

Selective detection of the rotational dynamics of the protein-associated lipid hydrocarbon chains in sarcoplasmic reticulum membranes

Thomas C. Squier and David D. Thomas

Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455

ABSTRACT We have developed a saturation transfer EPR (ST-EPR) method to measure selectively the rotational dynamics of those lipids that are motionally restricted by integral membrane proteins and have applied this methodology to measure lipid-protein interactions in native sarcoplasmic reticulum (SR) membranes. This analysis involves the measurement of spectral saturation using a series of six stearic acid spin labels that are labeled with a nitroxide at different carbon atom positions. A large amount of spectral saturation is observed for spin labels in

native SR membranes, but not for spin labels in dispersions of extracted SR lipids, implying that the motional properties of those lipids interacting with the Ca-ATPase, i.e., the boundary or annular lipid, can be directly measured without the need for spectral subtraction procedures. A comparison of the motional properties of the restricted lipid, measured by ST-EPR, with those measured by digital subtraction of conventional EPR spectra qualitatively agree, for in both cases the Ca-ATPase restricts the rotational mobility of a population of lipids, whose rota-

tional mobility increases as the nitroxide is positioned toward the center of the bilayer. However, the ability of ST-EPR to directly measure the motionally restricted lipid in a model-independent means provides the greater precision necessary to measure small changes in the rotational dynamics of the lipid at the protein-lipid interface, providing a valuable tool in clarifying the relationship between the physical nature of the protein-lipid interface and membrane function.

INTRODUCTION

Biological lipids play an important role in modulating a range of biological phenomena, and have been implicated as functionally significant in stabilizing or modulating different structures of the Ca-ATPase in sarcoplasmic reticulum (SR)¹ (Moore et al., 1978; Almeida et al., 1982; Bigelow et al., 1986; Bigelow and Thomas, 1987; Squier et al., 1988*a* and *b*; Squier and Thomas, 1988; reviewed by Hidalgo, 1985, 1987) and other membrane bound enzymes (Sinensky et al., 1979; Marsh et al., 1981; Fong and McNamee, 1987; reviewed by Shinitzky, 1984; Gordon and Mobley, 1985; McElhaney, 1985; Deuticke and Haest, 1987; Carruthers and Melchoir, 1988). Early studies have suggested a specific role for the lipids

adjacent to membrane proteins in modulating function (Jost et al., 1973*a*; reviewed by Marsh, 1985), and it has been suggested that protein-protein interactions are often modulated by the weak interaction energies between integral membrane proteins and their associated lipids (Owicki et al., 1978; Owicki and McConnell, 1979; Pearson et al., 1984). Furthermore, many hydrophobic drugs (e.g., anesthetics) may act at the lipid-protein interface (Franks and Lieb, 1987; Evers et al., 1987; Bigelow and Thomas, 1987), emphasizing the importance of developing methods for the selective study of this biological interface.

An important approach to identifying the physical features of biological lipids that are relevant to modulating the function of membrane-bound enzymes has been to directly measure the membrane's lipid and protein dynamics (reviewed by Thomas et al., 1985; Thomas, 1985, 1986). Electron paramagnetic resonance (EPR) spectroscopy of spin-labeled lipid molecules is widely used for this purpose, and conventional EPR is capable of spectrally resolving the lipids in biological membranes into two populations through the digital subtraction of optimally matched single-component reference spectra, thereby providing a first approximation for quantitating the relative amounts and motional properties of these lipid populations. The lipid component at the protein-lipid

¹Abbreviations: SR, sarcoplasmic reticulum; EPR, electron paramagnetic resonance; ST-EPR, saturation-transfer EPR; *S*, apparent order parameter; τ_c , apparent correlation time; σ , in-phase ST-EPR intensity parameter; *N*-SASL, stearic acid spin label where *N* is the relative position of the nitroxide on the stearic acid; T_{is} , the isotropic hyperfine splitting constant; T_{\perp} and T_{\parallel} , and principal values of the hyperfine constant for an axially symmetric system; T'_{\perp} and T'_{\parallel} , the measured inner and outer splitting resolved in the EPR spectrum; T_{\parallel} , the threshold temperature below which spectral saturation is minimal for stearic acid spin labels in extracted SR lipids.

Address correspondence to Thomas C. Squier, whose present address is Department of Biological Chemistry, University of Maryland, School of Medicine, Baltimore, MD 21201.

interface has thus been identified for a number of membrane systems (including SR; Thomas et al., 1982; Silvius et al., 1984; Bigelow and Thomas, 1987) through the comparison of spectra from membranes with those from extracted lipids, and on the conventional EPR time scale this annular lipid component has a reduced mobility relative to the bulk lipid (reviewed by Marsh and Watts, 1982; Marsh, 1985; Hidalgo, 1985, 1987).

However, it has been difficult to obtain accurate measurements of the rotational dynamics of the lipid restricted by membrane proteins on the conventional EPR time scale, due to (a) the difficulty of obtaining reference spectra that accurately model and motional properties of either component resolved in the conventional EPR spectrum, and (b) the tendency of the more mobile component to dominate the conventional EPR spectrum, resulting in a low signal-to-noise ratio for the motionally restricted spectral component subsequent to spectral deconvolution. As a result of these problems, considerable uncertainty remains regarding the properties of the restricted lipid surrounding the Ca-ATPase in sarcoplasmic reticulum membranes. Therefore, we have been motivated to investigate a more direct approach to measure the rotational dynamics of the restricted lipids using ST-EPR.

ST-EPR extends the EPR technique into the biologically important microsecond time domain (time scale of T_1), and is therefore sensitive to the dynamics of the lipid immobilized on the conventional (nanosecond) EPR time scale (time scale of T_2). In practice, the large population of mobile probes, undergoing large amplitude nanosecond rotational motions near the end of the hydrocarbon chains of the bulk lipids has interfered with the analysis of the ST-EPR spectral lineshape (line-height) parameters that are normally used to calculate rotational correlation times (Thomas et al., 1976), because the component spectra resulting from each population of spin labels are superimposed (Squier and Thomas, 1986 *a* and *b*). As a result, previous applications of ST-EPR to the study of lipid rotational dynamics have been limited mainly to homogeneous populations of gel phase lipids (Thomas and Hidalgo, 1978; Delmelle et al., 1980; Marsh, 1980; Marsh and Watts, 1980; Koole et al., 1981; Watts and Marsh, 1981; Johnson et al., 1982; Fajer and Marsh, 1983*a* and *b*; Fajer, 1983; reviewed by Robinson et al., 1985). However, in the present study we have taken advantage of the ability of the in-phase ST-EPR intensity parameters (spectral integration) to suppress the contribution from more mobile spin labels over a wide time scale (i.e., $3 \times 10^{-9} < \tau_r < 10^{-6}$ s for isotropic rotational motion; Squier and Thomas, 1986*a*), thereby allowing the selective measurement of rotational motions whose effective rotational correlation time is on the ST-EPR time scale (i.e., $\tau_r > 10^{-6}$ s for isotropic rotational motion or $\tau_r > 10^{-9}$ s for anisotropic rotational motion). We have thereby selec-

tively measured the dynamics of the motionally restricted population of lipids surrounding the Ca-ATPase, and compare the rotational mobility obtained from ST-EPR with that obtained with digital subtractions of conventional EPR spectra.

MATERIALS AND METHODS

Membrane preparation

Vesicles are prepared from rabbit skeletal white (fast twitch) muscle, essentially as described previously (Fernandez et al., 1980). The membrane vesicles were suspended in 0.3 M sucrose, 20 mM MOPS (pH 7.0), and stored in liquid nitrogen. The SR preparation utilized in these experiments had 77 ± 5 phospholipids per mole of Ca-ATPase (i.e., 560 ± 40 nmol phospholipid per mg SR), and was determined by comparing the molar concentration of phospholipids, determined from phosphorous assays (Chen et al., 1956), with that of the Ca-ATPase, determined by dividing the protein concentration by a molecular weight of 110 kD and multiplying by the fraction of the total protein that had this molecular weight, as determined from densitometer scans of polyacrylamide gels, as described previously (Bigelow et al., 1986).

Lipids were extracted by a modification (Hidalgo et al., 1976) of the method of Folch et al. (1957), using nitrogen-saturated solvents to prevent oxidation. The lipids were stored in chloroform-methanol (2:1) at -20°C . Before liposome formation the extracted SR lipid was dried under nitrogen and placed under a vacuum overnight to remove any remaining solvent. The liposomes were formed by vortex mixing in 0.3 M sucrose, 20 mM MOPS (pH 7.0). The fatty acid composition and cholesterol concentration (5% [mol/mol]) in extracted SR lipids was similar to the native vesicles (Bigelow et al., 1986).

Enzymatic assays

To ensure that the SR membrane dynamics were characteristic of the functional preparation, we measured the Ca^{+2} -dependent ATPase activity. The ATPase activity was measured in a solution containing 0.05 mg of protein per ml^{-1} , 60 mM KCl, 6 mM MgCl_2 , 25 mM MOPS (pH 7.0), and either 0.1 mM CaCl_2 or 2 mM EGTA; 2 μM A23187, a calcium ionophore, was added in some cases to obtain a measure of the coupling efficiency between ATP hydrolysis and calcium transport. The reaction was started by the addition of 5 mM ATP, and the initial rate of release of inorganic phosphate was measured by the method of Lanzetta et al. (1979). Activity assayed in the presence of EGTA (basal activity) was subtracted from that assayed in the presence of CaCl_2 (total ATPase activity) to obtain the calcium-dependent ATPase activity. The basal activity was $<5\%$ of the total ATPase activity, indicating that our preparation contains essentially only the longitudinal component of the SR membrane (Fernandez et al., 1980). The ionophore-stimulated calcium-dependent ATPase activity was typically 3–4 $\mu\text{mol P}_i \text{ mg}^{-1} \text{ min}^{-1}$, and the ionophore stimulation was typically threefold, indicating that ATP hydrolysis is coupled to calcium transport. Protein concentrations were determined by the biuret method (Gornall et al., 1949), using bovine serum albumin as a standard.

Spin labeling

4-Maleimido-2,2,6,6-tetramethyl piperidinoxyl (MSL; Fig. 1) was covalently attached to hemoglobin (Hb) so as to provide a model system for the study of microsecond rotational motion (Thomas et al., 1976). 4-Hydroxy-2,2,6,6-tetramethylpiperidinoxyl (TANOL) was utilized as a model system to study nanosecond rotational motion (Squier and

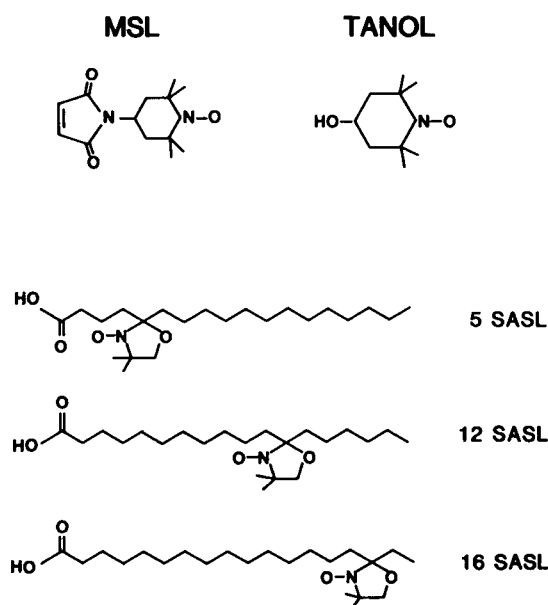


FIGURE 1 Spin labels used in this study. MSL (4-maleimido-2,2,6,6-tetramethyl piperidinoxyl) was bound covalently to the model system hemoglobin (Hb) for the study of microsecond rotational motion, whereas TANOL (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl) in aqueous glycerol and sec-butyl benzene solvents was used to study nanosecond rotational motions. 5-, 12-, and 16-SASL (stearic acid spin label), which are *N*-oxyl-4',4'-dimethyloxazolidine derivatives of stearic acid respectively, are examples of the stearic acid spin labels used to monitor the hydrocarbon chain dynamics.

Thomas, 1986a). Hydrocarbon chain rotational mobility in membrane vesicles was measured with fatty acid spin labels, i.e., 4',4'-dimethyloxazolidine-*N*-oxyl derivatives of stearic acid, which are designed N-SASL (Aldrich Chemical CO., Milwaukee, WI, or Molecular Probes Inc., Junction City, OR), where *N* is the nitroxide position relative to the α -carbon. The stearic acid spin labels were diluted from a stock solution in DMF into ethanol before addition to SR (or liposomes of extracted SR lipids) at a ratio of less than one spin label to 200 phospholipids, with a final ethanol concentration <1% (vol/vol). The lipid concentration was made sufficiently high (>50 mM) so that the conventional EPR spectrum contained a negligible contribution from unbound spin labels.

Stearic acid spin labels provide a means of estimating membrane heterogeneity, because they are randomly distributed in the plane of the bilayer, position themselves so that the α -carbon of the fatty acyl chain is aligned with the head group region of the bilayer, and faithfully reflect the degree of orientational order and dynamics of the lipid molecules (Godici and Landsberger, 1974; Hauser et al., 1979; Taylor and Smith, 1981; Lange et al., 1985; Esmann et al., 1988; Moser et al., 1989), as evidenced by the virtually identical spectra obtained from stearic acid and phospholipid spin labels whose nitroxide is located at the same position relative to the carboxyl terminal of the fatty acyl chain (Bigelow and Thomas, 1987, and Fig. 4). This suggests that stearic acid spin labels, like phospholipids (London and Feigensohn, 1981), partition uniformly between the annular and bulk lipids in SR membranes. The nitroxide on the stearic acid spin label is localized over a distribution of intramolecular locations relative to the membrane normal for each position along the chain (Ellena et al., 1988), consistent with earlier studies that have indicated stearic acid spin labels undergo verticle fluctuations (Feix et al., 1984, 1987; Davoust et al., 1983) and in

agreement with both statistical mechanical and molecular dynamics calculations that indicate the widest distribution of locations for positions near the chain terminus (Meraldi and Schlitter, 1981; Wendolowski et al., 1989).

EPR spectroscopy

EPR spectra were obtained with a model E-109 spectrometer (Varian Associates, Inc., Palo Alto, CA) and spectra were digitized and analyzed with a microcomputer interfaced to the spectrometer (Lipscorn and Salo, 1983) as described previously (Squier and Thomas, 1986a). The rotational motion of stearic acid spin labels (SASL) was detected using either conventional or saturation-transfer EPR, using 100 kHz field modulation with a peak-to-peak amplitude (H_m) of 2.0 Gauss. In the case of conventional EPR a microwave field amplitude (H_1) of 0.032 Gauss was used, thereby ensuring that the spectral shape is not distorted by saturation effects and is an accurate representation of the rotational dynamics on the nanosecond time scale. Under these conditions ($H_1 = 0.032$ Gauss) the spectral intensity depends linearly on H_1 (essentially no saturation effects). Submillisecond rotational motion was detected by in-phase saturation-transfer EPR (i.e., σ) using saturating conditions ($H_1 = 0.25$ Gauss). The normalized loss in spectral intensity provides an accurate measurement of the effective rotational correlation time (τ_r ; see below).

The accurate and reproducible setting of the microwave intensity incident upon the sample (H_1) is critical to the measurement of ST-EPR spectral intensity parameters and requires a correction for the dielectric loss of the sample. This was done by measuring and comparing the cavity *Q* for the sample with that of a standard of known saturation properties (Fajer and Marsh, 1982; Squier and Thomas, 1986a). All saturation studies were done subsequent to the removal of molecular oxygen, because O_2 will artificially shorten the spin-lattice relaxation time (T_1). Oxygen was removed from reference and experimental samples using gas-permeable sample cells purged with N_2 for 30 min at 25°C (Popp and Hyde, 1981). Temperature was controlled to within 0.5°C with a model V4540 variable temperature controller (Varian Associates, Inc.).

During data acquisition the temperature was monitored with a digital thermometer (model BAT-12; Sontek Co., Clifton, NJ), using a thermocouple probe (IT-21) positioned outside the sample cell in the center of the cavity.

Spectral subtraction

Subtractions of conventional EPR spectra was done by computer analysis of digitized spectra obtained under nonsaturating conditions (i.e., $H_1 = 0.032$ Gauss) using the single-component subtraction method of Jost and Griffith (1978a), as described previously (Bigelow and Thomas, 1987). This method involves a search for a spectrum that matches that of the more mobile (bulk lipid) component, which is subtracted from the observed composite spectrum to obtain the spectrum of the restricted component and its mole fraction. The bulk lipid component was approximated by the spin label in aqueous dispersions of extracted SR lipid at a slightly lower temperature to match the splittings of the composite spectrum.

Analysis of conventional EPR spectra

An empirical motion parameter, *S* (apparent order parameter), was used to measure the dynamics of single-component spectra obtained using stearic acid spin labels in dispersions of extracted SR lipids or,

alternatively, the motionally restricted spectral component obtained from spectral subtractions (see above). The apparent order parameter is normally calculated from the formula (Gaffney and Lin, 1976; Gaffney, 1976):

$$S = \{[T'_1 - (T'_\perp + C)]/[T'_1 + 2(T'_\perp + C)]\} \times 1.66, \quad (1)$$

where T'_1 and T'_\perp are the measured outer and inner splitting resolved in the EPR spectrum in Gauss and where $C = 1.4 - 0.053(T'_1 - T'_\perp)$. The correction factor (C) takes into account the small nonlinearity between the measured parameters and S .

In some cases the anisotropy of motion is poorly resolved, thereby preventing the reliable measurement of the order parameter from Eq. 1. This can result from either (a) a small residual anisotropy and resulting poorly resolved outer extrema (i.e., T'_1) of highly mobile stearic acid spin labels or (b) the loss of poorly resolved spectral features (e.g., T'_\perp) subsequent to spectral deconvolution. Therefore, to prevent systematic errors in the calculation of S , we have made use of the equivalent relationship (Gaffney, 1976):

$$S = \frac{T_o - T'_\perp}{T_o - T'_1} = \frac{T'_1 - T_o}{T'_1 - T'_\perp}, \quad (2)$$

where T_o is the isotropic hyperfine splitting constant in the absence of anisotropic effects. T'_\perp and T'_1 are the principal values of the hyperfine tensor for an axially symmetric system (e.g., a lipid bilayer). These intrinsic constants are characteristic of a particular probe environment and must be measured for each experimental system. Values of T_o and T'_\perp (or T'_1) correspond to values of T'_\perp (or T'_1) at $S = 0.0$ (isotropic motion) and $S = 1.0$ (complete order), respectively (Gaffney, 1976). Therefore, the value of T_o for each stearic acid spin label is determined in two ways: (a) the distance between the low-field and high-field cross-over points in Gauss (i.e., $2T_o$) from a spectrum where S approaches zero (i.e., increasing the sample temperature does not further narrow the spectrum) and (b) the extrapolation to $S = 0$ of T'_\perp and T'_1 plotted as a function of S (using spectra with well-resolved extrema, i.e., $S > 0.3$). The determination of the isotropic hyperfine splitting constant, T_o , from the extrapolation of T'_\perp to $S = 0$ required a correction because the measured inner splitting (i.e., T'_o at $S = 0$) underestimates the actual splitting (Gaffney, 1976), such that:

$$T_o = T'_o + 1.33 \text{ Gauss}. \quad (3)$$

In the case of T'_1 the actual and apparent splitting is the same, resulting in no systematic error. For stearic acid spin labels in vesicles of extracted SR lipids, T_o was found to be essentially independent of the nitroxide position of the stearic acid spin label (for 5- through 16-SASL) and was determined to be 14.3 ± 0.2 Gauss. In agreement with earlier reports (Seelig and Hasselbach, 1971), this indicates that there are no significant differences in the polarity at the different nitroxide positions (where $N > 5$) on the stearic acid spin labels in SR lipids. Equivalent results were obtained upon measurement of the distance between the low-field and high-field cross-over points (i.e., $2T_o$) obtained from spectra corresponding to the motionally restricted lipid component in SR, suggesting that there are no significant differences between the polarity of the bulk lipid's hydrocarbon environment and those lipids adjacent to the Ca-ATPase (i.e., annular lipids). In other membranes polarity gradients have been reported relative to the membrane normal (e.g., Pates and Marsh, 1987), and presumably arise as a result of a difference in the structure of the membrane, because the shape of the barrier to water penetration will vary with the composition of the polar head groups and lipid side chains (Griffith and Jost, 1976).

Similarly, determinations of T'_\perp or T'_1 were made for each stearic acid spin label in vesicles made from extracted SR lipids in two ways, i.e., (a)

by measuring the distance between either the inner extrema ($2T'_\perp$) or outer extrema ($2T'_1$) from a spectrum where $S = 1.0$ (using Eq. 1), and (b) the extrapolation to $S = 1.0$ of either T'_\perp or T'_1 plotted as a function of S (see above). Values for T'_\perp and T'_1 for the stearic acid spin labels in vesicles made from extracted SR lipids were determined to be 6.3 ± 0.3 Gauss and 33.5 ± 0.4 Gauss, respectively, and was essentially independent of the nitroxide position on the stearic acid, again indicating that there are no large differences in the polarity at the different nitroxide positions on the stearic acid spin labels. In some cases, only T'_\perp or T'_1 were measurable, so that only one of the two methods (Eqs. 1 and 2) could be used to calculate the order parameter. However, in the cases where both equations could be used, identical order parameters were obtained.

Analysis of ST-EPR spectra

The effective correlation times obtained from in-phase ST-EPR spectra were measured by comparing experimental ST-EPR spectra with reference spectra of known correlation times, obtained from isotropically tumbling model systems (Figs. 2 and 3). This involved either the measurement of the rotational diffusion of spin-labeled hemoglobin (Hb) or that of TANOL in solutions of varying viscosity, as previously described (Squier and Thomas, 1986a). In both cases the spin label's (and thus hemoglobin's in the case of MSL-Hb) rotational correlation time (in seconds) was assumed to undergo Brownian diffusion, so that

$$\tau_r = \eta / T \times Z, \quad (4)$$

where η is the viscosity in poise and T is the temperature in degrees kelvin. The constant Z was determined to be 7.6×10^{-4} for MSL-Hb (Hyde and Thomas, 1973) and 2.9×10^{-7} for TANOL (Squier and Thomas, 1986a). This empirical calibration plot has been validated by saturation recovery EPR measurements, which have revealed that (a) MSL-Hb undergoes isotropic rotational diffusion, (b) the rate of rotational motion for MSL-Hb is in agreement with hydrodynamic theory, and (c) that spectral relaxation (i.e., T_1) remains approximately constant over the ST-EPR time scale (Hyde, 1978; Fajer et al., 1986). However, T_1 for the rigid limit sample (i.e., ammonium sulfate precipitated MSL-Hb) is anomalously low (Fajer et al., 1986), resulting in an overestimation of spectral saturation at the rigid limit. For this reason, we approximate the rigid limit (no motion) value as that of MSL-Hb in 95% (wt/wt) glycerol at -20°C ($\tau_r = 10^{-3}$ s).

Relationship between spectral saturation and rotational motion

The extent of saturation depends primarily on the intensity of microwave radiation H_1 (where the microwave radiation field is $2H_1 \cos \omega t$) that depletes the ground state, and only secondarily on saturation transfer that reduces the effects of this radiation by transferring saturation to other (not irradiated) spectral positions. Saturation reduces the steady-state magnetization, M_z , below the Boltzmann equilibrium distribution value, M_o , according to

$$M_z/M_o = 1/(1 + \sigma). \quad (5)$$

The saturation factor σ equals $\gamma^2 H_1^2 T_1 T_2$ in the absence of spectral diffusion, and is less than this value if spectral diffusion occurs on the timescale of T_1 (Thomas, 1986). γ is the gyromagnetic ratio of the electron ($1.76 \times 10^7 \text{ rad s}^{-1} \text{ Gauss}^{-1}$), H_1 is the microwave field amplitude at the sample, T_1 is the spin-lattice relaxation time, and T_2 is the spin-spin relaxation time.

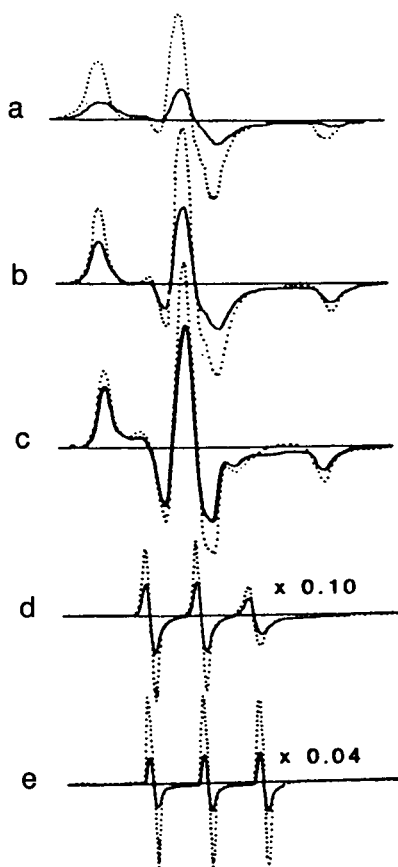


FIGURE 2 Relationship between EPR spectral lineshapes and intensities and the rotational correlation time. Spectra were recorded in-phase (i.e., V_1) using 100 kHz field modulation with a peak-to-peak amplitude of 2 Gauss at either $H_1 = 0.25$ Gauss (solid line) or $H_1 = 0.032$ Gauss (dashed line), which correspond to saturating and nonsaturating microwave fields, respectively. Spectra are normalized to the same spin concentration and microwave field intensity (i.e., all samples are divided by $\int V_1 \times \text{gain} \times H_1$, where $H_1 = 0.032$ or 0.25 Gauss), except where a different scale is indicated. The samples and their respective correlation times are: (a) MSL-Hb in 95% (vol/vol) glycerol, -20°C ; $\tau_r = 10^{-3}$ s, (b) MSL-Hb in 69% (vol/vol) glycerol, -12°C ; $\tau_r = 10^{-5}$ s, (c) MSL-Hb in 50% (vol/vol) glycerol, 30°C ; $\tau_r = 10^{-7}$ s, (d) TANOL in 90% (wt/wt) glycerol, 30°C ; $\tau_r = 10^{-9}$ s, and (e) TANOL in H_2O , 20°C ; $\tau_r = 10^{-11}$ s. The horizontal axis is the resonance field and the baseline represents a scan range of 100 Gauss for all spectra. The TANOL spectra are scaled down relative to the MSL-Hb spectra as indicated in the figure.

The in-phase ST-EPR intensity parameter, σ (which was previously defined as S'_1), is defined (Squier and Thomas, 1986a) as:

$$\sigma = \left\{ \frac{\int V_1(H_1 = 0.032 \text{ Gauss})}{H_1} \right\} / \left\{ \frac{\int V_1(H_1 = 0.25 \text{ Gauss})}{H_1} \right\} - 1. \quad (6)$$

The rationale for this definition is as follows. Neglecting passage effect, $\int V_1$ should be proportional to M_s , so that σ is the apparent value of the saturation factor (Eq. 5). The extent to which σ is less than the rigid

limit (no motion) value (i.e., ≈ 1.0) is a direct measure of saturation transfer, and thus of rotational motion on the microsecond time scale. When expressed in this form, this parameter has essentially the same motional dependence as other ST-EPR intensity parameters (e.g., $\int V_2$; Squier and Thomas, 1986a), which is expected because the latter is roughly proportional to saturation. The in-phase spectral intensity is superior to the $\int V_2$ (out-of-phase spectral intensity) in suppressing large amounts of a spectral component whose effective rotational correlation time is in the submicrosecond time regime. However, when the rate of rotational motion approaches that of the Larmor frequency (i.e., $\tau_r \leq 3 \times 10^{-9}$ s), the spectral saturation increases due to an increase in T_2 (Squier and Thomas, 1986a), indicating that σ suppresses the contribution of mobile probes only over a specific motional region, i.e., $3 \times 10^{-9} < \tau_r < 10^{-6}$ s for isotropic rotational motion (Fig. 3).

RESULTS

EPR spectra at different bilayer depths

EPR spectra are shown for stearic acid spin labels (SASL) in both native sarcoplasmic reticulum (SR) membranes as well as vesicles made from extracted SR lipids (Fig. 4); these spin labels vary in the position of the nitroxide relative to the membrane normal (Fig. 1). The spectra were recorded under nonsaturating and saturating microwave field intensities to obtain motional information on both the conventional and ST-EPR time scales. The unsaturated spectral line shape is sensitive only to motion on the nanosecond time scale, as determined by the magnetic anisotropy of the spin-label (on the time scale of T_2 ; reviewed by Marsh, 1985). Saturating condi-

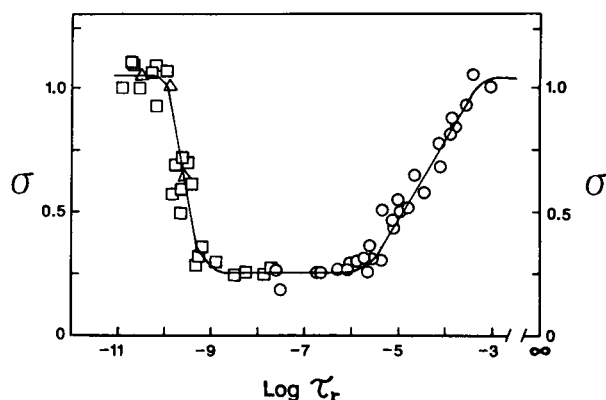


FIGURE 3 Sensitivity of ST-EPR to isotropic rotational motion. The rotational correlation time of the isotropic model systems MSL-Hb in glycerol/water solutions (circles) or TANOL in either glycerol/water (squares) or sec-butyl-benzene (triangles) solutions were determined with Eq. 4. Spectral saturation (σ) is defined in Eq. 6. The rotational correlation time was altered by varying either the glycerol concentration and the temperature (circles, squares) or by varying the temperature only (triangles).

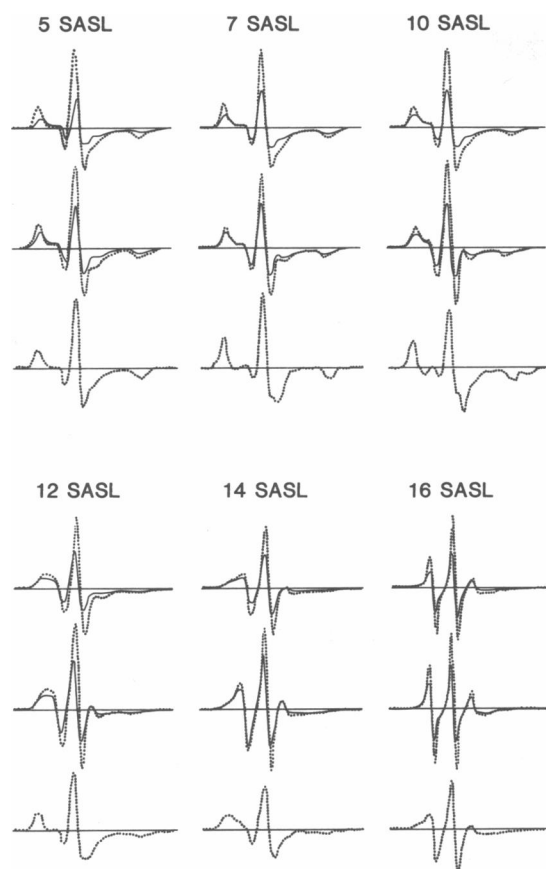


FIGURE 4 EPR spectra for positional isomers of stearic acid spin labels. Spectra were recorded at microwave field intensity $H_1 = 0.032$ Gauss (unsaturated, dotted lines) and $H_1 = 0.25$ Gauss (saturated, solid lines). From left to right, the position of the nitroxide is varied from near the top of the bilayer (5-SASL) toward the center of the bilayer. For each spin label, the first row corresponds to the spectra obtained in native SR at 0°C . The second row represents spectra obtained from vesicles made from extracted SR lipids at a slightly lower temperature (-4°C). The third row corresponds to the motionally restricted spectral component for $H_1 = 0.032$ Gauss and represents the result of digitized subtraction of the spectra in the second row (modeling the mobile fraction) from the composite spectrum in the first row (native SR). The spectra originating from the different stearic acid spin labels are all normalized to the same spectral height. Within a given column spectra are normalized to the same spin concentration and microwave field intensity (obtained by division of $\int V_1 \times \text{gain} \times H_1$, where $H_1 = 0.032$ or 0.25 Gauss). All spectra were recorded in-phase using 100 kHz field modulation with a peak-to-peak amplitude of 2 Gauss and a 100 -Gauss scan range. The buffer conditions consisted of 10% (wt/vol) sucrose, 20 mM MOPS (pH 7.0).

tions ($M_z/M_o < 1.0$ in Eq. 5) result in a decrease in spectral intensity relative to the normalized spectrum obtained under nonsaturating conditions, and the relative spectral intensity is sensitive to the spin label's effective rotational correlation time on the microsecond time scale (i.e., motion on the time scale of the spin-lattice relaxation time T_1 ; Fig. 3).

Motion on the conventional EPR time scale

For purposes of analyzing rotational motion on the nano-second time scale we need only consider the spectra obtained under nonsaturating conditions (i.e., $H_1 = 0.032$ Gauss; Fig. 4). As the nitroxide is positioned toward the center of the bilayer the spectra become motionally narrowed, i.e., the spin label's anisotropy becomes spectrally averaged, indicating an increased mobility of the spin label. This reflects the gradient of increased fluidity normally observed toward the terminal methyl group and the center of the bilayer (Hubbell and McConnell, 1969, 1971; Seelig and Hasselbach, 1971). The spectra obtained in native SR cannot be duplicated with those obtained from vesicles of extracted SR lipids by reducing the sample temperature, indicating that spectra in native SR contain at least two populations of lipids that are in slow exchange on the conventional EPR time scale (Marsh, 1985).

Through the use of digital subtraction procedures, we have deconvoluted these components, using the spectra of each spin label in vesicles of extracted SR lipids as a model for the more mobile component (see Methods). It was necessary to lower the temperature (by 4°C in all cases) at which these model spectra were recorded to obtain an optimal fit. The necessity of using a slightly lower temperature to model the bulk lipids indicates that while the Ca-ATPase primarily alters the rotational mobility of the annular lipids, all the lipids are affected to some extent (reviewed by Jost and Griffith, 1978a and b; Marsh, 1985).

The result obtained by subtracting the mobile spectral component (see above) from the composite spectrum (obtained from native SR vesicles) is shown in the bottom row of Fig. 4. We find that the spectra of these lipid hydrocarbon spin labels show a restricted component that is $43 \pm 6\%$ of the total ($\approx 33 \pm 4$ lipids per protein), irrespective of the nitroxide's position on the fatty acyl chain. These restricted components appear only in the presence of integral membrane proteins and are normally assumed to correspond to the first shell of lipid (i.e., boundary lipid) surrounding the protein (Marsh and Watts, 1982; Marsh, 1985). These spectra, in comparison to those obtained from stearic acid spin labels in vesicles of extracted SR lipids (second row in Fig. 4), have broader splittings and significantly more intensity in the spectral wings, corresponding to less rotational mobility.

Positional profile on the conventional EPR time scale

Independent of the position of the nitroxide on the fatty acyl chain, spectra obtained from either stearic acid spin

labels in vesicles of extracted SR lipids or spectra corresponding to the restricted lipid have significant spectral intensity in both the central regions of the spectrum and in the wings (Fig. 4), and correspond to relatively little mobility on the conventional EPR time scale. Such spectra are usually analyzed by an empirical formula describing an order parameter (see Methods), which depends on the positions of these extrema (Gaffney, 1976; Squier et al., 1988b).

The relationship between order parameter (at 0°C) and the position of the nitroxide on the stearic acid spin label is shown for single component spectra obtained under nonsaturating conditions representing the bulk and restricted lipid (modeled by extracted SR lipids at a slightly reduced temperature), as well as for extracted SR lipids at the same temperature (Fig. 5). In all cases the fatty acyl chain order parameter decreases as the nitroxide is positioned toward the center of the bilayer, reflecting the well-characterized fluidity gradient (Hubbell and McConnell, 1969). The shape of the positional profile relative to the membrane normal is approximately the same for the different populations of lipids, although the motionally restricted lipid has a significantly larger order parameter relative to that of extracted SR lipids. In contrast, the order parameter of the mobile spectral component is only slightly larger than that of extracted SR lipids at the same temperature, indicating that the mobile spectral component is only slightly affected by the Ca-ATPase. The similar mobility gradient of the restricted lipids in comparison to the bulk lipids indicates that the degree of interaction between lipid hydrocarbon

chains and the Ca-ATPase does not reflect specific interactions with particular amino acid side chains.

Motion on the ST-EPR time scale

The spectra demonstrating the saturation properties of stearic acid spin labels probing various depths in the bilayer are shown for native SR and vesicles of extracted SR lipids in Fig. 4. Under nonsaturating conditions (i.e., $H_1 = 0.032$ Gauss) the signal intensity varies linearly with the spin label concentration, and these spectra serve as a no-saturation reference point. Under saturating field intensities (i.e., $H_1 = 0.25$ Gauss), the relative spectral intensity decreases. The difference between the normalized spectral intensity is a direct measurement of spectral saturation (i.e., σ ; Eq. 5), and thus of motion on the ST-EPR time-scale (Fig. 3; Squier and Thomas, 1986a).

Temperature dependence of spectral saturation

Spectral saturation is minimal for isotropic rotational motion with an effective rotational correlation time (τ_r) in the submicrosecond, but not subnanosecond, time region (i.e., $1/\omega_L \leq T_1$; Fig. 3). Therefore, our experimental strategy is to identify experimental conditions where the more mobile lipid population can be suppressed. The variation of the sample temperature is a convenient mechanism to alter the rotational mobility of SR lipids and thereby allows us to search for a window where the population of lipids that are motionally restricted on the conventional EPR time scale can be selectively measured. It should be noted that at 0°C essentially all the SR lipid is in the liquid crystalline fluid state (i.e., L_α) (Davis et al., 1976; Martonosi, 1974; Bigelow et al., 1986), indicating that the reduction in temperature to 0°C simply reduces the mobility of the hydrocarbon chains.

We find that for stearic acid spin labels in both native SR and vesicles of extracted SR lipids that for temperatures greater than a certain threshold temperature (T_i) that there is a proportional relationship between spectral saturation (σ) and temperature that is not significantly different for either membrane sample (Fig. 6), indicating that for both SR and vesicles of extracted SR lipids the increase in σ largely results from the bulk lipid population (i.e., the mobile component in the spectrum from native SR). The increase in σ for all $T > T_i$ is almost certainly due to the increase in T_2 (see Eq. 5) that occurs as the rotational motion of stearic acid spin labels approaches the Larmor frequency (ω_L) (Fig. 3 and Squier and Thomas, 1986a). Furthermore, simulations indicate that while the effect of decreasing the amplitude of rotational motion is to increase σ , σ remains relatively independent

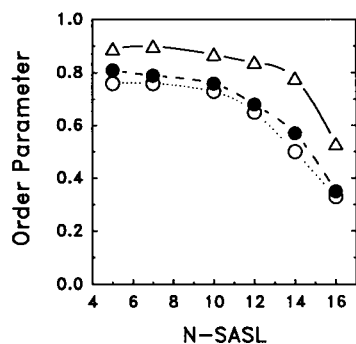


FIGURE 5 Apparent order parameter profile. Solid circles represent measurements of stearic acid spin labels in extracted SR lipids at -4°C , used to model the bulk lipids in native SR membranes at 0°C (Fig. 4, second row, dotted lines). Open triangles represent the restricted spectral component obtained subsequent to the spectral subtraction of the bulk lipid component (Fig. 4, third row). Open circles correspond to extracted SR lipids at 0°C . N-SASL refers to the position of the nitroxide spin label relative to the α -carbon of the stearic acid. Order parameters are calculated from the observed spectral anisotropy subsequent to spectral deconvolution using Eqs. 1 and 2 (see Methods).

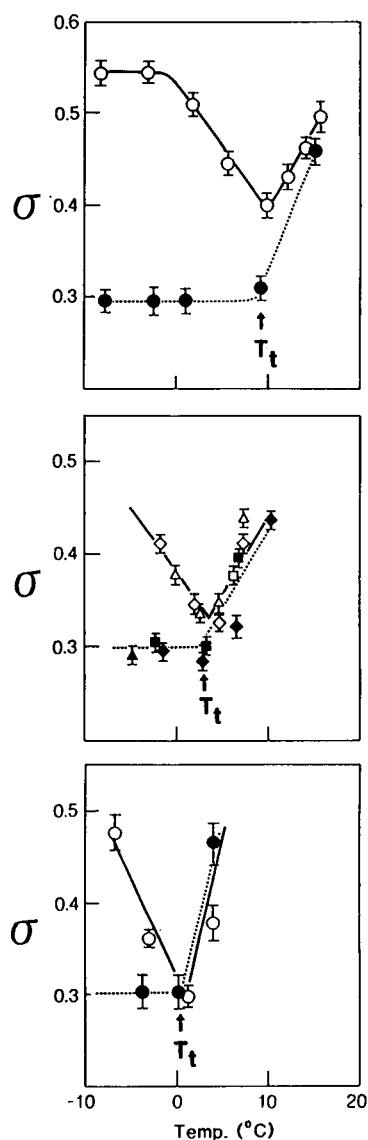


FIGURE 6 Temperature-dependence of spectral saturation. Spectral saturation (σ) for native SR (open symbols) and vesicles made from extracted SR lipids (solid symbols) as a function of temperature for the following stearic acid spin labels. (Top) 5-SASL; (middle) 7-SASL (diamonds), 10-SASL (squares), 12-SASL (triangles); (bottom) 16-SASL. T_t is the threshold temperature below which spectral saturation is minimal for stearic acid spin labels in extracted lipid.

of τ_r for $10^{-10} \text{ s} \leq \tau_r \leq 10^{-5} \text{ s}$ (Polnaszek, C., and D. D. Thomas, unpublished observations). Therefore, the occurrence of T_t at a lower temperature as the position of the nitroxide is moved toward the center of the bilayer is indicative of faster rotational motion on the nanosecond time scale.

The large amount of spectral saturation observed at temperatures above T_t precludes the selective measurement of the rotational mobility of the motionally

restricted lipid component (Fig. 6). However, the spectral saturation in vesicles of extracted SR lipids is minimal below T_t for all stearic acid spin labels examined (i.e., 5-, 7-, 10-, 12-, 14-, and 16-SASL; Figs. 6 and 7), and is comparable with that observed for fast isotropic rotational motion (i.e., $3 \times 10^{-9} \leq \tau_r \leq 1 \times 10^{-6} \text{ s}$; Fig. 3). This minimal level of saturation indicates that σ is insensitive to changes in the rotational mobility of the more mobile population of lipids for temperatures below T_t . However, native SR demonstrates a significant, temperature-dependent level of spectral saturation below T_t (Fig. 6). The saturation reaches a plateau for 5-SASL at temperatures $< -3^\circ\text{C}$, but σ is significantly less (i.e., 0.55) than the rigid limit value (i.e., $\sigma \approx 1.0$; Fig. 3). However, when the contribution of mobile lipids is subtracted, the calculated saturation value for the restricted lipid component (i.e., 0.9 ± 0.1) approaches that obtained in the rigid limit (no microsecond motion), as the temperature decreases (see legend to Fig. 7).

Positional profile on the ST-EPR time scale

We have replotted the data in Fig. 6 as a function of N , the position of the nitroxide on the stearic acid spin label, for spin labels in both SR and vesicles of extracted SR lipids at 0°C (Fig. 7), a temperature within the dynamic range of σ for all the stearic acid spin labels (i.e., a

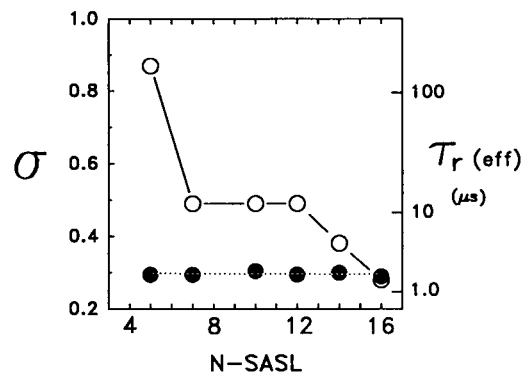


FIGURE 7 Rotational mobility of positional isomers of stearic acid spin labels on the ST-EPR time scale. Spectral saturation (σ) at 0°C is plotted as a function of the position of the nitroxide on the stearic acid spin label for the motionally restricted SR component (open circles) and for the mobile SR component (i.e., vesicles of extracted SR lipids; solid circles), where $\sigma(\text{observed}) = \{[\sigma(\text{restricted}) \times f_i] + [\sigma(\text{mobile}) \times (1 - f_i)]\}$. f_i is the fraction of lipids motionally restricted on the conventional EPR time scale as determined from the digital subtraction of conventional EPR spectra (Fig. 4). $\sigma(\text{mobile})$ is the measured saturation in vesicles of extracted SR lipids (row 2 in Fig. 4). Effective correlation times obtained from a comparison with isotropic reference compounds are shown on the right axis.

temperature at which all nitroxide probes are sensitive to the degree of motional restriction; Fig. 6). We have deconvoluted the spectral saturation corresponding to the mobile and restricted lipid components, because we know (a) the relative amounts of each spectral component from digital subtractions (Fig. 4) and (b) the spectral saturation (σ) associated with the mobile component (see Fig. 7 legend). Effective rotational correlation times (τ_r) obtained from a comparison with isotropic reference spectra are indicated only as a reference meant to illustrate the relative degree of lipid rotational mobility on the ST-EPR time scale, and probably does not correspond to actual correlation times because they neglect the effects of anisotropic rotational motion (see below). Only the spin labels restricted by the Ca-ATPase demonstrate appreciable saturation below T_i . Whereas σ is approximately the same for both lipid populations at the 16-SASL position, this is coincidental because at lower temperatures the membranes containing the Ca-ATPase likewise display appreciable saturation at the 16-SASL position (Fig. 6). Thus, in general, as the nitroxide is positioned toward the center of the bilayer σ decreases, indicative of an increased hydrocarbon chain mobility, i.e., a fluidity gradient in the boundary layer on the ST-EPR time scale. However, the spectral saturation observed at the 7-, 10-, and 12-SASL position is virtually the same, suggesting a constant degree of hydrocarbon chain mobility at these nitroxide positions. A similar positional profile is observed at other temperatures $<T_i$, for whereas σ (lipids only) is constant and minimal for all temperatures $<T_i$, σ (SR) increases in a fairly uniform manner for nitroxide positions $N \geq 7$ as the temperature is further lowered (Fig. 6).

DISCUSSION

Summary of results

We have quantitated the rotational dynamics of the lipid chains adjacent to the Ca-ATPase in sarcoplasmic reticulum membranes at varying depths normal to the bilayer (a) indirectly, with digital subtractions of conventional EPR spectra, and (b) directly by saturation-transfer EPR. We find that these two techniques are complementary: Digital subtraction of conventional EPR spectra provides an accurate measurement of the number of lipids motionally restricted by the Ca-ATPase, but often does not yield spectra with the well-resolved lineshapes necessary for accurate measurements of the rotational dynamics of the restricted lipids. ST-EPR avoids this problem by directly and selectively measuring only the dynamics of the motionally restricted lipid; however the determination of the spectral saturation associated with

each lipid population requires knowledge of the fraction of each population obtained from digital subtractions. Therefore, the combined use of conventional and ST-EPR offers greater precision in the measurement of small changes in the rotational dynamics of this population of lipids.

Resolution of the lipid dynamics in SR

Whereas the analysis of the restricted component by spectral subtractions of conventional EPR spectra is somewhat subjective, the determination of the mole fractions associated with each motional component can be determined with a high degree of accuracy, independent of the model used to approximate the mobile component (see below) (Thomas et al., 1982; Silvius et al., 1984; East et al., 1985; Bigelow and Thomas, 1987; Horvath et al., 1988b). The uncertainty in the spectral deconvolution of multicomponent spectra primarily arises due to the broadening of the mobile spectral component relative to the spectrum obtained at the same temperature for the same stearic acid spin label in extracted SR lipids. Typically, two models are applied to the deconvolution of multicomponent EPR spectra: (a) the propagation of motional restriction through the annular lipid, in analogy to the ability of cholesterol (a rigid molecule) to decrease the rotational mobility of neighboring fatty acyl chains, and (b) exchange between the mobile and restricted components (Knowles et al., 1979; Davoust and Devaux, 1982; East et al., 1985; Horvath et al., 1988a; reviewed by Marsh 1985, 1987). However, while the experimental uncertainty regarding the appropriate model spectrum can occlude small changes in the rotational dynamics, it does not affect the qualitative features regarding the rotational dynamics of the restricted lipid population, as evidenced by the fact that both models qualitatively agree (Thomas et al., 1982; East et al., 1985; Pates and Marsh, 1987). Furthermore, ST-EPR measurements likewise indicate that the presence of the Ca-ATPase reduces the mobility of a fraction of lipid spin labels along the entire hydrocarbon chain (Figs. 5 and 7).

The major technical advantage of ST-EPR measurements is the ability to directly and selectively measure the rotational dynamics associated with the restricted lipid population, without any complications arising from exchange with the bulk lipids (see Fig. 7), thereby allowing the accurate detection of small changes in the lipid's dynamics. Together, the digital subtractions of conventional EPR spectra and ST-EPR demonstrate that (a) the rotational mobility of a fraction (i.e., $43 \pm 6\%$) of the membrane lipids is restricted by the Ca-ATPase (Fig. 4), (b) both lipid populations display substantially less rota-

tional mobility in the upper half of the fatty acyl chain ($N < 12$; Figs. 5 and 7) relative to that observed in the lower half, in analogy to the positional profile generally observed by ^2H -NMR in many biological membranes (Seelig and Seelig, 1974), and (c) that the annular lipids undergo essentially the same degree of nonspecific interactions with the Ca-ATPase along their entire hydrocarbon chain, as demonstrated by the nearly constant difference in the apparent order parameter for the bulk and restricted lipid components along the fatty acyl chain (Fig. 5).

Effects of anisotropic rotational motion

In general the rotational mobility of the fatty acyl chains in biological membranes is expected to be highly anisotropic, because the two-dimensional organization of lipids in biological membranes provides a considerable energy barrier to isotropic rotational motion. Therefore, the spectroscopic effects of anisotropic rotational motion must be considered. When correlation times are faster than $\approx 3 \times 10^{-9}$ s, the line positions of conventional EPR spectra depend only on the time average of the orientation of the nitroxide axis relative to the bilayer normal (Marsh, 1981; Gaffney, 1976). Under these conditions the lipid dynamics are generally characterized in terms of an order parameter.

ST-EPR is able to identify the presence of subnanosecond rotational motion, because under these conditions the entire spin population becomes motionally averaged, resulting in an increased amount of saturation (Fig. 3 and Squier and Thomas, 1986a). Therefore, ST-EPR can be used to test whether the rate of rotational motion is sufficiently fast so as to average the spectral anisotropy, thereby permitting the use of an order parameter model (Eq. 1 or 2), that assumes that only the amplitude of the rotational motion is measured (i.e., $\tau_r^{-1} \gg \gamma [T_{\parallel} - T_{\perp}]$ implies that $\tau_r \ll 10^{-8}$ s).

Because increasing the temperature is predicted to increase the rate of rotational motion, the temperature-dependent increase in σ above a certain threshold temperature, T_c , for both SR and SR lipids indicates that the different stearic acid spin labels reporting the rotational dynamics of the bulk lipid undergo subnanosecond rotational motion for temperatures near or $> T_c$ (Figs. 3 and 6), permitting the application of the order parameter model. However, in native SR, where the additional contribution from the motionally restricted component is observed, there is a large amount of spectral saturation at temperatures $< T_c$, that varies inversely with the temperature, indicating that $\tau_r > 10^{-9}$ s (Figs. 3 and 6). Therefore, the spectral anisotropy of the annular lipids is not completely averaged at 0°C on the conventional EPR time

scale, indicating that the order parameter model is sufficient only for qualitative comparisons.

In the case of the motionally restricted lipid, where $\tau_r > 10^{-9}$ s, ST-EPR measurements of the dynamics of fatty acyl chains in biological membranes are sensitive to both the rate and amplitude of rotational motion; the effect of decreasing the angular amplitude of the rotational motion on spectral saturation (σ) is similar to the effect of decreasing the rate of rotational diffusion (Thomas et al., 1985; Lindahl et al., 1985). In most membrane systems, including SR, both the exchange between the bulk and annular lipid populations and the rotational motion with respect to the membrane normal of both lipid populations is generally considered to be fast (i.e., $\tau_{ex} \approx 10^{-6}$ s; $\tau_r \leq 10^{-8}$ s) in comparison to the ST-EPR time scale ($\tau_r \approx 10^{-4}$ s for isotropic rotational motion; Paddy et al., 1981; Davoust et al., 1983; East et al., 1985; Pates et al., 1985; reviewed by Devaux et al., 1985; Thomas, 1985, 1986; Marsh, 1985, 1987; Robinson et al., 1985). Therefore, the increased amount of spectral saturation characteristic of those lipids associated with the Ca-ATPase (σ in Fig. 7; under conditions where rotational motion is sufficiently less than the Larmor frequency so that T_{\parallel} is constant) is probably dominated by the degree of motional restriction (i.e., the amplitude of rotational dynamics), rather than the rotational rate of diffusion of these lipids.

Relationship to other work

Our results, indicating that on both the conventional and ST-EPR time scales the rotational motions of a population of lipids are motionally restricted, are consistent with previous results using conventional EPR, ^2H - and ^{31}P -NMR, fluorescence anisotropy, and Raman spectroscopy, which all indicate some degree of motional restriction due to the influence of the Ca-ATPase on the surrounding lipids (Thomas et al., 1982; Jahnig et al., 1982; reviewed by Hidalgo, 1985, 1987). Although spectral components have been observed that may correspond to phospholipid head groups immobilized on the ^{31}P NMR time scale (Selinsky and Yeagle, 1984; Yeagle et al., 1984), this remains controversial (Ellena et al., 1986). However, ^2H -NMR measurements (comparable with the stearic acid spin labels in that the probe is on the fatty acyl chains) resolve only a single motional component in SR membranes (Seelig et al., 1981; Paddy et al., 1981). The ability of conventional EPR spectra to resolve the motionally restricted population of fatty acyl chains is normally ascribed to its time scale (i.e., $\approx 10^{-8}$ s for EPR vs. $\approx 10^{-4}$ s for ^2H -NMR; reviewed by Watts, 1981; Seelig et al., 1982; Marsh, 1985), because diffusive exchange averages the motional dynamics of the lipid populations on the NMR time scale. However, while the time scale of ST-EPR (i.e., $\tau_r \approx 10^{-4}$ s) is analogous to that of

^2H -NMR, the superior orientational resolution of the nitroxide reporter group (as little as 8° ; Thomas et al., 1985) apparently enables ST-EPR to selectively measure those lipids motionally restricted on the conventional EPR time scale.

There is general agreement that membrane proteins (including the Ca-ATPase) reduce the rate of the internal modes of lipid rotational mobility (Jost et al., 1973b; Seelig et al., 1981; Jahnig et al., 1982; Pates and Marsh, 1987). The small increase in apparent lipid disorder in SR membranes on the ^2H -NMR time scale (Rice et al., 1979; Seelig et al., 1981) may arise from static disorder (i.e., a heterogeneous distribution of mean chain orientations with respect to the membrane normal), consistent with the suggestion that both the rate and amplitude of the rotational motion of the individual lipids adjacent to the Ca-ATPase is reduced relative to the bulk lipids (Jahnig, 1979; Jahnig et al., 1982).

Conclusion

The in-phase spectral intensity (σ) can be used to measure selectively the rotational mobility of lipids that are motionally restricted on the conventional EPR time scale, and is complementary to digital subtraction methods using conventional EPR spectra for the characterization of the physical properties of the lipid-protein interface. While the mobile component dominates the conventional EPR spectrum, the motionally restricted component is the dominant spectral feature observed in the ST-EPR spectrum, over a wide range of conditions. ST-EPR thus provides a sensitive method to measure selectively the rotational dynamics of annular lipids. However, a quantitative description of this motion will require both more detailed spectral simulations and the development and application of time-resolved EPR, so as to distinguish between the rate and amplitude effects.

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