A SPIN LABEL STUDY OF PURPLE MEMBRANES FROM *Halobacterium halobium*

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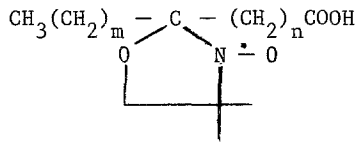
Received November 20, 1974

Summary: N-Oxyl-4',4'-dimethyloxazolidine derivative of 5-ketostearic acid, 12-ketostearic acid and 16-ketostearic acid were used to probe the molecular organization of "purple membranes" (PM) isolated from *Halobacterium halobium*. Electron spin resonance measurements suggested that PM are extremely rigid structures in which a large proportion of the lipid is present as boundary lipid closely associated with bacteriorhodopsin, the only protein present in the membranes. The PM showed a single transition at about 29°C which was probably due to some reversible change in the structure of bacteriorhodopsin.

The isolation of a purple-colored fragment from the cell membrane of the extreme halophile *Halobacterium halobium* represents one of the first successful attempts to obtain a simple membrane capable of translocating hydrogen ions (1). Purple membrane (PM) contains a single protein, bacteriorhodopsin, which constitutes about 75% of its dry weight. Oesterhelt and Stoeckenius (2) have shown that the purple color (absorption maximum 570 nm) of PM is due to retinaldehyde bound covalently to rhodopsin and evidence has accumulated that this light-induced proton translocating membrane bears a close relationship to the visual receptor membrane, although the ions translocated are different. Cone (3) and Brown (4) have demonstrated that the rhodopsin molecules in rod outer segments are highly mobile, the viscosity of the phospholipids being low enough to allow rotation and translation of the molecules within the membrane. This is consistent with the low degree of saturation of the fatty acid side chains found in rod outer segment phospholipids. By contrast, the side chains of PM phospholipids are highly saturated, consisting of dihydrophytol groups linked to phosphatidylglycerol by ether, instead of ester, linkages. The degree of fluidity of PM therefore becomes a question of interest.

Spin labels have been found to be extremely useful as probes of membrane structure. In particular, spin-labeled fatty acids with the general formula



I (m,n)

have provided information concerning the molecular organization, phase transitions and fluidity of various natural and synthetic membranes (5-7). The structure of mammalian visual receptor membranes has also been studied with the aid of spin labels (8-10). To date only one spin label study of membranes from halophilic bacteria has been reported. Hsia and co-workers (11) have detected salt-dependent conformational changes in the cell membrane of *Halobacterium salinarium* using a covalently bound maleimide spin label and a stearic acid label (I 1,14). We now report a study of the fluidity and molecular organization of PM using three stearic acid probes (I 1,14; 5,10; 12,3).

EXPERIMENTAL

Materials: The N-oxyl-4',4'-dimethyloxazolidine derivatives of 5-ketostearic acid (I 12,3), 12-ketostearic acid (I 5,10) and 16-ketostearic acid (I 1,14) were purchased from Syva, Palo Alto, California. Purple membranes were prepared from *H. halobium* in continuous culture using a modification[1] of the method of Oesterhelt and Stoeckenius (2). It showed one band on SDS gel electrophoresis with an estimated molecular weight of 26,000. The spectrum was identical with the published data (1).

Methods: Electron spin resonance (ESR) spectra were recorded with a Varian E-4 spectrometer operating at 9.5 GHz. Samples were introduced into the cavity in a quartz micro flat cell. Temperatures were maintained with a Varian variable temperature accessory (Varian E-257) and were measured with a Yellow Springs Instrument Co. Telethermometer model 42SC. All solutions contained 10 mM sodium phosphate buffer (pH 7.0). The PM were crosslinked with glutaraldehyde by the method of Brown (4).

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[1] Chignell, D. A., and Gray, I., manuscript in preparation.

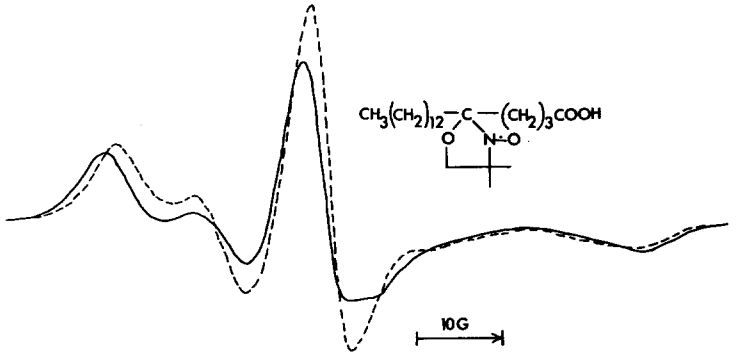

Fig. 1. The ESR spectrum of stearic acid label I (12,3) (2 x 10^{-5} M) bound to purple membranes (1.2 mg/ml) from *Halobacterium halobium*. 25°, ——; 37°, - - -.

RESULTS AND DISCUSSION

Stearic acid label I (12,3) exhibited maximum hyperfine splitting ($2T_m$) of 62 G and 59 G when bound to PM at 25°C and 37°C, respectively (Fig. 1). A comparison of the maximum hyperfine splitting of I (12,3) bound to PM with the splitting of the same label bound to other membrane systems clearly shows (Table 1) that PM is the most rigid of all the membranes hitherto probed with this label. Only the membranes of *Mycoplasma* have rigidities which are comparable to that of PM (Table 1). The $2T_m$ value of I (12,3) bound to PM decreased from 65.5 G at 5° to 57.8 G at 40°C. By contrast, a much steeper temperature dependence has been reported for I (12,3) bound to membranes from *Mycoplasma laidlawii* (18) and *E. coli* (17). An Arrhenius plot of the $2T_m$ values of I (12,3) bound to PM shows a single discontinuity at about 29°C (Fig. 2). Other workers have suggested that such discontinuities represent phase transitions occurring in membranes (17,20). When the PM was cross-linked with glutaraldehyde, the Arrhenius plot became monophasic (Fig. 2). Jost and co-workers have shown that glutaraldehyde fixation does not alter the ESR spectrum of I (12,3) bound to phospholipid multilayers (19). Thus it would appear that the discontinuity in the Arrhenius plot of untreated PM is probably due to a phase change in bacteriorhodopsin rather than to an alteration in the molecular organization of the phospholipids. Glutaraldehyde also

TABLE 1

The maximum hyperfine splitting ($2T_m$) of stearic acid spin label I (12,3) bound to various membrane systems

Membrane system	Temp. (°C)	$2T_m$ (G)	Ref.
L-cells	23	54.7	12
Human lymphocytes	23	53.7	12
Human erythrocytes	23	59.8	12
Human erythrocyte ghosts	--[a]	56.2	13,14
Human erythrocytes	--[b]	55.4	14
Human erythrocyte ghosts	--[a]	55.8	15
Influenza virus	--[a]	59.3	13
Submitochondrial particles	--[a]	57.8	15
Sarcoplasmic vesicles	22	53.8	16
E. coli membrane vesicles	20	56.7	17
Mycoplasma laidlawii membranes	15	58.0[c]	18
Mycoplasma laidlawii membranes	15	62.5[d]	18
Mycoplasma galliseptiaum membranes	15	61.75	18
Hallobacterium halobium purple	15	63.7	--
membranes	20	63.0	--
	25	62.0	--

[a] Temperature not stated
[b] Room temperature
[c] Cells grown at 15°
[d] Cells grown at 37°

produced a small but reproducible decrease in the rigidity of PM (Fig. 2). The significance of this observation is at the present moment unclear.

Stearic acid spin label I (5,10) was also highly immobilized when bound to PM with $2T_m$ values of 62.7 G, 60.7 G and 58.7 G at 5°C, 25°C and 37°C, respectively (Fig. 3). In other membrane system, it has been found that the

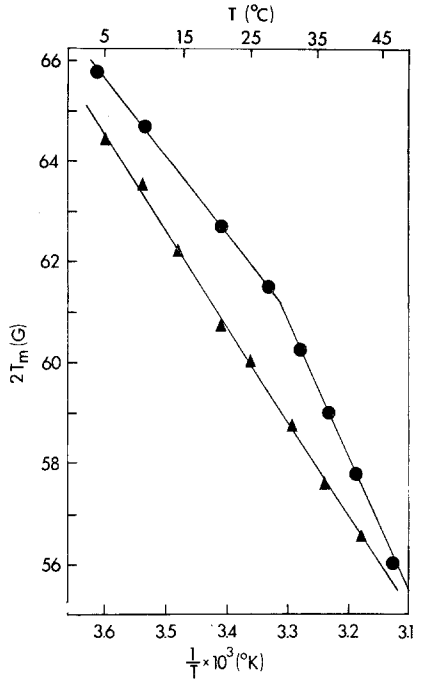

Fig. 2. The effect of temperature on the maximum hyperfine splitting ($2T_m$) of stearic acid label I (12,3) bound to normal (—●——●—) and glutaraldehyde fixed (—▲——▲—) purple membranes from *Halobacterium halobium*.

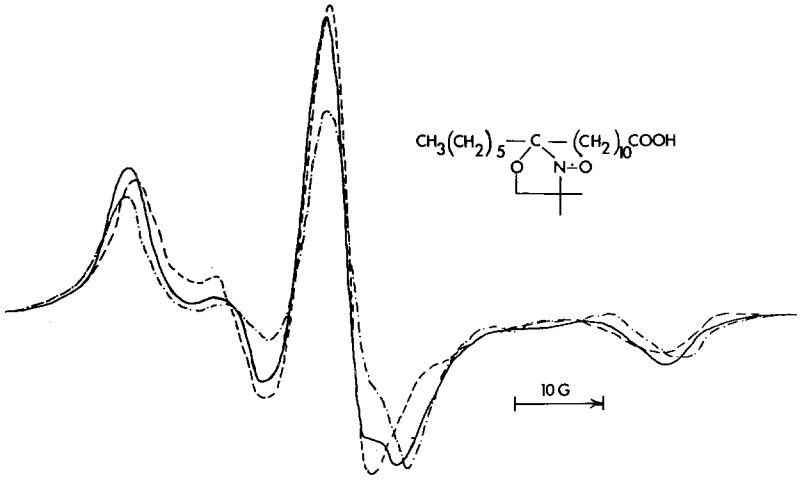

Fig. 3. The ESR spectrum of stearic acid label I (10,5) (2×10^{-5} M) bound to purple membranes (1.2 mg/ml) from *Halobacterium halobium*. 5°, -•-•-; 25°, ——; 37°, - - -.

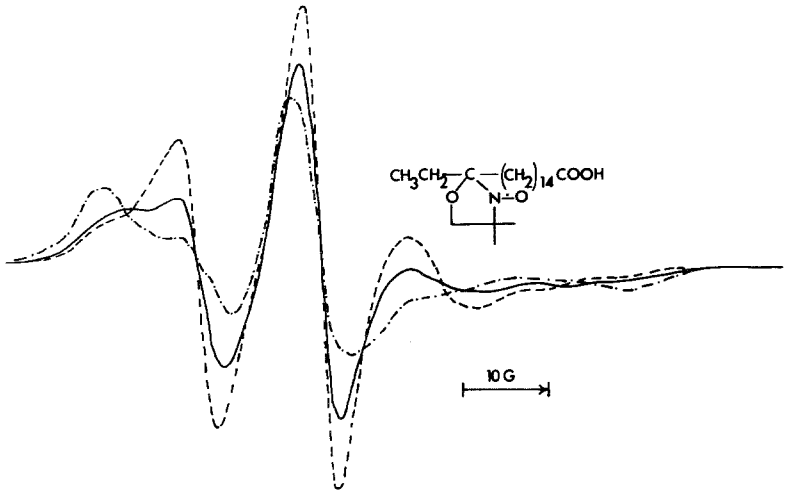

Fig. 4. The ESR spectrum of stearic acid label I (1,14) (2×10^{-5} M) bound to purple membranes (1.2 mg/ml) from *Halobacterium halobium*. 5^{o}, -•-•-; 25^{o}, ———; 37^{o}, - - -.

hyperfine splittings of the stearic acid spin labels decrease as the nitroxide radical is moved away from the polar head group of the fatty acid. Indeed, for such membrane systems as sarcoplasmic vesicles (16), phospholipid dispersions (16), mitochondrial membranes (20), L-cells (12) and lymphocytes (12), the motion of the nitroxide group of I (5,10) is almost isotropic. In contrast, at 37^{o}C, the $2T_m$ values of labels I (12,3) and I (5,10) bound to PM are almost identical. Thus it would appear that the rigidity of the PM extends deep into the interior of the membrane.

The ESR spectrum of stearic acid label I (1,14) revealed the presence of two populations of bound spin label, one of which was more highly immobilized than the other (Fig. 4). Jost and co-workers have reported similar results for I (1,14) bound to beef heart mitochondrial cytochrome oxidase (21,22). They have suggested that the highly immobilized labels are bound to boundary lipid, which is closely associated with the membrane protein. Jost and co-workers were able to prepare cytochrome oxidase containing differing amounts of lipid and to analyze the ESR spectra of bound I (1,14) in terms of the proportions of boundary and non-boundary (fluid) lipid. Unfortunately, it was not possible to perform a similar analysis with PM, since isolated bac-

teriorhodopsin did not bind the stearic acid labels. In addition, lipids isolated from PM would not form liposomes. Nevertheless, when the ESR spectrum of I (1,14) bound to PM is compared to the spectrum of the label bound to the cytochrome oxidase system containing the same lipid to protein ratio (see Fig. 2c in ref. 21), it is obvious that the PM contain a higher proportion of boundary lipid.

In conclusion, the evidence presented here clearly points to an extremely rigid structure for PM with two populations of phospholipids, one of which is tightly bound to the protein in the membrane. Immobilization of the protein by glutaraldehyde has little effect on this rigidity. Consistent with the findings of Racker and Hinkle on PM incorporated into vesicles of varying phospholipid composition (23), these experiments suggest that proton translocation occurs via a pore mechanism rather than being dependent on the mobility of the protein molecules.

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