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ESR DETERMINATION OF MEMBRANE ORDER PARAMETER IN YEAST STEROL MUTANTS

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Summary

ESR investigations designed to determine membrane order parameter in sterol mutants of *Saccharomyces cerevisiae* were conducted using the membrane probe, 5-doxyl stearic acid. These mutants are blocked in the ergosterol biosynthetic pathway and thus do not synthesize ergosterol, the end product sterol. They do not require exogenous ergosterol for growth and, therefore, incorporate ergosterol biosynthetic intermediates in their membrane. Increasing order parameter is reflective of an increase in membrane rigidity. Single mutants involving B-ring $\Delta^8 \rightarrow \Delta^7$ isomerization (*erg 2*) and C-24 methylation (*erg 6*) showed greater membrane rigidity than wild-type during exponential growth. A double mutant containing both lesions (*erg 6/2*) showed an even greater degree of membrane rigidity. During stationary phase the order of decreasing membrane rigidity was *erg 6* > *erg 6/2* > *erg 2* = wild-type. The increased membrane order parameter was attributed to the presence of substituted sterols rather than increased sterol content or altered fatty acid synthesis.

Introduction

Sterols are an important component of biological membranes influencing membrane organization which in turn influences membrane functions such as

Abbreviations: 5DS, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; YEPD, 2% Difco yeast extract, 2% Bacto-peptone, and 2% dextrose.

transport and the activity of membrane-bound enzymes [1—3]. The precise nature of the interactions of sterols with proteins and with other lipids in membranes is not as yet understood. Electron spin resonance (ESR) studies using spin-labeled fatty acids intercalated into the membrane have provided much valuable data concerning the nature of this interaction [4—6]. Analysis of ESR spectra provides information about the degree of motional freedom of the fatty acid chain to which the nitroxyl group is attached. The result is usually reported as an order parameter, S, where lower values of S mean less restricted motion or a more fluid membrane; higher values of S mean more restricted motion or a more ordered membrane. In model systems using cholesterol and various phosphatidylcholines, cholesterol is found to have a condensing effect resulting in reduced chain mobility of lipid bilayers in the liquid-crystalline state [1,7]. These studies have also shown cholesterol to have a liquifying effect on membranes in the gel or crystalline state. In addition, cholesterol has been shown to lower the temperature of the phase transition between the liquid crystalline and gel states [1,3,8,9]. There have been a limited number of studies reported using intact biological membranes. The lack of information on the effects of sterol concentration on membrane structure and function is due to the fact that the lipid content of most cells is scrupulously regulated [9]. Hence, studies have been restricted to those systems where variable sterol concentrations are possible. Guinea pig erythrocytes [8] and *Mycoplasma mycoides* [10] membranes containing variable sterol composition have been shown to yield results similar to those shown for the model systems.

In this investigation we report ESR order parameter results on the membranes of wild-type and sterol mutants of *Saccharomyces cerevisiae* with altered membrane sterol composition. Using the membrane spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5DS) significant differences in membrane order parameter among several sterol mutants are reported. The mutants used in this study are blocked in sterol biosynthesis and are unable to synthesize the end product sterol, ergosterol, but do not require exogenous ergosterol for growth. The altered sterol compositions of these mutants have been characterized genetically and biochemically [11—14].

TABLE I

PRESUMPTIVE ENZYME DEFECTS AND PRINCIPAL STEROL INTERMEDIATES ACCUMULATED IN WILD-TYPE AND STEROL MUTANTS OF *S. CEREVISIAE*

Strain	Enzymatic step blocked	Sterold intermediate(s) accumulated
A184 D		ergosterol (ergosta-5,7,22-trien-3β-ol) zymosterol (cholesta-8,24-dien-3β-ol)
erg 2	$\Delta^8 \rightarrow \Delta^7$ isomerization	ergosta-8-en-3β-ol ergosta-8,22-dien-3β-ol ergosta-5,8,22-trien-3β-ol
erg 6	C-24 transmethylation	zymosterol cholesta-5,7,22,24-tetraen-3β-ol
erg 6/2	$\Delta^8 \rightarrow \Delta^7$ isomerization and C-24 transmethylation	zymosterol

Table I shows the mutants, the enzymic step blocked, and the sterol intermediates accumulated in each strain. Previous ESR results [15] using a cytoplasmic spin label have shown one mutant, *erg 6/2*, to be permeable to nickel while the wild-type strain shows the typical impermeability to this cation [16]. Bard et al. [17] have shown that the sterol mutants used in this study show increased sensitivity to various cations and are more permeable to crystal violet dye. The use of sterol mutants provides a very effective way of investigating the effects of altered sterol composition on membrane structure and function. We have investigated the physical alterations of the membrane due to the substitution of sterol intermediates for ergosterol and have attempted to relate these changes with the reported alteration of permeability characteristics of yeast sterol mutants. The availability of genetically well-defined mutants whose accumulated sterol intermediates are known will provide insight into the contributions of various sterol molecules to membrane order.

Materials and Methods

Strains. The sterol mutants used in this investigation are derived from the wild-type strain of *S. cerevisiae*, A184D, and have been described previously [11—14]. *Erg 6* is unable to transmethylate zymosterol at the C-24 position and *erg 2* is blocked at the $\Delta^8 \rightarrow \Delta^7$ isomerization step in ergosterol biosynthesis. *Erg 6/2* is a double mutant obtained by genetically combining the single mutants *erg 6* and *erg 2* into the same strain.

Growth. Cells were grown aerobically at 30°C in a medium containing 1% Difco yeast extract, 2% Bacto-peptone, and 2% dextrose (YEPD). All growth was monitored using a Klett-Summerson colorimeter with a No. 66 red filter. Exponential phase cells were harvested at Klett readings ranging from 100 to 150. Stationary cells were harvested at 350 Klett units or greater.

Viability. 60 μl (5 mM) of spin label (5DS, Syva, Palo Alto, CA) in ethanolic solution was evaporated by a stream of nitrogen to a thin film on the bottom of a 15 ml conical centrifuge tube. A 100 μl cell sample suspended in a small volume of medium (approximately 20 μl) was added to the tube using a 100 μl micropipet. The mixture was vortexed and left to stand 2 min at 25°C. The mixture was centrifuged and then left to stand another 2 min. The fluids were then removed and the pellet suspended in 5 ml of 0.9% NaCl. The suspension was serially diluted in 0.9% NaCl and plated in duplicate on YEPD plates (2% agar). A control sample was prepared without spin label. The total time of exposure to spin label is comparable to the total time of exposure for ESR samples.

Sample preparation. Spin label was prepared in 15 ml conical centrifuge tubes as described for viability studies. After addition of the cell samples and vortexing, the labeled mixture was transferred into a flame-sealed 100 μl micropipet (Clay Adams No. 4625). The mixture was centrifuged (800 × *g* at 25°C, 1 min) and the tip containing the sample was broken off and excess liquid above the sample was removed.

ESR data acquisition and analysis. ESR spectra were obtained on a conventional X-band spectrometer comparable to a Varian V4502. A Varian V4535 large sample access cavity fitted with a quartz variable temperature dewar was

used. The temperature was maintained at 25 ± 2°C for all measurements and the data were corrected to 25.0°C using a correction factor of $dS/dT = -0.007°C^{-1}$. The spectrometer operating parameters were: microwave frequency/power = 91. GHz/13 mW, sweep time/width = 5 min/100 gauss, and phase-sensitive detector time constant = 0.6 s. For phase-sensitive dectection a modulation frequency of 50 kHz and a modulation amplitude of 1.3 or 2 gauss were used. The order parameter S was independent of modulation amplitude up to these levels. For 5DS in membranes S is computed as follows [18]

$$S = \frac{T_{\parallel} - T_{\perp} - C}{T_{\parallel} + 2T_{\perp} + 2C} \quad (1.66)$$

$$C = 1.4 \text{ gauss} - 0.053\,(T_{\parallel} - T_{\perp})$$

where $T_{\parallel}$ and $T_{\perp}$ are the apparent parallel and perpendicular hyperfine splitting parameters of the spectrum (Fig. 1). The constant C corrects for the difference between the true and apparent values of $T_{\perp}$ and the factor 1.66 is the solvent polarity correction factor. Theoretically, S can vary from 0 to 1 with biomembranes typically in the range from $S = 0.5$—0.7. Lower values of S correspond to more fluid membranes while higher values of S correspond to more ordered membranes.

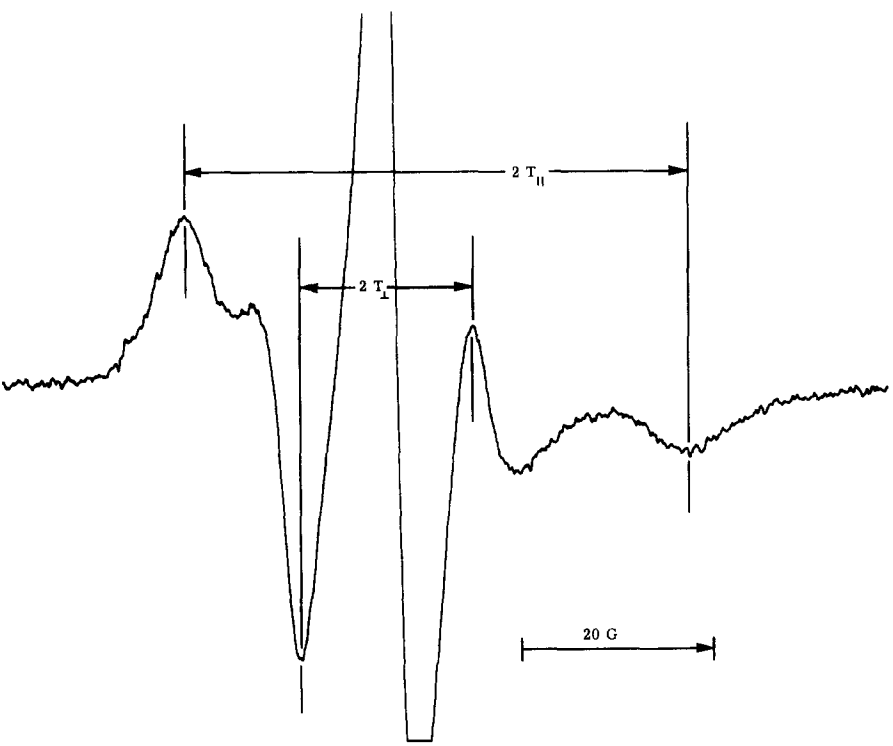

Fig. 1. ESR spectrum of exponential phase *erg 2* labeled with 5DS. The parameters used to calculate S are indicated.

The isotropic hyperfine coupling constant a is computed as follows [4]:

$$a = \tfrac{1}{3}(T_{\parallel} + 2T_{\perp} + 2C)$$

The value of a is dependent on the polarity of the solvent (or environment) and therefore provides information about the local environment of the spin label.

Results and Discussion

Viability studies showed that a 60 μl concentration of the spin label 5DS did not result in a loss of viability in any of the yeast strains employed in this study. All yeast samples prepared for ESR analysis contained this concentration, or less, of spin label. Thus, differences in the order parameter, S, among the strains cannot be attributed to changes in membrane structure resulting from cell death. The isotropic hyperfine coupling constant, a, for all samples ranged between 15.2 and 15.4 gauss indicating that the nitroxyl probe was located within the lipid bilayer.

The order parameter results for all yeast strains in exponential and stationary growth phases are shown in Table II. The order parameter values obtained for these strains are lower than S values obtained with rabbit and mouse polymorphonuclear leukocytes (0.65 and 0.62, respectively [19]), and intact human erythrocytes (0.7; Refs. 20, 21). Exponential wild-type (A184D) cells showed the greatest degree of membrane fluidity. The order parameter decreased in the exponential phase cells in a pattern of *erg 6/2* > *erg 2* > *erg 6* > A184D. Stationary phase cells showed an increase in order parameter when compared with exponential values for all four strains. During this phase the order of decreasing order parameter is *erg 6* > *erg 6/2* > *erg 2* = A184D. Increasing order parameter with increasing culture age has been shown to occur in *Acholeplasma laidlawii* [6]. This change in membrane physical structure has been attributed to an increase in the ratio of saturated to unsaturated fatty acids. Bard (Bard, M., unpublished results) has shown that stationary phase sterol mutants derived from a different wild-type (strain S288C) but allelic to *erg 6* and *erg 2* possessed fatty acid compositions that were identical to that of the wild-type strain. Lomb et al. [22] have shown that polyene-resistant cultures of *Torulopsis glabrata* which do not synthesize ergosterol, the end product sterol, have fatty acid compositions which do not differ significantly from the fatty acid composition of the wild-type strain. Thus, the differences

TABLE II

MEMBRANE ORDER PARAMETER, S, OF WILD-TYPE AND STEROL MUTANTS OF *S. CEREVISIAE* IN EXPONENTIAL AND STATIONARY GROWTH PHASES

Data are mean ± S.D. Number of replicas in parentheses.

Strain	Exponential phase	Stationary phase
A184D	0.591 ± 0.002 (29)	0.616 ± 0.001 (30)
erg 2	0.611 ± 0.001 (30)	0.616 ± 0.004 (38)
erg 6	0.609 ± 0.002 (29)	0.642 ± 0.004 (24)
erg 6/2	0.621 ± 0.003 (26)	0.630 ± 0.005 (24)

in S for exponential versus stationary phases may be due to alterations in fatty acids but the differences among the strains in the same growth phase are due to altered sterol composition.

Several studies using model membrane systems [1,3,9] and biological membrane systems [1,3,8,10] have shown that increased membrane sterol content results in reduced lipid chain mobility (increased order parameter) when membranes are in a liquid-crystalline state. This alteration has been attributed to the membrane-condensing effect of sterols. Molzhan and Woods [14] have shown, using stationary phase cultures, that the sterol content of A184D is 1.6% of total cell dry weight while the sterol contents of *erg 6*, *erg 2* and *erg 6/2* are 3.7, 3.3, and 3.8% of total cell dry weight, respectively. Since stationary A184D and *erg 2* cultures yield the same order parameter, the amount of sterol present cannot alone be responsible for the membrane structural changes in yeast sterol mutants. Thus, the structural changes in the membranes are likely to be due to the substitution of sterol intermediates for ergosterol, the end product sterol.

Differences in membrane fluidity as indicated by elevated S values would be expected to affect several physiological processes. For example, several reports [3,8,9] have shown cell permeability alterations in cells with increased membrane sterol content (higher order parameter). Increased cholesterol content of *A. laidlawii* membranes reduces cell permeability to glycerol and erythritol [3]. Kleinhans et al. [15] have reported that *erg 6/2* is significantly more permeable to nickel than A184D. Bard et al. [17] have shown that *erg 6/2*, *erg 6*, and *erg 2* are more permeable to crystal violet dye and mono-, di-, and trivalent cations than A184D. The permeability changes in the sterol mutants have been attributed to the presence of substitute sterol molecules in the membranes of these mutants. In addition, these mutants have been observed to exhibit increased sensitivity to the detergent tergitol, a two-fold increase in generation time at 30°C, and further increase in generation time at 35°C (Lees, N.D., unpublished results).

Permeability differences among the sterol mutants [17] have indicated that the ability to methylate the C-24 position is more important than the B-ring $\Delta^8 \rightarrow \Delta^7$ isomerization step in maintaining normal membrane permeability. During exponential growth the C-24 methylation lesion results in an S value of 0.609 while the isomerization lesion increases the S value still further to 0.611. When present in the same strain, (*erg 6/2*), the mutations produce an even higher order parameter (0.621). When compared with A184D, stationary phase cells show order parameter increases for *erg 6* (0.642) and *erg 6/2* (0.630) but not for *erg 2*. Previous studies [3,9] have shown that the presence of a 3β-hydroxyl group, a planar ring structure, and a hydrophobic side chain at C-17 are critical for normal sterol activity in membranes. All sterol intermediates accumulated by the strains used in this study possess each of these moieties. The altered S values and permeability characteristics suggest that these mutants may be useful in elucidating an important role for an additional critical site(s) on the sterol molecule necessary for the maintenance of normal membrane function.

Our results indicate that altered sterol composition in yeast cell results in significant changes in the physical properties of the yeast membrane. The

altered membranes will be investigated further under variable temperature conditions using fatty acid probes with the nitroxyl group at different positions on the hydrocarbon chain (carbons 12 and 16) and with sterol nitroxyl probes. These investigations will probe different parts of the membrane and may provide information on phase transitions.

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