Biochimica et Biophysica Acta, 298 (1973) 32–38

BBA 76190

EFFECT OF PROTEINS ON THE MOTION OF SPIN-LABELED FATTY ACIDS IN MYCOPLASMA MEMBRANES

SHLOMO ROTTEM and AMRAM SAMUNI

Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School and Department of Physical Chemistry, The Hebrew University, Jerusalem (Israel)

(Received August 31st, 1972)

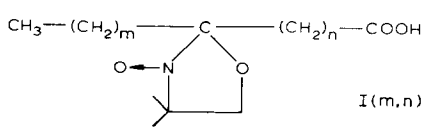
SUMMARY

Spin-labeled fatty acids have been incorporated as structural probes into *Acholeplasma laidlawii* and *Mycoplasma hominis* native membranes and into pronase-treated membranes from which up to 75% of the protein had been removed. The electron paramagnetic resonance (EPR) spectra showed the mobility of the spin labels in the native and pronase-digested membranes to be temperature dependent, increasing as the nitroxide radical is moved away from the polar head group of the fatty acid. The changes were more pronounced in *A. laidlawii* than in *M. hominis* membranes. The mobility of the spin-labeled fatty acids in the pronase-digested membranes increased with protein digestion which was accompanied by a decrease in the isopycnic density of the membranes. On binding lysozyme or cytochrome *c* to the pronase-digested membranes, the mobility of the spin-labeled fatty acids decreased with a corresponding increase in membrane density. These changes were almost completely reversed when the soluble proteins were removed by 1 M NaCl. Our findings indicate that membrane proteins, including those bound electrostatically to membrane lipids, influence the physical state of membrane lipids.

INTRODUCTION

Electron paramagnetic resonance (EPR) studies with mycoplasma have shown that spin-labeled fatty acids have greater freedom of motion in membrane lipid dispersions than in native membranes. It accordingly seems that the protein in the membrane affects the fluidity of the lipids[1,2].

The purpose of the present investigation was to further elucidate the effect of proteins on the physical state of mycoplasma membrane lipids by examining the freedom of motion of spin-labeled fatty acids after removal of the membrane proteins by pronase and the binding of positively charged soluble proteins to the digested membranes. The spin labels used were *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of stearic acid with the general formula I (m, n):



I(m,n)

The EPR spectra of such labels have been thoroughly investigated in a model system[3–5] and their hyperfine splitting 2 T maximum ($2T_m$) has been related to the freedom of motion of the nitroxide radical[4] greater freedom of motion being associated with smaller values of $2T_m$.

MATERIALS AND METHODS

Acholeplasma laidlawii (oral strain) and *Mycoplasma hominis* (ATCC 15056) were grown in a modified Edward medium[6]. *M. hominis* was growing in the medium supplemented with 20 mM L-arginine and its pH was adjusted to 6.5. To label membrane lipids, 50 μCi of [9,10–^{3}H]oleic acid were added to each l of the medium. The organisms were grown for 18–24 h at 37 °C, and membranes were isolated by osmotic lysis of the organisms, washed as described previously[7], and resuspended in β-buffer[8] diluted 1:20 in deionized water (diluted β-buffer). Digestion of *A. laidlawii* membranes (2 mg membrane protein per ml) by pronase (200 μg/ml) was carried out at 45 °C for different periods of time in a final volume of 2 ml. To stop the digestion the samples were chilled to 4 °C and centrifuged at $100000 \times g$ for 30 min. The supernatant was separated from the pellet which was resuspended in 2 ml of the diluted β-buffer.

To bind lysozyme or cytochrome *c* to pronase-digested membranes, equal volumes of the resuspended pellet of digested membranes and each of the soluble proteins (2 mg/ml) were incubated at 37 °C for 30 min. The resulting membrane-soluble protein complex formed was sedimented by centrifugation at $100000 \times g$ for 30 min, washed once with diluted β-buffer and resuspended in the same buffer. The attached basic protein was removed by washing the membrane-soluble protein complex twice in a cold solution containing 0.01 M sodium citrate in 1 M NaCl (pH 8.5).

For isopycnic density gradient analysis, samples (0.2 ml, 200 μg protein) of labeled membrane preparations were layered over 11 ml linear 20–40% sucrose gradients. The gradients were centrifuged at 35000 rev./min in the SW 41 rotor of a Spinco Model L-2 ultracentrifuge for 2 h at 4 °C. Fractions (0.02 ml) collected by puncturing the bottom of the tube were assayed for protein and/or radioactivity.

The *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid and 12-ketostearic acid were synthesized according to Keana *et al.*[9]. The keto methyl esters were prepared according to Jones[10]. Membranes were spin-labeled by exchange from bovine serum albumin[4]. EPR spectra of spin-labeled membranes contained in a sealed Pasteur pipette were obtained with a Varian E-4 spectrometer. Unless otherwise stated all spectra were recorded at 20 °C. Hyperfine splitting ($2T_m$) was measured to within ± 0.5 G.

Lipids were extracted from native or pronase-digested membranes by 5 successive extractions with acetone–water–ammonia (90:10:0.03, by vol.) at 4 °C for 30 min. Protein was determined by the Folin phenol method of Lowry *et al.*[11].

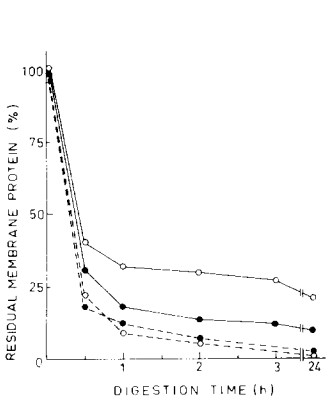
Radioactivity of tritiated materials was measured in a Packard Tri-carb liquid scintillation spectrometer using toluene–dioxane scintillation liquor[7].

RESULTS

Exposure of mycoplasma membranes (2 mg membrane protein per ml) to pronase (200 μg/ml) at 45 °C resulted in the digestion of up to 80% of *A. laidlawii*,

and up to 90% of *M. hominis* membrane proteins (Fig. 1). The remaining 10–20% were unaffected by the enzyme even after prolonged incubation (up to 48 h). No more than 1% of the membrane lipids were released during the pronase incubation. Removal of about 85% of the lipids by 5 successive extractions with acetone–water–ammonia (90:10:0.03, by vol.) at 4 °C, before exposing the membranes to pronase resulted in the digestion of almost all the proteins of *A. laidlawii* or *M. hominis* membranes (Fig. 1); similarly lipid extraction of pronase-digested membranes rendered the residual undigested protein susceptible to pronase.

The EPR spectra of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-keto-stearic acid [spin label I (12, 3)] in *A. laidlawii* native membranes and in pronase-digested membranes from which 65% of the proteins were removed were similar (Fig. 2). The large hyperfine splitting ($2T_m$), however, was different indicating a higher freedom of motion of spin label I(12,3) in the pronase-digested membranes.



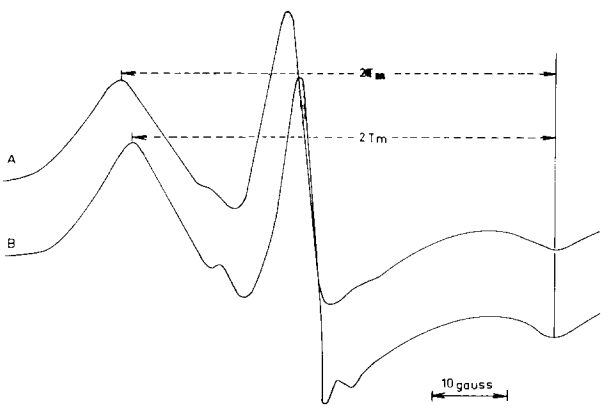


Fig. 1. Pronase digestion of native (solid lines) and lipid-depleted (broken lines) mycoplasma membranes. ○, *A. laidlawii* membranes; ●, *M. hominis* membranes. Digestion was carried out as described under Materials and Methods.

Fig. 2. The EPR spectra of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid [spin label I(12,3)] in *A. laidlawii* native membranes (A) and pronase-digested membranes (B).

The freedom of motion of spin label I(12,3) in the pronase-digested membranes of *A. laidlawii* increased with the extent of protein digestion, which was accompanied by a concomitant decrease in the isopycnic density of the membranes (Fig. 3). Similar results were obtained with *M. hominis*, where removal of 75% of the proteins after 2 h of exposure to pronase resulted in the isopycnic density of the preparation decreasing from 1.167 to 1.095 g/cm^3 and the freedom of motion of spin label I (12,3) increasing, as judged from the decrease in the hyperfine splitting ($2T_m$) from 59.7 to 57.2 G.

The hyperfine splitting ($2T_m$) of spin-labeled fatty acids in native or pronase-digested *A. laidlawii* or *M. hominis* membranes showed a pronounced dependence on temperature (Fig. 4) and on the position of the nitroxide group in the fatty acid chain; decreasing the further this group moved away from the polar end of the molecule (Table I).

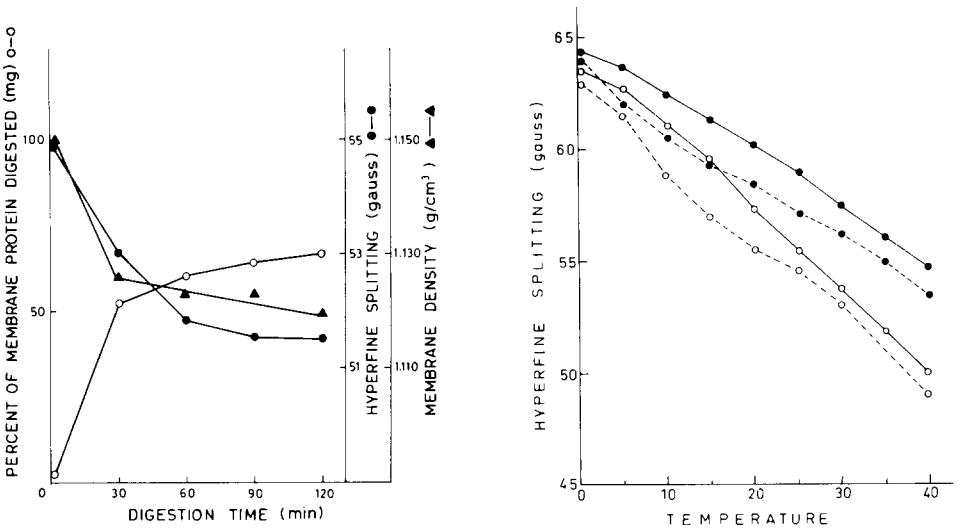


Fig. 3. The effect of pronase digestion of *A. laidlawii* membranes on the density of the membranes and the hyperfine splitting ($2T_m$) of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid [spin label I(12,3)] in the membranes.

Fig. 4. Temperature dependence of the hyperfine splitting ($2T_m$) of *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5 ketostearic acid [spin label I(12,3)] in *A. laidlawii* membrane preparations (○) and in *M. hominis* membrane preparations (●). Solid lines, native membranes; broken lines, pronase-digested membranes.

TABLE I

HYPERFINE SPLITTING ($2T_m$) VALUES OF SPIN LABEL I INCORPORATED INTO NATIVE AND PRONASE-DIGESTED MYCOPLASMA MEMBRANES

Membranes (2 mg protein/ml) were exposed to pronase (200 μg/ml) for 2 h, at 37 °C. Paramagnetic spectral analysis was carried out at 10 °C using *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid [spin label I(12,3)] and 12-ketostearic acid [spin label I(5,10)]. The hyperfine splittings were determined as described under Materials and Methods.

Organism	*Preparation*	*Hyperfine splitting (G)*	
		Spin label I(*12,3*)	*Spin label* I(*5,10*)
A. laidlawii	Native membranes	60.5	54.2
	Digested membranes	58.5	51.5
M. hominis	Native membranes	62.0	59.7
	Digested membranes	60.2	57.2

The addition of positively-charged soluble proteins, such as lysozyme or cytochrome *c* to pronase-digested membranes of *A. laidlawii* resulted in the binding of the protein to the membranes. The amount of protein bound to the pronase-digested membranes increased with the amount of soluble protein added, reaching

maximum levels of about 0.8 mg cytochrome *c* and 1.4 mg lysozyme per mg membrane lipid. The membrane-soluble protein complex showed a single band in a sucrose density gradient where isopycnic density was higher than that of the digested membranes. The EPR spectra of spin-labeled fatty acids in the membrane-soluble protein complex as in pronase-digested membranes, were sensitive to temperature and to change in the position of the nitroxide group on the fatty acid chain. The freedom of motion of the nitroxide radical in the membrane-soluble protein complex was, however, less than in the digested membranes, as appeared from the increase in the hyperfine splitting ($2T_m$) upon binding of cytochrome *c* or lysozyme to the pronase-digested membranes (Table II). The binding of both cytochrome *c* and lysozyme to the digested membrane was inhibited by increasing the NaCl concentration. The addition of 1 M NaCl moreover removed over 95% of the previously bound basic proteins. Their release was accompanied by a decrease in the isopycnic density of the preparation and an increase in the freedom of motion of the nitroxide radical in the membrane (Table II).

TABLE II

EFFECT OF BINDING AND RELEASE OF SOLUBLE PROTEINS TO *A. LAIDLAWII* MEMBRANES ON THE HYPERFINE SPLITTING ($2T_m$) OF SPIN LABEL I(12,3) IN THE MEMBRANES

Membranes were digested with pronase (200 μg/ml) for 2 h at 37 °C. The binding of soluble proteins, and the analyses of membrane density and EPR spectra were determined as described under Materials and Methods.

Preparation	*Protein (mg/ml)*	*Lipid/ protein (cpm/mg)*	*Membrane density (g/cm^3)*	*Hyperfine splitting (G)*
Native membranes	1.63	119 470	1.152	56.7
Digested membranes	0.59	301 140	1.124	54.5
Digested membranes + lysozyme	1.67	101 270	1.170	56.0
Digested membranes + lysozyme, washed with 1 M NaCl	0.57	247 352	1.138	54.7
Digested membranes + cytochrome *c*	1.21	143 700	1.146	55.5
Digested membranes + cytochrome *c*, washed with 1 M NaCl	0.56	292 400	1.128	54.2

DISCUSSION

Our finding that pronase treatment, when applied to lipid-depleted mycoplasma membranes, digested almost all membrane proteins but when applied to native membranes digested only 80–90% of them, the residual 10–20% becoming accessible to the proteolytic enzyme when the lipids were removed, supports the suggestion of Morowitz and Terry[12] that some of the proteins are buried within the lipid matrix of the membrane and are inaccessible to pronase. Further support for this suggestion was obtained by iodinating membrane proteins with $K^{125}I$ by a lactoperoxidase-dependent process in which only membrane proteins that had access to the enzyme became labeled[13]. When iodinated membranes of *A. laidlawii*

or *M. hominis* were digested with pronase, the undigested protein residue had a lower specific label (cpm/mg protein) than the native membrane protein (S. Rottem, unpublished results). One cannot, however, exclude the possibility that acetone made membrane proteins more accessible to pronase, or that lipids partially inhibit the pronase so that their removal by acetone enables the membrane proteins to be completely digested.

The spectra of spin-labeled fatty acids in pronase-digested membranes in which most of the proteins have been digested resemble the spectra of native membranes, showing an increase of nitroxide mobility when the radical was moved towards the methyl end of the hydrocarbon chain of the fatty acid and when temperature was raised. These characteristics suggest that the local environment of the spin-labeled fatty acid in the digested membranes is an associated lipid structure having the properties of a bilayer. The temperature dependent increase in mobility was more pronounced in membrane preparations of *A. laidlawii* than of *M. hominis*. This might be due to the marked differences in cholesterol content (up to 40% of total lipid), in *M. hominis* and (2–4%), in *A. laidlawii*. The high cholesterol content of *M. hominis* might alter the physical state of the membrane lipids by increasing the viscosity of the hydrocarbon region[14].

The higher freedom of motion of spin-labeled fatty acid in pronase digested than in native membranes corroborates the assumption that in mycoplasma membrane proteins have a marked influence on the physical state of the membrane lipids[1,2,15]. Similar conclusions were reached in other systems[16,17]. It is not yet known what exactly are the protein–lipid interactions in native myoplasma membrane that interfere with the mobility of the hydrocarbon chains of membrane lipids, since the nature of the bonds holding the lipids and proteins together is still obscure. Our present findings, however: (a) that alterations in the physical state of membrane lipids occur by attaching soluble basic proteins (cytochrome *c* or lysozyme) to membrane preparations, apparently to the negatively charged phospholipids predominant in mycoplasma; (b) that these alterations are reversible by high salt solutions, indicate that electrostatic attractions may also affect the mobility of the hydrocarbon chains of membrane lipids. At the same time, one cannot rule out the possibility that the positively charged proteins may have some hydrophobic regions in their molecule which, though not affecting their binding to the membrane, do affect the mobility of hydrocarbon chains.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Joint Research Fund of the Hebrew University and Hadassah.

We would like to thank S. Razin, B. G. McFarland and H. M. McConnell for valuable advice and W. L. Hubbell for providing us with spin-labeled fatty acids.

REFERENCES

1 Rottem, S., Hubbell, W. L., Hayflick, L. and McConnell, H. M. (1970) *Biochim. Biophys. Acta* 219, 104–113
2 Tourtellotte, M. E., Branton, D. and Keith, A. (1970) *Proc. Natl. Acad. Sci. U.S.* 66, 909–916
3 Hubbell, W. L. and McConnell, H. M. (1968) *Proc. Natl. Acad. Sci. U.S.* 61, 12–16

4 Hubbell, W. L. and McConnell, H. M. (1969) *Proc. Natl. Acad. Sci. U.S.* 64, 20–27
5 McConnell, H. M. and McFarland, B. G. (1970) *Q. Rev. Biophys.* 3, 91–120
6 Razin, S. (1963) *J. Gen. Microbiol.* 33, 471–475
7 Rottem, S., Stein, O. and Razin, S. (1968) *Arch. Biochem. Biophys.* 125, 46–56
8 Razin, S., Morowitz, H. J. and Terry, T. M. (1965) *Proc. Natl. Acad. Sci. U.S.* 54, 210–225
9 Keana, J. W., Keana, S. B. and Beetham, D. (1967) *J. Am. Chem. Soc.* 89, 3055–3056
10 Jones, R. G. (1947) *J. Am. Chem. Soc.* 69, 2350–2355
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
12 Morowitz, H. J. and Terry, T. M. (1969) *Biochim. Biophys. Acta* 183, 276–294
13 Phillips, D. R. and Morrison, M. (1970) *Biochem. Biophys. Res. Commun.* 40, 284–289
14 Long, R. A., Hruska, F., Gesser, H. D., Hsia, J. C. and Williams, R. (1970) *Biochem. Biophys. Res. Commun.* 41, 321–327
15 Chapman, D. and Urbina, J. (1971) *FEBS Lett.* 12, 169–172
16 Berger, K. U., Barratt, M. D. and Kamat, V. B. (1970) *Biochem. Biophys. Res. Commun.* 40, 1273–1280
17 Hammes, G. G. and Schullery, S. E. (1970) *Biochemistry* 9, 2555–2563