

# APPLICATIONS OF NEW SATURATION TRANSFER ELECTRON PARAMAGNETIC RESONANCE METHODOLOGY TO THE ROTATIONAL DYNAMICS OF THE Ca-ATPase IN SARCOPLASMIC RETICULUM MEMBRANES

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**ABSTRACT** The presence of small amounts of weakly immobilized probes can result in large systematic errors in the measurement of correlation times ( $\tau_r$ ) from saturation transfer EPR spectra. However, we have recently developed experimental methodology to minimize these errors (Squier and Thomas, *Biophys. J.*, 49:921–935). In the present study we have applied this methodology to the measurement of the rotational motion of the Ca-ATPase in sarcoplasmic reticulum. This analysis involves the estimate of  $\tau_r$  from line-shape parameters (spectral line-height ratios) and intensity parameters (spectral integral), coupled with digital subtractions to remove spectral components corresponding to weakly immobilized probes. We have analyzed the ST-EPR spectra of the Ca-ATPase over a range of temperatures and find that, unlike line-shape parameters, intensity parameters are little affected by the subtraction of the weakly immobilized spectral component (W). Thus,  $\tau_r$  values from intensity parameters are a more reliable measurement of rotational motion. As reported previously, an analysis with line-shape parameters yields a nonlinear Arrhenius plot of protein mobility. However, the plot is linear when intensity parameters or corrected spectra are used, consistent with the theory for the hydrodynamic properties of a membrane protein of unchanging size and shape in a fluid bilayer. An analysis with line-shape parameters yields different effective  $\tau_r$  values in different spectral regions, and these  $\tau_r$  values are temperature-dependent. However, correction of spectra for W yields temperature-independent  $\tau_r$  ratios, indicating that the motional anisotropy is temperature-independent. Obtaining a good match for the weakly immobilized spectral component remains a major difficulty in the quantitative analysis of ST-EPR spectra using line-shape parameters. This study shows that intensity parameters can be used to avoid this problem, making the ST-EPR technique applicable in cases that were previously resistant to analysis.

## INTRODUCTION

In order to understand the physical mechanisms of membrane function, it is important to measure macromolecular motions directly, using spectroscopic probe techniques in the functional membrane. Saturation transfer EPR (ST-EPR) is useful in the measurement of the microsecond rotational motions characteristic of membrane proteins, provided the probe can be immobilized on the protein surface, so as to provide an exclusive measurement of overall protein rotational motions (reviewed in Thomas, 1985, 1986; Dalton, 1985).

Even a simple system like sarcoplasmic reticulum (SR), containing only a single major integral membrane protein, is difficult to label with complete specificity. Thus it is difficult to avoid the presence of a small fraction of "weakly immobilized probes" (defined as having sufficient nanosecond rotational mobility that their spectral contribution, W, is resolved from that of rigidly attached or "strongly immobilized probes," S) is usually unavoidable. These weakly immobilized probes can interfere with the analysis of ST-EPR spectra, particularly if line-shape parameters are used in the spectral analysis (Squier and Thomas, 1986). When the weakly immobilized spectral

component (W) changes, it becomes difficult to compare ST-EPR spectra even qualitatively (Thomas, 1985, 1986).

The temperature dependence of Ca-ATPase rotational motion in SR has been studied by ST-EPR of a maleimide spin label covalently attached to the Ca-ATPase (Thomas and Hidalgo, 1978; Kirino et al., 1978; Kaizu et al., 1980). Arrhenius plots of protein mobility have shown apparent "breaks" (changes in slope), leading to the interpretation that either a lipid phase transition or a large-scale protein conformational change occurs as a function of temperature (Hoffman et al., 1979; Kirino et al., 1978; Kaizu et al., 1980; King and Quinn, 1983).

However, it has been suggested that the apparent breaks could be artifacts due to the temperature-dependent motions of W (Thomas, 1985, 1986). It has previously been suggested that the integrated intensity of the ST-EPR spectra suppresses the contribution of W (Evans, 1981; Horvath and Marsh, 1983), since these spectral components have negligible integrated intensity. Although the latter is not strictly true (Squier and Thomas, 1986), spectral intensity parameters can be useful in suppressing many of these interfering signals, provided that an estimate of the mole fraction and rate of motion of the W is obtainable.

In the present study, we have used ST-EPR intensity parameters to measure the rotational dynamics of the Ca-ATPase in SR, providing an unambiguous interpretation regarding the temperature dependence of protein rotational mobility even in the presence of contributions from W.

## MATERIALS AND METHODS

### Fragmented SR

Vesicles were 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, 1 mM  $\text{Na}_2\text{S}_2\text{O}_3$ , 20 mM MOPS, (pH 7.0) and stored in liquid nitrogen.

### Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) as described previously (Bigelow et al., 1986). Densitometer scans of coomassie blue stained gels were used to quantitate the protein present. Such gels indicated that  $80\% \pm 5\%$  of the proteins in our SR preparation migrated as a 100 kD band, presumably the Ca-ATPase. Phosphorylase b was found to be  $<4\%$  of the protein in this preparation.

### Arrhenius Analysis

Lines in Arrhenius plots were fit using linear regression least-squares analyses, using appropriate statistical analysis to determine the number and slopes of lines fitting the data (Bigelow et al., 1986).

### Spin Labeling

To monitor the rotational motion of the Ca-ATPase protein, SR was labeled with a short-chain maleimide spin label (MSL) (Fig. 1), *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidiny)-maleimide (Aldrich Chemical Co., Metuchen, NJ) as described previously (Bigelow et al., 1986).

### EPR Spectroscopy

EPR spectra were obtained with a Varian E-109 spectrometer (Varian Associates, Palo Alto, CA) and spectra were digitized and analyzed with a microcomputer interfaced to the spectrometer (Lipscomb and Salo, 1983) as described previously (Squier and Thomas, 1986). Submicrosecond rotational motion of spin labels was detected by conventional EPR (first harmonic absorption in-phase, designated  $V_1$ ), using 100 kHz field modulation (with a peak-to-peak amplitude of 2 Gauss) and a microwave field amplitude of 0.032 Gauss, thereby assuring us that  $V_1$  spectra depend linearly on  $H_1$  (no saturation effects). Submillisecond rotational motion was detected by saturation-transfer EPR (second harmonic absorption out-of-phase, designated  $V_2'$ ) using 50 kHz field modulation (with a modulation amplitude of 5 Gauss) and a microwave field intensity of 0.25 Gauss. The accurate and reproducible setting of the microwave intensity incident on the sample ( $H_1$ ) required 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, 1986). All saturation studies were done in the absence of oxygen. Oxygen was removed from reference and experimental samples using gas-permeable sample cells purged with  $\text{N}_2$  (Popp and Hyde, 1981). Temperature was controlled to within  $0.5^\circ\text{C}$  with a Varian V4540 variable temperature controller (Varian Associates, Inc.).

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

## Spectral Analysis

ST-EPR spectra from SR were analyzed by comparison with reference spectra obtained from isotropically tumbling spin-labeled hemoglobin in solutions of known viscosity ( $\eta$ ) and temperature ( $T$ ), in which the rotational correlation times,  $\tau_r$ , are known (Squier and Thomas, 1986; see Fig. 2). Two types of experimental parameters were used to compare spectra: line-shape parameters and integrated intensity parameters (Squier and Thomas, 1986). Briefly, line-shape ratios involve the comparison of peak heights in the  $V_2'$  spectrum (e.g.  $L''/L$ ), while integrated intensity parameters are obtained by integrating the spectrum ( $V_1$  or  $V_2'$ ) and are designated  $S_1'$  or  $S_2'$ , respectively. The effective correlation time is defined, for a given spectral parameter, as the value of the rotational correlation time,  $\tau_r$ , for which the hemoglobin spectrum has the same parameter value.

Spin concentration was determined by double integration of the digitized conventional ( $V_1$ ) EPR spectra, recorded at low (nonsaturating) microwave power. The relative number of spins per sample was determined by comparison of the number obtained from double-integration of the  $V_1$  spectrum, with the number obtained for a 0.1 mM MSL standard, whose  $V_1$  spectrum has been digitized and double-integrated in the same manner. Conventional and ST-EPR ( $V_2'$ ) spectra were all normalized to the same number of spins, by dividing each spectrum by a number proportional to the double integral of the  $V_1$  spectrum.

Spectra of the spin-labeled Ca-ATPase containing multiple populations of probe mobilities were deconvoluted by computer subtraction of the digitized spectra, using a model system (i.e., TANOL, 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl, in aqueous glycerol) to simulate the weakly immobilized component (W; see Fig. 1 A). The  $\tau_r$  of the model spectrum was varied by altering both the temperature ( $T$  in  $^\circ\text{K}$ ) and the viscosity ( $\eta$  in Poise), where  $\tau_r = (\eta/T) \times 2.9 \times 10^{-7}$  s (Squier and Thomas, 1986). The criteria for an appropriate fit to W were obtained through the use of both the  $V_1$  and  $V_2'$  spectra. The  $V_1$  spectra were used to determine the appropriate model spectrum for W. The decreased polarity of glycerol resulted in a narrower hyperfine splitting than in water. Before subtraction, the model spectrum was aligned to match the low-field region of the SR spectrum. For each candidate model spectrum, varying mole fractions were subtracted digitally from the SR spectrum, in an attempt to obtain an endpoint spectrum corresponding to the rigid limit, i.e., a homogeneous population of probes undergoing no motion on the  $V_1$  timescale. If two model spectra produced equally good  $V_1$  endpoint spectra, the quality of the  $V_2'$  endpoints was used to choose between the two models. The corrected  $V_2'$  spectrum of MSL-SR was obtained by subtracting the  $V_2'$  model spectrum, using the same mole fraction determined from the  $V_1$  spectral titration.

## RESULTS AND DISCUSSION

### Temperature-Dependence of Uncorrected Spectra

EPR spectral line-shapes and intensities (Fig. 1 A) show that the spin-labeled Ca-ATPase undergoes microsecond rotational motion at all temperatures studied, in agreement with previous observations (Hidalgo et al., 1978; Thomas and Hidalgo, 1978; Thomas et al., 1982). The  $V_2'$  spectral lineshape and intensity change as a function of temperature, indicating that the rotational mobility of the Ca-ATPase increases with temperature. However, both the measured  $\tau_r$  and changes in  $\tau_r$  as a function of temperature, when measured from the uncorrected spectra, (Fig. 1 A) are very different for the two types of parameters (Table I).

When the temperature is increased, the ST-EPR ( $V_2'$ )

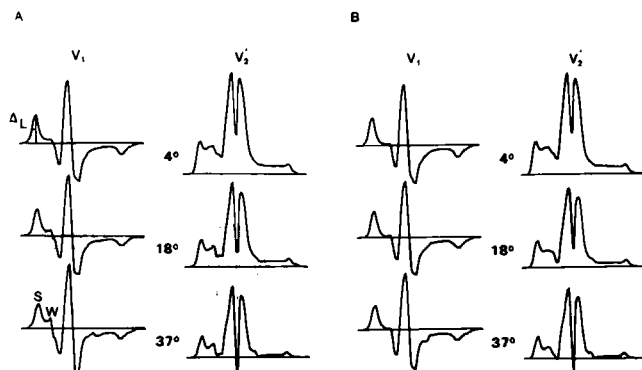


FIGURE 1 Conventional ( $V_1$ ) and saturation transfer ( $V_2$ ) EPR spectra of MSL-NEM-CaATPase in SR as a function of temperature, before (A) and after (B) subtraction of the weakly immobilized spectral component. The spectra of the model for this component ( $10^{-4}$  M TANOL in 90% (wt/wt) glycerol at 20°C) are shown as dotted curves and are normalized to  $0.1 \times$  gain of the other spectra. From the top, the corrected spectra were obtained after the subtraction of 1.0%, 1.8%, and 4.1% of the model spectrum. All spectra shown are normalized to the same spin concentration. Baselines are 100 Gauss wide.

spectral intensity decreases (Fig. 1 A), indicating that the protein mobility increases. In contrast, the lineshape parameters ( $L''/L$  is shown in Fig. 1 A) change much less. The majority of the labels are motionally restricted on the conventional EPR time scale (ns), i.e., the spectral line-shape is characteristic of the rigid limit. This population of strongly immobilized probes has a constant value for the outer half width at half height of the low-field line ( $\Delta_L = 3.2 \pm 0.2$  Gauss; see Fig. 1 A) over the entire temperature range studied, indicating that these probes either have no nanosecond mobility or have highly restricted nanosecond mobility that is independent of temperature (Mason and Freed, 1974). That is, any temperature-dependent change in the mobility of these probes detected by  $V_2'$  reflects

TABLE I  
EFFECT OF WEAKLY IMMOBILIZED  
PROBES ON  $\tau_r$

Temp. (°C)	$W/S^\dagger$	$\tau_r$ from ST-EPR Parameter*				
		$L''/L$	$C'/C$	$H''/H$	$fV_2'$	$S_1'$
4°	0.16	$78 \pm 5$	$17 \pm 2$	$100 \pm 20$	$35 \pm 6$	$17 \pm 9$
	0.06	$70 \pm 5$	$18 \pm 2$	$110 \pm 20$	$30 \pm 5$	$17 \pm 9$
18°	0.25	$72 \pm 5$	$7 \pm 1$	$60 \pm 10$	$12 \pm 2$	$5 \pm 3$
	0.15	$29 \pm 2$	$11 \pm 1$	$70 \pm 10$	$9 \pm 2$	$5 \pm 3$
37°	0.45	$66 \pm 5$	$2 \pm 0$	$47 \pm 8$	$4 \pm 1$	$2 \pm 1$
	0.09	$11 \pm 1$	$4 \pm 1$	$32 \pm 5$	$2 \pm 1$	$2 \pm 1$

All experimental uncertainties are  $\sigma$ , the standard deviation for a single measurement (see Table IV, Squier and Thomas, 1986).

\*ST-EPR spectral parameters are defined in Methods. Effective correlation times ( $\tau_r$ ) are obtained from reference spectra corresponding to isotropic motion.

<sup>†</sup>The two rows at each temperature show the result before and after subtraction of the model spectrum for the weakly immobilized component, obtained from  $10^{-4}$ M TANOL in 90% (wt/wt) glycerol at 20°C.

microsecond rotational motion, probably corresponding to overall rotational mobility. However, there is a second more mobile spectral component, designated W in  $V_1$ , whose relative contribution to both the  $V_1$  and  $V_2'$  spectra increases with temperature, interfering with the goal of detecting microsecond motions selectively (Squier and Thomas, 1986). These two components are well resolved in the spectrum, implying that the probes are either (a) independent or (b) exchanging with each other slowly compared with the nanosecond time scale. It is therefore legitimate to correct the experimental spectra (Fig. 1 A) by subtracting a model spectrum for the weakly immobilized component.

### Correction for Weakly Immobilized Probes

In Fig. 1 the effect of W on the ST-EPR spectral line-shape and intensity is evaluated. Glycerol solutions of TANOL were used to obtain model spectra (both  $V_1$  and  $V_2'$ ) for W by varying both the glycerol concentration and temperature. Since W was resolved from the component due to strongly immobilized probes (S), particularly at

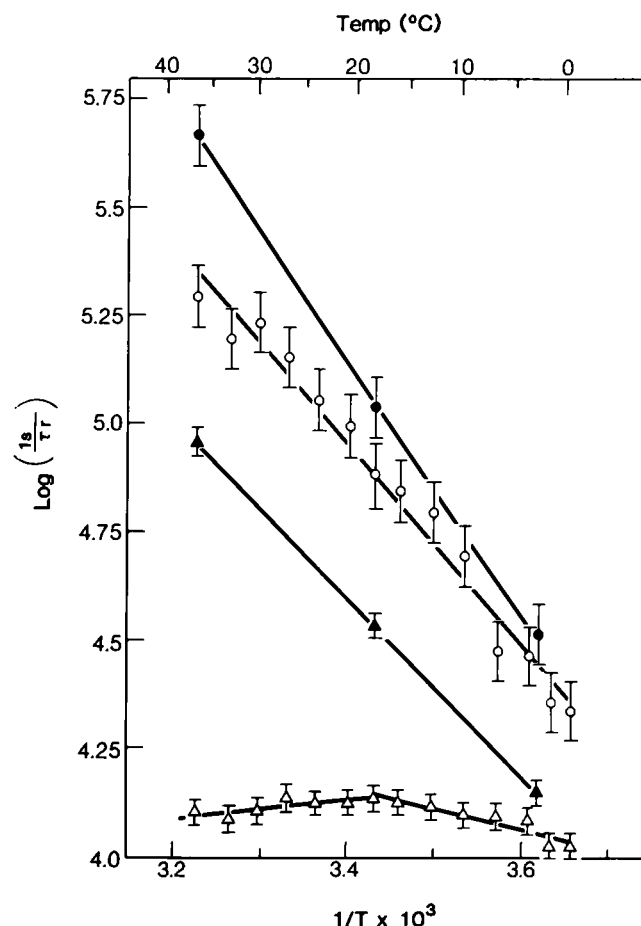


FIGURE 2 Arrhenius plots of protein rotational mobility using lineshape ( $L''/L$ , triangles) and intensity ( $fV_2'$ , circles) parameters. Open and closed symbols represent  $\tau_r$  values obtained before and after subtraction of the spectrum of the weakly immobilized probe, respectively.

higher temperatures, it was a relatively straightforward procedure to obtain a model spectrum matching W, as determined from digital subtraction of TANOL spectra from SR spectra, as described in Methods. The best fits at all temperatures were for the same weakly immobilized model (i.e., TANOL in 90% (wt/wt) glycerol at 20°C,  $\tau_r = 3.0$  ns, Fig. 1 A, ----), whose mole fraction increased with temperature (1.0%, 1.8%, and 4.1% of the total spin concentration at 4°C, 18°C, and 37°C respectively). The resulting corrected  $V'_2$  spectra (Fig. 1 B), as well as the uncorrected  $V'_2$  spectra (Fig. 1 A), were analyzed using both spectral lineshapes and intensities (see Table I). The  $\tau_r$  measured from lineshape parameters (particularly  $L''/L$ ) are very sensitive to the weakly immobilized component. Unlike lineshape parameters, the integrated intensity parameters were little affected by the spectral correction, indicating that they provide a more reliable measurement of protein rotational mobility in the presence of small amounts of weakly immobilized probes. After spectral correction, all parameters show a similar increase (within experimental error) in protein mobility (decreased  $\tau_r$ ) as the temperature is increased.

### Arrhenius Plots

When the apparent  $\tau_r$  obtained from the  $L''/L$  line-shape parameter of the uncorrected spectrum is plotted in an Arrhenius plot, we observe the previously reported change in slope near 20°C (Fig. 2). Similar changes in slope are observed with the  $H''/H$  parameter, although the apparent break point is near 30°C (not shown). This break has been previously interpreted to indicate either a lipid phase change or a change in the protein's global conformation or oligomeric state (Kirino et al., 1978; Kaizu et al., 1980; King and Quinn, 1983). However, when we subtract W from the ST-EPR spectra, an Arrhenius plot of protein mobility ( $\tau_r$ )<sup>-1</sup> calculated from line-shape parameters is linear (Fig. 2). Similar results were observed in Arrhenius plots of  $\tau_r$ <sup>-1</sup> obtained from other line-shape parameters (e.g.  $H''/H$ , not shown). When intensity parameters are used, the Arrhenius plot of protein mobility is linear, regardless of whether W is subtracted, and the slope is changed only slightly. This agreement between corrected line-shape parameters and integrated intensity parameters strengthens the conclusion that spectral intensity parameters can often be used to calculate  $\tau_r$  accurately in the presence of small amounts of W. This avoids the difficulty of finding an appropriate model spectrum that seems to fit the W component. This latter problem is nontrivial, since the EPR model spectrum depends on the rate and amplitude of the motion as well as the polarity of the medium (Griffith and Jost, 1976), making it very difficult to obtain a reliable model spectrum that can be used to analyze the ST-EPR spectral line-shape. The large spectral intensity that can result from some W components, requires that some estimate be obtained for  $\tau_r(W)$  (Squier and Thomas, 1986).

### Anisotropic Rotational Motion

Because of the orientational resolution within the nitroxide EPR spectrum, anisotropic rotations are expected to have different effects from those of isotropic rotations. Disagreement in the effective  $\tau_r$  value determined from different line-shape parameters in the  $V'_2$  spectrum has frequently been taken as evidence that the motion is anisotropic (Thomas et al., 1975), and has been proposed as a quantitative measure of the degree of anisotropy (Hyde and Thomas, 1980; Marsh, 1980; Johnson et al., 1982; Fajer and Marsh, 1983). This principle has been supported by theoretical simulations of ST-EPR spectra in the absence of W (Robinson and Dalton, 1980; Robinson and Dalton, 1981; Lindahl and Thomas, 1982; Polnaszek and Thomas, 1985). However, small amounts of W can result in experimental spectra whose effective  $\tau_r$  values, obtained from ST-EPR line-shape parameters in different spectral regions, are very different, even if the majority of probes are known to be undergoing isotropic microsecond rotational motion (Fig. 7 in Squier and Thomas, 1986). Thus it is essential to correct for the effect of W, as we have in the present study, before attempting to analyze spectra in terms of anisotropic motions.

Before correction for W, our data (Table I) indicate a large temperature-dependence in the ratios of  $\tau_r$  values obtained for different spectral regions (e.g. [ $\tau_r(L''/L)/\tau_r(C'/C)$ ], suggesting that the motional anisotropy is temperature-dependent. After correction, the values of  $\tau_r$  obtained from different line-shape parameters are still different, but have a similar temperature-dependence, i.e., the ratios of  $\tau_r$  values are nearly temperature-independent. Thus, the correction for W indicates a decrease in the apparent motional anisotropy and an elimination of its temperature-dependence, consistent with motional models in which the anisotropic mobility of the entire protein about the membrane normal is constant (e.g., axial motion; Polnaszek and Thomas, 1985). However, a definitive analysis will require both (a) determination of the probe's orientation relative to the membrane normal and (b) a quantitative comparison with simulations. This result is consistent with other data indicating that W-corrected ST-EPR experiments measure the overall rotational motion of the Ca-ATPase (Squier et al., 1985; Bigelow et al., 1986). We cannot, however, rule out models that invoke two populations of probe molecules which have different  $\tau_r$  values on the microsecond time scale.

### Conclusions

We have analyzed the ST-EPR of the spin-labeled Ca-ATPase spectra as a function of temperature and find that  $\tau_r$  values measured from integrated spectral intensity parameters are little affected by the weakly immobilized spectral component, yielding linear Arrhenius plots of protein mobility. The correction for the weakly immobilized component in the spectrum at higher temperatures

(when  $W$  is a problem) clarifies much of the ST-EPR work done previously on the Ca-ATPase in SR. At higher temperatures, typically  $T > 10^\circ\text{C}$ , this weakly immobilized component often results in a spectrum whose line-shape parameters (e.g.,  $L''/L$ ) indicate erroneously longer  $\tau_r$  values. Studies involving the analysis of the temperature-dependence of  $\tau_r$  have been subject to systematic errors, depending on the ST-EPR parameter and the variable weakly immobilized component (Kirino et al., 1978; Thomas and Hidalgo, 1978; Kaizu et al., 1980). Under conditions where the weakly-immobilized probes represent a small and constant mole fraction of the spin-probes (e.g., at  $4^\circ\text{C}$ ), experimental results using line-shape parameters have provided reliable evidence for microsecond changes in  $\tau_r$  (Hidalgo et al., 1978; Thomas et al., 1982; Squier et al., 1985).

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