BBA 76059

COMMON PEPTIDES IN *MICROCOCCUS LYSODEIKTICUS* MEMBRANE PROTEINS

YOSHIO FUKUI[a] AND MILTON R. J. SALTON[b]

[a]*Department of Microbiology, Hiroshima University School of Dentistry, Hiroshima (Japan) and* [b]*Department of Microbiology, New York University School of Medicine, New York, N.Y. 10016 (U.S.A.)*

(Received April 4th, 1972)

SUMMARY

The contents of common peptides previously reported in three proteins isolated from *Micrococcus lysodeikticus* membranes (Y. Fukui, M. S. Nachbar and M. R. J. Salton, *Biochim. Biophys. Acta*, 241 (1971) 30) have been determined on membrane fractions prepared from cells grown on a defined medium containing ^{14}C-labeled protein hydrolysate. Common peptides on tryptic digest fingerprints accounted for 53% of the purified ATPase (EC 3. 6. 1. 3), 92% of the fast-moving component, and about 50% of the original membranes and the "stripped" EDTA-shock washed membranes.

The molecular weights of the fast-moving component and sodium dodecylsulfate-treated ATPase were determined by sodium dodecylsulfate–polyacrylamide-gel electrophoresis and values of 31000 and 62000, respectively, were obtained. The amino acid composition of the fast-moving component which exhibits a common antigenic specificity with sodium dodecylsulfate-treated ATPase was determined and the hydrophobic amino acids account for 42% of its residues.

INTRODUCTION

In our previous studies[1,2], the antigenic analysis of *Micrococus lysodeikticus* membrane fractions isolated by polyacrylamide-gel electrophoresis suggested the presence of a specificity common to each fraction. Accordingly, with the use of the purified enzymes ATPase and NADH dehydrogenase and a fast-moving component of unknown function obtained from the membranes, the presence of a common antigen in these purified proteins was demonstrated by treatment with sodium dodecylsulfate[2]. Moreover, trypsin digestion of the purified proteins yielded peptide "fingerprints" showing the presence of common, major peptides which appeared to be characteristic for the membranes and distinct from the peptide pattern seen for the purified cytoplasmic enzyme catalase[2]. During these investigations, it appeared likely that the common peptides seen in the fingerprint patterns would account for a substantial proportion of the membrane proteins. Thus, in the present work, we have attempted to determine the contents of the common peptides in the membranes and the membrane proteins by the fingerprint method. We have also determined the

molecular weight and amino acid composition of the fast-moving component which possesses the common antigenic specificity.

MATERIALS AND METHODS

Organism and growth medium

The organism used was *M. lysodeikticus* (NCTC 2665). The defined medium described by Salton[3] was prepared from 20 ml of basal salts, 10 ml of 0.2 M phosphate buffer, pH 7.5, 4 ml of 10 % ammonium acetate, 4 ml of 10 % sodium glutamate and 0.4 ml of biotin (100 μg/ml), and after autoclaving, 0.4 ml of Millipore filtered catechol (final concentration, $2 \cdot 10^{-3}$ M) was added to the medium. For isotope labeling, uniformly ^{14}C-labeled protein hydrolysate purchased from the Radiochemical Center, Amersham, Great Britain, was added to the medium. The cells were grown at 30 °C for 30 h in 100 ml of the above medium supplemented with 0.5 μCi ^{14}C-labeled protein hydrolysate in 500 ml erlenmeyer flasks, using a 5 % (v/v) inoculum which had been grown for 24 h in the defined medium[3].

Preparations of membranes

Cells were washed and lysed with lysozyme, and membranes were prepared as previously described[4,5]. The membrane fractions were washed 4 times with 50 mM Tris–HCl buffer (pH 7.5). In certain experiments, the membrane fractions were washed further with buffer containing 5 mM EDTA (EDTA wash) or were subjected to the osmotic shock wash with 5 mM Tris–HCl buffer as described previously[6].

Analytical polyacrylamide gel electrophoresis

Electrophoresis in polyacrylamide gel was performed in the MS equipment (MS Industrial Corporation, Osaka, Japan) by the methods originally described by Davis[7]. For most the electrophoretic examinations of fractions, the 7.5 % polyacrylamide gel and standard buffer were employed[2,4]. Sodium dodecylsulfate–polyacrylamide-gel electrophoresis was also used to obtain the molecular weight of samples according to the procedure of Shapiro *et al.*[8] and Dunker and Rueckert[9]. The proteins used as standards were bovine serum albumin, ovalbumin, chymotrypsinogen A (beef pancreas) and myoglobin (sperm whale) purchased from Mann Research Laboratories, New York. The migration of the sodium dodecylsulfate-treated proteins was measured in 5 % and 10 % polyacrylamide gels[8,9]. Before application, all proteins were treated with 1 % sodium dodecylsulfate, 1 % 2-mercaptoethanol, 1 vol. of 4 M urea added, and then incubated at 45 °C for 60 min. The electrophoresis buffer contained 0.1 M phosphate and 0.1 % sodium dodecylsulfate. Gels were stained in Coomassie blue.

Purification of ATPase and fast-moving component

The aqueous phase of the *n*-butanol-extracted membranes was used as the starting material for purification of the ATPase, and ATPase was purified by $(NH_4)_2SO_4$ fractionation, gel filtration on Sephadex G-200 and polyacrylamide-gel electrophoresis, as described in our previous paper[2]. The purity of the fractions was monitored by electrophoresis in polyacrylamide gel and by staining for protein and enzymatic activity[2]. The aqueous phase after *n*-butanol extraction of the membranes was

also used as the initial source of the fast-moving component, which was purified as described in the preceding paper[2]. The purity of the fast-moving component was judged by immunodiffusion and by polyacrylamide gel electrophoresis.

Tryptic digestion and peptide mapping

Proteins, purified as described above, and membranes were heat-denatured at 100 °C for 10 min. The samples were suspended in 0.5 M ammonium acetate buffer (pH 7.2) and trypsin which had been treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Worthington Biochemical Corp.), was added at a ratio of one part trypsin to 60 parts of protein and incubated at room temperature for 24 h. The tryptic digests from 50–100 μg of initial protein were applied to a 20 cm × 20 cm precoated glass plate (Avicel Micro Crystalline, Cellulose powder, supplied by Brinkman Instruments), and developed as described in the preceding paper[2]. For ^{14}C-labeled fractions, the common peptide spots corresponding to those detectable by ninhydrin staining of the "peptide map"[2] were carefully removed from the plate and the powders collected in centrifuge tubes. The powders containing the common peptides were extracted with three consecutive extractions with 0.3 ml of 0.001 M NaOH. All of the other areas which appeared to contain peptide spots other than the common peptides were also collected from the plate and extracted as described above. The resulting extracts were dissolved in 10 ml of Insta-gel (Packard Instrument Co.) and the radioactivity was determined in the Aloka Liquid Scintillation Counter (Nippon Musen Co.). Counts for the pooled common peptides and the remaining peptide areas were totalled in order to determine the recovery of ^{14}C-labeled digest material from the plates and the corresponding recoveries are noted for each preparation in the Results. In general, the ^{14}C-labeled fractions subjected to peptide mapping contained 400–600 cpm.

Sonication

A Tomy model UR-150 P sonicator was employed. Before tryptic digestion of the original membranes and EDTA-shock washed membrane, the fractions were sonicated for 1 min and sonicated again for 1 min after 16 h of tryptic digestion.

Ultracentrifugation

A Hitachi model 55 p-2 ultracentrifuge was employed. After tryptic digestion of the membrane fractions and EDTA and shock washed membranes, they were centrifuged at 105000 × g for 120 min to remove insoluble material, and the supernatant fractions were applied to the plate for fingerprinting.

Protein determination

The method of Lowry *et al.*[10] was used with crystalline bovine serum albumin as a standard.

Amino acid composition

The amino acid composition of the fast-moving component was determined with a model 120 C Beckman Amino Acid analyzer. Protein samples were hydrolyzed in 6 M HCl under nitrogen in sealed tubes for 24 and 48 h at 110 °C prior to analysis.

The half-cystine content was determined as cysteic acid after performic acid oxidation[11].

RESULTS

Common peptides in ATPase and fast-moving component

Fingerprints of the tryptic digest of the fast-moving component in our previous study suggested that it may be composed almost entirely of the "common peptides". For the present quantitative study it was selected for comparison with ATPase which showed a number of additional peptide spots after fingerprinting. Purified ATPase and fast-moving component from membranes isolated from cells grown in the ^{14}C-labeled amino acid supplemented medium, were subjected to fingerprinting and the ^{14}C-labeled peptides (common and total peptide components) estimated as desaribed in Materials and Methods. Table I summarizes the results of the determinations cnd indicate that 92 % of the fast-moving component can be accounted for as the common peptides and that the corresponding value for ATPase is 53 %. The overall recovery of ^{14}C-labeled materials in the tryptic digests from the fingerprint plates after electrophoresis, chromatography, and elution from excised spots was 90–93 %.

While the amount of radioactivity recovered in the "common peptide" spots (a total of some 15 spots as reported previously[2]) is surprisingly high, it appeared to us to be unlikely that they could be derived from adsorbed amino acids or peptides. However, this possibility has been considered further and many fingerprint plates of heat-denatured, undigested ATPase and membrane fractions have been examined for the appearance of the "common peptides." Under these conditions of electrophoresis and chromatography of undigested protein only very faint traces of any identifiable amino acids have been detected, thus confirming our previous observation that the peptides were not derived from the acrylamide gel, trypsin or buffer systems used[2], nor from the undigested protein fractions. Control fingerprints of mixtures of amino acids including most of the major ones found in the fast-moving component (*e.g.* lysine, arginine, aspartic acid, glutamic acid, serine, proline, glycine, alanine and valine) indicated that the major common peptides occupied different positions on the fingerprint maps. Traces of ninhydrin-positive spots corresponding to several of the free amino acids were detectable in tryptic digests, but from their intensity it appeared

TABLE I

CONTENT OF COMMON PEPTIDES IN ATPASE AND FAST-MOVING COMPONENT DETERMINED BY FINGERPRINTING ^{14}C-LABELED PROTEINS

	% *Total ^{14}C-labeled peptides*
ATPase*	
Common peptide spots	52.6
Total peptide spots	100.0
Fast-moving component**	
Common peptide spots	92.0
Total peptide spots	100.0

* 91% recovery of ^{14}C counts applied to fingerprints.
** 93% recovery of ^{14}C counts applied to fingerprints.

unlikely that they accounted for more than 5 % of the material. Although it is unlikely that the common peptides originate from adsorbed contaminating proteins and peptides, the unequivocal identity of these can only come from isolation and characterization and experiments on this aspect of the problem are proceeding.

Common peptides in original membranes, EDTA and shock washed membranes

As shown in earlier studies, *M. lysodeikticus* membranes subjected to the low ionic strength shock wash release most of the ATPase activity[6] and the EDTA wash releases NADH dehydrogenase[12]. Up to about 30 % of the original membrane protein can be released by the combined wash procedures and the residual membranes have a "stripped" appearance when examined as negatively stained preparations in the electron microscope[12]. It was therefore of interest to compare the common peptide contents of original and EDTA-shock washed membranes. In order to compare the contents of common peptides in these membrane fractions, the ability of trypsin to "solubilize" the membrane proteins was first checked, any insoluble residues remaining having been removed by centrifugation (105000 × *g* for 2 h). As shown in Table II,

TABLE II

EFFECT OF TRYPTIC DIGESTION ON MEMBRANES

After tryptic digestion the fractions were centrifuged, and protein contents of supernatants were determined. The precipitates were suspended in the original volume of buffer, sonicated and protein determined; the residual protein was accounted for in the precipitates.

	Protein before trypsin digestion (mg/ml)	*Protein in supernatants after trypsin digestion (mg/ml)*	*Percent digestion*
Whole membrane	1.7	1.5	88
EDTA–shock washed membrane	1.5	1.47	98

TABLE III

CONTENTS OF COMMON PEPTIDES IN WHOLE MEMBRANES AND EDTA–SHOCK WASHED ("STRIPPED") MEMBRANES DETERMINED BY FINGERPRINT ^{14}C-LABELED MEMBRANE FRACTIONS

Recoveries of peptide materials from the plates were 90% and 85% from whole membrane and EDTA–shock washed membranes respectively. Both membrane fractions contained ^{14}C label in the lipid extract and as the tryptic digest fingerprints were performed on unextracted membranes, non-peptide ^{14}C-labeled material present separated from the peptides as a yellow region on the fingerprints. The total ^{14}C-labeled material added to the plates was accordingly adjusted by subtracting the non-peptide cpm, in order to determine peptide distribution and recoveries.

	% Total ^{14}C-labeled peptides
Whole membrane	
Common peptide spots	50.9
Total peptide spots	100.0
EDTA–shock washed membranes	
Common peptide spots	49.0
Total peptide spots	100.0

88 % and 98 % of the protein was released into the supernatant fractions for original and EDTA-shock washed fractions, respectively, thus indicating that digestion by trypsin under these conditions was fairly complete. The common peptides were quantitated with ^{14}C-labeled membranes and the data for the original and stripped membranes is summarized in Table III. Thus the common peptides released by trypsin digestion account for about one half of the original and "stripped" membrane fractions.

Molecular weights of fast-moving component and sodium dodecylsulfate subunit of ATPase

The molecular weights of sodium dodecylsulfate-treated fast-moving component and ATPase were estimated by electrophoresis in 5 % and 10 % polyacrylamide

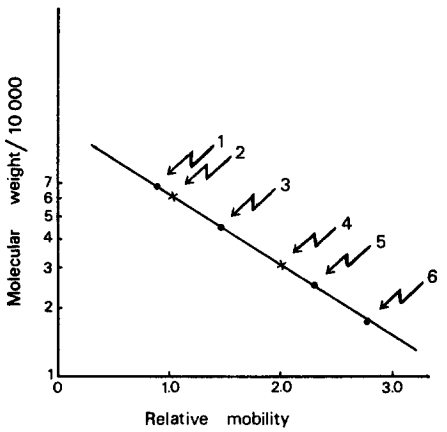

Fig. 1. Molecular weight determination of fast-moving component and sodium dodecylsulfate subunit of ATPase by electrophoresis in sodium dodecylsulfate–5 % polyacrylamide gel according to the method of Dunker and Rueckert[9]. 1, bovine serum albumin; 2, sodium dodecylsulfate-treated ATPase; 3, ovalbumin; 4, fast-moving component; 5, chymotrypsinogen A; 6, myoglobin.

TABLE IV

AMINO ACID COMPOSITIONS OF FAST-MOVING COMPONENT

Amino acid	*Residues per 31 000 molecular weight*
Lysine	8.4
Histidine	4.9
Arginine	15.4
Aspartic acid	28.2
Threonine	15.0
Serine	12.5
Glutamic acid	33.9
Proline	11.9
Glycine	26.0
Alanine	36.0
Half-cystine	1.6
Valine	22.8
Methionine	4.9
Isoleucine	8.6
Leucine	24.2
Tyrosine	7.0
Phenylalanine	8.3

gels containing sodium dodecylsulfate as described by Shapiro *et al.*[8] and Dunker and Rueckert[9]. The linear calibration curve relating the subunit molecular weights of reference proteins and the sodium dodecylsulfate-treated membrane proteins is shown in Fig. 1. A value of 31 000 was obtained for the fast-moving component and the corresponding value for the sodium dodecylsulfate subunit of ATPase was 62 000. Although only one band was found when ATPase was examined by the electrophoresis method of Dunker and Rueckert[9], it should be noted that two distinct bands quite close to one another are given in the Weber and Osborn[13] system (M. R. J. Salton and M. T. Schor, unpublished observations).

Amino acid composition of fast-moving component

The amino acid composition of the fast-moving component purified as described in Materials and Methods was determined and the results are presented in Table IV. It is of interest to note that the hydrophobic amino acids account for approximately 42 % of the amino acid residues of this membrane protein.

DISCUSSION

There is now a growing body of evidence suggesting that a number of membrane proteins are composed of common peptide units. Thus Yang and Criddle[14] found similar peptide fingerprints and amino acid compositions for three purified yeast mitochondrial membrane proteins. Our previous results with *M. lysodeikticus* membrane proteins[2] have been confirmed by quantitation of the common peptides in purified fast-moving component and ATPase. Other membranes containing relatively large amounts of miniproteins[15] and spectrin[16] have been reported. It is unlikely that the fast-moving component of *M. lysodeikticus* membrane with a mol. of 31 000 is directly related to the miniproteins[15], although it is conceivable that some of the common peptides released by trypsin may be related. It is evident that common peptides can account for a substantial proportion of membrane fractions and membrane proteins and it will be of great interest to see to what extent this will be generally true for other membrane systems. The existence of common peptide chains containing high proportions of hydrophobic amino acids would provide an ideal mechanism for anchoring membrane proteins as the growing peptide chains leave the ribosomes. Further evidence to support this suggestion is being sought at the present time.

Although the possibility that the common peptides originate from a contaminating protein bound to each purified protein was considered previously[2], the present data showing that they account for a substantial amount of the protein make this less likely. However, it should be pointed out that the possible existence of contaminant protein association has not been completely excluded and further investigations are required to resolve this problem. The recent discovery by Baron and Abrams[17] of nectin, a specific protein involved in the attachment of ATPase to the membrane of *Streptococcus faecalis*, may be related to our observations of common antigenic specificities and peptide components in several membrane proteins[2].

The existence of a surface antigen on the membrane of *M. lysodeikticus* was shown by immunochemical and it showed a common antigenic specificity with sodium dodecylsulfate -treated ATPase[1]. The internal location of the ATPase was, moreover, deduced from antibody absorption studies with protoplasts[1]. The assymetric distri-

bution of membrane proteins has attracted more attention in recent years and Phillips and Morrison[18] have shown that only one molecular weight class of protein (mol. wt 90000) is exposed on the outer membrane surface of the intact erythrocyte. By chemical labeling methods, Bretscher[19,20] has recently reported the presence of two proteins on the outside surface of the erythrocyte. These are major membrane components and part of component A resides on each side of the membrane and it has been suggested that this protein extends right across the membrane[19,20]. However, in the light of our detection of a common antigen on the surface of the membrane and in sodium dodecylsulfate-treated ATPase and NADH dehydrogenase[1,2], it may not necessarily follow that components detectable on both faces of the membrane actually span the membrane structure.

The significance of the preponderance of common peptides in tryptic digests of membrane proteins[2,14] must be explored more fully before we can understand the implications these observations have for membrane structure, function and biogenesis. The existence of common peptide chains, especially if they were of a hydrophobic character, could provide a mechanism for ensuring the anchoring and functional organization of membrane proteins (*e.g.* the nectin–ATPase system[17]) and at the same time provide a degree of specialization in membrane protein biosynthesis.

ACKNOWLEDGMENTS

These investigations were supported by a grant, GB 17107 from the National Science Foundation and in part by the Research Fund from the Ministry of Education of Japan. We are grateful for the technical assistance of Misses Ikuko Sasaki and Yoko Matsui, for the help of Mr Charles Harman in the amino acid analyses and for the help of Mrs Marian Schor in the purification of the fast-moving component.

REFERENCES

1 Y. Fukui, M. S. Nachbar and M. R. J. Salton, *J. Bacteriol.*, 105 (1971) 86.
2 Y. Fukui, M. S. Nachbar and M. R. J. Salton, *Biochim. Biophys. Acta*, 241 (1971) 30.
3 M. R. J. Salton, *Biochim. Biophys. Acta*, 86 (1964) 421.
4 M. R. J. Salton, *Trans. N. Y. Acad. Sci.*, 29 (1967) 764.
5 M. R. J. Salton and J. H. Freer, *Biochim. Biophys. Acta*, 107 (1965) 531.
6 E. Muñoz, M. S. Nachbar. M. T. Schor and M. R. J. Salton, *Biochem. Biophys. Res. Commun.*, 32 (1968) 539.
7 B. J. Davis, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404.
8 A. L. Shapiro, E. Vinuela and J. V. Maizel, Jr, *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.
9 A. K. Dunker and R. R. Rueckert, *J. Biol. Chem.*, 244 (1969) 5074.
10 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
11 C. H. W. Hirs, *Methods in Enzymology*, Vol. XI, Academic Press, New York, 1967 p. 59.
12 M. S. Nachbar and M. R. J. Salton, *Biochim. Biophys. Acta*, 223 (1970) 309.
13 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
14 S. Yang and R. S. Criddle, *Biochemistry*, 9 (1970) 3063.
15 M. T. Laico, E. I. Ruoslahti, D. S. Papermaster and W. J. Dreyer, *Proc. Natl. Acad. Sci U.S.*, 67 (1970) 120.
16 S. L. Marchesi, E. Steers, V. T. Marchesi and T. W. Tillack, *Biochemistry*, 9 (1970) 50.
17 C. Baron and A. Abrams, *J. Biol. Chem.*, 246 (1971) 1542.
18 D. R. Phillips and M. Morrison, *Biochemistry*, 10 (1971) 1766.
19 M. S. Bretscher, *J. Mol. Biol.*, 58 (1971) 775.
20 M. S. Bretscher, *J. Mol. Biol.*, 59 (1971) 351.