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A Passage Saturation Transfer Paramagnetic Resonance Study of the Rotational Diffusion of the Sarcoplasmic Reticulum Calcium-ATPase

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Abstract

A study was made of the dependence on temperature of the saturation transfer ESR spectra obtained from sarcoplasmic reticulum vesicles labeled with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide. When the spin-labeled preparation was heated, some change occurred that was accompanied by an increase in the spectral parameters L''/L and H''/H (Thomas *et al.* (1976). *J. Chem. Phys.* **65,** 3006–3024). This heat-induced increase in L''/L and H''/H competed with the reduction in these parameters that would normally accompany a reduction in τ_2, with the result that a biphasic response to increasing temperature was observed. The heat-induced perturbation was partially irreversible. Consequently, although the preparation also exhibited a biphasic response to cooling, the heating and cooling curves did not coincide. It is suggested that a heat-induced re-orientation of the nitroxide probe with respect to the membrane normal, together with the anisotropic nature of the rotational motion, could be the cause of the departure from the expected monotonic relationship between the spectral parameters and temperature.

Key Words: Saturation transfer ESR; sarcoplasmic reticulum; calcium-ATPase; Arrhenius behavior.

Introduction

The sarcoplasmic reticulum membrane has a relatively simple protein and lipid composition and can be isolated as a microsomal fraction which retains its capacity for Ca^{2+} translocation. This has prompted many researchers to study the biophysical and biochemical properties of the fragmented membrane preparation, particular attention having been paid to the mutual

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interaction between the Ca^{2+}-ATPase and its surrounding lipids. One of the most interesting biochemical features of the isolated membrane is the nonlinear Arrhenius behavior of the Ca^{2+}-ATPase first reported by Inesi *et al.* (1973). These workers attributed this effect to an order–disorder phase transition of the membrane lipid which, in turn, causes a perturbation of the ATPase molecule. In support of their hypothesis they obtained ESR spectroscopic evidence of a structural change in the lipids occurring at about 20°C. Since then a variety of alternative explanations have been suggested to account for the nonlinear Arrhenius behavior, including the formation of lipid clusters (Lee *et al.*, 1974), a reduction in the anisotropy of the motion in parts of the lipid molecule (Davis *et al.*, 1976), and some interaction between the ATPase molecule and a tightly bound lipid annulus (Hesketh *et al.*, 1976). The possibility that the break in the Arrhenius plot is a reflection of some structural change within the protein itself and not a property of the surrounding lipid was suggested by experiments showing that delipidated, detergent-activated Ca^{2+}-ATPase preparations also exhibit nonlinear Arrhenius behavior (Dean and Tanford, 1978). Furthermore, Moore *et al.* (1978) failed to observe nonlinearity in the Arrhenius plot of the diphenylhexatriene-derived "microviscosity" of the sarcoplasmic reticulum membrane.

The widely reported observation that the sarcoplasmic reticulum Ca^{2+}-ATPase only exhibits maximum enzyme activity in a fluid environment led a number of researchers to make measurements of the motional freedom of the ATPase molecule. Rotational correlation times have been calculated using passage saturation transfer ESR spectroscopic data (Hidalgo *et al.*, 1978; Thomas and Hidalgo, 1978; Kirino *et al.*, 1978, 1979) and from measurements made using flash photolysis (Hoffmann *et al.*, 1979, 1980). The observation that the correlation between ATPase activity and protein rotational mobility may be better than the correlation between enzyme activity and lipid "fluidity" (Hidalgo *et al.*, 1978; Thomas and Hidalgo, 1978) may indicate that the need for a fluid environment is merely a reflection of the protein molecule requiring motional freedom to function properly.

The temperature dependence of the rotational motion of the sarcoplasmic reticulum Ca^{2+}-ATPase has been studied using both saturation transfer ESR spectroscopy (Kirino *et al.*, 1978, 1979; Thomas and Hidalgo, 1978) and flash photolysis (Hoffmann *et al.*, 1979). Both techniques yield data indicating that although rotational mobility increases with increasing temperature in the range 0 to 15–20°C, some anomaly in the rotational motion occurs as the temperature is further increased. This anomalous behavior may be related to the discontinuity in the Arrhenius plot of ATPase activity. Hoffmann *et al.* (1979) attributed the departure from classical behavior to an association of monomeric protein species while Kirino *et al.* (1978, 1979) favor an explana-

tion based on a protein conformational change and a subsequent reorientation of the probe molecule with respect to the rotation axis.

It has recently been shown that the thermal stability of the sarcoplasmic reticulum ATPase, as reflected by its capacity for Ca^{2+} translocation, depends on the presence of dithiothreitol and/or phenylmethylsulfonyl halide in the isolation buffers (Johannsson *et al.*, 1981; Madden *et al.*, 1981). Furthermore, the response to changing temperature is different in vesicles prepared in dithiothreitol/phenylmethylsulfonyl halide-containing buffers (our unpublished observations). We therefore made a saturation transfer ESR spectroscopic study of the rotational behavior of the Ca^{2+}-ATPase isolated in the presence of these two reagents.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared in the presence of 1 mM dithiothreitol and 5 μM phenylmethylsulfonyl chloride from the white muscle of rabbit hind leg according to the method of Warren *et al.* (1974) and Robinson *et al.* (1972) but using *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer instead of histidine. The final suspension of sarcoplasmic reticulum vesicles was dialyzed at 4°C for 16 h against 300 mM sucrose, 1 M KCl, 50 mM K_2HPO_4:KH_2PO_4, pH 8.0 buffer (1 liter/10 ml of suspension containing 40–50 mg protein/ml; one change of dialysis buffer) and then rapidly frozen in liquid nitrogen at a protein concentration of 40–50 mg/ml. The suspension was stored at about −30°C.

The ATPase was pretreated with *N*-ethylmaleimide and subsequently labeled with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide at 0–5°C as described by Thomas and Hidalgo (1978) except that the pretreatment was carried out using the higher ratio of 1.5 mole *N*-ethylmaleimide per 10^5 g protein (cf. Kirino *et al.*, 1978). The spin-labeled vesicles were sedimented by centrifugation and, in order to improve the signal-to-noise ratio, the spectroscopic measurements were made on the pellet.

Second harmonic, 90° out-of-phase (V_2') ESR spectra were recorded using a Varian E-104 spectrometer as described by Hidalgo *et al.* (1978). No direct measurement was made of the microwave power incident on the sample, but a nominal power of 60 mW was used (cf. Kirino *et al.*, 1978). The phase-sensitive detector was set 90° out of phase using the self-null method (Thomas *et al.*, 1976; Hidalgo *et al.*, 1978). The temperature in the cavity was regulated using a Varian variable temperature controller, and the sample was held in a quartz flat cell. The temperature of the sample was continuously monitored using a copper:constantan thermocouple which was fixed to the

outer surface of the cell. It should be emphasized that temperature profiles were plotted using data taken from thermocouple recordings and not the controller settings. The heating effect of the 60 mW microwave field was therefore not a problem. Spectral analysis was performed using the parameters L''/L and H''/H as suggested by Thomas *et al.* (1976).

Results

The effect of temperature on the saturation transfer ESR spectrum of spin-labeled sarcoplasmic reticulum vesicles, previously isolated in the presence of dithiothreitol and phenylmethylsulfonyl chloride, was examined to establish whether this preparation exhibited the anomalous rotational behavior observed in previous studies of the sarcoplasmic reticulum membrane, as obtained in the absence of these reagents. A striking feature of the preparation obtained in the presence of dithiothreitol and phenylmethylsulfonyl chloride was that some irreversible change took place in the spin-labeled material when it was heated. The spectra we obtained that first drew our attention to this phenomenon are shown in Figs. 1a and 1b. The first of these is a V_2' spectrum, recorded at 15°C, of an ATPase preparation that had been maintained at 0–5°C during its isolation and subsequent labeling. The peak-height ratio L''/L is approximately 0.29, which, when compared with the calibration curve given in Fig. 6 of Thomas *et al.* (1976), indicates an apparent rotational correlation time of about 5 μsec. The term "apparent rotational correlation time" is used because it has been demonstrated that isotropic calibration curves do not yield absolute rotational correlation times if the motion is anisotropic (Delmelle *et al.*, 1980). The spectrum shown in Fig. 1b was obtained from the same sample after it had been heated to 35°C and then re-equilibrated at 12°C. The peak-height ratio $L''/L = 0.48$ corresponds to an apparent rotational correlation time greater than 10 μsec. The difference between the correlation times obtained before and after heating is much greater than expected for the 3°C difference in the temperatures at which the two spectra were recorded. The effect of heating the preparation was particularly obvious at temperatures above 30°C. If, for example, samples were rapidly heated to 35°C and then held at that temperature while spectra were repeatedly recorded, a continual increase in the L''/L peak-height ratio (i.e., increase in the apparent rotational correlation time) was observed.

Figure 1c shows the V_1 ESR spectrum recorded at 35°C. This typical "two-component" spectrum indicates that a significant percentage of the nitroxide probe is bound to a site that allows the molecule to move independently of the protein. The mobile component was not prominent at temperatures lower than 10°C (Fig. 1d). The appearance of a mobile V_1 signal at

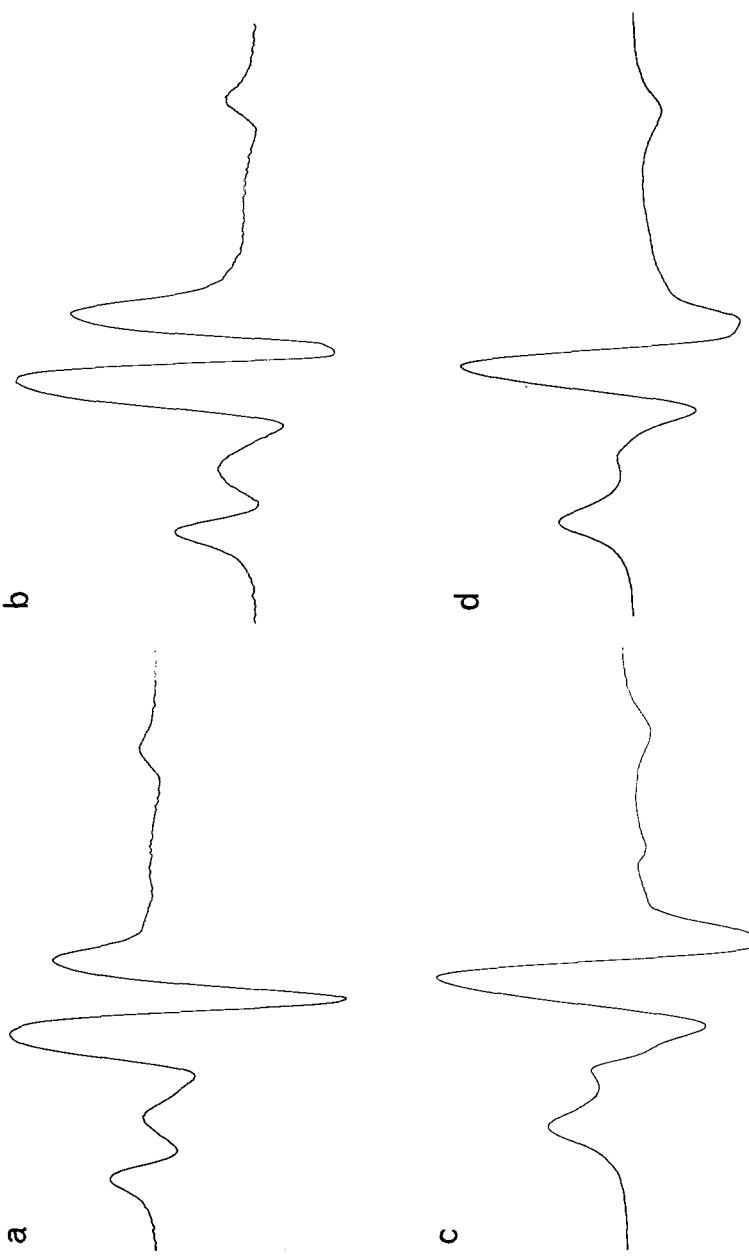

Fig. 1. Passage saturation transfer (V_2') and conventional (V_1) ESR spectra of sarcoplasmic reticulum vesicles labeled with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-malcimide. The sarcoplasmic reticulum vesicles were isolated at between 0 and 5°C, rapidly frozen in liquid nitrogen, and stored at about −30°C. The vesicles were subsequently spin-labeled at 0–5°C and maintained at this temperature prior to recording the spectra. All four spectra were obtained from the same sample. (a) V_2' ESR spectrum recorded at 15°C prior to warming to 35°C; (b) V_2 ESR spectrum recorded at 12°C after heating the sample to 35°C; (c) V_1 ESR spectrum recorded at 35°C; (d) V_1 ESR spectrum subsequently recorded at 9°C.

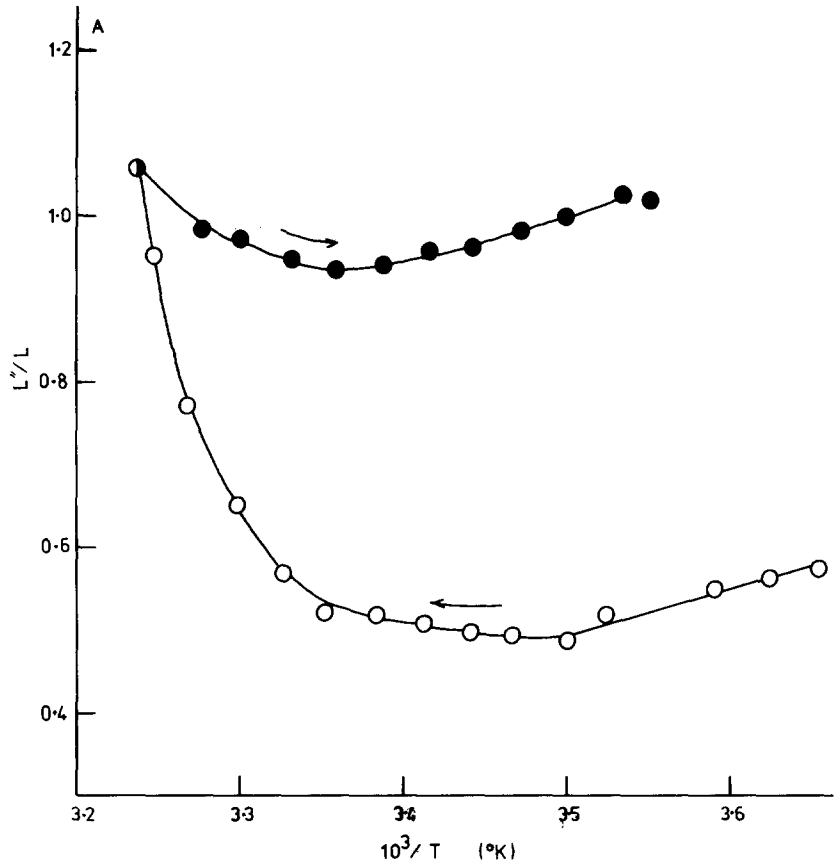

Fig. 2. Temperature dependence of the spectral parameters L''/L and H''/H. A sample of spin-labeled sarcoplasmic reticulum was placed in the cavity at 0°C and heated to 36°C in 2–3°C steps, allowing sufficient time for the sample to equilibrate before recording the V_2' spectrum at each temperature. V_2' spectra were similarly recorded as the same sample was cooled. (a) L''/L versus reciprocal temperature; (b) H''/H versus reciprocal temperature.

higher temperatures is important since it can interfere with the V_2' signal, particularly if the phase-sensitive detector is not precisely 90° out of phase or if there is inhomogeneity of phase in the sample (see Thomas *et al.*, 1976).

To exclude the possibility that a fraction of the maleimide probe was bound to residual dithiothreitol or some other nonprotein sulfhydryl, the V_1 spectrum of the labeled ATPase was recorded, at room temperature, before and after solubilization of the vesicles in Triton X-100 detergent. No freely tumbling nitroxide was detected after the Triton X-100 treatment (spectra not shown). The "immobile" spectrum was, however, converted to a three-line

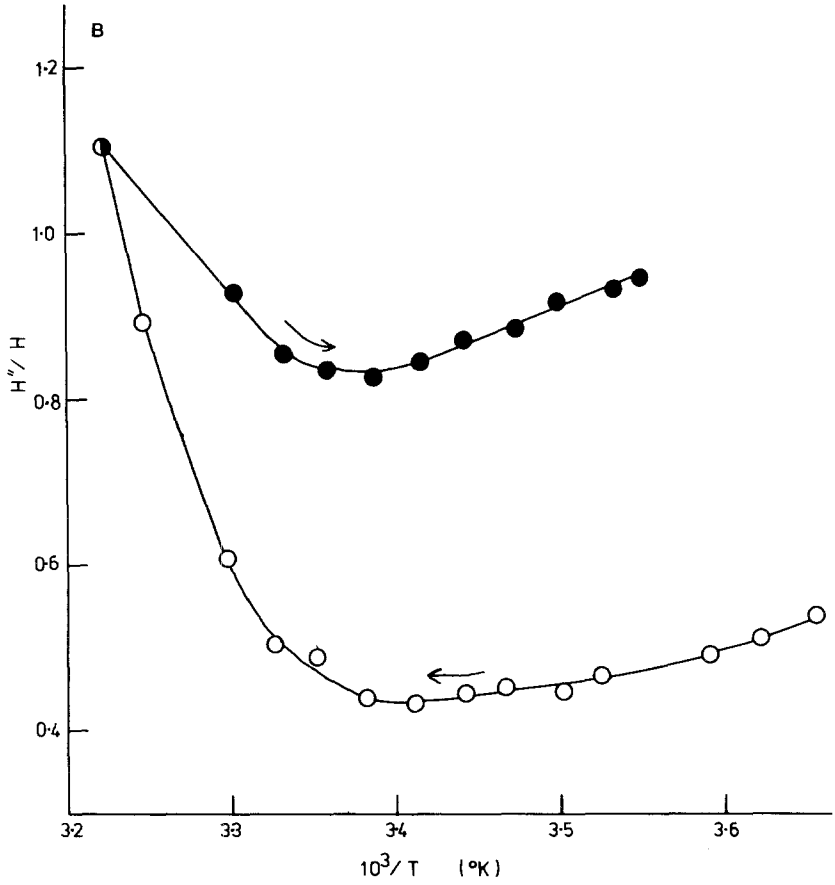

Fig. 2. Continued.

spectrum typical of a rapidly tumbling nitroxide after trypsin digestion of the solubilized protein.

Experiments were performed in which V_2' spectra of the labeled sarcoplasmic reticulum ATPase were recorded at temperature intervals in the range 0–36°C. A time-dependent change in the peak heights was again observed above room temperature. Peak-height ratios were therefore calculated using measurements made after the sample had been left for a time sufficient for the peak heights to become steady. The spectral parameters L''/L and H''/H are shown in Fig. 2 plotted against the inverse of temperature. Heating the preparation from 0°C initially caused a relatively small decrease in L''/L and H''/H (equivalent to a decrease in the apparent

rotational correlation time), the two parameters reaching a minimum in the temperature range 15–19°C. A further increase in temperature was accompanied by an increase in L''/L and H''/H. The cooling curves did not coincide with the heating curves but did exhibit minima in the region 22–25°C.

Discussion

The origin of the anomalous rotational behavior we observed in our spin-labeled sarcoplasmic reticulum ATPase preparation is not known. Some indication of its cause can, however, be obtained by an examination of the expected relationship between temperature and the two spectral parameters, L''/L and H''/H.

The Debye equation for Brownian rotational diffusion is

$$\tau_2 = 4\pi nR^3/3kT \tag{1}$$

where n is viscosity and R the radius of the rotating species. If viscosity changes with temperature according to the equation

$$n = n_0 \exp(E_A/RT) \tag{2}$$

where E_A is the "energy of activation for flow" (Barrer, 1943), Eq. (1) can be rewritten as

$$\tau_2 = (4\pi R^3/3kT)n_0 \exp(E_A/RT) \tag{3}$$

The temperature range over which the present experiments were performed was such that, given typical values for E_A/R (see, for example, Fig. 4 of Moore *et al.*, 1978), the temperature dependence in (3) is expected to be dominated by the exponential function. It therefore follows that

$$\ln \tau_2 \simeq \ln K + (E_A/R)/T \tag{4}$$

where, taking a mean value of $T = 20$°C in the denominator of (3), we have $K = 4\pi R^3 n_0/879k$. It is important to note that E_A is a property of the membrane and independent of the size of the rotating species (protein). In the case of isotropic rotational diffusion in the range $10^{-5} < \tau_2 < 10^{-3}$ sec the spectroscopic parameters L''/L and H''/H are, approximately, linear functions of $\ln(\tau_2)$ with positive gradients (see Fig. 6 of Thomas *et al.*, 1976). It therefore follows that

$$\left.\begin{aligned} L''/L &\simeq a + b/T \\ H''/H &\simeq c + d/T \end{aligned}\right\} 10^{-5} < \tau_2 < 10^{-3} \text{ sec} \tag{5}$$

where a, b, c, and d are constants, the gradients b and d being positive.

The two most striking features of the plots shown in Fig. 2 are that the heating and cooling curves are not coincident and that the L''/L and H''/H ratios do not exhibit the monotonic increase with increasing reciprocal temperature indicated by Eq. (5). The noncoincidence of the heating and cooling curves is caused by the heat-induced, irreversible change mentioned above. We have examined the dependence on temperature of the ATPase activity exhibited by the unlabeled microsomal sarcoplasmic reticulum preparation, as obtained in the presence of dithiothreitol, and observed no biphasic or irreversible Arrhenius behavior in the temperature range 3–37°C. One explanation for the irreversible, heat-induced change in the spin-labeled preparation is based on the assumption that before the sample was heated the nitroxide possessed some motional freedom that was independent of the protein to which it was covalently bound. A discrepancy between the heating and cooling curves would then occur if, as the temperature of the protein was raised, some irreversible conformational change occurred that forced the nitroxide into an environment such that its independent motion was lost when the sample was subsequently cooled. Alternatively, a conformationally driven re-orientation of the nitroxide molecule with respect to the membrane normal, if it were partially irreversible, could give rise to the same discrepancy. This possibility is discussed below.

The failure to observe a decrease in L''/L and H''/H with increasing temperature throughout the range 0–36°C contrasts with the results obtained by Thomas and Hidalgo (1978), who found that the ratio L''/L continued to decrease as the temperature was increased from 20 to 35°C, albeit at a reduced rate. Kirino *et al.* (1978), on the other hand, observed no marked change in τ_2 in the temperature range 15–35°C. Our failure to see a monotonic increase in the ratios L''/L and H''/H with increasing reciprocal temperature is not entirely attributable to the irreversible change responsible for the noncoincidence of the heating and cooling curves since biphasic curves were also obtained on cooling.

Hoffmann *et al.* (1979), who have studied the temperature dependence of the rotational diffusion rates of the ATPase molecule using flash photolysis, also observed a departure from classical Arrhenius behavior in the region 15–35°C. These researchers attributed this to a heat-induced volume change of the rotating species brought about by an association of monomeric protein species. The increase in τ_2 caused by this aggregation would oppose the decrease in τ_2 brought about by the increase in temperature. They suggest that aggregation is triggered by a change in protein conformation.

Kirino *et al.* (1978) have demonstrated that those nitroxides that give rise to the "mobile component" of the two-component V_1 spectrum interfere with the V_2' signal. It therefore follows that an alternative explanation for our failure to obtain data consistent with Eq. (5) is that it was caused by the

increasing presence of partially mobile probe molecules as the temperature was increased (Fig. 1). The out-of-phase spectrum should, in theory, be relatively unaffected by freely tumbling or partially mobile nitroxides (Hyde, 1978). In practice, however, a failure to set the phase-sensitive detector precisely 90° out of phase or inhomogeneity of phase over the sample probably gives rise to a significant level of in-phase interference. Kirino *et al.* (1978) made a careful study of this interference and found that although the effect was significant in relation to the parameter L''/L, the peak-height ratio H''/H was not significantly affected. In our experiments, both H''/H and L''/L exhibited a biphasic response to changing temperature. This may indicate that weakly immobilized nitroxides were not the sole cause of the anomalous behavior.

Another explanation for the departure from Eq. (5) is that it is caused by the anisotropic nature of the rotational diffusion, the rotational motion probably being restricted to rotation about the membrane normal. If, as suggested by Hoffman *et al.* (1979), a change in protein conformation was to occur as the temperature is increased, then this could bring about a re-orientation of the nitroxide probe with respect to the axis of rotation. This could, in turn, have a marked effect on the temperature dependence of the spectral parameters L''/L and H''/H. If this heat-induced probe re-orientation was *partially* irreversible, then it could also be the cause of the noncoincidence of the heating and cooling curves.

Robinson and Dalton (1980) have used computer-simulation techniques to make a detailed study of the effect of anisotropy in the rotational diffusion tensor on the first-harmonic, 90° out-of-phase dispersion spectrum. They found that the apparent rotational correlation times obtained from L''/L and H''/H and calibration curves constructed using isotropic model systems deviate from τ_{iso}. In their treatment the y axes of the rotational diffusion and nitroxide frames are coincident and a rotation through γ about this axis transforms one frame into the other. The quantity τ_{iso} is then defined by $\tau_{iso} = 1/(6d_+)$, where $d_+ = (d_x \cos^2 \gamma + d_z \sin^2 \gamma + d_y)/2$ and d_i, $i = x, y, z$, are the three diffusion coefficients along the principal rotation axes. Robinson and Dalton (1980) did not examine the effect of rotational anisotropy on the V_2' signal, presumably because such a study is too demanding of CPU time. It is, however, easy to show, in a semiquantitative manner, that the V_2' signal must be sensitive to anisotropy in the rotational motion and to changes in the orientation of the nitroxide probe with respect to the rotational diffusion frame.

In order to explain how a conformationally driven re-orientation of the nitroxide molecule can affect the V_2' spectrum, it is necessary to briefly consider the reason why the V_2' signal is sensitive to changes in τ_2. This sensitivity arises partly because the V_2' signal is affected by the rate at which

resonance is swept. A rotationally driven re-orientation of the probe molecule can cause a passage through resonance because the g and hyperfine coupling tensors are anisotropic. The magnetic field is modulated at a high frequency (50 kHz in the present experiments) and hence the overall passage rate is determined by the interaction between the sinusoidal 50-kHz sweeping of the magnetic field and the irregular but repetitive sweeping of the resonance condition caused by Brownian rotational diffusion. The rotationally driven movement of a subset of spins from one portion of the spectrum to another is referred to as spectral diffusion. In addition to its effect on the passage rate, this rotationally induced shift in the resonance condition competes with the saturating effect of the high-intensity microwave field. This competition between rotationally induced relaxation and the partially saturating field also contributes to the V_2' signal's sensitivity to changes in rotational correlation time. The shape of the V_2' spectrum (and hence L''/L or H''/H) is a useful index of τ_2 because no spectral diffusion occurs at several points in the spectrum while a maximum spectral diffusion occurs within the intermediate regions. This may be shown mathematically. It should be noted, however, that the simple treatment outlined below assumes axial symmetry of the magnetic interactions. Although the effect of the deviation from axial symmetry is negligible at the wings of the spectrum, the effect is important in the central region. The following comments are therefore expected to apply to the spectral parameters L''/L and H''/H but not to C'/C.

The transition energies (ΔE) for a given orientation of the nitroxide molecular coordinate system with respect to the dc magnetic field are

$$\Delta E(M) = g_{\text{eff}}\beta_e H_0 + A_{\text{eff}}M, \qquad M = 0, \pm 1 \tag{6}$$

(see, for example, Nordio, 1976), where β_e and H_0 are the electron Bohr magneton and the dc magnetic field, respectively. If the g and hyperfine tensors are axially symmetric, then

$$g_{\text{eff}} = (g_\parallel^2 \cos^2\theta + g_\perp^2 \sin^2\theta)^{1/2} \tag{7}$$

and

$$A_{\text{eff}} = (A_\parallel^2 \cos^2\theta + A_\perp^2 \sin^2\theta)^{1/2} \tag{8}$$

where θ is the angle between the symmetry axis and the dc magnetic field direction, $g_\parallel$ and $g_\perp$ are the g components for H_0 parallel and perpendicular to the symmetry axis, respectively; $A_\parallel$ and $A_\perp$ are the hyperfine couplings parallel and perpendicular to the symmetry axis. Equations (6)–(8) enables us to write an expression for the orientation dependence of the resonance condition. If we take as an example the high field ($M = -1$) nuclear spin configuration and, for simplicity, neglect the relatively unimportant aniso-

tropy in g, then

$$\Delta E = g_e\beta_e H_0 - (A_\parallel^2 \cos^2\theta + A_\perp^2 \sin^2\theta)^{1/2} \tag{9}$$

Since $H_0 = H_{res}$ when $\Delta E = h\nu$ it follows that

$$H_{res} = (h\nu/g_e\beta_e) + (H_\parallel^2 \cos^2\theta + H_\perp^2 \sin^2\theta)^{1/2} \tag{10}$$

where $H_\parallel = A_\parallel/g_e\beta_e$ and $H_\perp = A_\perp/g_e\beta_e$ [cf. Eq. (1) of Hyde and Dalton (1979)]. Differentiation of (10) yields

$$dH_{res}/d\theta = \sin\theta\cos\theta\,(H_\perp^2 - H_\parallel^2)/(H_\perp^2 \sin^2\theta + H_\parallel^2 \cos^2\theta)^{1/2} \tag{11}$$

from which it follows that the rate of passage through resonance, caused by a re-orientation of the probe, varies across the spectrum. In particular, the spectral diffusion rate is zero at $\theta = 0$ and $\pi/2$ (see Fig. 3 of Thomas *et al.*, 1976). For a given orientation θ, the spectral diffusion rate is

$$dH_{res}/dt = (dH_{res}/d\theta)(d\theta/dt) \tag{12}$$

In order to obtain an expression for $d\theta/dt$ we consider an ideal situation in which one spin probe molecule is rigidly bound to the same site on each protein molecule. The symmetry axis will then be fixed at some angle (α) with respect to the rotation axis. All orientations of the rotation axis with respect to the dc magnetic field may be adopted, but, for a given protein molecule, this angle does not change on the saturation transfer ESR time scale. Let us consider a single protein molecule whose rotation axis (R) is at some angle β with respect to the dc magnetic field direction. We define **i**, **j**, and **k** to be mutually perpendicular unit vectors with **k** aligned with R and H_0 in the **i**–**k** plane (Fig. 3). The projection of the nitroxide symmetry axis on the **i**–**j** plane forms an angle ψ with **i**. If **z** is a unit vector lying along the symmetry axis and **h** is a unit vector aligned with H_0, it follows that

$$\mathbf{z} = \cos\alpha\,\mathbf{k} + \sin\alpha\cos\psi\,\mathbf{i} + \sin\alpha\sin\psi\,\mathbf{j} \tag{13}$$

$$\mathbf{h} = \cos\beta\,\mathbf{k} + \sin\beta\,\mathbf{i} \tag{14}$$

and

$$\cos\theta = \mathbf{z}\cdot\mathbf{h} \tag{15a}$$

$$= \cos\alpha\cos\beta + \sin\alpha\cos\psi\sin\beta \tag{15b}$$

Differentiating (15) with respect to time, we obtain

$$(d\theta/dt)\sin\theta = \sin\alpha\sin\beta\sin\psi\,(d\psi/dt) \tag{16}$$

Thus, at a given instant in time, the rate at which this single probe molecule

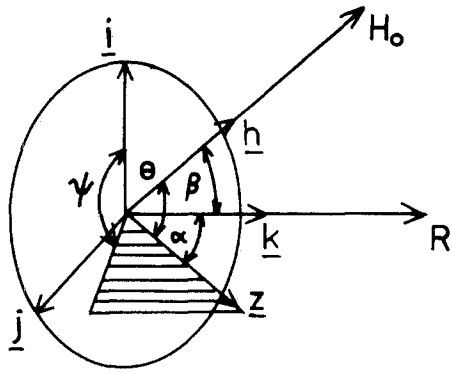

Fig. 3. A vector diagram of the spin-labeled Ca^{2+}-ATPase. **i**, **j**, and **k** are mutually perpendicular unit vectors with **k** lying along the rotation axis (R) and the dc magnetic field H_0 in the **i**–**k** plane. **z** is a unit vector aligned with the nitroxide symmetry axis and, at any instant in time, its projection on the **i**–**j** plane forms an angle ψ with **i**. α is the fixed angle between the symmetry axis and the rotation axis, and β the fixed angle between the dc magnetic field direction and the rotation axis.

"diffuses across the spectrum" is given by

$$\frac{dH}{dt}_{\text{res}} = \sin\beta \sin\alpha \sin\psi \frac{d\psi}{dt} \frac{\cos\theta\,(H_\perp^2 - H_\parallel^2)}{(H_\perp^2 \sin^2\theta + H_\parallel^2 \cos^2\theta)^{1/2}} \tag{17}$$

It is clear that the rate of the rotationally driven passage through resonance is dependent upon the magnitude of both α and β. In particular, a reduction in the angle α will be accompanied by a reduction in the spectral diffusion rate and will thus lead to a change in the shape of the V_2' spectrum.

Having established that the passage rate is sensitive to changes in the angle α, it is easy to see how a conformationally driven change in this angle could give rise to the biphasic Arrhenius behavior seen in the present experiments. Should an increase in temperature be accompanied by a change in protein conformation and this, in turn, cause α to become smaller, then the resulting reduction in the rate of the rotationally driven passage through resonance would oppose the increase in passage rate that would normally accompany the reduction in τ_2. If the former effect is dominant, then negative gradients will be obtained when L''/L and H''/H are plotted against the inverse of temperature. The magnitude of the effect on the passage rate of a change in the angle α is, by differentiation of Eq. (17), proportional to $\cos\alpha$. The minima in the Arrhenius curves shown in Fig. 2 may therefore correspond to a transition from a region in which the change in τ_2 is dominant in

determining the rate at which the rotationally driven spectral diffusion rate changes with temperature, to a region in which α becomes sufficiently small that the heat-induced change in probe orientation is dominant. Only in the former region would the V_2' signal be expected to respond to changes in temperature in a manner that is (qualitatively) the same as that observed with isotropic rotational diffusion.

If the above interpretation of our data is correct it is clear that the passage saturation transfer ESR technique cannot, at present, be used to obtain absolute rotational correlation times for the sarcoplasmic reticulum Ca^{2+}-ATPase, because the rotational diffusion is anisotropic. A relative rotational correlation time is the best that may be obtained. Alternatively, changes in τ_2 may be completely swamped by probe re-orientation effects. For this reason the present authors feel that the increase in L''/L and H''/H observed in this study as samples were warmed from 15–20°C to 36°C does not provide unequivocal evidence for an increasing rotational correlation time brought about, for example, by the formation of protein aggregates. Probe orientation effects would first have to be excluded.

Changes in the orientation of the probe molecule may also complicate the measurement of rotational correlation times using the flash photolysis method since the capacity of axial rotational diffusion to bring about depolarization must depend on the angle (γ) formed between the rotation axis and the transition dipole [see Eqs. (3) and (4) of Cherry, 1979]. Although Hoffmann *et al.* (1979) have interpreted the break in their Arrhenius plot as evidence for the formation of protein aggregates, a re-orientation of the triplet probe cannot be excluded as the cause of the effect. In this context it is interesting to note that in their flash photolysis study of eosin-labeled ATPase reconstituted in dipalmitoylphosphatidylcholine, Hoffmann *et al.* (1980) observed a significant temperature dependence in the quotient r_∞/r_0, r_0 and r_∞ being the zero-time and time-independent absorption anisotropies, respectively. Since this quotient is a function of the angle γ ($\gamma = \cos^{-1}[(1 \pm 2(r_\infty/r_0)^{1/2})/3]^{1/2}$; Heyn *et al.*, 1977) this observation is consistent with the hypothesis that the orientation of the probe is temperature dependent. Hoffmann *et al.* (1980) have, however, put forward an alternative hypothesis, namely, that the temperature dependence in r_∞/r_0 is caused by an increase in the fraction of protein molecules present as aggregates that are so large that their rotational motion is too slow to bring about a detectable depolarization. Neither hypothesis can be ruled out on the basis of the available experimental evidence.

In this paper we have sought an explanation for the anomalous Arrhenius behavior we observed in our saturation transfer ESR study of the rotational motion of the sarcoplasmic reticulum Ca^{2+}-ATPase. To this end we have briefly looked at probe re-orientation effects. In our derivation of Eq. (17) a number of simplifying assumptions were made. In particular we have assumed

that the ATPase molecule undergoes Brownian rotational diffusion about a single axis, that the nitroxide probe is bound to a homogeneous population of binding sites, and that, at a given temperature, the orientation of the probe molecule is rigidly fixed with respect to the rotation axis. Our main conclusion is that because anisotropic systems give rise to V_2' spectra that are sensitive to changes in probe orientation, the rotational correlation times derived from these spectra are inherently unreliable. The apparently anomalous rotational behavior of the spin-labeled ATPase does not therefore provide unequivocal evidence for a volume change in the rotating species. The assumed homogeneity of the binding site is undoutedly an oversimplification since the sarcoplasmic reticulum membrane contains several distinct populations of sulfhydryl groups (Hasselbach and Seraydarian, 1966). Nevertheless, Hidalgo and Thomas (1977) have shown that, providing the membrane is pretreated with *N*-ethylmaleimide, the spin label effectively binds only to the Ca^{2+}-ATPase, and it has been estimated that less than 2% is bound to other membrane proteins (Thomas and Hidalgo, 1978). It does not necessarily follow, however, that the population of bound spin label is homogeneous, since the ATPase has the capacity to bind more than one nitroxide per protein molecule (Hidalgo and Thomas, 1977). This does not compromise our main conclusion. On the contrary, lack of homogeneity in the ATPase binding sites further complicates the analysis and renders the task of obtaining absolute rotational correlation times from V_2' spectra more difficult.

It has been suggested to us that the anomalous Arrhenius behavior could be caused by nonuniform labeling together with a selective thermal reduction of a subset of probe molecules. We have not yet examined the effect of heat on the total signal intensity, and a selective loss of nitroxide molecules bound to a subset of binding sites could well have given rise to the noncoincidence of the heating and cooling curves. This cannot, however, be the sole cause of the biphasic response to changing temperature since this was observed during both the heating and cooling phases of the experiment.

The treatment that led to Eq. (17) cannot be dismissed by arguing that the nitroxide may possess considerable motional freedom independent of the protein and cannot be considered fixed with respect to the membrane normal since, if this were the case, the use of saturation transfer ESR spectroscopy to measure the rotational correlation time would itself not be justified. The assumed uniaxial nature of the rotational motion may be an oversimplification, although there is some evidence that the sarcoplasmic reticulum Ca^{2+}-ATPase does not require freedom of movement about an axis in the plane of the membrane to exhibit its Ca^{2+} pumping or hydrolytic activity (Martonosi and Fortier, 1974; Dutton *et al.*, 1976). Even if the rotational diffusion is not entirely restricted to a rotation about the membrane normal, the motion must be highly anisotropic, and it therefore follows that the V_2' signal will be sensitive to changes in the orientation of the nitroxide.

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