

Saturation transfer electron spin resonance of Ca^{2+} -ATPase covalently spin-labeled with β -substituted vinyl ketone- and maleimide-nitroxide derivatives

Effects of segmental motion and labeling levels

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ABSTRACT The Ca^{2+} -ATPase in native sarcoplasmic reticulum membranes was selectively spin-labeled for saturation transfer electron spin resonance (ESR) studies by prelabeling with *N*-ethylmaleimide and by using low label/protein ratios. Results with the nitroxide derivative of the standard sulphhydryl-modifying reagent, maleimide, were compared with a series of six novel nitroxide β -substituted vinyl aryl ketone derivatives which differed (with two exceptions) in the substituent at the ketone position. The two exceptions had a different electron withdrawing group at the α -carbon, to enhance further the electrophilic character of the β -carbon. Although differing in their reactivity, all the conjugated unsaturated ketone nitroxide derivatives displayed saturation transfer ESR spectra indicative of much slower motion than did the maleimide derivative. The saturation transfer ESR spectra of maleimide-labeled Ca^{2+} -ATPase therefore most likely contain substantial contributions from segmental motion of the labeled group. The effects of the level of spin labeling were also investigated. With increasing degree of spin label incorporation, the linewidths of the conventional ESR spectrum progressively increased and the intensity of the saturation transfer spectrum dropped dramatically, as a result of increasing spin-spin interactions. The hyperfine splittings of the conventional spectrum and the outer lineheight ratios of the saturation transfer spectrum remained relatively unchanged. Extrapolation back to zero labeling level yielded comparable values for the effective rotational correlation times deduced from the saturation transfer spectrum intensities and from the lineheight ratios, for the vinyl ketone label. For the maleimide label the extrapolated values from the integral are significantly lower than those from the lineheight ratios, probably because of the segmental motion. Comparison is made of the effective rotational correlation time for the vinyl ketone label with the predictions of hydrodynamic models for the protein diffusion, in a discussion of the aggregation state of the Ca^{2+} -ATPase in the native sarcoplasmic reticulum membrane. The implications for the study of protein rotational diffusion and segmental motion, and of the proximity relationships between labeled groups, using saturation transfer ESR spectroscopy are discussed.

INTRODUCTION

The calcium activated adenosinetriphosphatase (Ca^{2+} -ATPase) is the major integral membrane protein of the sarcoplasmic reticulum. The relationship between the structure and Ca^{2+} transport function is the focus of intensive research. Using spin-labeled maleimide, a covalently attached spin probe, the molecular dynamics of the Ca^{2+} -ATPase was followed both in the native membrane and in reconstituted vesicles (for reviews see Thomas, 1985; Hidalgo, 1987). The aggregation state of the Ca^{2+} -ATPase and its changes during the transport cycle can be followed by rotational diffusion measurements. Saturation transfer electron spin resonance (STESR)¹ spectroscopy extends the motional sensitivity

of conventional spin labeling ESR to slow motions such as the rotational diffusion of a 100-kD protein in the membrane. In spite of its good dynamic sensitivity, STESR can only be applied successfully to measurements of overall protein rotation provided that the covalently attached spin label: (a) has negligible segmental motion with respect to the protein backbone, and (b) reacts selectively with the protein in question. For selective labeling of the Ca^{2+} -ATPase in the native sarcoplasmic reticulum membrane, the fast reacting sulphhydryl groups of a minor glycoprotein must be masked with *N*-ethylmaleimide (NEM) before labeling the Ca^{2+} -ATPase (Ikemoto et al., 1976; Hidalgo and Thomas, 1977; Thomas

¹Abbreviations used in this paper are: ESR, electron spin resonance; NEM, *N*-ethylmaleimide; STESR, saturation transfer ESR; V_1 , first harmonic ESR absorption signal detected in-phase with respect to the field modulation; V_2 , second harmonic absorption signal detected 90° out-of-phase with respect to the field modulation; 5-BzVSL, 1-oxyl-2,2,5,5-tetramethyl-3-[2-(benzoyl)ethenyl]pyrroline; 5-DiBzVSL, 1-oxyl-2,2,5,5-tetramethyl-3-[2,2-(dibenzoyl)ethenyl]pyrroline;

5-HBzVSL, 1-oxyl-2,2,5,5-tetramethyl-3-[2-(4'-hydroxybenzoyl)ethenyl]pyrroline; 5-HNBzVSL, 1-oxyl-2,2,5,5-tetramethyl-3-[2-(3'-nitro-4'-hydroxybenzoyl)ethenyl]pyrroline; 5-InVSL, 2-[(1-oxyl-2,2,5,5-tetramethylpyrrolin-3-yl)methenyl]indane-1,3-dione; 5-MSL, 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxyl; 6-MSL, 4-maleimido-2,2,6,6-tetramethylpiperidinoxyl; 5-(2)PyVKSL, 1-oxyl-2,2,5,5-tetramethyl-3-[2-(2'-pyridylcarbonyl)ethenyl]pyrroline tosylate.

and

Hidalgo, 1978). Typically, a small fraction of maleimide labels ($\approx 3\text{--}7\%$) reacts with weakly immobilized superficial sulfhydryl groups, and thus two-component ESR spectra are obtained (Thomas, 1985; Bigelow et al., 1986). Because the lineheight ratio method of STESR is not additive, the integral method previously suggested for handling multicomponent STESR spectra (Evans, 1981; Horváth and Marsh, 1983; Squier and Thomas, 1986a; Horváth and Marsh, 1988) has been used for the analysis of such spectra (Squier and Thomas, 1986b).

In practice, the segmental motion of the spin label sometimes sets a lower dynamic limit and slower motional modes cannot be followed. A comparison of different covalent labels should help in choosing the most suitable labeling conditions. Esmann et al. (1989) studied the rotational diffusion of the Na,K-ATPase using spin-labeled maleimide and benzoyl vinyl derivatives and found that the latter label reported more faithfully the overall protein rotation. Here we compare a series of new covalent labels similar to the benzoyl vinyl derivative with spin-labeled maleimide and describe a labeling condition which gave a better estimate of the rate of protein rotation as a whole.

There is currently no evidence to suggest that the strongly immobilized spin probes are bound to a single specific sulfhydryl group of the Ca^{2+} -ATPase (cf. Bigelow and Inesi, 1989). The composite spectrum from two (or more) labeling sites in different regions of the protein cannot be distinguished easily from the spectrum of a single specific labeling site on the basis of the STESR spectral parameters. For simplicity, spectral changes associated with temperature or ligand induced conformational changes of the protein were interpreted in terms of the single site model (Squier and Thomas, 1986b; Lewis and Thomas, 1986; Napier et al., 1987). In the present paper, we address this particular problem and demonstrate by varying the label/protein ratio that, even at low labeling levels ($\text{MSL}_{\text{bound}}/\text{Ca}^{2+}\text{-ATPase} < 0.5 \text{ mol/mol}$), spin-spin interaction between labels occurs. The spectral consequences of spin-spin interactions are discussed and their effects on the saturation transfer spectra are evaluated to obtain consistent effective rotational correlation times from lineheight and ST-intensity parameters.

MATERIALS AND METHODS

Materials

N-(1-oxy-2,2,6,6-tetramethyl-4-piperidiny) maleimide (6-MSL) was from the Institut Jozef Stefan (Ljubljana, Yugoslavia). 1-oxy-2,2,5,5-tetramethyl-3-[2-(2-benzoyl)ethenyl]pyrroline (5-BzVSL) and 1-oxy-2,2,5,5-tetramethyl-3-[2-(2'-pyridylcarbonyl)ethenyl]pyrroline tosylate, 5-(2)PyVKSL, were synthesized as described by Hankovszky et al. (1982). The other covalent modifying nitroxide derivatives (see Fig. 1)

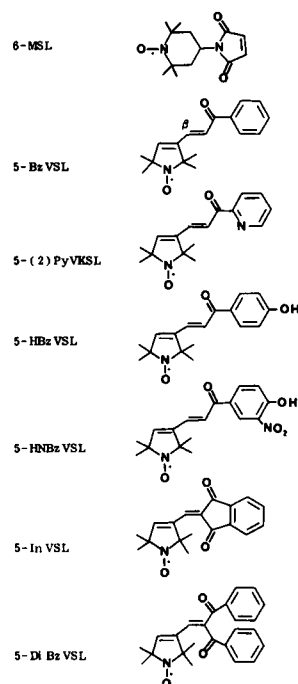


FIGURE 1 Structures of nitroxide spin label derivatives designed for the covalent modification of proteins.

were synthesized as described by Hankovszky et al. (1989). All other chemicals were of analytical grade purity.

Preparation of fragmented sarcoplasmic reticulum vesicles and enzymatic activity measurements

Sarcoplasmic reticulum (SR) vesicles were prepared from the white skeletal muscles of rabbits according to the method of Nakamura et al. (1976). The freshly isolated SR vesicles were suspended in 0.25 M sucrose, 10 mM Tris maleate, pH 7.4 at a concentration of 25 mg of protein/ml, divided into 0.5 ml aliquots and stored at -70°C until use. The Ca^{2+} , Mg^{2+} -activated ATPase activity was measured according to the method of Nakamura et al. (1976) by colorimetric determination of inorganic phosphate using the method of Fiske and Subbarow (1925). Protein concentrations were determined by the standard procedure (Lowry et al., 1951).

Spin labeling

Thawed SR suspensions were prelabeled with 1 mol NEM/mol Ca^{2+} -ATPase (100 kD protein) at 22°C for 10 min to mask the fast-reacting glycoprotein (Ikemoto et al., 1976; Hidalgo and Thomas, 1977). Then 0.5–3 mol 6-MSL/mol Ca^{2+} -ATPase was added and allowed to react at 22°C for 30 min. (Essentially similar conditions were used for labeling with the 2-[(1-oxy-2,2,5,5-tetramethylpyrrolin-3-yl)methenyl]indane-1,3-dione (5-InVSL) label, except that the reaction time was 40 min. For the other labels, which were much less reactive, it was necessary to increase the reaction time to 12 h, with continuous stirring. The labeling reaction was stopped by adding a 100-fold excess of ice-cold

buffer and the vesicles were washed to remove any unreacted label as recommended by Bigelow et al. (1986). The NEM-prelabeled enzyme had 80–90% of the activity of the native enzyme. Reaction with the spin label at a level of 1:1 mol/mol added gave rise to a 30% reduction in activity in the case of the MSL label, and very little reduction in activity for the 5-InVSL label, or with the other (less reactive) vinyl ketone spin labels (see Table 1).

The labeled SR vesicle suspension was pelleted at 100,000 *g* for 30 min and filled into ESR capillaries (I.D. 1 mm). The glass capillaries were centrifuged at full speed in a bench centrifuge and excess supernatant was removed to obtain membrane pellets of 5 mm height. All buffer solutions were first degassed and then kept under argon. The sample capillaries were flushed with argon before filling with the labeled SR suspensions. In all conventional and STESR experiments the above standard sample configuration was used. The 1-mm i.d. glass capillaries were centered in a standard 4-mm ESR quartz tube containing light silicone oil for thermal stability.

Conventional ESR measurements

ESR spectra were recorded at 9 GHz on a E-12 Century Line spectrometer (Varian Associates, Palo Alto, CA). The temperature was regulated by a nitrogen gas flow system. All spectra were recorded at 4°C. The spectrometer was interfaced to an IBM PC computer via a Labmaster interface card (Tecmar Inc., Cleveland, OH) and data were collected using software written by Dr. M. D. King (Max-Planck-Institut, Göttingen, FRG). Conventional, in-phase (V_1), absorption ESR spectra were recorded at nonsaturating microwave power ($H_1 = 0.032$ G) and a modulation amplitude of 1.6 G p-p; in parallel each conventional spectrum was recorded at the high microwave power used for the STESR experiments ($H_1 = 0.25$ Gauss). Quantitative measurements of spin label concentration were performed by calibrating the spectrometer with a secondary intensity standard (Sachse et al., 1987). For this purpose 1–3 μ g 6-MSL (in ethanol) was filled into thin walled glass micropipettes and placed into an unlabeled SR vesicle suspension (5-mm sample in 1-mm i.d. capillary), to obtain identical cavity Q-factors to those of the STESR samples. All conventional spectra were recorded under critical coupling conditions at the above two microwave power settings. Labeling levels are quoted in terms of effective mol/mol ratio which is equivalent to 10 nmol label/mg membrane protein.

TABLE 1 Enzymatic activity (μ mol P_i /mg protein/min) of sarcoplasmic reticulum Ca^{2+} -ATPase after covalently labeling with various nitroxide derivatives*

SL/protein	5-MSL	6-MSL	5-InVSL	5-BzVSL	5-(2)PyVKSL
Control	0.88	0.77	0.81	0.95	0.95
1:1	0.59	0.50	0.81	0.95	0.79
5:1	0.23	0.18	0.53	0.87	0.87
10:1	0.17	0.15	0.18	0.79	0.51
20:1	0.15	0.03	0.14	0.67	0.47

*Thawed SR suspensions were prelabeled with 1 mol NEM/mol Ca^{2+} -ATPase (100 kD protein) at 22°C for 10 min, and then incubated with 1–20 mol spin label/mol Ca^{2+} -ATPase for 30 min, except for 5-BzVSL and 5-(2)PyVKSL, which were incubated overnight. Activity was measured by coupled assay.

Saturation transfer ESR measurements

A standard sample configuration was used in all STESR experiments (Fajer and Marsh, 1982; Hemminga et al., 1984). The spectrometer was calibrated by measuring the continuous wave saturation properties of peroxyamine disulphonate (Fremy's salt) as described by Kooser et al. (1969) and Fajer and Marsh (1982). STESR spectra were recorded in the second harmonic, 90° out-of-phase (V_2'), absorption mode at a modulation frequency of 50 kHz and a modulation amplitude of 5 G p-p. STESR measurements were performed under critical coupling conditions at a microwave power giving an average H_1 field over the sample of 0.25 G (Thomas et al., 1976). For details of corrections for changes in cavity Q see Fajer and Marsh (1982) and Hemminga et al. (1984). Quadrature phase settings were adjusted by the "self-null" method (Thomas et al., 1976; de Jager and Hemminga, 1978).

Spectral analysis

The outer hyperfine splitting $2A_{max}$ and the linewidth parameters ΔH_1 and ΔH_2 of the extrema were evaluated by fitting Lorentzian absorption curves from the outer ends of the spectrum to the extremum positions (McConnell and McFarland, 1970; Freed, 1976). STESR spectra were analyzed by the lineheight ratio and the integral method. The low-field (L'') and high-field (H'') diagnostic resonance positions were specified as the points $1/3$ of the distance ($\theta = 45^\circ$, $\phi = 35^\circ$) from the outer turning points L and H toward the (x, y) average spectral position for the $m_1 = +1$ and -1 manifolds, respectively, after allowing for the averaging of the axial tensorial values. For definition of the lineheight ratio parameters see Thomas et al. (1976) and Robinson and Dalton (1980). The integrated ST-intensity I_{ST} was normalized with respect to the second integral of the high power conventional (V_1) ESR spectrum as previously described (Horváth and Marsh, 1983):

$$I_{ST} = \int V_2'(H) dH / \int \int V_1(H) d^2H. \quad (1)$$

Effective rotational correlation times, τ_R^{eff} , were evaluated from STESR calibrations of the lineheight ratios and the ST-intensity, deduced from the isotropic rotation of 6-MSL labeled haemoglobin samples in glycerol-water mixtures. Accurate τ_R^{eff} values were determined by polynomial interpolation of the $\tau_R^{eff}(x)$ (where x denotes L''/L , H''/H , and I_{ST}) inverse functions (Horváth and Marsh, 1988).

RESULTS

Labeling stoichiometry

The incorporation of spin-labeled maleimide (6-MSL) into the Ca^{2+} -ATPase of SR vesicles was analyzed by quantitative spin label concentration measurements. The MSL_{bound}/Ca^{2+} -ATPase molar ratio is given as a function of the total amount of 6-MSL added in Fig. 2. In the range studied (up to three-fold molar excess of 6-MSL), a gradually increasing label incorporation was observed with an average labeling level of 50%. Rather similar results were obtained with the spin-labeled indandiono derivative (5-InVSL, Fig. 1), except that the labeling level was slightly lower than that with 6-MSL, probably because of a lower reactivity. All the other spin labels

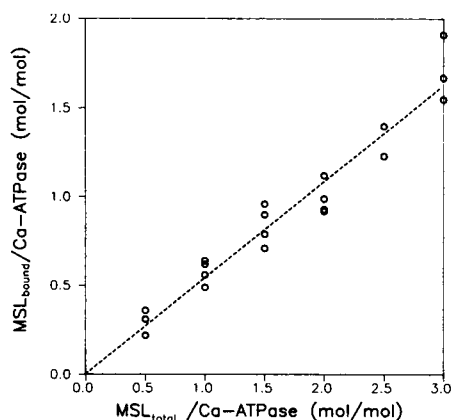


FIGURE 2 Stoichiometry of spin labeling of SR vesicles using sulfhydryl reagent spin-labeled maleimide (6-MSL). SR was pre-labeled with 1 mol NEM/mol of Ca^{2+} -ATPase (100 kD protein) at 22°C for 10 min, and then reacted with various amounts of 6-MSL at 22°C for 30 min. The binding of 6-MSL to the enzyme was assayed by quantitative spin label concentration measurements, in conjunction with protein determinations.

required much longer incubation (typically 12 h) and their level of incorporation was only 25% of that of the above two labels.

Conventional ESR spectra

A series of ESR spectra of the different spin labels covalently attached to the Ca^{2+} -ATPase is shown in Fig. 3. To obtain pure immobilized lineshapes the aqueous component observed with the 5-BzVSL and 1-oxyl-2,2,5,5-tetramethyl-3-[2,2-(dibenzoyl)ethenyl]pyrroline (5-DiBzVSL) labels was digitally subtracted; no such processing was necessary with the highly reactive 6-MSL and 5-InVSL. In the ESR spectrum of 6-MSL, two spectral components could be resolved in agreement with previous studies (Thomas, 1985; Bigelow et al., 1986): 93–96% of the total intensity was assigned to a strongly immobilized component and 4–7% to a weakly immobilized component. In the case of 5-InVSL, a pure strongly immobilized component was observed, whereas the other labels displayed small amounts of weakly immobilized component. The 1-oxyl-2,2,5,5-tetramethyl-3-[2-(4'-hydroxybenzoyl)ethenyl]pyrroline (5-HBzVSL) label gave a more complex lineshape, probably as a result of more than the above two components (spectrum not shown).

Because all these spectra possessed close to rigid limit lineshapes the outer hyperfine splitting, $2A_{\text{max}}$, and linewidths at half-height, ΔH_1 and ΔH_h , of the outer extrema were evaluated as described by Freed (1976). All the β -substituted vinyl ketone spin labels gave larger outer splittings than 6-MSL; $2A_{\text{max}}$ values at 4°C were: 6-MSL,

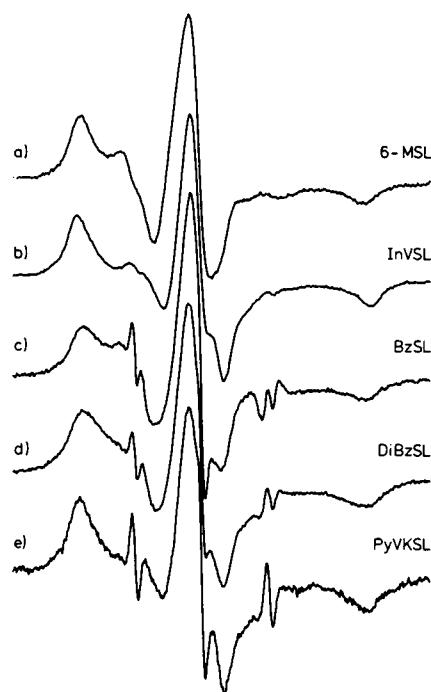


FIGURE 3 Conventional, in-phase (V_1) ESR spectra of SR vesicles covalently labeled with various spin labels at a labeling level of <0.2 mol/mol $\text{label}_{\text{bound}}/\text{Ca}^{2+}$ -ATPase (100 kD protein). NEM prelabeling was carried out as described in Materials and Methods. Spectra are recorded at 4°C. (a) Maleimide spin label, 6-MSL; (b) indandiono spin label, 5-InVSL; (c) benzoyl vinyl spin label, 5-BzVSL; (d) dibenzoyl vinyl spin label, 5-DiBzVSL; (e) pyridoyl vinyl spin label, 5-(2)PyVKSL. Total scan width = 100 G.

67.9 G; 5-BzVSL, 68.1 G; 5-DiBzVSL, 68.5 G; 5-(2)PyVKSL, 69.4 G; and 1-oxyl-2,2,5,5-tetramethyl-3-[2-(3'-nitro-4'-hydroxybenzoyl)ethenyl]pyrroline (5-HNBzVSL), 69.9 G. 5-InVSL gave an unusually large outer splitting (70.4 G) similar to that from the low temperature spectra of MSL hydrogen bonded in hemoglobin (Johnson, 1978).

In the case of 6-MSL and 5-InVSL, the above lineshape parameters were studied for a wide range of label incorporation levels. From a comparison of spectra recorded at different $\text{SL}_{\text{bound}}/\text{Ca}^{2+}$ -ATPase ratios a substantial linebroadening of the strongly immobilized component with increasing labeling level was evident. Both linewidth parameters displayed an approximately linear dependence on the $\text{SL}_{\text{bound}}/\text{Ca}^{2+}$ -ATPase ratio, as illustrated for 6-MSL in Fig. 4 b. No parallel change in the outer splitting with level of labeling was observed (cf. Fig. 4 a). Parameter values extrapolated to $\text{MSL}_{\text{bound}}/\text{Ca}^{2+}$ -ATPase = 0 were measured from the linear regressions in Fig. 4, and the following values obtained: $2A_{\text{max}} = 67.65$ G, $\Delta H_1 = 2.95$ G, and $\Delta H_h = 3.75$ G. According to calibrations for slow isotropic motion (Freed,

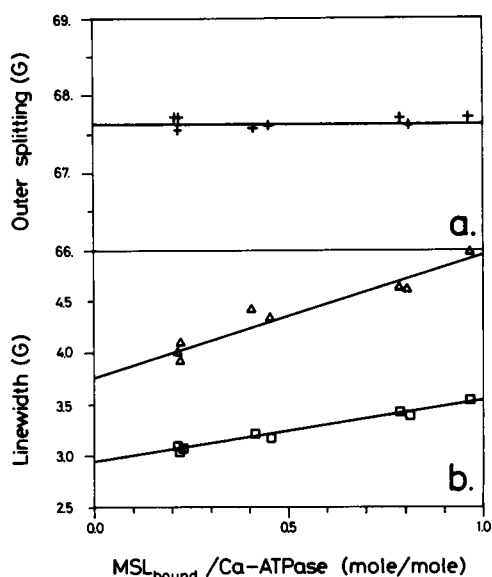


FIGURE 4 Spectral parameters of conventional, in-phase (V_1), ESR spectra of SR vesicles labeled with various amounts of 6-MSL. (a) Outer hyperfine splitting, $2A_{\max}$ (+); (b) low-field linewidth, ΔH_l (\square), and high-field linewidth, ΔH_h (Δ) are given in Gauss; for definition of these parameters see Freed (1976). Full lines represent linear regression curves.

1976), and using the single crystal values of 6-MSL labeled haemoglobin (Johnson, 1978), all the effective correlation times were $\sim 10^{-7}$ s and thus near to the slow motion limit. Similar results were obtained for the linewidths of the 5-InVSL label, and the outer splitting remained constant with label concentration, at a value of 70.4 G. Therefore, none of these labels can undergo rapid large-scale segmental motion when covalently bound to the Ca^{2+} -ATPase.

Saturation transfer ESR spectra

A series of second harmonic, 90° out-of-phase, absorption (V_2) STESR spectra of the different spin labels covalently bound to Ca^{2+} -ATPase is shown in Fig. 5. These spectra were obtained at a labeling stoichiometry of <0.2 mol/mol. There are substantial differences among these spectra and all the β -substituted vinyl ketone spin labels indicate significantly slower motion than 6-MSL. This is seen from the STESR parameters (L''/L , H''/H , C'/C , and I_{ST}) and effective rotational correlation times, $\tau_R^{\text{eff}}(m_1)$, listed in Table 2. With the exception of 5-InVSL, the lower reactivity of the new labels dictated overnight incubation at 22°C to obtain reasonable signal-to-noise ratios, as compared with a labeling time of only 30–40 min for the 6-MSL and 5-InVSL labels. It is possible that the prolonged incubation may therefore give rise to some

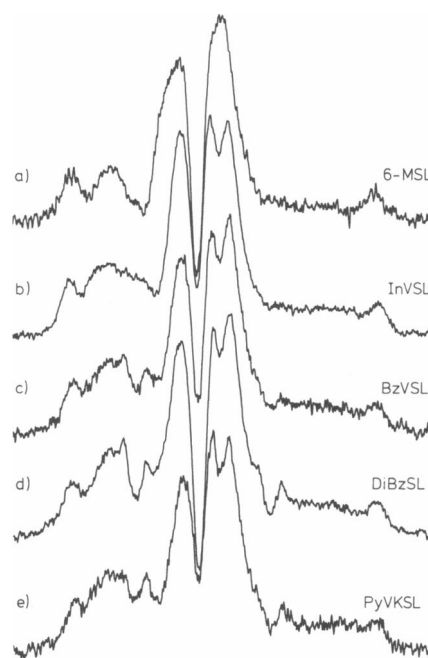


FIGURE 5 Second harmonic, 90° out-of-phase absorption (V_2) STESR spectra of SR vesicles covalently labeled with various spin labels at a labeling level of <0.2 mol/mol $\text{label}_{\text{bound}}/\text{Ca}^{2+}$ -ATPase (100 kD protein). NEM prelabeling was carried out as described in Materials and Methods. Spectra are recorded at 4°C . (a) Maleimide spin label, 6-MSL; (b) indandiono vinyl spin label, 5-InVSL; (c) benzoyl vinyl spin label, 5-BzVSL; (d) dibenzoyl vinyl spin label, 5-DiBzVSL; (e) pyridoyl vinyl ketone spin label, 5-(2) PyVKSL. Total scan width = 100 G.

time-dependent changes for the less reactive labels. The effective rotational correlation times derived from L''/L and H''/H are in reasonable agreement; those deduced from C'/C are consistently lower. The reason for the latter discrepancy lies mostly in the spectral overlap with the mobile spectral component and/or unreacted spin label, which makes the central region less reliable for spectral analysis.

The effects of increasing levels of spin labeling on the STESR spectra were studied for the 6-MSL and 5-InVSL labels. The limited reactivity precluded such studies for the other labels. The STESR lineshapes for different $\text{MSL}_{\text{bound}}/\text{Ca}^{2+}$ -ATPase ratios are given in the lower part of Fig. 6. The first integrals are given above the spectra and are seen to decrease with increasing $\text{MSL}_{\text{bound}}/\text{Ca}^{2+}$ -ATPase ratio, whilst no change is apparent in the low-field and high-field lineheight ratios. Essentially similar results were obtained with the 5-InVSL label (spectra not shown). Effective rotational correlation times were determined from the low-field and high-field STESR lineheight ratios and from the normalized STESR spectral intensities, using calibration curves from isotropically rotating spin-labeled haemoglobin. The normalized ST-

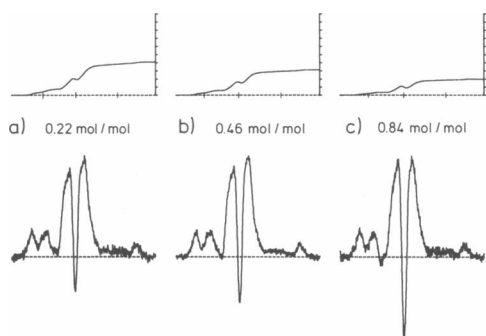


FIGURE 6 (Lower row) Second harmonic, 90° out-of-phase, absorption (V'') STESR spectra of SR vesicles labeled with various amounts of 6-MSL. $MSL_{bound}/Ca^{2+}\text{-ATPase}$ (100 kD protein): (a) 0.22 $MSL/Ca^{2+}\text{-ATPase}$ (mol/mol); (b) 0.46 $MSL/Ca^{2+}\text{-ATPase}$ (mol/mol); (c) 0.84 $MSL/Ca^{2+}\text{-ATPase}$ (mol/mol). (Upper row) First integral of the STESR spectra in the lower row. The maximum ordinate corresponds to a normalized integrated intensity (I_{ST}) of 5.10^{-3} . Total scan width = 100 G, $T = 4^\circ\text{C}$.

intensity (I_{ST}) of 6-MSL labeled $Ca^{2+}\text{-ATPase}$ decreased from $2.11 \cdot 10^{-3}$ to $1.36 \cdot 10^{-3}$, on increasing the label to protein ratio from 0.2 to 1.0 mol/mol, corresponding to effective correlation times of $1.8 \cdot 10^{-6}$ s and $2.3 \cdot 10^{-7}$ s, respectively. In contrast, the lineheight ratios remained essentially independent of the labeling level, having values of $L''/L = 0.71$ and $H''/H = 0.47$, corresponding to an effective correlation time of $2.1 \cdot 10^{-5}$ s. Using the 5-InVSL label the ST-intensity varied between $6.5 \cdot 10^{-3}$ and $3.8 \cdot 10^{-3}$ s on increasing the labeling level from 0.2 to 0.8 mol/mol, corresponding to effective correlation times of $4.5 \cdot 10^{-5}$ s and $1.5 \cdot 10^{-5}$ s, respectively, whereas the lineheight ratios remained constant with values of $L''/L = 1.0$ and $H''/H = 0.9$, corresponding to an effective rotational correlation time of $8 \cdot 10^{-5}$ s.

The data for the effective correlation times calculated from the different STESR parameters for the 6-MSL and 5-InVSL labels are summarized in Fig. 7, as a function of $SL_{bound}/Ca^{2+}\text{-ATPase}$ ratio. Effective rotational correlation times extrapolated to zero labeling level were obtained by linear regression from the log-linear plots. For 6-MSL and 5-InVSL effective correlation times of $3.4 \cdot 10^{-6}$ s and $5.1 \cdot 10^{-5}$ s, respectively, were obtained from ST-intensity (I_{ST}) measurements (Fig. 7 b). From lineheight ratio measurements, extrapolated values of $2.3 \cdot 10^{-5}$ s and $8.3 \cdot 10^{-5}$ s were calculated for the 6-MSL and 5-InVSL labels, respectively (Fig. 7 a). It should be noted that the decreasing ST-intensity I_{ST} is not accounted for by the decreasing central ratio C'/C which sensitively depends on the fraction of weakly immobilized component, but rather by a true decrease in the out-of-phase/in-phase ratio of the second harmonic spectra.

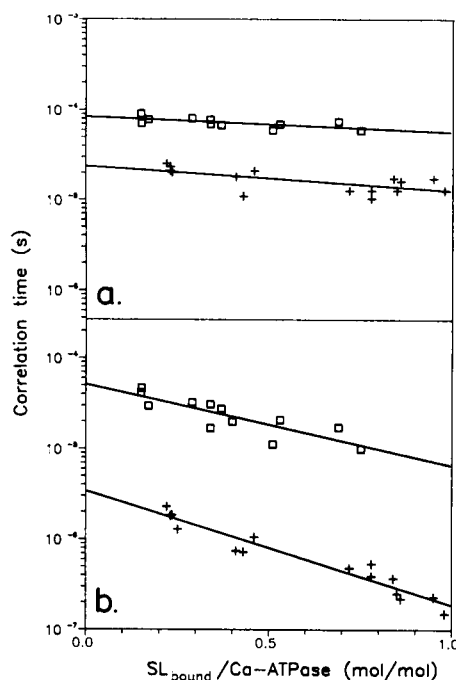


FIGURE 7 Effective rotational correlation times of 6-MSL and 5-InVSL covalently bound to SR vesicles, deduced from the STESR spectra as a function of the $SL_{bound}/Ca^{2+}\text{-ATPase}$ ratio. Effective rotational correlation times were calculated using (a) the lineheight ratio L''/L for 5-InVSL (\square) and 6-MSL ($+$) and (b) the ST intensity for 5-InVSL (\square) and 6-MSL ($+$). Full lines represent linear regression curves.

DISCUSSION

The aims of the present investigation were severalfold. Firstly, to assess the suitability of a new series of nitroxide derivatives for the covalent labeling of proteins, with regard both to reactivity and tightness of labeling. Secondly, to make intercomparisons between the different labels, particularly with reference to the conventional maleimide derivative, to estimate the degree of segmental motion of the labeled group. Thirdly, to study the effects of the level of spin-labeling on the saturation transfer ESR spectra. Finally, the potentiality of these various experimental strategies in the use of STESR to study both overall protein diffusion and segmental motion, and also the proximity relationships between the various groups were to be evaluated.

Vinyl ketone spin label series

The β -substituted vinyl ketone labels all display a considerably reduced mobility relative to the conventional maleimide label (Fig. 5 and Table 2), suggesting that the

TABLE 2 Saturation transfer ESR lineheight ratios and effective rotation correlation times for sarcoplasmic reticulum Ca²⁺-ATPase covalently spin-labeled with various nitroxide derivatives

Label	L''/L	$\tau_R^{eff}(+1)$	C'/C	$\tau_R^{eff}(0)$	H''/H	$\tau_R^{eff}(-1)$	$I_{ST} \times 10^2$	$\tau_R^{eff}(I)$
		μs		μs		μs		μs
6-MSL	0.62	24	-0.34	1.1	0.45	22	0.21	3.5
5-InVSL	1.15	88	0.22	7.5	0.77	65	0.65	51
5-BzVSL	1.33	151	0.20	7.0	0.86	84	0.69	63
5-DiBzVSL	0.99	60	-0.17	2.1	0.82	73	0.40	53
5-(2)PyVKSL	1.39	187	0.43	16.0	0.93	102	0.70	69

T = 4°C.

former are more suitable for studying the overall rotational diffusion of the protein. The agreement between the extrapolated values for the effective rotational correlation times deduced from the ST integral and from the lineheight ratios for the 5-InVSL label (Fig. 7) support this suggestion (cf. Esmann et al., 1987, 1989). The less reactive labels register somewhat lower rotational mobility than does the more reactive 5-InVSL derivative (see Table 2). However, this may reflect time-dependent decreases in the protein mobility arising from the long incubation times required for labeling with the less reactive compounds. Previous studies with MSL labeled Ca²⁺-ATPase have detected time- and temperature-dependent irreversible changes in the STESR spectra (Bigelow et al., 1986; King and Quinn, 1983).² Of the different β -substituted vinyl ketones, the indandiono derivative (5-InVSL) appears to be the label of choice for STESR studies because of its favorable reactivity and tightness of binding.

Segmental mobility of MSL

Comparison of the STESR parameters of the maleimide derivative with those of the new series of vinyl ketone labels suggests that the MSL label has considerable segmental mobility on the microsecond timescale, when attached to the Ca²⁺-ATPase. This conclusion is also supported by the fact that the extrapolated effective correlation time deduced for MSL from the ST integral is considerably shorter than that deduced from the lineheight ratio, in contrast to the results with the 5-InVSL label (Fig. 7). In principle, these differences might alter-

²In this connection, it is interesting to note that previous workers (e.g., Squier and Thomas, 1986b) have obtained correlation times with 6-MSL, after incubation at 30°C for a period of 30 min, that are close to those reported here for 5-InVSL without this incubation procedure. Our own measurements with 6-MSL have indicated that the value of I_{ST} increased from 0.16 to 0.32 after incubation. Similar time- and temperature-dependent irreversible effects have also been observed with MSL-labeled Na⁺, K⁺-ATPase (Esmann et al., 1987, 1989).

natively be explained by a difference between the MSL and aroyl-VSL labels in the orientation of the nitroxide axis relative to the rotation axis (cf. later). However, the differences are present over a relatively large range of labeling levels of a heterogeneous population of sulphhydryl groups, and also are found for all the various VSL labels tested. In addition, similar differences have also been found between the 5-MSL and 5-BzVSL labels attached to a different membrane protein, the Na⁺, K⁺-ATPase (Esmann et al., 1989). Thus it seems most likely that the differences can be attributed to segmental motion of the MSL label. Previous studies with MSL-labeled Ca²⁺-ATPase have reported only small ($\approx 70\%$) increases in the effective STESR rotational correlation time on inducing two-dimensional protein crystals (Lewis and Thomas, 1986; Napier et al., 1987). This again would be consistent with a dominant contribution to the STESR spectrum of MSL arising from segmental motion. Our own studies on various partially crystalline SR preparations have indicated a much greater decrease in the rotational mobility relative to the native membranes, as deduced from the STESR spectra of the Ca²⁺-ATPase labeled with 5-InVSL (Horváth, L. I., L. Dux, and D. Marsh, manuscript in preparation). Evidence for additional segmental motion with MSL has also been provided by cross-linking studies with the Ca²⁺-ATPase (Squier et al., 1988).

Spin-spin interactions: conventional ESR

The spectral changes observed in both conventional and saturation transfer ESR with increasing levels of protein labeling can be attributed to spin-spin interactions arising from the proximity of the labeled groups. The lack of any change in the outer hyperfine splitting of the conventional spectrum, or in the lineheight ratios of the STESR spectrum, demonstrates that the changes do not arise from motional effects. Although there are -SH groups which are situated very close together in the primary sequence of the Ca²⁺-ATPase (MacLennan et al., 1985),

these are not the groups which display the highest reactivity to maleimide. Therefore, it seems likely that the intramolecular spin-spin interactions arise primarily from the longer range dipolar interactions rather than from the contact exchange interaction. This suggestion is supported by the gradual uniform increase in linebroadening with labeling level, but nevertheless still remains to be verified in detail.

For rigid lattice dipolar broadening, the dependence of the linewidth and lineshape on spin concentration varies with the concentration range. Statistical theories for high dilutions yield a Lorentzian broadening with a linear dependence on spin concentration (Abragam, 1961):

$$\Delta H_{dd} = (4\pi^2/3\sqrt{3})g\beta \cdot n, \quad (2)$$

where ΔH_{dd} is the full width at half-height, g is the spin label g -factor, β is the Bohr magneton, and n ($\approx 1/r^3$) is the effective density of spin labeling. Such a concentration dependence is qualitatively in agreement with the results of Fig. 4 *b*, and yields a number density of approximately one label per $1-2 \cdot 10^5 \text{ \AA}^3$, at a labeling level of 1 MSL_{bound}/Ca²⁺-ATPase. For a statistical distribution, this implies that neighboring spin labels should lie within a mean radius of $\sim 30 \text{ \AA}$ of one another. This is clearly an upper estimate for the distance of closest approach, particularly because the model corresponds to an extended three-dimensional distribution, rather than labels localized solely to the Ca²⁺-ATPase proteins. Labeling experiments with NEM have shown that the primary reaction targets are residues Cys 344 and Cys 364 of the Ca²⁺-ATPase (Saito-Nakatsuka et al., 1987), and recent energy transfer measurements with fluorescently labeled maleimides have revealed sulphhydryl sites that are located 36 \AA apart in the protein structure (Bigelow and Inesi, 1989; D. Bigelow, private communication). The gradual broadening of the ESR spectra suggests that other residues in addition to these two may be labeled by 6-MSL (cf. Marsh and Smith, 1972).

It is also of interest to estimate the linebroadening which could arise from intermolecular dipolar interactions, resulting from modulation by translational diffusion of the labeled proteins. The linewidth is then given by (Abragam, 1961; Sachse et al., 1987):

$$\Delta H_{dd}(\text{inter}) = (38\pi g^3 \beta^3 / 450\hbar) (n/r_c D_T), \quad (3)$$

where \hbar is Planck's constant/ 2π , $2r_c$ is the distance of closest approach of the two spin dipoles, and D_T is the translational diffusion coefficient of the protein. Assuming a lipid/protein ratio of 70:1 mol/mol in the sarcoplasmic reticulum and a labeling level of 1 MSL/Ca²⁺-ATPase, the mean number density of spin dipoles is $n \approx 4 \cdot 10^{18} \text{ cm}^{-3}$. Taking $r_c = 33.5 \text{ \AA}$ as the maximum outer radius of the Ca²⁺-ATPase molecule (Dux et al., 1985),

the corresponding value for the diffusion coefficient to give a linebroadening of $\Delta H = 1 \text{ G}$ would have to be: $D_T \approx 2 \cdot 10^{-8} \text{ cm}^2 \text{ s}^{-1}$. A translational diffusion coefficient of $9.9 \cdot 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ has been measured at 13°C for the Ca²⁺-ATPase reconstituted into sarcoplasmic reticulum lipids at very high lipid/protein ratio (Vaz et al., 1982). This comparison suggests that intermolecular spin-spin interactions could also influence the linebroadening, but only if the diffusion is sufficiently rapid to fulfill the dynamic rather than the static limit for dipolar interaction.

Spin-spin interactions: STESR

The effects of the spin-spin interaction on the saturation transfer spectral intensities almost certainly arise from an increase in the spin lattice relaxation rate. Theoretical simulations have shown that, when all other parameters are maintained constant, the intensity of the out-of-phase spectrum is directly proportional to the product $\omega_m T_1$, where T_1 is the spin-lattice relaxation time and ω_m is the modulation frequency (Thomas and McConnell, 1974; Thomas et al., 1976).

Induction of relaxation by increasing spin concentration requires a modulation of the spin-spin interactions by molecular motion. The dominant motion is the rotational diffusion of the protein which, for a STESR experiment, lies in the same time regime as the intrinsic T_1 of the nitroxide (see e.g., Thomas, 1985). This may preclude a straightforward calculation of the relaxation rate using motional narrowing theory. For correlation times, τ_R , and angular resonance frequency, ω_o , such that $\omega_o \tau_R \gg 1$, fast motional theory would predict a dipolar relaxation rate of (Carrington and McLachlan, 1966):

$$(1/T_1)_{dd} = 3(g^4 \beta^4 / \hbar^2 r^6) / (5\omega_o^2 \tau_R), \quad (4)$$

where r is the separation of the two spins within the protein. An order of magnitude calculation shows that the spins must come very close together ($r \leq 1.7 \text{ \AA}$) for the dipolar relaxation rate to approach that of a nitroxide in the STESR regime ($T_1 \approx \tau_R$). This result may be a consequence of the nonapplicability of fast motion theory in this correlation time regime. However, because solid state dipolar relaxation mechanisms are unlikely to be more effective, dipole rotation is probably not the dominant contribution to the observed reduction in STESR intensity.

Estimates of the effects of modulation of the intermolecular dipolar interaction by translational diffusion in the dynamic limit (Abragam, 1961) suggest that this is unlikely to contribute appreciably to T_1 relaxation. The spin-lattice relaxation time could, however, be limited by possible spin exchange on collision of labeled protein molecules. The contribution to the spin-lattice relaxation

rate would then be related to an effective bimolecular collision rate constant, k_{coll} , by a statistical factor (cf. Sachse et al., 1987):

$$(1/T_1)_{\text{ex}} = (1/3)k_{\text{coll}} \cdot \sigma \cdot c, \quad (5)$$

where the effective concentration is best expressed as an area fraction: $c \approx 0.2$, corresponding to a lipid/protein ratio of 70:1 mol/mol. The orientational factor, $\sigma \leq 1$, is included to allow for the fact that the colliding species may not be oriented such that the label groups are sufficiently close for spin exchange to take place. To obtain a T_1 relaxation time in the region of 1 μs , $k_{\text{coll}} \cdot \sigma \approx 1.5 \cdot 10^7 \text{ s}^{-1}$ would be required. For comparison, with the same definition: $k_{\text{coll}} \approx 4 \cdot 10^8 \text{ s}^{-1}$ for lipid diffusion in fluid bilayers. Thus, depending on the orientational factor, collision-induced exchange could be a factor in reducing T_1 . Exchange at this rate would have little influence, however, on the linebroadening of the conventional ESR spectrum (cf. above).

Rotational diffusion of the Ca^{2+} -ATPase

Use of the 5-InVSL label and extrapolation to zero labeling level allows estimation of parameters relating to the overall rotational diffusion of the Ca^{2+} -ATPase in the SR membrane. These may then be compared with hydrodynamic estimates of the oligomeric size of the protein. As mentioned above, previous STESR measurements with MSL-labeled protein partly reflect the segmental motion of the protein and are therefore unsuitable for such a comparison.

Simulations of first harmonic, out-of-phase dispersion STESR spectra for anisotropic rotational diffusion have been performed by Robinson and Dalton (1980). The effective rotational correlation times, $\tau_{\text{R}}^{\text{eff}}(\pm 1)$, deduced from the low-field and high-field lineheight ratios using calibrations for isotropic motion, were related to the principal elements, $D_{\text{R} //}$ and $D_{\text{R} \perp}$, of the diffusion tensor. For highly anisotropic diffusion ($D_{\text{R} //}/D_{\text{R} \perp} \gg 1$) in the time regime appropriate to membrane proteins, the following dependence was found:

$$\tau_{\text{R}}^{\text{eff}}(\pm 1) = 1/\{3[D_{\text{R} //} \sin^2\Theta + D_{\text{R} \perp}(1 + \cos^2\Theta)]\}, \quad (6)$$

where Θ is the orientation of the nitroxide z -axis relative to the rotational diffusion axis. Comparison of the high-field and total integral intensities suggests that the nitroxide z -axis has an intermediate orientation between $\Theta = 0^\circ$ and $\Theta = 90^\circ$ (cf. Horváth and Marsh, 1983; Fajer and Marsh, 1983). Thus the equivalent correlation time for axial rotation ($\tau_{\text{R} //} \equiv 1/6D_{\text{R} //}$) is given by: $\tau_{\text{R} //} \approx 1/2\tau_{\text{R}}^{\text{eff}}(\pm 1) \sin^2\Theta$, assuming $D_{\text{R} \perp} \approx 0$. The data of Fig. 7 therefore yield maximum values for $\tau_{\text{R} //}$ of $\approx 25\text{--}40 \mu\text{s}$ at

4°C , for the 5-InVSL label, corresponding to a spin label orientation of $\Theta = 90^\circ$.

Oligomeric state of the Ca^{2+} -ATPase

Saffmann and Delbrück (1975) have derived an expression for the rotational diffusion coefficient of a cylindrical protein in a membrane, using hydrodynamic theory, and the extension to proteins of elliptical cross-section has been given by Jähnig (1986):

$$D_{\text{R} //} = [kT/(4\pi\eta abh)]F_{\text{R} //}, \quad (7)$$

where a and b are the semi-axes of the cylinder, h is the height of the membrane-spanning region of the cylinder, η is the effective viscosity within the membrane, k is Boltzmann's constant, and T is the absolute temperature. $F_{\text{R} //}$ is a shape factor that is only weakly dependent on a/b for small ellipticities (Marsh and Horváth, 1989). From electron microscopy of two-dimensional Ca^{2+} -ATPase crystals, the intramembranous diameter of a single protein molecule is found to be: $2a \approx 2b \approx 40 \text{ \AA}$ (Taylor et al., 1986). For $h = 45 \text{ \AA}$ and values of the effective membrane viscosity within the realistic range (see Cherry and Godfrey, 1981) $\eta = 2.5\text{--}5 \text{ P}$, the calculated rotational correlation time is $\tau_{\text{R} //} = 2.5\text{--}5 \mu\text{s}$ for a monomer, with $F_{\text{R} //} = 1$. For a dimer, the cross-sectional area is twice that of the monomer and the predicted rotational correlation time is: $\tau_{\text{R} //} = 6\text{--}12 \mu\text{s}$, with $F_{\text{R} //} = 0.8$ (corresponding to $a/b = 2$).

In view of the uncertainty in the orientation of the spin label axes, it is difficult to make firm conclusions regarding the oligomer state. The maximum values of the measured correlation times correspond to a higher oligomerization state than the dimer. This would be consistent with the suggestion of probable heterogeneity in oligomer size (Fagan and Dewey, 1986; Squier et al., 1988). For the measured correlation times to be in agreement with the values predicted for the dimer would require that the nitroxide axes were oriented within an angular range of $\Theta = 25\text{--}45^\circ$ to the rotation axis, and within $\Theta = 15\text{--}25^\circ$ for the monomer. Evidence in favor of a dimeric form of the protein in the membrane has been advanced by other, independent methods (Napolitano et al., 1983; Napier et al., 1987; Squier et al., 1988), whereas fully active monomeric forms of the Ca^{2+} -ATPase have been obtained in detergent solution (reviewed in Hidalgo, 1987). However, the solubilized enzyme is known to possess somewhat different properties from those in the intact membranous state (see Squier et al., 1988, for a discussion). It is possible that no one single oligomer state corresponds to the active form of the enzyme in the native sarcoplasmic reticulum membrane (Squier et al., 1988).

CONCLUSIONS

(a) The MSL spin label displays considerable segmental mobility relative to the whole protein when covalently bound to the Ca^{2+} -ATPase.

(b) The 5-InVSL spin label is very well suited to STESR studies of the overall rotational diffusion of the Ca^{2+} -ATPase, and probably other proteins, because of its favorable reactivity and lack of independent segmental motion.

(c) Reliable estimates of the rotational correlation time, particularly from the ST integral, requires back extrapolation to zero labeling level, to correct for the effects of spin-spin interactions.

(d) STESR is a very sensitive method for detecting spin-spin interactions between immobilized spin labels and offers the potential for investigating proximity relationships and/or slow translational diffusion.

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