ *et al.*[9]. The purity of the fractions was checked by electrophoresis in polyacrylamide gel and by staining for protein and enzymatic activity[9]. Fractions containing the bulk of the ATPase activity were pooled, precipitated with $(NH_4)_2SO_4$ (50 % sat.) and dialyzed against 50 mM Tris–HCl buffer (pH 7.5) at 0°.

Sodium dodecyl sulfate-treated ATPase. The ATPase fraction partially purified by gel filtration on Sephadex G-200 was treated with 0.5 % sodium dodecyl sulfate at room temperature (approx. 24°) for 1 h. The treated ATPase was then applied to the Sephadex G-200 column and the dissociation product separated by gel filtration and the separation followed by polyacrylamide-gel electrophoresis and immunodiffusion tests of the fractions from the column. Fractions containing the antigenic activity of sodium dodecyl sulfate-treated ATPase were pooled and the product recovered by precipitation at 70 % satn. with $(NH_4)_2SO_4$.

NADH dehydrogenase. The 0.005 M EDTA-wash from the membranes[20] was used as the starting material for further purification of the NADH dehydrogenase. This fractions was precipitated with $(NH_4)_2SO_4$ and most of the activity was found in the 50 and 80 % satn. levels (specific activity about twice that of the starting fraction). Precipitation by 80 % satn. with $(NH_4)_2SO_4$ was selected for the partial purification of this membrane enzyme.

Fast-moving component. The aqueous phase after *n*-butanol extraction of the membranes was used as the initial source of the fast-moving component. Conditions for precipitation with $(NH_4)_2SO_4$ (30, 50 and 80 % satn.) were judged by polyacrylamide-gel electrophoresis and the 50 % level was chosen for precipitation of fast-moving component which was purified further by gel filtration on Sephadex G-200.

Elution from the column and purity of the fractions were monitored by immunodiffusion and electrophoresis. The fast-moving component was eluted from Sephadex after ATPase.

Catalase. The enzyme was partially purified by a simplified procedure modified from the method of HERBERT[21] (M. R. J. SALTON, unpublished). To the cytoplasmic fraction from *M. lysodeikticus* was added sodium acetate (0.05 M) and $(NH_4)_2\ SO_4$ to 50 % satn. The yellow precipitate of residual membrane fragments was removed by centrifugation and discarded. The supernatant was then brought to 85 % satn. by further addition of solid $(NH_4)_2SO_4$ and the precipitate of crude catalase was collected by centrifugation and the colorless supernatant discarded. The precipitate was dissolved in 50 mM Tris–HCl buffer (pH 7.5), dialyzed against buffer, re-precipitated twice at 75 % satn. with $(NH_4)_2SO_4$. The catalase was visible in polyacrylamide gels as a green-brown colored band and it could be visualized in the gels enzymatically with the starch–iodide indicator reagent[22].

Purification of proteins by polyacrylamide-gel electrophoresis

The partially purified fractions of ATPase, NADH dehydrogenase, fast-moving component, catalase or sodium dodecyl sulfate-treated ATPase were further separated by electrophoresis on the analytical-gel system under standard conditions[17,18]. Single gels were stained for zones of enzymatic activity or protein and the corresponding regions were cut out from unstained gels. Slices from 24–48 gels were combined, homogenized in an equal volume of 50 mM Tris–HCl buffer (pH 7.5) and the homogenates were electrophoresed again by the same analytical procedure. Protein staining of the second run demonstrated the efficacy of the purification method as only one or two bands were detectable, compared to 18–20 bands found in the original membrane extracts. Gel slices were again pooled and homogenized as before. A 7 % stacking gel 2 cm in height in a P-100 column of a Canalco Preparative Disc Gel Apparatus, was prepared and the total homogenate layered on top, following a 30-min equilibration run at 4 mA. The electrode buffer was the standard Tris–glycine at pH 9.6 and was changed following the preliminary run. A dialysis sac containing 1 ml of buffer was placed over the end of the column. A constant current of 4.5 mA was applied and the column cooled by circulating tap water through the jacket. Electrophoresis was continued for 1–2 h depending upon the rate of migration of the material through the gel, or for 10–15 min after faintly visible bands of protein had moved off the column into the dialysis sac. The material collected in the 1 ml of buffer in the dialysis sac was analyzed for protein content, enzymatic activity, checked in the analytical polyacrylamide-electrophoresis system or used for immunochemical studies or for peptide mapping. Starting concentrations of crude protein were approx. 1 mg/ml and the final yields of purified protein ranged between 1 and 5 % of the starting material. Despite the low yield this procedure provided the fastest and most convenient method for the preparation of small quantities of highly purified membrane proteins.

Examination of the final fractions purified by the above procedures is illustrated in Fig. 1, which shows the migration of ATPase (a), NADH dehydrogenase (b) and fast-moving component (c) in the analytical disc-gel electrophoresis system. The location of the NADH dehydrogenase stained for protein (d) and dichlorophenolindophenol reduction[23] (e) is also shown. It should be noted that the fast-moving com-

ponent frequently showed a tendency to aggregate, giving rise to two bands near the gel front as seen in Fig. 1(c). The purification of the catalase is shown in Fig. 2 which illustrates the initial cytoplasmic fraction (a), partially purified (b) and the final catalase fraction purified by the procedure described above (c).

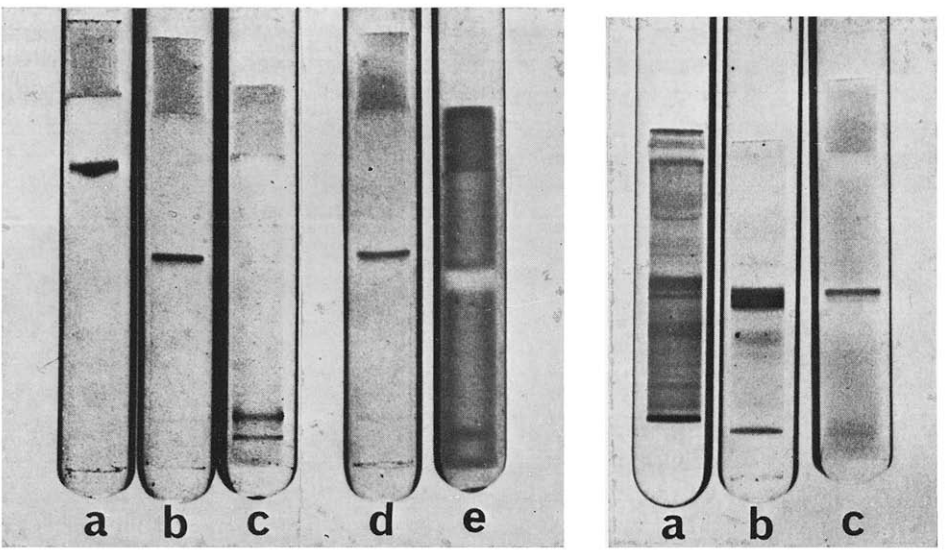

Fig. 1. Electrophoresis in polyacrylamide gel of three proteins purified from *M. lysodeikticus* membranes as described in MATERIALS AND METHODS. (a) ATPase; (b) NADH dehydrogenase; (c) fast-moving component (d) and (e) two gels of purified NADH dehydrogenase run simultaneously and stained for protein with coomassie blue and for enzymatic reduction of dichlorophenolindophenol, respectively. The difference in gel size between (d) and (e) is due to the respective staining procedures. Electrophoresis performed in 7 % gel, stacking at pH 8.9 and separating at pH 9.5.

Fig. 2. Electrophoresis in polyacrylamide gel of *M. lysodeikticus* cytoplasmic fraction (a), partially purified catalase (b), and the final catalase preparation (c), purified as described in MATERIALS AND METHODS. All gels stained for protein with coomassie blue, after electrophoresis under standard conditions as for Fig. 1.

Tryptic digestion and peptide mapping

Proteins, purified as described above, were heat denatured at 100° for 10 min. The protein was suspended in 0.5 M ammonium acetate buffer (pH 7.2). Trypsin, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Worthington Biochemical Corp.), was added at a ratio of 1 part trypsin to 60 parts of protein and the mixture incubated at 37° for 24 h. The digests were then evaporated under vacuum and kept in the dried state until thin-layer electrophoresis and chromatography were carried out. The tryptic digests from 50–100 μg of initial protein were suspended in 10 % (v/v) isopropyl alcohol in water and placed as a 1 inch band on a 20 cm × 20 cm precoated glass plate (Avicel Micro Crystalline, Cellulose powder, supplied by Brinkman Instruments). Electrophoresis was carried out in a Shandon TLE Apparatus at 825 V for 25 min. The buffer system was glacial acetic acid–98 % formic acid–water (85:25:1400, by vol.)[24]. The plate was air-dried and then placed in a tank containing 1 % (v/v) acetic acid. The solvent was allowed to rise by capillary action to

compress the peptide spots and the plates were again air-dried. Ascending chromatography was then performed for 7 h using *n*-butanol–acetic acid–pyridine–water (5:1:4:4, by vol.) as the solvent[24]. The plates were air-dried and dipped into a 0.5 % ninhydrin solution in acetone containing 0.01 M pyridine, dried and then developed in an oven at 80° for 30 min. Control fingerprint plates were prepared with trypsin alone, contents of dialysis sac following electrophoresis in polyacrylamide gels without protein, and undigested proteins alone. The maps obtained from tryptic digests of proteins could not be attributed to any of the reagents or preparative procedures as judged from the examination of the above control preparations.

RESULTS

Immunochemical analysis of purified membrane proteins

The immunodiffusion test of the ATPase, NADH dehydrogenase and fast-moving component prepared as described in MATERIALS AND METHODS, following reaction against membrane antiserum is shown in Fig. 3(a). The intersecting lines indicate that each protein has a unique specificity. When the sodium dodecyl sulfate -treated proteins (0.3–1.0 % sodium dodecyl sulfate, overnight at about 24° prior to addition to the wells) were reacted against membrane antiserum a continuous line of precipitate was observed as seen in Fig. 3(b). Thus ATPase, NADH-dehydrogenase and fast-moving component possess a common antigen which is released or demonstrable after the isolated proteins have been dissociated with sodium dodecyl sulfate. As only a single line of precipitate was formed under these conditions it would follow that the individual antigenic specificities indicated in Fig. 3(a) were either destroyed by sodium dodecyl sulfate or were in too low a concentration to give rise to precipitates in the agar gels. However, the presence of the common antigenic specificity and the individual specificities indicated by spur formation are shown in Fig. 3(c) where sodium dodecyl sulfate-treated ATPase, ATPase and sodium dodecyl sulfate-treated fast-moving component are placed in adjacent wells.

That the antigenic determinants of ATPase and fast-moving component are of peptide nature was suggested by their sensitivity to pronase, trypsin and heat. ATPase, sodium dodecyl sulfate-treated ATPase and fast-moving component were digested overnight with trypsin or pronase, each enzyme at 100 μg/ml in 50 mM Tris–HCl buffer (pH 7.5) at room temperature (about 24°). Preparations of antigens were also heated for 10 min at 100°. No precipitation bands were observed in immunodiffusion tests following heating or digestion of the antigens with proteolytic enzymes. The loss of reactivity of fast-moving component with membrane antiserum by such treatments is illustrated in Fig. 3(d).

Catalase is a soluble, cytoplasmic enzyme in *M. lysodeikticus* and the membranes can be freed from the enzyme by several washes in buffer (M. S. NACHBAR, unpublished observation). The catalase purified as outlined in MATERIALS AND METHODS showed no reaction when tested against membrane antiserum and no reaction was observed with sodium dodecyl sulfate-treated catalase. As anticipated, however, catalase gave a strong precipitin reaction when tested by immunodiffusion against antiserum prepared to the cytoplasmic fraction (Fig. 3(e)). These results and those of our previous study[11] suggest that the "common antigen" may be unique for the membrane proteins rather than the cytoplasmic proteins of *M. lysodeikticus*.

Although "structural protein" and phenol-soluble protein fractions from such diverse sources as beef-heart mitochondria, erythrocyte membranes and *Escherichia coli* envelopes[25] have been found to cross react serologically, major bacterial membrane antigens have generally been observed to be highly specific[11,26-28]. Membranes were isolated from *Bacillus megaterium*, strain KM, and were tested against *M. lysodeikticus* membrane antiserum for the presence of a "common antigen" by treating

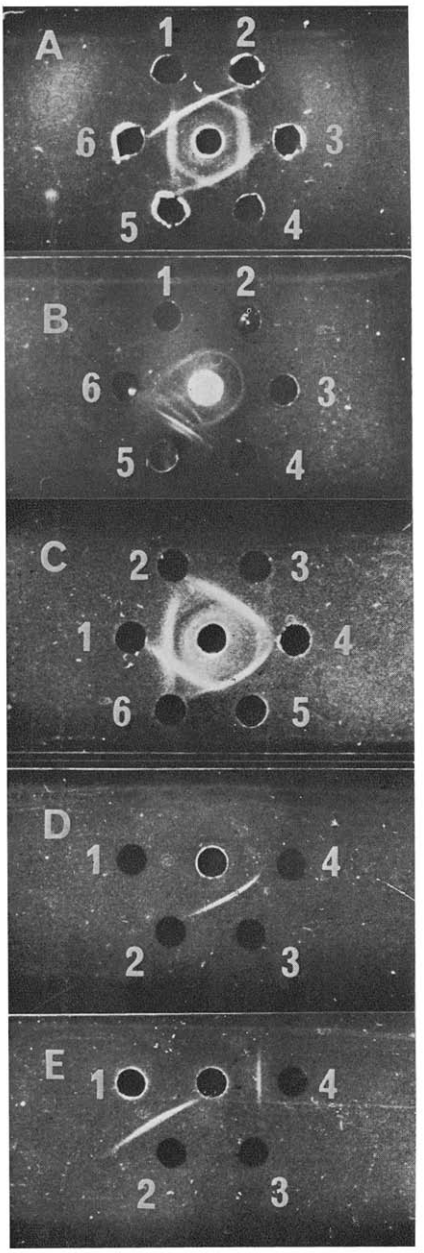

Fig. 3. Reaction of *M. lysodeikticus* proteins and treated proteins against antisera in agar diffusion tests. (a) ATPase (Wells 1 and 4), NADH dehydrogenase (Wells 2 and 5) and fast-moving component (Wells 3 and 6), reacted against membrane antiserum in the center well. (b) Sodium dodecyl sulfate-treated fast-moving component (Wells 1 and 4), sodium dodecyl sulfate-treated ATPase (Well 2), sodium dodecyl sulfate-treated NADH dehydrogenase (Well 3), and membrane sonicated at pH 9 (Well 5) reacted against membrane antiserum (center well). (c) Reaction of untreated fast-moving component (Well 1), treated with 1.3 M guanidine·HCl (Well 2) and sodium dodecyl sulfate-treated fast-moving component (Well 4), against membrane antiserum (center well), and compared with sodium dodecyl sulfate-treated ATPase (Wells 3 and 6) and untreated ATPase (Well 5). (d) Loss of reactivity of fast-moving component (Well 3) upon digestion with pronase (Well 1), trypsin (Well 4) or upon heating at 100° for 10 min (Well 2). The membrane antiserum is in the centrally placed well. (e) Reaction of purified catalase (Well 2) against antiserum to *M. lysodeikticus* cytoplasm (Well 1). No reaction is observed against membrane antiserum (Well 5) since the line of precipitate continues to the edge of the well. Sodium dodecyl sulfate-treated catalase (Well 3) shows no reaction against membrane antiserum (Well 5), whereas the "common antigen" reaction is indicated by the line of precipitate with sodium dodecyl sulfate-treated ATPase in Well 4. Well 5 is the centre well.

the membranes with 1 % sodium dodecyl sulfate but no precipitation bands were detectable.

Peptide mapping of purified proteins, membrane fractions and catalase

ATPase, purified sodium dodecyl sulfate-treated ATPase 'subunit', fast-moving component and NADH dehydrogenase were subjected to fingerprinting by two-dimensional separation of the tryptic digests by electrophoresis and chromatography as described in MATERIALS AND METHODS and the resulting peptide maps are shown in Figs. 4(a)–(d), respectively. It can be seen from the comparison of these maps that a number of the major peptides are common to all fractions and that the principal differences reside in the 'background' of minor peptides and/or the rela-

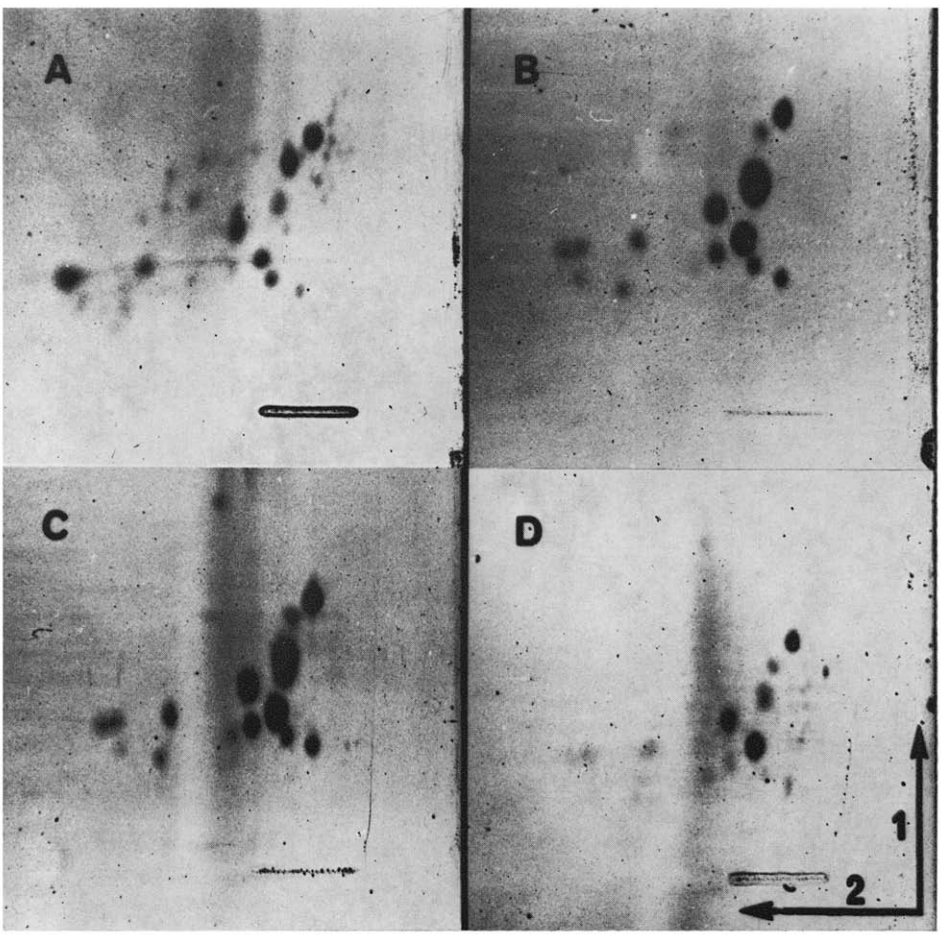

Fig. 4. "Fingerprints" of tryptic digests of purified membrane proteins prepared as described in MATERIALS AND METHODS. Directions of electrophoresis (1) and chromatography (2) are indicated and all plates were developed with ninhydrin. Each digest was prepared from approx. 100 μg protein. (a) ATPase; (b) the sodium dodecyl sulfate 'subunit' of ATPase purified by Sephadex filtration as described in MATERIALS AND METHODS; (c) fast-moving component; (d) NADH dehydrogenase.

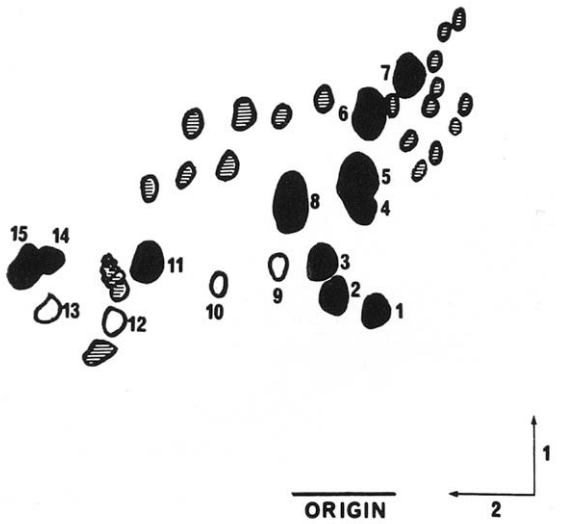

Fig. 5. A composite map of spots of the peptides found in fingerprints of tryptic digests of membrane proteins. The fifteen common peptides have been assigned numbers arbitrarily as indicated and the black "spots" represent the major common peptides found in the digests. These peptides were found in ATPase, sodium dodecyl sulfate-treated ('subunit') ATPase, fast-moving component, NADH dehydrogenase and the deoxycholate-insoluble fraction previously characterized[29]. The 'unique' minor peptides are indicated by the shaded spots, some of which were characteristic for ATPase and others for NADH dehydrogenase. The direction of electrophoresis is indicated by '1' and chromatography by '2'.

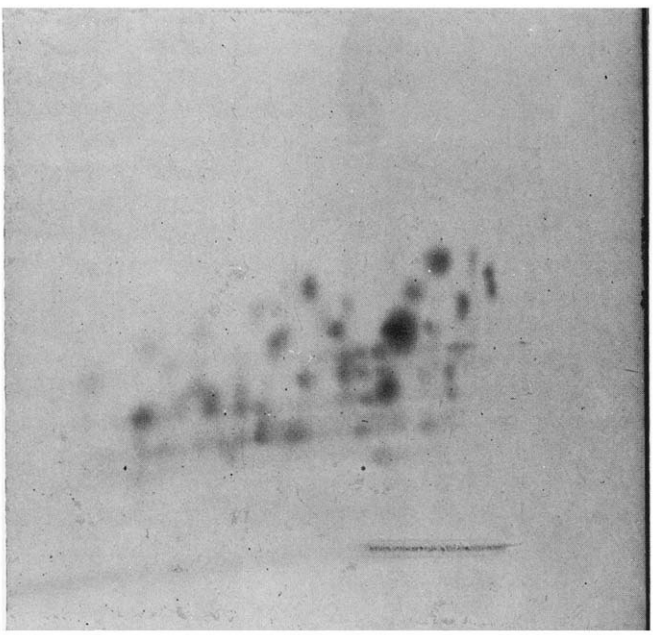

Fig. 6. Fingerprint of the tryptic digest of purified catalase (approx. 100 μg protein). Conditions of digestion and peptide mapping as for other proteins as described in MATERIALS AND METHODS.

tive proportions of major peptides as judged by the intensity of staining with ninhydrin. The similarity of the map of the purified sodium dodecyl sulfate-subunit of ATPase (Fig. 4(b)) to that of fast-moving component (Fig. 4(c)) is striking. The tryptic digest of the deoxycholate-insoluble residues containing components of the electron transport system of *M. lysodeikticus*[29] also showed a number of the common peptides. A diagram of the composite map of peptide 'spots' is presented in Fig. 5 and major common and minor peptides of each of the fractions is indicated. It is worthy of note that the peptide maps were highly reproducible from preparation to preparation of each of the protein fractions.

The purified catalase which gave a single band on polyacrylamide-gel electrophoresis and a single band of precipitate in the immunodiffusion test with antiserum to the cytoplasmic fraction yielded a completely different peptide map as shown in Fig. 6. Most of the peptides on the catalase map appear to be unique with relatively few peptides in common with those of the membrane proteins.

The results obtained with the peptide mapping reinforce the observations suggesting the existence of common antigens in the three different membrane proteins investigated in this study.

DISCUSSION

Despite the distinctive separation on polyacrylamide-gel electrophoresis, of *M. lysodeikticus* membrane ATPase, NADH dehydrogenase and a fast-moving component of unknown function, all three purified proteins exhibited in addition to their own specific antigens a common antigenic specificity on treatment with sodium dodecyl sulfate and they possessed similar major peptides in "fingerprints" of tryptic digests. Just as we had completed the above studies, YANG AND CRIDDLE[30] reported that peptide "fingerprints" of three major proteins of mitochondrial membranes from *Saccharomyces carlsbergensis* were extremely similar. Thus the results of YANG AND CRIDDLE[30] and those from the present study with bacterial membrane proteins suggest that at least certain membrane proteins may possess very similar peptide regions or chains. Moreover, from our evidence of the presence of a common antigenic component in the three individual proteins, it can be anticipated that the yeast mitochondrial membrane proteins of YANG AND CRIDDLE[30] may show similar immunochemical properties.

At this state of our studies it is difficult to rule out the possibility that the common antigen and tryptic peptides are due to a contaminant bound to each protein. Although the proteins in our study and that of YANG AND CRIDDLE[30] have been separated by electrophoresis on polyacrylamide gels, it is still too early to critically assess the possibility that the observations are due to firmly bound peptide contaminants. It will be recalled that mitochondrial structural protein also possessed strong affinities for certain proteins[31,32]. Moreover, at this preliminary stage it is not possible to conclude that the "common specificity" is indeed due to identical antigenic determinants. It is evident that further investigation will be required to resolve this problem and studies are being carried out to determine quantitatively, the contribution of the 'common antigen' to each protein. In addition we are determining the yields and nature of the peptides obtained by tryptic digestion. The number of peptides found on trypsin digestion is compatible with the residues of lysine and arginine

determined by amino acid analysis of ATPase and fast-moving component (unpublisved observations), a result very similar to that reported by YANG AND CRIDDLE[30].

Further studies will be required to account for the apparent disappearance of the minor "specific" peptides of ATPase (Fig. 4(a)) when the sodium dodecyl sulfate-ATPase subunit is purified (*cf.* Fig. 4(a) with Fig. 4(b)). Although fast-moving component, and the sodium dodecyl sulfate subunit of ATPase, occupy different positions on polyacrylamide-gel electrophoresis, as shown in Figs. 4(b) and (c) their peptide maps are remarkably similar. The relationships of these two components to one another and the possible function of fast-moving component are being explored.

The significance of common peptide sequences in membrane proteins has yet to be established. It is conceivable that membrane proteins may have a preferred sequence of peptides, perhaps of a hydrophobic character, so that as they come off the ribosomes their anchoring in the membrane is ensured. Alternatively, the existence of common peptides in *M. lysodeikticus* membrane proteins may represent the 'evolution' of a degree of biosynthetic economy in this organism arising from the limitations imposed by the high G+C contents (72 %) of the DNA of this organism[33]. The resolutions of these problems should broaden our understanding of the organization and biogenesis of membrane systems.

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