Rotational motion of the sarcoplasmic reticulum Ca²⁺-ATPase

(saturation transfer electron paramagnetic resonance/spin labels/membranes)

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ABSTRACT   Using saturation transfer electron paramagnetic resonance, we have detected the rotational motion of a spin label rigidly attached to the sarcoplasmic reticulum Ca²⁺-ATPase (ATP phosphohydrolase, EC 3.6.1.3). At 4°C, the spectrum indicates an effective rotational correlation time of 60 μsec, determined by comparison with reference spectra obtained from theoretical calculations and from experiments on model systems. This motion appears to correspond to rotation of the enzyme with respect to the membrane, because the motion persists when the membrane fragments are immobilized by sedimentation and the motion stops when the polypeptide chains, but not the membrane vesicles, are crosslinked by glutaraldehyde. The rotational mobility of the enzyme increases with increasing temperature, and this increase becomes more gradual when the temperature exceeds 20°C; the same kind of temperature dependence has been observed previously for lipid fluidity and enzymatic activity.

The dynamic nature of many processes occurring in biological membranes suggests strongly that molecular motions are extremely important aspects of membrane function. Therefore, direct measurements of motions of the lipid and protein components of membranes are essential to the understanding of the molecular details of membrane function. Translational and rotational motions of membrane lipids have been investigated extensively, as exemplified by the studies of spin-labeled lipids by McConnell and coworkers (1, 2). Some measurements have been made on the translational motion of membrane proteins (3, 4), but few measurements on their rotational motion have been reported (5, 6). Until recently, the magnetic resonance and optical spectroscopic techniques used to study the rotational motions of membrane lipids, usually in the nanosecond time range, were not sensitive to the much slower rotational motions that might be expected for membrane proteins. For example, the conventional electron paramagnetic resonance (EPR) technique, using nitroxide spin labels, can provide information only about motions characterized by rotational correlation times much less than 1 μsec, whereas the rotational motion of a relatively small protein, rhodopsin, in a relatively fluid visual photoreceptor membrane is characterized by a rotational correlation time of the order of microseconds (5, 7). Saturation transfer EPR spectroscopy extends the time range of sensitivity to correlation times as long as 1 msec; this technique is therefore well-suited for studying the slow rotational motion of membrane proteins. The utility of the saturation transfer technique in the study of rotational motion in the microsecond to millisecond time range has been demonstrated in theoretical and model system studies (8, 9) and in studies on large-scale rotational dynamics in assemblies of muscle proteins (10). These and other applications of saturation transfer spectroscopy have been described in several recent reviews (11–14).

Transport enzymes represent a particularly important kind of membrane protein in which rotational mobility may play

a key role. We have focused our attention on the Ca²⁺-ATPase (ATP phosphohydrolase, EC 3.6.1.3) of sarcoplasmic reticulum (SR). Because this enzyme apparently requires a fluid lipid environment in order to function (15), it is likely that some degree of protein mobility, such as rotational mobility, is required for enzyme activity. In order to make possible the study of protein mobility as a function of enzymatic activity and lipid fluidity (16, 17) we have carried out a study of the rotational motion of the spin-labeled Ca²⁺-ATPase. We have attached a maleimide spin label selectively and rigidly to the enzyme in SR vesicles and used saturation transfer EPR to measure an effective rotational correlation time of 60 μsec at 4°C, corresponding to rotation of the enzyme within the membrane. A preliminary account of some aspects of this work has appeared (17).

EXPERIMENTAL PROCEDURES

Membrane Preparations. Fragmented SR was prepared from rabbit white skeletal muscle as described (15). A purified Ca²⁺-ATPase preparation obtained by solubilization of SR with Triton X-100 (18), containing the endogenous SR lipids and a single 100,000-dalton polypeptide, will be designated SR-ATPase. A purified Ca²⁺-ATPase preparation in which the lipids in SR-ATPase have been replaced by dipalmitoyllecithin (DPL), as described (16), will be designated DPL-ATPase.

Labeling with *N*-Ethylmaleimide. Unless otherwise indicated, the solution contained 0.3 M sucrose and 20 mM Tris maleate, pH 7.0 (SR buffer). SR, at a protein concentration of 10 mg/ml, was incubated with 1 mol of *N*-ethylmaleimide (MalNEt) per 10⁵ g of protein for 1 hr at 0°C and then was centrifuged at 100,000 × g for 1 hr to remove unreacted reagent. The pellet was rinsed and resuspended at a protein concentration of 10 mg/ml and then was incubated with maleimide spin label as described below. To determine the specificity of labeling under these conditions, the glycoprotein was separated from the ATPase by Triton X-100 solubilization of the SR vesicles (19). In order to determine the degree of MalNEt labeling, [¹⁴C]MalNEt (New England Nuclear) was used as described (19). Protein concentrations were determined by the method of Lowry *et al.* (20), and ATPase activities were measured at 32°C as described (8, 16).

Spin-Labeling. A maleimide spin label derivative, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide (MSL), was used to label the enzyme polypeptide. A fresh solution of spin label in ethanol was diluted 1:10 with buffer, added to Mal-NEt-treated SR, SR-ATPase, or DPL-ATPase (final ethanol concentration, <1%) at a ratio of 1.25 mol of label per 10⁵ g of

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Abbreviations: SR, sarcoplasmic reticulum; DPL, dipalmitoyllecithin; MalNEt, *N*-ethylmaleimide; EPR, electron paramagnetic resonance; MSL, maleimide spin label [i.e., *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide].
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protein, and incubated overnight at 0°C. The reaction was stopped by 1:30 dilution with ice-cold SR buffer. The labeled enzyme was sedimented by centrifugation at 100,000 × g for 1 hr and the pellet was resuspended in SR buffer. The resulting preparations were designated MSL-SR, MSL-SR-ATPase, and MSL-DPL-ATPase. To determine the concentration of bound spin label, EPR spectra were double-integrated with a Nicolet 1074 computer.

EPR Spectroscopy. A Varian E-109E EPR spectrometer was used in the absorption mode. The temperature was controlled to within 0.5°C with a V4540 variable temperature controller. Conventional spectra, designated V_1, were obtained with 100-kHz field modulation, a peak-to-peak modulation amplitude of 2 gauss, and a microwave power setting of 10 mW. Saturation transfer spectra, designated V_2', were obtained as described (9, 14, 16). Unless otherwise stated, all spectra were obtained from preparations in SR buffer at 4°C and containing 10–20 mg of protein per ml.

Protein Crosslinking. We treated SR vesicles with glutaraldehye as follows. SR was suspended in 10% (vol/vol) glycerol/0.25 M morpholinopropanesulfonic acid, pH 7.0, at 4°C. A solution of 0.8 M glutaraldehyde (Polysciences) in water was added to a final concentration of 40 mM. The reaction was quenched by addition of 0.5 M glycylglycylglycine (Calbiochem), pH 8.5, to a final concentration of 80 mM; addition was gradual to keep the pH at 7.0 ± 0.1. The extent of protein crosslinking was controlled by varying the concentration of protein and the reaction time as described below.

To characterize the crosslinked products, the vesicles were analyzed by electron microscopy of negatively stained preparations, or by electrophoresis in 6% polyacrylamide gels containing sodium dodecyl sulfate (21).

RESULTS AND DISCUSSION

Spin-Labeling. The usual method of spin-labeling SR with sulfhydryl-directed probes, generally with the purpose of studying Ca^{2+}-ATPase (22–24), has been simply to incubate the membranes with the label and then to wash away the unreacted label, resulting in EPR spectra of SR with at least two spectral components. However, when we applied such a procedure in labeling SR-ATPase, which contains no proteins besides the Ca^{2+}-ATPase, we obtained only one spectral component (16, 19). We have recently shown (19) that a minor glycoprotein component of SR, which is partially exposed to the external surface of the membrane (25), contains highly reactive sulfhydryl groups that are readily labeled either by MalNEt or by a maleimide spin label (MSL). If SR is incubated first with MalNEt and then with MSL, the fast-reacting sulfhydryl groups of the glycoprotein are specifically labeled with MalNEt and MSL is selectively attached to the Ca^{2+}-ATPase (19). The resulting single-component spectra are virtually identical to those obtained from the purified SR-ATPase (19). Therefore, in the present study, to ensure that the spin label was only attached to the Ca^{2+}-ATPase, SR was always labeled with MalNEt prior to spin-labeling.

For each of four preparations of spin-labeled SR, 0.8–1.0 mol of MalNEt and 1.0–1.2 mol of MSL were bound per 10^5 g of protein. Under these conditions, only 0.1–0.2 mol of MSL per 10^5 g of protein was bound to the glycoprotein isolated from the spin-labeled SR preparations. This is about ⅒th of the glycoprotein labeling obtained when the fast-reacting sulfhydryl groups are not first blocked by MalNEt (19). Because the amount of glycoprotein present in SR is ⅒th that of the Ca^{2+}-ATPase and because these are the only two SR proteins that react with MSL, more than 98% of the label is bound to the Ca^{2+}-ATPase. Therefore, the EPR spectra below can be reliably

interpreted in terms of the physical state of the enzyme. The Ca^{2+}-ATPase activity of the labeled SR was 50–80% of the activity of the unlabeled control.

EPR Spectra. The parameters $2T_\parallel'$ and L''/L (see Fig. 1 and refs. 9, 14, and 26) will be used to characterize the conventional and saturation transfer spectra, respectively. Effective correlation times can be estimated from these parameters by assuming isotropic rotational motions. Because it is almost certain that the observed motions are not isotropic, these values should not be interpreted as accurate correlation times describing complex molecular motions (14). For example, if rotational motion occurs about a single molecular axis and the principal axis of the spin label is aligned approximately parallel to the axis of diffusion, the effective correlation time estimated from the spectrum will be greater than the actual value. Thus, in the absence of experiments on oriented planar membranes, it remains possible that a change in the effective τ_2 is due to a change in the probe's orientation rather than a change in the rate of the protein's rotational motion. Nevertheless, these measurements provide good estimates of the approximate time ranges of the motions, and they are useful for comparative purposes. The parameter $2T_\parallel'$, the separation of the outer peaks in the conventional spectrum, is sensitive only to rotational correlation times (τ_2) less than 1 μsec, whereas L''/L, from saturation transfer spectra, is sensitive to τ_2 values as long as 1 msec (9, 14, 26) (Fig. 1). Therefore, $2T_\parallel'$ is useful for the characterization of relatively rapid motions such as those of fluid lipid hydrocarbon chains and proteins in solution, whereas L''/L is useful for the characterization of slower motions such as those of lipid hydrocarbon chains in the gel phase or of proteins assembled into restrictive structures such as membranes (14).

The conventional (V_1) and saturation transfer (V_2') EPR spectra of MSL-SR are shown in Fig. 2A. The conventional spectrum has the characteristic shape of a "strongly immobilized" spin label, implying that the label undergoes no significant
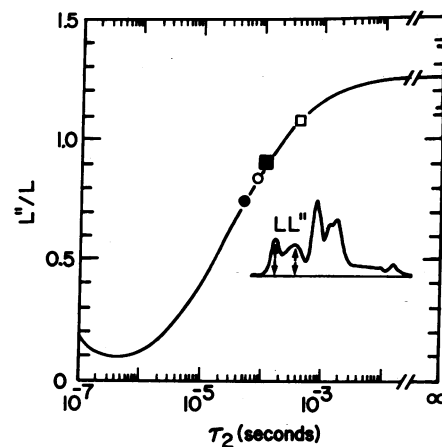

FIG. 1. Plot of the spectral parameter L''/L vs. the rotational correlation time τ_2, illustrating the procedure used to determine effective correlation times from nitroxide spin label saturation transfer EPR spectra (V_2'). The curve was obtained from theoretical studies and model system experiments on isotropic Brownian rotational diffusion (9, 14, 16). The plotted symbols represent data from the present study and have been placed on the curve according to the L''/L values observed in V_2' saturation transfer experiments. The L''/L values are given in Table 1. ●, MSL-SR; ○, MSL-SR + 10 mM $CaCl_2$; ■, MSL-SR + glutaraldehyde (Fig. 2C); and □, MSL-SR + glutaraldehyde (Fig. 2D). The precision of each L''/L determination is indicated by the size of the symbol. The error bar at $\tau_2 = \infty$ indicates the range of values that have been observed for immobilized proteins, implying some uncertainty in the absolute τ_2 determination as the spectrum approaches the rigid limit.
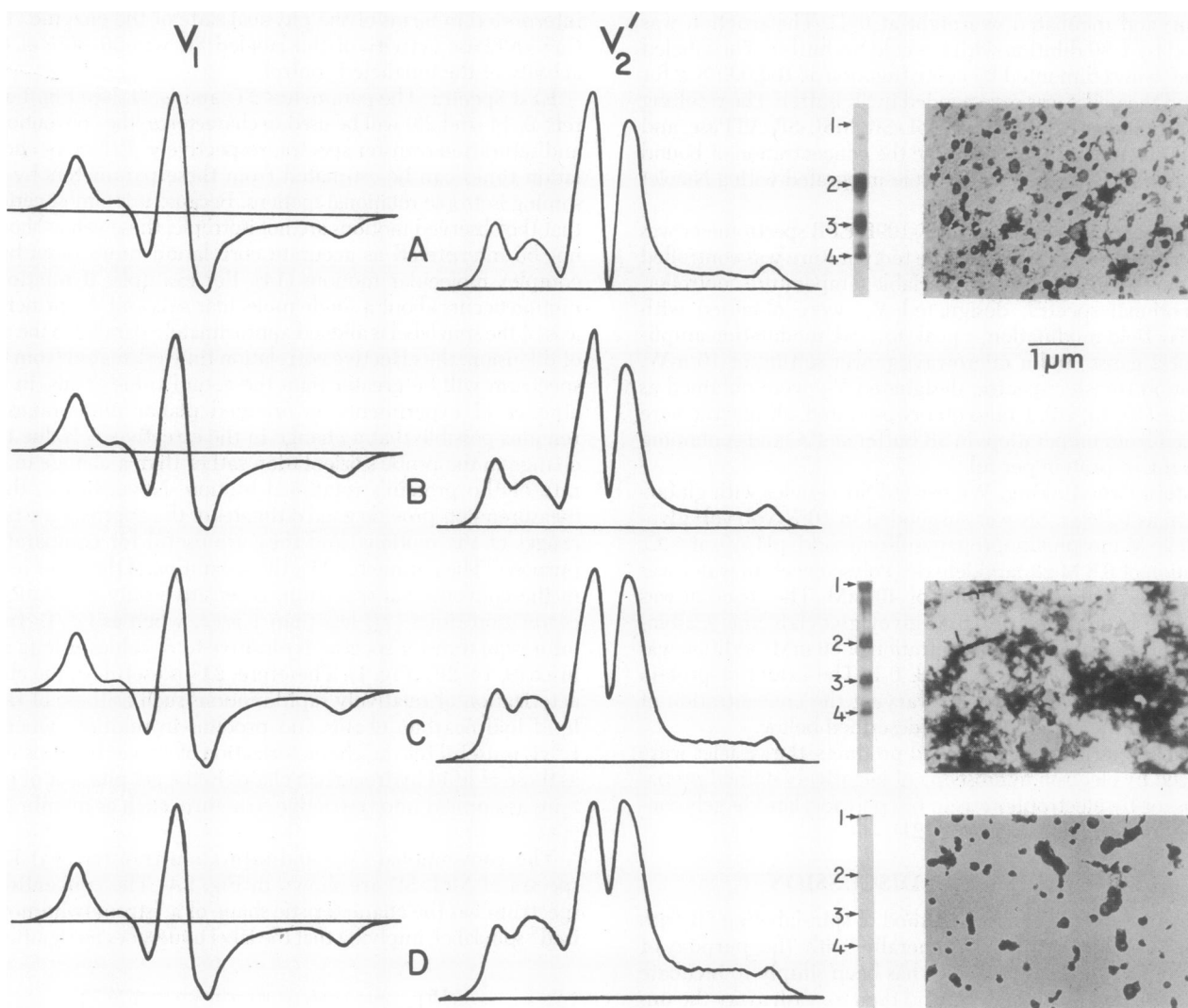
FIG. 2. Conventional (V_1) and saturation transfer (V_2') EPR spectra, sodium dodecyl sulfate/polyacrylamide gels, and electron micrographs obtained from spin-labeled SR. (*A*) Vesicles in suspension. (*B*) Pellet obtained by sedimenting vesicles at 100,000 × *g* for 1 hr. (*C*) Vesicles treated with glutaraldehyde for 10 min at a protein concentration of 50 mg/ml. (*D*) Vesicles treated with glutaraldehyde for 12 hr at a protein concentration of 1 mg/ml. Spectral baselines are 100 gauss wide. Gel positions: 1, >10^6 daltons; 2, 10^5 daltons (ATPase); 3, 5.5 × 10^4 daltons(calsequestrin); 4, 3 × 10^4 daltons(glycoprotein). The electron micrographs were from the same preparations shown in the gels, negatively stained with sodium silicotungstate.

degree of rotational motion with a correlation time less than 0.1 μsec. Therefore, the spin label is rigidly attached to the protein, and any slower motion, not detectable by the conventional spectrum, is probably large-scale rotational motion such as that of the entire enzyme or a large segment of it (14). Evidence for such a slow rotational motion is provided by the saturation transfer spectrum of SR (Fig. 2*A*, V_2'). As indicated in Table 1, the value obtained for L″/L is 0.75, implying an effective rotational correlation time of $\tau_2 = 60$ μsec on the basis of Fig. 1.

This correlation time is in the same range as that obtained for the rotational diffusion of rhodopsin in rod outer segment disk membranes (5, 7). It is likely that, as in the case of rhodopsin, this time characterizes the rotational diffusion of the protein in the membrane lipids. The remaining spectra in Fig. 2 provide evidence in support of this interpretation. The spectra of a pellet of spin-labeled SR vesicles (Fig. 2*B*) are similar to those of the vesicle suspension (Fig. 2*A*). The conventional spectra (V_1) are virtually identical in shape and the values observed for $2T_∥'$ are not significantly different (Table 1), implying the absence of any localized change in the environment of the spin label. The central region of the saturation transfer spectrum

(V_2') is affected slightly by pellet formation, but the value of L″/L is not significantly changed, implying that the effective correlation time (τ_2) is still 60 μsec. This result rules out the possibility that the motion evident from Fig. 2*A* is due to tumbling of the membrane vesicles. Accordingly, we can conclude that the observed motion corresponds to motion within the membrane.

Further support for this conclusion is provided by the following experiments. Spin-labeled SR vesicles were crosslinked with glutaraldehyde briefly (for 10 min) at a high protein concentration (50 mg/ml), to maximize intervesicular crosslinking and minimize intravesicular crosslinking. After crosslinking, the membranes were visibly aggregated so that centrifugation at 500 × *g* for 10 min sedimented most of the material. This pellet was resuspended and used for spectral measurements. The electron micrograph (Fig. 2*C*) shows that the vesicles are highly aggregated, as opposed to the untreated control (Fig. 2*A*). Analysis of the crosslinked MSL-SR by sodium dodecyl sulfate/polyacrylamide gel electrophoresis showed only partial protein–protein crosslinking; some protein remained in the position of the 100,000-dalton enzyme and of the lower molecular weight SR proteins, and the rest stayed on top of the

Table 1.   EPR spectral parameters of maleimide spin-labeled
SR preparations

	$2T'_\parallel$	L''/L
MSL-SR (Fig. 2A)	67.7 ± 0.2 (7)	0.75 ± 0.02 (6)
MSL-SR + 10 mM CaCl₂	67.6 ± 0.2 (1)	0.83 ± 0.02 (1)
MSL-SR pellet (Fig. 2B)	67.9 ± 0.1 (2)	0.74 ± 0.03 (2)
Glutaraldehyde-MSL-SR (Fig. 2C)	68.0 ± 0.2 (1)	0.91 ± 0.03 (1)
Glutaraldehyde-MSL-SR (Fig. 2D)	68.4 ± 0.2 (1)	1.08 ± 0.02 (1)
MSL-SR-ATPase	67.8 ± 0.1 (3)	0.74 ± 0.02 (2)
MSL-SR-ATPase + 10 mM CaCl₂	67.9 ± 0.1 (1)	0.85 ± 0.02 (2)
MSL-DPL-ATPase	67.9 ± 0.1 (2)	1.13 ± 0.01 (2)

$2T'_\parallel$ was determined from conventional spectra (V_1) and L''/L was determined from saturation transfer spectra (V'_2), as indicated in Fig. 1. The first, third, fourth, and fifth rows correspond to the four pairs of spectra in Fig. 2, as indicated. Data on SR-ATPase, containing the purified Ca^{2+}-ATPase in endogenous SR lipids, and DPL-ATPase, containing the purified ATPase in DPL, are from ref. 16. The number of different preparations studied is given in parentheses.

gel (Fig. 2C). The control MSL-SR gel showed no protein at the top of the gel (Fig. 2A). The conventional EPR spectrum (V_1) of the crosslinked preparation (Fig. 2C) was essentially the same as that observed for untreated SR membranes (Fig. 2 A and B). The saturation transfer spectrum (V'_2) revealed a decrease in rotational mobility, but the value of 0.91 for L''/L is significantly less than the value corresponding to the rigid limit (see Fig. 1). Thus, despite the complete immobilization of the membrane vesicles, partial protein–protein crosslinking results in only partial immobilization, permitting considerable rotational motion in the submillisecond time range.

To maximize protein–protein crosslinking, MSL-SR vesicles were incubated with glutaraldehyde for 12 hr, at a protein concentration of 1 mg/ml. Most of the material that crosslinked under these conditions remained in the supernatant after centrifugation at 500 × g for 15 min, and this supernatant was used to obtain the spectra, gel, and electron micrograph shown in Fig. 2D. Crosslinking at this low protein concentration minimized aggregation due to intervesicular crosslinking, as shown in the electronmicrograph, but the long reaction time resulted in complete protein–protein crosslinking within each vesicle, as evidenced by the gel pattern. The conventional EPR spectrum (Fig. 2D, V_1) is like the other spectra in Fig. 2, but the saturation transfer spectrum (Fig. 2D, V'_2) indicates a higher degree of immobilization than do the other three V'_2 spectra. The value of L''/L (1.08) is, in fact, at or near the rigid limit, as shown in Fig. 1. Thus, although the vesicles remain dispersed, extensive intramembrane crosslinking eliminates most or all of the submillisecond rotational motion.

The data in Fig. 2 indicate that the observed submillisecond rotational motion in SR at 4°C reflects primarily motions of the Ca^{2+}-ATPase molecules within the membrane. Vesicle tumbling is clearly not an important contributor to the motion in this time range. This result is expected, because electron micrographs show that the mean vesicle diameter in our SR preparations is 1200 Å. According to the Stokes–Einstein–Debye equation (27), the predicted rotational correlation time for a 1200-Å sphere in 0.3 M sucrose at 4°C [viscosity, 0.022 poise (28)] is 0.51 msec. This value is in good agreement with that obtained from the V'_2 spectrum of the glutaraldehyde-treated but dispersed vesicles ($L''/L = 1.08$, $\tau_2 = 0.5$ msec, as shown in Fig. 1), but it is an order of magnitude longer than the τ_2 value observed for untreated SR ($L''/L = 0.75$, $\tau_2 = 60$ μsec, as shown in Fig. 1). Although it seems most likely that the slow

rotational motion inhibited by crosslinking is the rotational motion of the entire enzyme or its possible oligomeric assemblies, the possibility remains that we are observing large-scale intramolecular motions, such as the rotation of a large segment of the enzyme (14). Intramolecular crosslinking might slow down such motions, producing spectral changes such as those observed in Fig. 2D.

We recorded the saturation transfer spectra of the SR pellet (used in Fig. 2B) from 0°C to 34°C. The parameter L''/L increased with decreasing temperature (Fig. 3), indicating that the rotational mobility of the enzyme decreased. The motion decreased more rapidly with cooling at temperatures below about 20°C than above 20°C, correlating well with similar changes in slope in plots of lipid fluidity and enzymatic activity as a function of temperature (15, 22). These results suggest that the observed rotational motion might have a role in regulating the enzymatic activity of the Ca^{2+}-ATPase.

In order to investigate further the possible functional significance of the observed motion, we carried out a study relating Ca^{2+}-ATPase activity to the protein's rotational mobility (16, 17). Spectroscopic data from that study have been included in Table 1 for comparison with the results obtained in this work. Note that the spectral parameters from the purified SR-ATPase at 4°C are essentially the same as those observed for SR, implying that the observed submillisecond rotational motion of the Ca^{2+}-ATPase in SR is not affected by the other proteins present in the SR membrane. However, we found that replacing the endogenous SR lipids with DPL, producing DPL-ATPase, strongly inhibited both enzymatic activity and rotational motion at 4°C, a temperature at which the DPL molecules are much more rigid than are the endogenous SR lipids (16, 17). The saturation transfer spectrum of DPL-ATPase yields a value of L''/L that is similar to that obtained in the present study for extensively crosslinked SR (Table 1), implying little or no submillisecond rotational mobility for both preparations.

When [Ca^{2+}] is increased from the micromolar range to the millimolar range, the ATPase activity of this enzyme decreases, due to inhibition of the hydrolysis of the phosphoenzyme intermediate (18, 29), the same reaction step that is inhibited by DPL substitution (15, 16). We have shown that the inhibition at 10 mM Ca^{2+} in the purified SR-ATPase is accompanied by a decrease in the rotational mobility of the protein (16), as evidenced by an increase in L''/L (Table 1). Ca^{2+} at 10 mM produced a similar decrease in protein mobility in SR vesicles
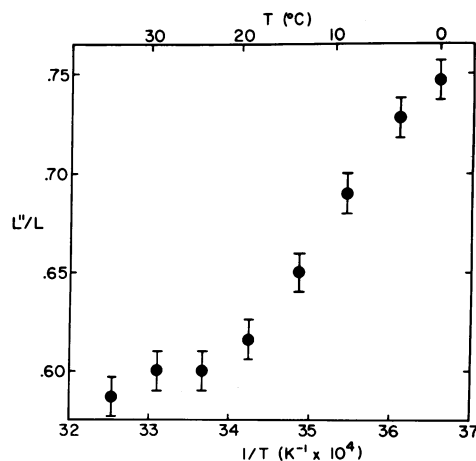

FIG. 3.   Temperature dependence of the parameter L''/L obtained from saturation transfer spectra of spin-labeled SR. The spectra, like those of Fig. 2B, were obtained from a membrane pellet. Effective rotational correlation times may be determined from L''/L by referring to Fig. 1.

(compare the L''/L values for SR, in the first and second rows of Table 1, with the L''/L values of SR-ATPase, in the sixth and seventh rows).

Our previous results have indicated that a fluid lipid environment is required for Ca^{2+}-ATPase activity in SR, because decreases in lipid fluidity achieved by cooling or by DPL substitution also inhibit enzymatic activity (15). Our saturation transfer studies in the present work and in the studies of purified enzyme systems (16, 17) have shown that these decreases in lipid fluidity are accompanied by decreases in the rotational mobility of the protein. In fact, ATPase activity appears to correlate better with protein mobility than with lipid fluidity, because inhibition by high $[Ca^{2+}]$ is accompanied by a decrease in the rotational mobility of the protein but not of the lipids (16). Therefore, fluid lipids, although required, are not sufficient for optimal ATPase activity, and the correlation between enzymatic activity and lipid fluidity (15, 22) could be due to the effect of the lipids on the submillisecond protein rotational motion that may be required for optimal function of this enzyme.

Our observations of rotational motion of Ca^{2+}-ATPase have been made in the absence of ATP. Further experiments in the presence of ATP are required in order to analyze whether the observed motions are consistent with a model in which the enzyme functions as a rotary carrier to carry out Ca^{2+}-transport coupled to ATP hydrolysis (24, 30, 31). It should be noted that the motions observed in SR in the present study are much faster than the motions required to account for the observed values of ATPase activity, assuming a tightly coupled rotary carrier model (23). A technique sensitive to $\tau_2 > 1$ msec may be required to investigate the possibility of such a model. Because little or no inhibition of ATPase activity and Ca^{2+} transport were observed after attachment of bulky molecules to the enzyme (32, 33), it seems unlikely that Ca^{2+}-ATPase activity requires a 180° rotation about an axis in the plane of the membrane. In any case, it remains likely that Ca^{2+}-ATPase activity requires some change in protein conformation, orientation, or supramolecular arrangement that involves substantial movement of the enzyme (or of a large segment of it) relative to nearby lipids.

A more precise description of the rotational motion of the Ca^{2+}-ATPase should be possible in the future. In the present experiments, the membranes are randomly oriented with respect to the magnetic field, and the orientation of the probe relative to the membrane plane is unknown, so the geometric details of the motion are difficult to determine. Experiments on uniformly oriented planar membranes, combined with theoretical simulations of the effects of anisotropic motions on V_2' spectra, should provide good tests for specific proposed motions. In addition, more detailed information on the structure of the ATPase molecule should help in indicating what motions are likely to occur, particularly in the case of segmental motions within the enzyme.

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