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Lipid-Protein Interactions in Membranes: Effect of Lipid Composition on Mobility of Spin-Labeled Cysteine Residues in Yeast Plasma Membrane

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In order to gain direct evidence for lipid-dependent protein conformation in membrane, effects of modification of lipid composition on mobility of spin-labeled cysteine residues were investigated in the plasma membrane of the yeast Saccharomyces cerevisiae. Conversion of the bulk of phospholipids to diglycerides by treatment of the membrane with phospholipase C substantially enhanced spectral anisotropy. However, alteration of the viscosity of the lipid-bilayer by enriching the membrane with palmitelaidic or oleic acid had no effect on mobility of spin-labeled cysteine residues. These observations indicate that while the spin-labeled residues are not in direct contact with the lipid core of the membrane, there are lipid-protein interactions to the extent that removal of the polar portion of the bulk of phospholipids induces conformational changes in proteins, which in turn restrict mobility of these residues. It is concluded that conformation of membrane proteins depends on lipid structure and that phospholipids have a role in preserving the native conformation of proteins.

Key words: **protein conformation, phospholipids, diglycerides, lipid fluidity**

Although the general features of the organization and dynamics of lipids of the biological membrane are well-established [1–6], the precise nature of lipid-protein interactions that are necessary for expression of membrane function remains to be elucidated. Studies of membrane systems in which apolar and/or polar portions of phospholipids were altered and the effect of such alterations on membrane properties were investigated [7–12] emphasize the importance of lipid-protein interactions in membrane function. These interactions may involve a modification of protein conformation, the evidence for which, so far, has come indirectly, ie, by measurement of kinetic parameters [13]. In the present paper we have investigated the effect of modification of lipids in yeast (Saccharomyces cerevisiae) plasma membrane on the mobility of spin-labeled sulfhydryl residues of

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proteins. We have found that conversion of phospholipids to diglycerides by treatment of the membrane with phospholipase C restricts the mobility of the spin-labeled residues. However, altering the "fluidity" of the lipid bilayer does not affect the motion of these groups. Taken together, these observations indicate that while the spin-labeled residues are not in direct contact with the lipid core of the membrane, there are lipid-protein interactions to the extent that conformational changes are induced in proteins by removing polar portions of the bulk of phospholipids.

MATERIALS AND METHODS

Materials

Glusulase was obtained from Endo Laboratories, Garden City, New York. B cereus phospholipase C type V, NADPH (nicotinamide adenine dinucleotide, reduced) ATP (adenosine triphosphate), imidazole, Triton-X 100, N-ethyl-maleimide, oleic acid, palmitelaidic acid, and oligomycin were products of Sigma Chemical Company, St. Louis. 4-Maleimido-2,2,6,6-tetramethylpiperidinooxyl (4-maleimido-TEMPO) and 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl (12-doxylstearic acid) were purchased from Syva Research Chemicals, Palo Alto, California. All other materials were of reagent grade and were obtained from various commercial sources.

Yeast Strains and Growth Conditions

Saccharomyces cerevisiae, strain NYC 366 [14], and the unsaturated fatty acid auxotroph KD115 [15] were kindly provided by Dr. A.H. Rose, Bath University, Great Britain, and Dr. A.W. Linnane, Monash University, Australia, respectively. The cells were grown aerobically in the mineral salt medium of Wallace et al [16] at 30°C. For growth of NYC 366 the medium was supplemented with Bacto-Yeast extract (0.2%), glucose (0.05%), and glycerol (2%). Growth medium of strain KD115 contained 0.2% yeast extract, 0.5% ethanol, 0.5% glucose, 0.02% fatty acid, and 1% Triton-X 100. Cells were harvested at the logarithmic phase of growth and were washed once at 4°C with 0.02 M Tris-HCl (pH 8.0) containing 0.01 M $MgSO_4$.

Isolation of Plasma Membrane

Plasma membrane was isolated according to the procedure of Wiley [17] except that DNAase, RNAase, and NaCl [18] were eliminated, and the pellet of the second-step centrifugation containing the plasma membrane fraction was not washed with phosphate buffer containing ethylene diamine tetracetic acid (EDTA). Instead, it was resuspended in 0.02 M Tris-HCl (pH 8.0) containing 0.01 M $MgSO_4$ at a protein concentration of 16–19 mg/ml. Aliquots of 5 ml each of this suspension were layered on discontinuous sucrose density gradients consisting of equal volumes of 60, 55, 50, and 45% (w/w) sucrose in the same Tris-Mg^{++} solution and centrifuged in a SW 27 rotor for 4 h at 25,000 rpm. Routinely, four discrete membrane bands, designated I, II, III, and IV, and a small pellet were obtained. Fraction I routinely banded at a distance one-fourth to one-third into the 45% sucrose layer; fraction II banded at the interface of 45 and 50%; and III and IV were located at the interfaces of 50 and 55%, and of 55 and 60% sucrose, respectively. The fractions were diluted each to 40 ml with 0.02 M Tris-HCl (pH 8.0) containing 0.01 M $MgSO_4$, and the membrane was pelleted by centrifugation for 15 min at 39,000g. Each pellet was washed twice with 40 ml of the same buffer containing 0.01 M $MgSO_4$ with homogenization and

then resuspended in the same solution. About 70–80% of protein applied to sucrose gradients was recovered in these bands.

As shown in Figure 1, band I had the highest specific activity of Mg^{++}-dependent oligomycin-resistant ATPase, a yeast plasma membrane enzyme [19–23], and the lowest levels of succinate oxidase (a mitochondrial marker [19, 23]), and NADPH-cytochrome C reductase (a microsomal enzyme [24]). This band was therefore identified as the plasma membrane fraction and was used throughout these studies. Fraction II also had a relatively high activity of oligomycin-resistant ATPase (Fig. 1), but it was discarded because of contamination with other particles.

Enzyme Determinations

Oligomycin-resistant Mg^{++}-stimulated ATPase activity was measured under described conditions [23] using 100 μg oligomycin per milligram protein. NADPH cytochrome C reductase activity was assayed according to the procedure of Yoshida et al [24]. Succinate

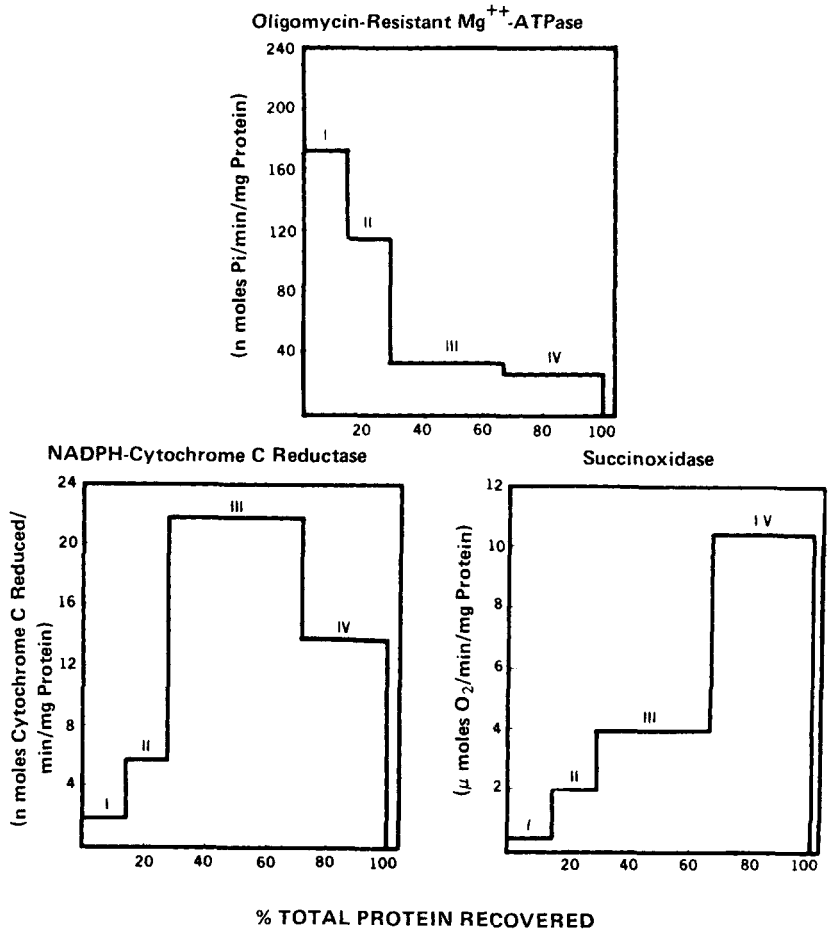

Fig. 1. Relative distribution of marker activities on discontinuous sucrose density gradients. Membranes from strain NYC 366 were fractionated, and activities of various enzymes were assayed in each fraction as described in Materials and Methods.

oxidase activity was determined as described previously [13]. All assays were carried out at 37°C. Protein was measured by the method of Lowry et al [25].

Lipid Analysis

Procedures for extraction of membrane lipids, isolation of phospholipid fraction, and preparation of fatty acid methyl esters have been described [26]. Methyl esters were chromatographed on a Hewlett-Packard series 700 gas chromatograph equipped with a 6-ft column of 10% diethylene glycol succinate (DEGS-PS) on Supelcoport (80–100 mesh) at 175°C. Lipid phosphorus was determined according to the procedure of Chen et al [27].

Treatment With Phospholipase C

To each suspension of plasma membrane (11 mg protein in 0.5 ml of 0.02 M Tris-HCl, pH 8.0), an equal volume of a solution containing 0.1 M $CaCl_2$ and 0.15 M NaCl [28] was added as well as 100 μl of 3.2 M ammonium sulfate, pH 6 (intact membrane), or 100 μl of ammonium sulfate containing 46 units of phospholipase C. After incubation for 1–2 h at 37°C, the contents of each tube were diluted with 30 ml of cold 0.02 M Tris-HCl (pH 8.0), and the membranes were pelleted by centrifugation at 39,000g for 15 min. Each pellet was washed once more with the same buffer. Phospholipid contents of these preparations were determined after they had been labeled with 4-maleimido-TEMPO and electron paramagnetic resonance (EPR) spectra had been recorded.

Labeling With 4-Maleimido-TEMPO

Sulfhydryl groups of the membrane were spin-labeled essentially according to Yu et al [28]. Membrane suspensions (8 mg protein each) were incubated for 30 min at room temperature in 5 ml of a solution of 0.02 M Tris-HCl (pH 8.5) containing 0.3 M sucrose, and 0.001 M 4-maleimido-TEMPO. To each suspension 30 ml of cold 0.02 M Tris-HCl were added, and the membranes were pelleted as described above. Each pellet was washed twice with 40 ml of the same buffer with homogenization. To each pellet a volume of 0.2 ml of 0.01 M imidazole buffer (pH 7.0) containing 0.1 M KCl was added [28]. The suspension was transferred to an aqueous EPR cell (Varian Associates), and spectra were recorded. Approximately 2 nmoles of nitroxide were bound per milligram protein.

Labeling With 12-Doxyl Stearic Acid

Membranes were labeled with 12-doxyl stearic acid as described previously [26] except that 10 mg of membrane protein and 112 μg of spin label were used. Based on phospholipid content (0.42 mg/mg protein) and assuming that all of the spin label was taken up, the concentration of 12-doxyl stearate represented about 2.5% of the total membrane lipids.

EPR Spectra

EPR spectra of preparations labeled with 4-maleimido-TEMPO were recorded at 22°C on a Varian E-3 EPR spectrometer operating at X-band. Spectra of membranes labeled with 12-doxyl stearic acid were recorded at various temperatures at X-band on a Varian E-12 spectrometer. Temperature was controlled with a variable temperature accessory and measured with a copper-constantan thermocouple. Samples were allowed to equilibrate for 10 min at the desired temperature before spectra were recorded.

RESULTS

Effect of Conversion of Phospholipids to Diglycerides With Phospholipase C on Mobility of Spin-Labeled Residues in Proteins

The cysteine residues in intact membrane as well as preparations digested with phospholipase C were reacted with 4-maleimido-TEMPO, and EPR spectra were recorded at 22°C. Representative spectra of these preparations are shown in Figure 2. The spectra revealed highly anisotropic motion of the nitroxide, but the degree of anisotropy increased with decreasing phospholipid content (see spectra B and C, Fig. 2). The hyperfine splitting ($2T_{//}$) remained the same (65 G) in all preparations. The amplitude of the high-field line (h-1), however, was substantially reduced in the preparation digested for 1 h with phospholipase C (spectrum B), and approached zero with further degradation of phospholipids (spectrum C).

The relationship between the phospholipid content of the membrane and the ratio of line height amplitudes (h-1/h-0) is shown in Table I. This ratio serves as a convenient empirical measure of motion of nitroxide [26, 29]. In intact membrane there was 17 μg lipid phosphorus per milligram protein, which decreased to 3 μg after 1 h and 0.8 μg after 2 h of treatment with phospholipase C, respectively. The corresponding value of the spec-

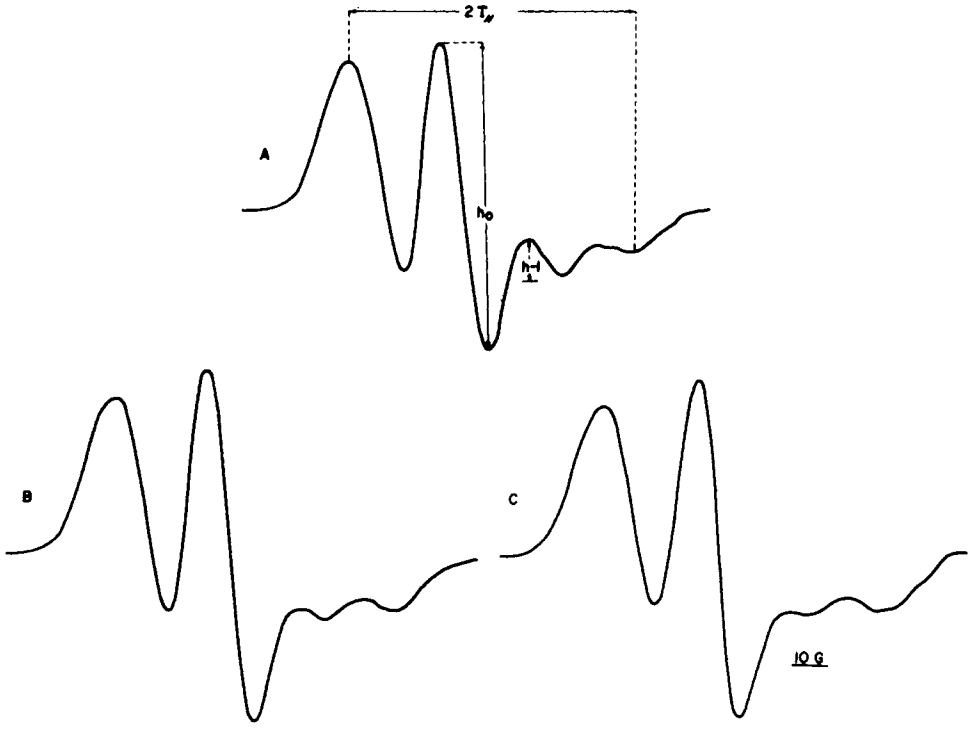

Fig. 2. Electron paramagnetic resonance spectra of membrane preparations digested with phospholipase C and labeled with 4-maleimido-TEMPO. Plasma membrane was isolated from strain NYC 366, treated with phospholipase C, and labeled with 4-maleimido-TEMPO, as described in Materials and Methods. A) Spectrum of intact membrane; B) spectrum of membrane digested with phospholipase C for 1 h; C) spectrum of membrane digested with phospholipase C for 2 h.

TABLE I. Effect of Phospholipid Content on h-1/h-0 Ratio in Spectra From Membranes Labeled With 4-Maleimido-TEMPO*

Preparation	Phospholipid content[a]	h-1/h-0[b]
Intact	17.0	0.15
Phospholipase C-treated		
Treated for 1h	3.0	0.03
Treated for 2h	0.8	0.01

*Each value is an average of two independent determinations.
[a]Micrograms lipid phosphorus per milligram protein.
[b]Ratio of amplitude of high-field line to that of midfield line.

tral parameter, h-1/h-0, of spin-labeled proteins was 0.15 in the intact membrane and 0.03 for the preparation depleted of phospholipids by 80%, and it approached zero when the phosholipid content was reduced by 95%. The results were independent of the sequence of phospholipase C digestion and spin-labeling since spectra of membranes first labeled and then digested with phospholipase C revealed as much spin immobilization as in membranes first digested and then labeled (shown in Fig. 2B and C, and Table 1). From these observations we conclude that conversion of the bulk of phospholipids to diglycerides induced conformational changes in proteins, which in turn restricted mobility of spin-labeled residues and enhanced spectral anisotropy.

Effect of Lipid Viscosity on Mobility of Spin-Labeled Residues in Membrane Proteins

The viscosity of the lipid bilayer of mycoplasma and of Escherichia coli membrane has been altered by growing the cells on oleate or elaidate [30, 31]. However, since the unsaturated fatty acid requirement of yeast cannot be met with elaidate [32], palmitelaidic acid was used in the present studies.

Plasma membranes were isolated from strain KD115 grown on oleate or palmitelaidate. The fatty acid composition of the phospholipid fraction from these membranes is shown in Table II. Both types of phospholipids contained 80% unsaturated fatty acids. Lipid "fluidity" was monitored by incorporating 12-doxyl stearic acid into the membranes and taking paramagnetic resonance spectra at various temperatures. Hyperfine splitting ($2T_{//}$), which is a useful parameter for measuring "fluidity" of the lipid environment of the doxyl group in the membrane [30], was determined from each spectrum. Results are shown in Figure 3. Over the temperature range of the experiment, $2T_{//}$ values for the nitroxide group in palmitelaidate membrane were higher than those obtained in oleate membrane. This indicates that in palmitelaidate membrane the reporter group has less rotational mobility because of a higher lipid viscosity [30].

We then investigated the effect of the viscosity of the lipid bilayer on the mobility of the spin-labeled residues in proteins. Plasma membranes, enriched with oleate or palmitelaidate, were labeled with 4-maleimido-TEMPO, and EPR spectra were recorded at 22°C (Fig. 4A and B). Although at this temperature lipids in palmitelaidate membrane were more viscous than those in oleate membrane (see Fig. 3), the spectra were quite similar and both showed a ratio of low field to midfield peak amplitude equal to 0.43. Therefore, mobility of the labeled sulfhydryl residues was not affected by altering viscosity of the lipid bilayer.

TABLE II. Fatty Acid Composition (percentage) of Membrane Phospholipids

Fatty acids in phospholipids	Fatty acid added to growth medium	
	Palmitelaidate	Oleate
$C_{12:0}$	–	1
$C_{14:0}$	–	3
$C_{16:0}$	20	13
$C_{16:1}$	80	14
$C_{18:0}$	–	4
$C_{18:1}$	–	66

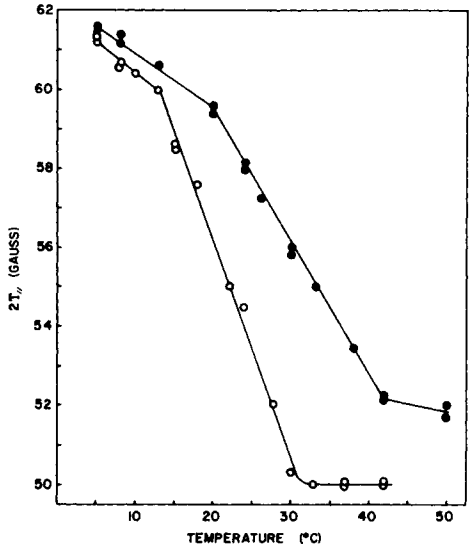

Fig. 3. Effect of membrane fatty acid composition on hyperfine splitting ($2T_{//}$) of 12-doxyl stearate. Oleate- or palmitelaidate-enriched membrane from strain KD115 was labeled with 12-doxyl stearate as described under Materials and Methods, and EPR spectra were recorded at various temperatures. ○) Oleate membrane; ●) palmitelaidate membrane.

DISCUSSION

From the alteration of kinetic parameters of some membrane-bound enzymes by phospholipids [13, 33, 34] and from the phospholipid specificity of several lipid-requiring enzymes [34–39], a possible role of lipids in the regulation of enzymatic activities in vivo may be speculated. This regulation may be exercised through lipid diffusion and phase segregation [4, 5, 40], or through the existence of boundary lipids [41, 42], and it may well involve modifications of protein conformation. The present studies were undertaken in order to obtain more direct evidence of lipid-dependent conformational changes in membrane proteins.

We found that conversion of the bulk of phospholipids to diglycerides resulted in a substantial enhancement of anisotropic motion of spin-labeled cysteine residues. This

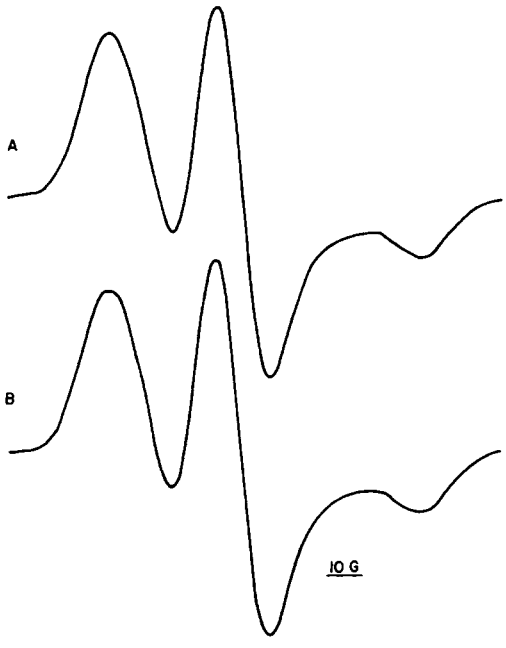

Fig. 4. Electron paramagnetic resonance spectra of membranes enriched with oleate or palmitelaidate and labeled with 4-maleimido-TEMPO. Plasma membranes from strain KD115 were labeled with 4-maleimido-TEMPO and spectra were recorded at 22°C. A) Spectrum from membrane enriched with palmitelaidate; B) spectrum from membrane enriched with oleate.

indicates that conformational changes had taken place in proteins that then restricted mobility of these residues. These changes were detected even though it appears that the residues are not in direct contact with the lipid core. Rather, they are either sequestered from the lipids or situated in the aqueous environment. The proximity of the labeled residues to the lipid phase was deduced from the observation that their mobility was the same in oleate-enriched membrane as that in palmitelaidate-enriched membrane, although the two membranes differed substantially in lipid "fluidity" (as evidenced by significantly higher freedom of motion of 12-doxyl stearate in oleate membrane).

4-Maleimido-TEMPO was synthesized in McConnell's laboratory as an SH-directed probe. It was first used to label β-93 cysteine of hemoglobin and deduce its orientation relative to the protein molecule [43]. The probe was subsequently used by other investigators who labeled sulfhydryl groups of proteins in erythrocyte and sarcoplasmic reticulum membranes and monitored changes produced by various biologically active agents [27, 28, 44–46]. Labeling specificity has been verified by blocking with sulfhydryl reagents [44–46]. Under some reaction conditions [46–48] amino groups can also be labeled. In such a case, a narrow component positioned about 10 G to the right of the center of the low-field peak appears in the spectrum and cannot be eliminated by treatment, prior to spin-labeling, with sulfhydryl reagents. This component was absent from our spectra. Furthermore, the spectra were of the powder type, with $2T_{//}$ of about 65 G, which are obtained when the spins are linked to sulfhydryl groups [43]. Finally, 95% of signal intensity was lost by a brief treatment of membranes with N-ethyl-maleimide (1 mM for

30 min) prior to labeling. These observations lead us to conclude that under the conditions we employed [27], amino groups were not labeled to any significant extent. Therefore, we referred to spin-labeled groups as cysteine residues.

Our investigation, evidently, deals with the effect of lipid composition on mobility of spin-labeled residues in general. At present it is not feasible to label proteins in these membranes selectively. Indeed, very few membrane proteins are amenable to specific labeling. One such protein is the M-protein of Escherichia coli membrane, which we have been able to selectively label by a procedure based on that of Fox and Kennedy [49]. Our preliminary findings indicate that the motion of the spin-labeled cysteine residue involved in β-galactoside transport is influenced by membrane lipid composition.

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