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Spin-Label Studies of Dynamics of Lipid Alkyl Chains in Biological Membranes: Role of Unsaturated Sites

(membrane structure/yeast membrane/spin-label reduction)

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ABSTRACT **Cultures of a yeast mutant, deficient in the synthesis of unsaturated fatty acids, were supplemented with either stearolic acid or with any one of three octadecanoic acids having a *cis* double bond 6, 9, or 11 carbons away from the acyl group. The resulting cells, with lipid alkyl chains well defined with respect to the position and nature of unsaturated sites, were then studied with spin-labeled stearic acids having a *N*-oxyl-oxazolidine ring located at 4, 6, 9, or 12 carbons away from the acyl group, and added *in vitro* to the cellular preparations.**

Differences in the molecular motion of each spin label were observed, as a function of the unsaturated site, in the intact yeast cells. Characteristic order to disorder phase transitions are inferred from data of temperature dependence. The results also indicate that triple bonds and *cis* double bonds inhibit a relatively ordered packing of lipid alkyl chains in the region between unsaturated sites and terminal methyl groups, leaving the hydrophobic region bounded by acyl groups and unsaturated sites unaffected.

The molecular organization and the physical mechanisms that govern protein-lipid interactions in biological membrane systems are still largely unknown. However, lipid fatty alkyl chain dynamics are apparently important in determining the biological activity of membrane-related functions. A study of *Mycoplasma laidlawii*, for example, reports different morphological and growth properties of the cells according to whether the temperature of the culture is above, equal to, or below that of a thermotropic phase transition observed in both intact cells and aqueous dispersions of their extracted lipids (1). Abrupt changes in the temperature dependence of mitochondrial respiratory activity in several organisms (2) correlate with similar changes in the temperature-dependent dynamic state of a spin-labeled fatty acid moiety solubilized in the mitochondrial lipids (3). Finally, the calcium-dependent ATPase activity, in fragmented sarcoplasmic vesicles isolated from rabbit skeletal muscles, depends on empirical dynamic parameters believed to describe the fluidity of hydrocarbon regions of membrane lipids (4). The connection between lipid dynamics and biological activity is possibly the reason why fatty acyl chains of bacteria, fungi, plants, insects, and marine organisms become richer in unsaturates as the growth (or habitat) temperature is lowered (5–9). The implication is that the organism adjusts the lipid composition so as to prevent lipid fluidity from decreasing below a point critical to the favorable functioning of membrane-bound enzymes. The purpose of this work is to elucidate the function of unsaturated alkyl sites in well-defined biological membrane systems that have unsaturated bonds at known positions along lipid alkyl chains.

Cells of a yeast mutant deficient in fatty acid desaturase activity (10) incorporate several supplemented fatty acids into their membrane lipids. Since no growth occurs in the absence of unsaturated supplements and since no unsaturates are biosynthesized, cells can be obtained containing membranes that are well-defined with respect to the positions of unsaturated bonds along lipid alkyl chains. We determined the enrichment in unsaturated chains after the fact in order to completely define the composition of the alkyl chains. The yeast mutant we used has a low reversion rate, is respiratory deficient, and, consequently, has reduced internal membrane structures. We believe that this mutant and others containing similar lesions (11) are superior to model membrane systems in some cases and that they may potentially bridge the large gap that exists between these systems and complex mammalian membranes.

Electron spin resonance (ESR) of nitroxide-free radicals has been used extensively to probe the physical state of artificial and biological membranes (3, 4, 11, 12). Fatty acids, labeled at various positions along the hydrocarbon chain and added *in vitro* to membrane preparations, readily associated with membrane lipids. ESR spectra of these probes provided conformational and dynamic information that may be interpreted in terms of the relative fluidity of the host matrix or used to infer the existence of temperature-dependent phase transitions.

MATERIALS

Yeast. The nuclear petite KD46 (*ole 2*) mutant of *Saccharomyces cerevisiae* is described genetically and biochemically elsewhere (10). The standard liquid culture medium contained (w/v) 1% yeast extract and 2% peptone (Difco Laboratories, Detroit, Mich.) and 2% dextrose (Sigma Chemical Co., St. Louis, Mo.). Tergitol (Union Carbide, Institute, W. Va.) was added at 1% (v/v) for solubilization of fatty acid sup-

Abbreviations: GLC, gas–liquid chromatography; NS, nitroxide stearate; Δ^{11}, Δ^{9}, and Δ^{6}, *cis*-Δ^{11}-, *cis*-Δ^{9}-, and *cis*-Δ^{6}-octadecanoate, respectively; $\Delta^{9\equiv}$, stearolate.

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plements. We used 1 mM each of four different fatty acids to supplement the growth medium: *cis*-Δ^6octadecanoate (18:1Δ^6 *cis*) was obtained from Analabs Inc., North Haven, Conn.; *cis*-Δ^9-octadecanoate (18:1Δ^9 *cis*) and *cis*-Δ^{11}-octadecanoate (18:1Δ^{11} *cis*) were obtained from Hormel Institute, Austin, Minn.; stearolic acid (18:1$\Delta^{9\equiv}$) was obtained from Lachat Chemicals Inc., Chicago Heights, Ill. All were better than 99% pure, as determined by the supplier or by gas–liquid chromatography (GLC) of the corresponding methyl esters. Finally, a pure phospholipid, 1-palmitoyl-2-oleoyl phosphatidylcholine, was provided by Dr. J. Tinoco. GLC on the corresponding methyl esters revealed that the ratio of 16:0 to 18:1Δ^9 *cis* is about 1.01.

Nitroxide Spin Probes. Stearic acid, labeled at various positions along the hydrocarbon chain with an *N*-oxyl oxazolidine ring, provided the various probes used in this work. These are denoted by 4, 6, 9, and 12NS where 4, 6, 9, or 12 refer to the carbon position, counted from the carboxyl group, on which the label is attached and NS is an abbreviation for nitroxide stearate. The methyl ester of 12NS was also used. Their synthesis is described elsewhere (13), and their purity was determined by analytical thin-layer chromatography.

METHODS

Cultures. The cultures were harvested in logarithmic or stationary phase by centrifugation of the growth medium at about 2000 × *g*. The pellets were washed with 1% Tergitol in water, then with distilled water. The liquid cultures were routinely tested for reversion to insure that the harvested cells were indeed desaturase mutants. Alkyl chain composition was determined by analytical GLC performed on the methyl esters of fatty acids isolated from the pellets. These were saponified in 2 M KOH and methylated in 0.5 M HCl in methanol. GLC was performed on a Varian Aerograph model 600D equipped with a disc integrator. The column was 0.3 × 244 cm stainless steel packed with 60/80 Chromosorb W, acid-washed and coated with 15% diethylene glycol succinate.

Model System. The model membrane system was an aqueous dispersion of 1-palmitoyl-2-oleoyl phosphatidylcholine (10 mg/ml) in Tris·HCl buffer (pH 7.2).

Electron Spin Resonance. The spin probes were dissolved in ethanol at 0.1 M; 0.2 μl were then added to about 0.1 ml of yeast pellet (about 2.5 mg of protein) to make an approximate bulk concentration of 0.2 mM. The spectra were recorded immediately after the mixture was stirred, as long incubation periods were found to be unnecessary. A Varian model E-3 electron paramagnetic resonance spectrometer equipped with a Varian variable temperature controller was used. The controller scale was calibrated with an iron-constantan thermocouple inserted in a dummy sample tube. The calibration was checked before every run, and the spectra were recorded at sample temperatures estimated accurate to better than ±1° overall.

RESULTS

Lipid Composition. The growth characteristics of the KD46 desaturase mutant in media supplemented with fatty acids have been described (14), and no anomalies were observed in the cultures retained for further experimentation. All our preparations were grown at 30°, and the minimum division times, determined from turbidity measurements with a Klett photometer, varied from 2–3½ hr, depending upon the supplemented fatty acid. Cultures grown in a medium supplemented with 18:1Δ^{11} *cis* were the fastest growing, followed by those supplemented with 18:1Δ^9 *cis*, 18:1$\Delta^{9\equiv}$, and 18:1Δ^6 *cis*, respectively. (The corresponding enrichments will heretofore be simply denoted by Δ^{11}, Δ^9, Δ^6, and $\Delta^{9\equiv}$.) Several samples were prepared from each of several cultures supplemented with a given fatty acid. The degree of enrichments of supplemented fatty acid all ranged between 55 and 78% of the total fatty acid composition. Results specific to any one batch are given together with the corresponding ESR results.

Electron Spin Resonance. The methyl ester of 12NS is insoluble in aqueous solutions, and the polarity of the *N*-oxyl group is not high enough to provide an effective anchoring of the molecule to the amphiphilic interface. Spectra of this spin probe in membrane systems exhibit the same solvent and local polarity effects as do spectra obtained in solvents such as octadecane (15). It is inferred from this, and from studies in aqueous lipid dispersions, that the probe is incorporated in the hydrophobic regions of membrane lipids.

A molecule such as 12NS is far from spherical; however, the measured spectra are very nearly those expected from isotropic motion. It has been shown that a rotational correlation time (τ_c) may be derived from spectral parameters when the motion is isotropic (16),

$$\tau_c = K\,(W_{-1} - W_0). \qquad [1]$$

K is obtained from the spin-label crystal parameters (12); W denotes a first derivative line width; and the subscripts −1 and 0 refer to the high- and mid-field lines, respectively. This formula is valid as long as $\tau_c < 10^{-9}$ s. In the range of temperatures used in this work, τ_c as given by Eq. 1 is about 2×10^{-9} s or more, and the equation no longer holds as K is no longer independent of nitroxide motion (16). An empirical motion parameter (τ_o) may be defined in this case by fixing K arbitrarily at its limiting value in the case of rapid isotropic tumbling. Assuming further that the curves are Lorentzian, Eq. 1 becomes

$$\tau_o = K\,W_0[(h_0/h_{-1})^{1/2} - 1] \qquad [2]$$

where h referes to first derivative line heights.

The temperature dependence of the motion parameter was measured in Δ^{11}, Δ^9, and Δ^6 and displayed on an Arrhenius plot in Fig. 1. Abrupt changes in the activation energies are evident. The measurements were repeated in several preparations differing only in the degree of lipid enrichment in unsaturated fatty acyl chains. In all experiments with a given fatty acid supplement, e.g., Δ^9, the discontinuity in the temperature dependence of τ_c occurred at the same temperature, 16 ± 1°, although the enrichments varied from 68–78% of total lipid alkyl chains. The slopes of the curves on either side of the discontinuity could not be reproduced but, at any given temperature above the transition, τ_o was lower for the higher enrichments in unsaturated chains.

Experiments with the same label were also done in the $\Delta^{9\equiv}$ system and the artificial system of phosphatidylcholine, to verify that both the position of unsaturated sites as well as the nature of the unsaturation determine the transition temperature. The artificial system is the pure phospholipid equivalent of Δ^9 systems enriched at 50%. The transition in the phosphatidylcholine system is also observed at 16°, but

that in $\Delta^{9\equiv}$ occurs at 27°. These results are displayed in Fig. 2. The transition temperatures observed in all systems were reproducible on the same samples and were unaltered by irreversible thermal protein denaturation.

Characteristics of spectra obtained with *n*NS probes in membranes and in sonicated lipid dispersions have been extensively described (4, 12). The shapes of the spectra may be interpreted in terms of rapid anisotropic rotation of the probing molecule about the equilibrium position of its long axis. Orientation experiments in phospholipid multilayers and in membrane systems (17, 18) have shown that this axis is perpendicular to the amphiphilic interface. It has also been shown that the degree of the nitroxide motional anisotropy decreases as the label's distance from the anchoring carboxylic group is increased. The flexibility of the fatty acid chain and the fluidity of the host membrane system may result in extensive transverse random motion at the level of the nitroxide ring. A possible quantitative measure of the mean angular deviation of these oscillations was introduced (19); an order parameter

$$S = {}^1/_2 \, (3 \, \langle \cos^2 \alpha \rangle - 1) \qquad [3]$$

may be obtained from the shape of the spectrum. Here, α denotes the angle between the nitrogen $2p\pi$ orbital and the equilibrium position of the fatty acid long molecular axis. Expressed in terms of spectral parameters, this quantity becomes

$$S = (T_{//} - T_{\perp})/(T_{zz} - T_{xx}) \qquad [4]$$

where $2T_{//}$ and $2T_{\perp}$ correspond to the separation of the two outer and two inner hyperfine extrema, respectively. The isotropic hyperfine splittings, T_{zz} and T_{xx}, are obtained from the crystal parameters (12). These are not known accurately, however, because of their small dependence on solvent polarity which may vary, for example, with the average distance between the nitroxide ring and the amphiphilic interface. Accurate values of the order parameter are thus difficult to obtain; this need not concern us, however, as our purpose is to show relative variations in S as measured in different membrane systems with identical spin probes.

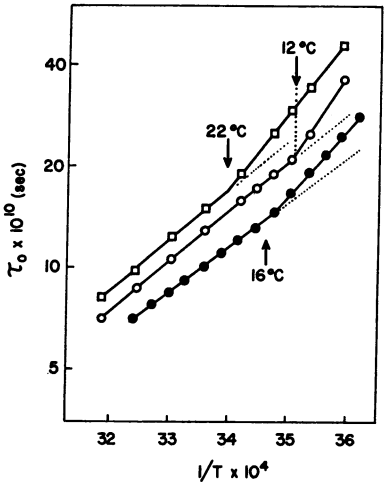

FIG. 1. Arrhenius plots of the motion of 12NS (methyl ester) in Δ^{11} (○), Δ^9 (●), and Δ^6 (□). Enrichments of these systems in unsaturated acyl chains are 76, 78, and 62%, respectively.

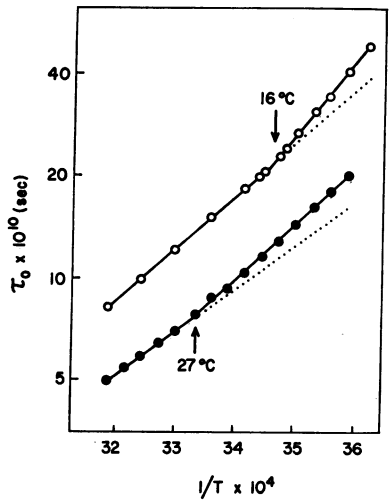

FIG. 2. Arrhenius plots of the motion of 12NS (methyl ester) in the artificial phosphatidylcholine system (○) and in $\Delta^{9\equiv}$ (●). Enrichment of the latter in stearolic acyl chains is 54%.

The order parameters obtained from spectra measured in the four yeast cell systems and in the artificial model system are given in Fig. 3.

Degradation of Spin Probes. A temperature-dependent reduction in amplitudes of the ESR signal was observed in all biological membrane systems. Most of this activity was irreversibly destroyed by heat denaturation of the cells before the introduction of spin probe molecules. The rates of destruction in any given system increased exponentially with temperature until denaturation became a competing process. The rates differed from sample to sample and were the greatest in cells harvested in the logarithmic growth phase and the least in cells harvested in the stationary phase. The signal from labeled bovine serum albumin, added to the cell preparations, remained unchanged in shape or amplitude. Since vitamin C destroys the ESR signal from bovine serum albumin while the yeast does not, we tentatively conclude that the observed nitroxide reductase activity is not localized at the outer surface of the yeast cells.

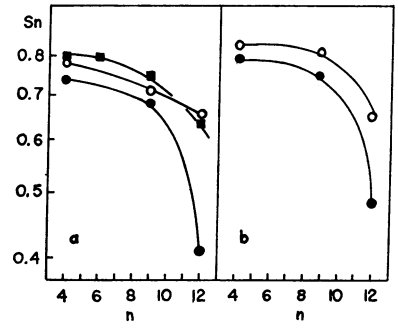

FIG. 3. (*a*) Order parameter *Sn* as a function of nitroxide ring position along the fatty acid chain of *n*NS probes in Δ^{11} (○), Δ^9 (●), and Δ^6 (■). (*b*) Same plot for the artificial phosphatidylcholine (○) and $\Delta^{9\equiv}$ (●). All measurements were made at 0° except for the Δ^6 system, which was measured at 5°. Enrichments in unsaturated acyl chains are 80, 78, 58, 50, and 49%, respectively.

DISCUSSION

Smooth freeze-fracture faces characteristic of lipid bilayer cleavage were observed by electron microscopy in similar preparations of the same mutant used in this work (14). Furthermore, the behavior of hydrophobic or amphiphilic spin probes in intact yeast cells has the same general features as that in aqueous dispersions of lipids extracted from these same cells (11). It is, thus, reasonable to assume that the organization of lipid moieties in our cellular preparations is similar to that of artificial bilayer models. Also, since internal membrane structures are reduced in this particular anaerobic mutant, and since the experiments were conducted immediately after introduction of the labels, we believe that our results pertain to the cellular plasma membranes.

Discontinuities in the Arrhenius behavior of spin label motion parameters, obtained from labeled mitochondria of temperature-sensitive organisms (3), correlate with nonlinearities in biological activities (2) and, for *M. laidlawii* (20), with a thermotropic phase transition (1, 21) also observed in the same temperature range by x-ray diffraction (22). Therefore, we interpret these discontinuities in terms of a phase transition of the lipid moieties, since they are observed in both the organisms and aqueous dispersions of the extracted lipids. The different systems studied in this work are compared with one another in terms of their measured transition temperatures in addition to other isothermic dynamic parameters. However, lipids exhibit several structural forms or mesomorphic lyotropisms. Thus, there is some uncertainty in the interpretation of the exact meaning of the transition temperature in the motional behavior of a spin label.

It is well known that double bonds in alkyl chains lower the bulk melting point. It is expected that double bonds provide a steric hindrance to the tight packing of alkyl chains and the ordered arrangement of methylene groups. In phospholipid bilayers, the axial symmetry imposed on the alkyl chains is also expected to be perturbed by the occurrence of *cis* double bonds. Consequently, an order to disorder phase transition temperature would be lowered by the addition of double bonds to a given membrane system. This is consistent with the observed results. In saturated phospholipid dispersions, phase transitions occur at higher temperatures than those reported here. For example, in aqueous dispersions of dipalmitoyl lecithin the transition occurs at about 40° (12). The perturbation by double bonds in the Δ^6 system is the least effective as these are the closest to the polar anchoring groups, leaving a wide area in the interior of the hydrophobic region constituted of methylene groups only. In the Δ^{11} system, the double bonds are the farthest from the anchoring groups and saturated areas are about the same width. This system, being the most effectively interrupted, has, as expected, the lowest of the three transition temperatures. The Δ^9 system is intermediate between these two extremes. Note that the transition in the $\Delta^{9=}$ system occurs at a higher temperature than in any of the three others but still at a lower temperature than in saturated systems. The greater perturbing influence of *cis*-unsaturated sites is probably due to the "kink" these bonds introduce in the static conformation of a hydrocarbon chain whereas triple bonds do not. It is interesting that the KD46 mutant will *not* grow on medium supplemented with either saturated or elaidic (18:1Δ^9 *trans*) fatty acids. Stearolic acid, elaidic acid, and stearic acid have similar static conformations; consequently, it is difficult to suggest physiological roles. Since 16:1Δ^9 *trans* and 18:2$\Delta^{9,12}$ *trans, trans* acids are both growth factors, temperature-dependent molecular geometry must be important to the host organism as well as the properties conferred by an acetylenic bond, a *trans* double bond, or a *cis* double bond. Finally, the growth rates are related to the phase transitions detectable by ESR. The slowest growing is the Δ^6 system, whose transition temperature is the closest to the growth temperature of 30°, presumably resulting in a system less fluid than Δ^9 or Δ^{11}. Absolute values of τ_o cannot be directly related to any motional correlation time of the host molecules. The same label in pure solvents, such as octadecane, shows that, on either side of the melting point, the motion of the probe molecule may be several orders of magnitude more rapid than that of the host molecules, as indicated by viscosity data.

The fatty acid probes, *n*NS, used for measurement of the order parameter S*n* in the various systems provide even more insight into the disruptive role of unsaturates. S*n* depends exponentially on *n* in smectic liquid crystals (19) and in aqueous dispersions of egg yolk lecithin and cholesterol (12). This behavior also agrees with theoretical considerations about the conformation of a long, saturated, hydrocarbon chain anchored at one end (12). In our experiments, any departure from exponential behavior that is correlated with the position of unsaturated sites must be ascribed to the perturbing influence of these sites. The results shown in Fig. 3 are striking in this respect. The Δ^{11} system is saturated from the polar group to $n = 11$; in the measured range, S*n* values display an exponential dependence on *n*. The 12NS label shows no drastic difference from what an 11NS label would be expected to measure since the double bond encompasses carbons 11 and 12. In contrast, the Δ^9 system displays an exponential behavior of S*n* up to $n = 9$ followed by a dramatic decrease at $n = 12$, indicating a greater freedom of the alkyl chains beyond the unsaturated sites. Similar effects are visible in the Δ^6, $\Delta^{9=}$, and artificial systems. This result is important. It indicates that the perturbing influence, of the conformation of the unsaturated bond on the ordered packing of alkyl chains, is more effective in the region between unsaturated sites and terminal methyl groups.

The effect of higher enrichment in unsaturated acyl chains somewhat decreases the order parameter at 0°. All preparations of Δ^9 were better enriched than the artificial system (equivalent to 50% enrichment), and all resulted in lower order parameters than in that system. This is consistent with previous observations that higher unsaturation increases lipid fluidity.

In the fatty alkyl chains of membranes having a single unsaturated site, e.g., in microbes, the position of the *cis* double bond defines the size of the ordered region between the polar interface and the unsaturated site; the alkyl chains are relatively disordered between this site and the methyl terminal. In mammalian systems, where double bonds are located everywhere from Δ^4 to Δ^{17} and many polyunsaturates occur, the role of unsaturated sites in determining the region and degree of alkyl chain ordering is not as important. We speculate that the condensing effect of cholesterol serves instead as the ordering element at the methylene groups near the polar interface.

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