Temperature Dependence of Rotational Dynamics of Protein and Lipid in Sarcoplasmic Reticulum Membranes†

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ABSTRACT: We have investigated the relationship between function and molecular dynamics of both the lipid and the Ca-ATPase protein in sarcoplasmic reticulum (SR), using temperature as a means of altering both activity and rotational dynamics. Conventional and saturation-transfer electron paramagnetic resonance (EPR) was used to probe rotational motions of spin-labels attached either to fatty acid hydrocarbon chains or to the Ca-ATPase sulfhydryl groups in SR. EPR studies were also performed on aqueous dispersions of extracted SR lipids, in order to study intrinsic lipid properties independent of the protein. While an Arrhenius plot of the Ca-ATPase activity exhibits a clear change in slope at 20 °C, Arrhenius plots of lipid hydrocarbon chain mobility are linear, indicating that an abrupt thermotropic change in the lipid hydrocarbon phase is not responsible for the Arrhenius break in enzymatic activity. The presence of protein was found to decrease the average hydrocarbon chain mobility, but linear Arrhenius plots were observed both in the intact SR and in extracted lipids. Lipid EPR spectra were analyzed by procedures that prevent the production of artifactual breaks in the Arrhenius plots. Similarly, using sample preparations and spectral analysis methods that minimize the temperature-dependent contribution of local probe mobility to the spectra of spin-labeled Ca-ATPase, we find that Arrhenius plots of overall protein rotational mobility also exhibit no change in slope. The activation energy for protein mobility is the same as that of ATPase activity above 20 °C; we discuss the possibility that overall protein mobility may be essential to the rate-limiting step above 20 °C.

Understanding the molecular mechanism of a transmembrane enzyme requires relating physical states of both the lipid and the protein to biochemical properties of the enzyme. Variation of temperature is an effective method of inducing functional perturbations, as well as physical perturbations in both lipid and protein components. In the case of the Ca-ATPase of sarcoplasmic reticulum (SR),[1] there is an intriguing change in the slope (apparent activation energy) near 20 °C of Arrhenius plots for both steady-state ATP hydrolysis and calcium transport (Inesi et al., 1973; Deamer, 1973; Madiera et al., 1974; Hidalgo et al., 1976), raising the following question: Can this nonlinearity ("break") be ascribed to specific physical changes in the protein or lipid components of the membrane? The answer promises to provide clues about the physical basis of active transport.

A sharp gel to liquid crystal transition is not predicted in SR lipids, since the fatty acyl chains and head groups are quite heterogeneous (Quinn, 1981). Differential scanning calorimetric measurements detect only a very broad endothermic transition in the hydrated lipids of SR near 4 °C (Martonosi, 1974). High-angle X-ray diffraction indicates that less than 3% of the lipids are in rigid crystalline order at 1 °C (Davis et al., 1976). Nevertheless, several laboratories have reported changes in the temperature dependence (usually expressed as a break, i.e., a change in slope on Arrhenius plots) of membrane fluidity near 20 °C, as measured by electron paramagnetic resonance (EPR) of spin-labeled fatty acids in SR (Eletr & Inesi, 1972; Inesi et al., 1973; Hesketh et al., 1976; Hidalgo et al., 1976) and by studies of fluorescent probes that partition into the lipid phase (Madeira & Antunes-Madeira, 1975; Almeida et al., 1982). These changes are often interpreted as evidence for a phase transition, although they are much less dramatic than those observed when a single-component lipid sample undergoes a first-order phase transition. These studies have been cited as evidence for the importance of lipid fluidity for enzymatic activity (Hidalgo et al., 1976). However, the magnitudes of the slopes and the direction of the change (increase or decrease) in slope have exhibited great variation, so that they present no coherent picture of the relationship of these parameters to enzymatic function. This may be explained, in part, by the work of Cannon and coworkers in mitochondrial membranes (1975), which showed that incorrect analysis of EPR spectra can lead to artifactual breaks in Arrhenius plots.

In contrast to these studies showing breaks, another study reported no change in slope of Arrhenius plots derived from spin-labeled fatty acids in SR but did report a break near 20 °C from a probe of boundary lipid, i.e., a spin-labeled hydrocarbon chain covalently attached to the protein (Kirino et al., 1981), suggesting that a change occurs in the protein itself, its boundary lipid, or both. This is consistent with studies of the ATPase reconstituted into pure lipids, which have suggested that protein perturbs the temperature dependence of the first shell of lipids (i.e., boundary lipid) surrounding the Ca-ATPase (Hesketh et al., 1976; Hidalgo et al., 1976; Lentz et al., 1983, 1985). Several other studies have reported evi-

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[1] Abbreviations: DMPC, 1,2-dimyristoylphosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; H_{II}, reverse hexagonal; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; MSL, maleimide spin-label; PE, phosphatidylethanolamine; SASL, stearic acid spin-label; SR, sarcoplasmic reticulum; ST-EPR, saturation-transfer EPR; TANOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.

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dence for Arrhenius breaks in protein physical properties in SR (Inesi et al., 1973; Kirino et al., 1977; Anzai et al., 1978; Hoffmann et al., 1979; Lippert et al., 1981).

Overall protein rotational mobility is a valuable measurement for assessing the temperature dependence of lipid fluidity, since this mobility should be proportional to the effective lipid fluidity that the protein experiences (Saffman & Delbruck, 1975; Peters & Cherry, 1982; Hughes et al., 1982; Thomas, 1985b). Thus, if the fluidity of the lipid phase surrounding a protein has an Arrhenius break, the protein mobility would also be expected to have one. In addition, if the lipid fluidity (defined as the inverse of viscosity) undergoes no transition, but the protein's shape or state of aggregation does, so should the overall rotational mobility. Our previous studies (Hidalgo et al., 1978; Thomas & Hidalgo, 1978; Thomas et al., 1982; Thomas, 1985a) have shown that lipid hydrocarbon chain mobility correlates with enzymatic activity under some, but not all, conditions, while overall protein mobility more consistently correlates with function, adding to the evidence that the measurement of overall protein mobility is important for the understanding of SR function.

Spectral parameters from saturation-transfer EPR (ST-EPR), a technique sensitive to microsecond protein rotational motion (Thomas, 1985a,b), have been reported to show breaks near 20 °C for spin-labeled Ca-ATPase in SR (Thomas & Hidalgo, 1978; Kirino et al., 1978; Kaizu et al., 1980). However, spectral analysis by line-shape parameters, as carried out in these previous studies, has suffered from the poorly defined contribution of a second, more mobile spin-label population, casting doubt on the assumption that these parameters accurately report overall protein rotational mobility (Thomas, 1985a,b). Protein mobility detected by optical spectroscopic methods (Hoffmann et al., 1979; Burkli & Cherry, 1981; Spiers et al., 1983) has resulted in Arrhenius plots with one or two breaks, some of which roughly coincide with breaks in enzymatic activity; the kind of motion measured in these optical studies has not been clarified.

There is no consensus as to whether any of these breaks represent changes in intrinsic protein properties or indirectly reflect changes in lipid structure and dynamics. Alternatively, the lipid changes observed may be a response to protein conformational fluctuations. In order to resolve the effects of protein and lipid, it is essential to perform EPR experiments on both native SR and vesicles of extracted endogenous lipids. Only a few studies have compared results from SR with those from extracted SR lipids, where there is no boundary lipid. These studies have found either no break (Madeira & Antunes-Madeira, 1975; Almeida et al., 1982) or a very different break temperature of 30 °C (Hesketh et al., 1976) in Arrhenius plots of lipid fluidity in extracted SR lipids.

Therefore, in the present study, we have used spin-labels to probe the temperature dependence of hydrocarbon chain mobility in both SR and extracted SR lipids, as well as the overall protein rotational mobility, using both conventional and saturation-transfer EPR, enabling us to measure a range of molecular motions from subnanoseconds to milliseconds. We have taken care to use the most rigorous methods in EPR spectral analysis that are currently available, including recent improvements in the analysis of ST-EPR spectra (Thomas, 1985b; Squier & Thomas, 1986), thus avoiding apparent temperature effects due to spectral artifacts.

Materials and Methods

Membrane Preparations. Vesicles of fragmented SR were prepared from rabbit skeletal white (fast twitch) muscle, essentially as described previously (Fernandez et al., 1980). All preparation was done at 4 °C. The membrane vesicles were suspended in 0.3 M sucrose–20 mM MOPS (pH 7.0) and stored in liquid nitrogen. Lipids were extracted by a modification (Hidalgo et al., 1976) of the method of Folch (1957), using nitrogen-saturated solvents to prevent oxidation. The lipid was stored in chloroform–methanol (2:1) at −20 °C. Liposomes were prepared by drying an aliquot of extracted lipid under nitrogen and then vortex mixing in 0.3 M sucrose–20 mM MOPS (pH 7.0).

Table I: Fatty Acid Composition (%) of SR Membranes and Extracted SR Lipids

fatty acid	preparation	
	SR membranes	extracted SR lipids
14:1	9.4	5.7
16:0	22.8	24.9
18:0	6.0	8.6
18:1	12.8	12.9
18:2	15.7	15.4
20:4	14.2	16.8
22:5	3.5	3.9
22:6	3.1	3.2
others	12.4	8.4

Lipid Analysis. Analysis of phospholipid composition of SR lipid extracts was made by two-dimensional thin-layer chromatography, using silica gel plates prepared by Supelco, Inc. (250 μm thickness). Plates were developed in the first dimension with chloroform–methanol–NH_4OH (65:25:5) and in the second dimension with chloroform–acetone–methanol–acetic acid–water (3:4:1:1:0.5). Spots were visualized by exposure to iodine vapor, identified with appropriate standards, and scraped from the plate for quantitation by phosphorus determination (Chen et al., 1956). Such analysis indicated that the phospholipid composition of our preparations is typical of other reported SR preparations (Martonosi, 1983), with the major components phosphatidylcholine and phosphatidylethanolamine being present as 58 and 16%, respectively, of the total lipids. Cholesterol was present as 5%.

Fatty acid composition was determined on a Packard 428 gas/liquid chromatograph (GLC), after lipid extraction and esterification, at the Hormel Institute (Austin, MN), essentially according to the method described previously (Ogburn et al., 1982). Extracted lipids were compared with SR membranes for fatty acyl chain composition (Table I), showing that aqueous dispersions of extracted lipids are representative of the SR membranes with respect to fatty acid composition.

The molar ratio of phospholipids per Ca-ATPase was determined by dividing the molar concentration of total lipids, determined from phosphorus assays, by that of the Ca-ATPase. The latter was determined by dividing the protein concentration by a M_r of 115K and multiplying by the fraction of the total protein that had this molecular weight, as determined from densitometer scans of polyacrylamide gels. This SR preparation typically has about 80 mol of phospholipid/mol of Ca-ATPase.

Enzymatic Assays. Ca-dependent ATPase activity was measured in a solution containing 0.05 mg of protein/mL, 60 mM KCl, 6 mM $MgCl_2$, 2 μM A23187, 25 mM MOPS (pH 7.0), and either 0.1 mM $CaCl_2$ or 2 mM EGTA. The reaction was started by the addition of 5 mM ATP, and the initial rate of release of inorganic phosphate was measured by the method of Lanzetta et al. (1979). Alternatively, ADP production was assayed by monitoring absorbance at 340 nm with an enzyme-linked assay (Warren et al., 1974). Activity assayed in the presence of EGTA (basal activity) was subtracted from that assayed in the presence of $CaCl_2$ (total Ca-ATPase activity), in order to obtain Ca-dependent ATPase activity.

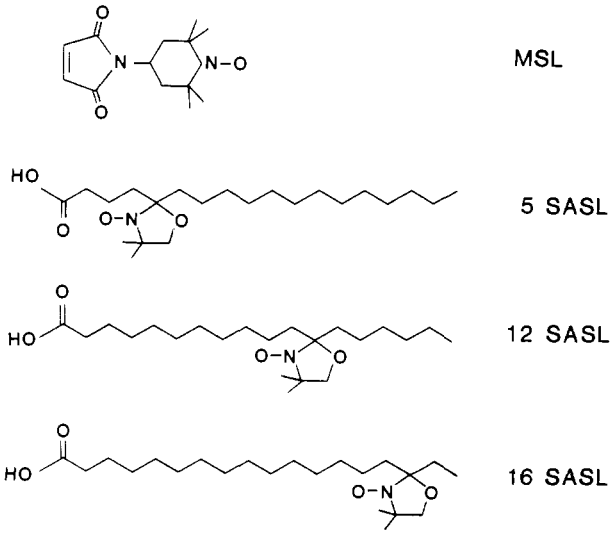

FIGURE 1: Spin-labels: MSL (maleimide spin-label), *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; 5-, 12-, and 16-SASL (stearic acid spin-label), which are 4′,4′-dimethyloxazolidine-*N*-oxyl derivatives of stearic acid labeled at the 5-, 12-, and 16-positions, respectively.

Protein concentrations were determined by the biuret method, using bovine serum albumin as a standard.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the general method of Laemmli (1970), using either 7.5% acrylamide gels with 3% stacking gels or 2.2% gels without a stacking gel (Louis & Holroyd, 1978). The former was utilized to resolve the Ca-ATPase from a 98-kdalton protein, presumably phosphorylase *b* (Pickart & Jencks, 1984), which we found to be less than 4% of the protein in this preparation. Before electrophoresis, samples (3 mg/mL) were incubated in 1% sodium dodecyl sulfate without heating. Gels were stained for protein with Coomassie Blue. Densitometer scans of these gels indicated that 80 ± 5% (by weight) of the proteins in our SR preparation migrated as a 100-kdalton band, presumably the Ca-ATPase.

Arrhenius Analysis. Lines and break points in Arrhenius plots were fit by linear regression least-squares analyses. The data were fit to either a single straight line or two lines intersecting at a break temperature, which was varied over the temperature range measured. Breaks were considered significant if (a) the difference in the two slopes was greater than the sum of their standard deviations and (b) the mean correlation coefficient of the two lines (weighted by the number of data points fit to each line) was greater than the correlation coefficient for a single line. Apparent activation energies were calculated according to the Arrhenius equation, where the slope of an Arrhenius plots is $(-E^*/R)$, where E^* is the apparent activation energy and R is the gas constant.

Spin-Labeling. Hydrocarbon chain rotational mobility was measured with fatty acid spin-labels, 4′,4′-dimethyloxazolidine-*N*-oxyl derivatives of stearic acid (Figure 1), which are designated 5-, 12-, and 16-SASL (Aldrich). They were diluted from a stock solution in DMF into ethanol before addition to SR (or liposomes of extracted SR lipids) at a ratio of less than one spin-label to 200 phospholipids, with the final ethanol concentration less than 1%. The lipid concentration was made sufficiently high (>50 mM) so that the EPR spectrum contained a negligible contribution from unbound (aqueous) spin-labels.

To monitor the rotational motion of the Ca-ATPase protein, SR was labeled with a maleimide spin-label (MSL) (Figure 1), *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (Aldrich), essentially according to the method of Thomas & Hidalgo (1978). *N*-ethylmaleimide (NEM) was added in a ratio of 1.0 mol/10^5 g of protein to SR to block fast-reacting sulfhydryl groups. After incubation for 30 min at 25 °C, MSL was added at a ratio of 2.5 mol/10^5 g of protein and incubated 50 min at 25 °C. This resulted in incorporation of 1.8 mol of spin-label/10^5 g of SR protein. The reaction was stopped by a 30-fold dilution into cold buffer (0.3 M sucrose, 20 mM MOPS, pH 7.0), and the membranes were washed twice, by centrifuging at 100000*g* for 45 min each, to remove any unbound label.

In order to prevent a time-dependent loss of spins at temperatures above 20 °C and the accompanying spectral hysteresis, membrane pellets were preincubated for 3 h at 37 °C before resuspension into buffer. This resulted in the reduction of 0.6 mol of spin-label/10^5 g of SR protein. Thus, all ST-EPR measurements of protein mobility were conducted on a sample containing 1.2 mol of spin-label/10^5 g of SR protein. Because the population of probes that were reduced by this preincubation were more mobile than the remaining population of spins, their reduction resulted in spectra that had less contribution from weakly immobilized probes. The 37 °C preincubation was required to obtain ST-EPR spectra that accurately measure overall protein mobility (Squier et al., 1985). This incubation at 37 °C resulted in no decrease in Ca-dependent ATPase activity, but presumably, the membranes suffer some leakiness, as evidenced by a 50% loss of Ca uptake activity and increased Ca-ATPase activity in the absence of ionophore. Arrhenius plots of Ca-dependent ATPase activity of spin-labeled preincubated SR are identical with those of untreated SR.

EPR Spectroscopy. EPR spectra were obtained with a Varian E-109 spectrometer (Varian Associates) as described previously (Thomas & Hidalgo, 1978; Thomas, 1985a,b; Squier & Thomas, 1986), and spectra were digitized and analyzed with a microcomputer (Northstar Co.) interfaced to the spectrometer (Lipscomb & Salo, 1983). Submicrosecond rotational motion of spin-labels was detected by conventional EPR (first harmonic absorption in phase, designated V_1), with 100-kHz field modulation (with a peak-to-peak amplitude of 2 G) and microwave field amplitude of 0.14 G. Submillisecond rotational motion was detected by saturation-transfer EPR (second harmonic absorption out of phase, designated V_2') with 50-kHz field modulation (with a modulation amplitude of 5 G) and a microwave field amplitude of 0.25 G. The accurate and reproducible setting of the microwave amplitude incident on the sample (H_1) required a correction for the dielectric loss of the sample. This was done by measuring and comparing the cavity Q for the sample with that of a standard of known saturation properties (Fajer & Marsh, 1982). All saturation studies were done in the absence of oxygen, which was removed from reference and experimental samples in gas-permeable sample cells purged with N_2 (Popp & Hyde, 1981). Temperature was controlled to within 0.5 °C with a Varian V4540 variable-temperature controller.

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

spectrum by a number proportional to the double integral of the V_1 spectrum.

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

Spectral Analysis. Two methods were used to evaluate fatty acid spin-label spectra. For spectra indicating nearly isotropic nanosecond motion (i.e., having three nearly symmetrical lines, implying that the order parameter $S \leq 0.3$), an empirical motion parameter, τ_r (effective correlation time) was calculated from the formula (Keith et al., 1970):

$$\tau_r = (6.5 \times 10^{-10}) W_0[(h_0/h_{-1})^{1/2} - 1] \quad (1)$$

where W_0 is the peak-to-peak line width of the midfield line and h_0 and h_{-1} are the peak-to-peak line heights in the midfield and high-field regions of the spectrum, respectively. Spectra having sufficiently resolved extrema were analyzed by measuring the effective order parameter S (Gaffney & Lin, 1976; Gaffney, 1976). The parameters τ_r^{-1} (for 16-SASL) and $(1 - S)/S$ (for 5-SASL) were used for the Arrhenius analysis of lipid hydrocarbon rotational motion (Grisham & Barnett 1973; Hidalgo et al., 1976).

The effective rotational correlation times for MSL-SR were determined by comparing experimental ST-EPR spectra with reference spectra of known correlation time, obtained from isotropically tumbling spin-labeled hemoglobin in aqueous glycerol solutions (Thomas et al., 1976). ST-EPR spectra are commonly interpreted by either the line shape (ratio of line heights) or the integrated intensity. In the present study, an integrated intensity parameter, the normalized integral of the V_2' spectrum, was used in order to suppress the interfering signals from weakly immobilized probes (i.e., probes having large-amplitude nanosecond mobility) that can distort line-shape parameters (Evans, 1981; Horvath & Marsh, 1983; Fajer & Marsh, 1983; Thomas, 1985b; Squier & Thomas, 1986). A valid analysis of ST-EPR line shapes in terms of microsecond mobility requires that the ST-EPR spectrum has no weakly immobilized component, or at least that this component be unchanging (Thomas, 1985a,b; Squier & Thomas, 1986). When line-shape parameters are used to analyze the ST-EPR spectra of MSL-SR, a small but temperature-dependent fraction of weakly immobilized spin-labels causes large errors in τ_r measurement, particularly at temperatures above 20 °C, whereas the V_2' integral (the integrated intensity parameter used in the present study) is virtually unaffected, making Arrhenius plots of protein mobility much more reliable (Thomas, 1985b; Squier & Thomas, 1986).

Results

Temperature Dependence of Ca-ATPase Activity. A break occurs at 20 °C in the Arrhenius plot of Ca-dependent ATPase activity (Figure 2) with apparent activation energies of 11.8 ± 0.4 and 23 ± 1 kcal/mol, above and below 20 °C, respectively. The same apparent activation energies are observed regardless of whether or not the Ca ionophore A23187 is included in the assay, indicating that no temperature-dependent activation occurs due to changes in vesicle permeability to calcium.

Temperature Dependence of Hydrocarbon Chain Rotational Mobility. Lipid chain mobility was probed at various depths in the bilayer by fatty acid analogues spin-labeled at several positions along the hydrocarbon chain. Conventional (V_1) EPR spectra were recorded at every 2–3 °C over a temperature range of 0–39 °C. No hysteresis was observed for any of the probes. Representative spectra spanning the temperature range

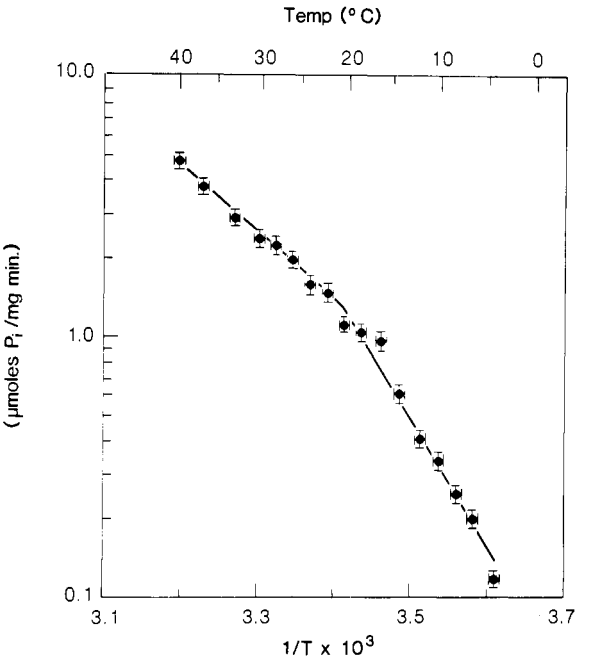

FIGURE 2: Arrhenius plot of Ca-dependent ATPase activity of SR assayed from 4 to 40 °C at 0.05 mg of protein/mL. The apparent activation energies were 11.8 ± 0.4 kcal mol^{-1} deg^{-1} and 23 ± 1 kcal mol^{-1} deg^{-1}, above and below the break point of 20 ± 1 °C. The correlation coefficients were 0.996 and 0.990, respectively. If the Arrhenius plot was fit to a single line, the activation energy was 17 ± 2 kcal mol^{-1} deg^{-1} and the correlation coefficient was 0.972, showing that this fit is significantly worse than the fit to two lines.

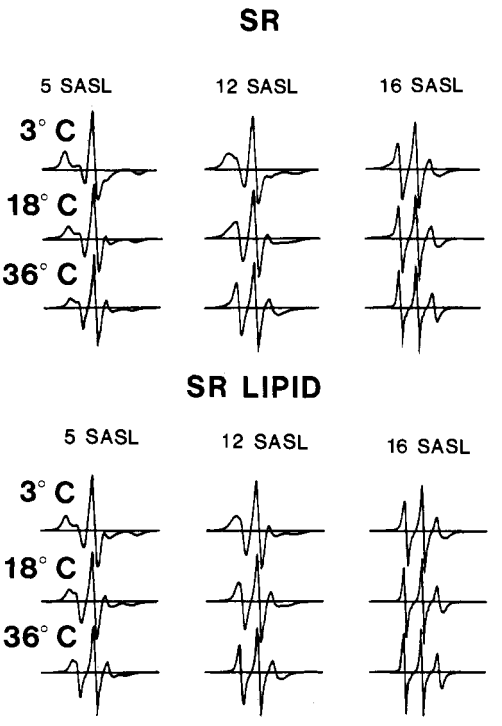

FIGURE 3: Conventional EPR spectra of 5-, 12-, and 16-SASL incorporated into SR membranes and vesicles of extracted SR lipids, all suspended in 0.3 M sucrose–20 mM MOPS (pH 7.0). All spectra were recorded with a 100-G scan range.

studied are shown in Figure 3. At any single temperature, as the nitroxide probe is placed further down the fatty acyl chain toward the center of the bilayer, the spectra display a rotational mobility gradient typical of lipid bilayers, from a relatively restricted polar head region to a more mobile terminal methyl region.

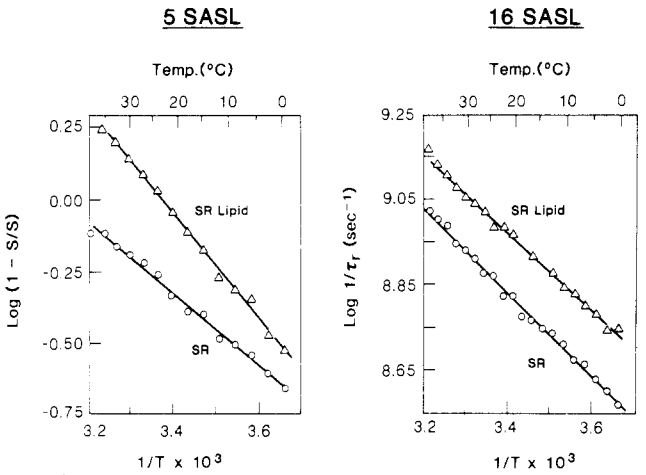

FIGURE 4: Arrhenius plots of lipid hydrocarbon chain mobility in SR (O) and in extracted SR lipids (Δ). Rates of motion were plotted as $(1 - S)/S$ for 5-SASL and τ_r^{-1} for 16-SASL. When data were fit to a single line, correlation coefficients determined for 5-SASL in SR and SR lipids were 0.995 and 0.998, respectively. The best fit of each data set to two lines (27 °C break point in each case), having significantly different slopes, gave correlation coefficients of 0.989 and 0.996, respectively. Correlation coefficients determined for data from 16-SASL in SR and SR lipids were 0.997 and 0.997 with activation energies of 4.5 ± 0.1 and 4.2 ± 0.1 kcal mol^{-1} deg^{-1}, respectively. If SR and SR lipid were fit to two lines, the best fits were for a break point at 16 and 12 °C with mean correlation coefficients of 0.995 and 0.993, respectively, indicating that the single-line fit was superior in each case.

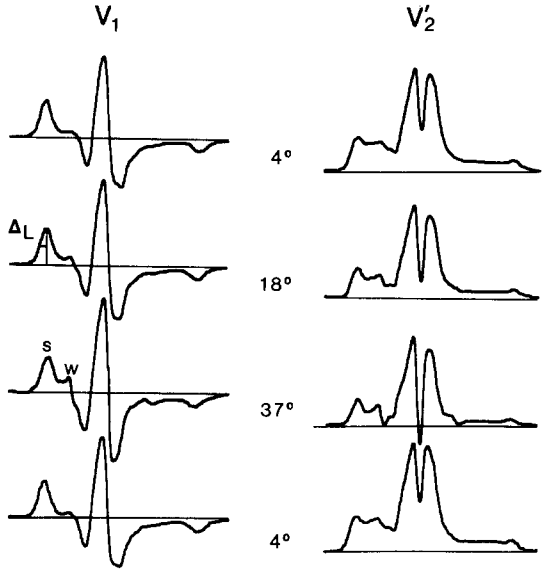

FIGURE 5: Conventional and ST-EPR spectra of maleimide spin-labeled Ca-ATPase in SR in 0.3 M sucrose–20 mM MOPS (pH 7.0) obtained at several temperatures. All spectra were recorded with a 100-G scan range. The low-field line width Δ_L of the V_1 spectrum is defined as the outer half-width at half-maximum and is constant over this temperature range.

Thus, spectra of 5-SASL show the most anisotropy, having resolved extrema both in the wings and in the central region. Such spectra are usually analyzed by an empirical formula describing an "order parameter" (S), which depends on the positions of these extrema (Gaffney, 1976). S depends only on the angular amplitude of the motion of the probe, assuming that the rate of motion is so rapid (the correlation time, τ_r, is less than 10^{-9} s) that an increase in rate (decreased τ_r) has little effect on the positions of extrema. Spectra of 16-SASL in bilayers, on the other hand, appear as narrow three-line spectra due to motional averaging of anisotropy, implying that S is very low (<0.3). Such spectra are most commonly characterized by a correlation time, assuming that the motion is isotropic ($S = 0$), i.e., that only the rate of motion has an effect on the spectrum. In this case, the positions of spectral extrema are not very sensitive to motion, so this effective correlation time, τ_r, is measured from line-height ratios (eq 1). The spectra of 12-SASL are intermediate in line shape and are, therefore, difficult to analyze with either of these models. At low temperatures, the spectra show enough anisotropy so that positions of extrema (and thus the effective order parameter S) are most easily measured, while at high temperatures the anisotropy is no longer resolved and only line heights (and thus, the effective correlation time, τ_r) can be precisely measured. This effectively prevents a reliable Arrhenius analysis with 12-SASL, since neither line positions nor line heights can be used to analyze spectra over the entire temperature range.

Arrhenius plots for the motion of 5-SASL and 16-SASL are shown in Figure 4. For each probe, there was no break in the Arrhenius plot in either SR membranes or vesicles of extracted SR lipids, indicating that there was no change in the temperature dependence of hydrocarbon chain mobility, either as an intrinsic lipid property or as a reflection of a changing protein conformation. Only the parameter τ_r^{-1}, measured to describe the motion of the 16-SASL spin-label, can be directly interpreted as a rate, and therefore, Arrhenius activation energies can only be meaningfully calculated for this probe. The activation energies for SR and SR lipid are 4.5 ± 0.1 and 4.2 ± 0.1 kcal mol^{-1} deg^{-1}, respectively.

Temperature Dependence of Protein Mobility. Protein mobility was measured by ST-EPR. Figure 5 shows conventional (V_1) and ST-EPR (V_2') spectra of one sample of spin-labeled Ca-ATPase, whose spectra were recorded sequentially at the various temperatures indicated in the figure. There is no hysteresis, as shown by the reproducibility of the 4 °C spectrum at the bottom of Figure 5.

Conventional EPR spectra (V_1) are sensitive to nanosecond molecular motion (Thomas, 1978) and have been used primarily for the study of nanosecond lipid dynamics. These spectra are useful as a control in the study of protein rotational motion since they measure nanosecond probe mobility, which can interfere with the detection of slower motions by ST-EPR. The V_1 spectra in Figure 5 are very similar. Spectral subtraction and double integration (see Materials and Methods) show that 96–99% of the probes are strongly immobilized ($\tau_r \geq 10^{-8}$ s). These spectra indicate that there is no temperature-dependent variation in the moderately slow (10^{-8} s $\leq \tau_r \leq 10^{-6}$ s) or highly restricted ($S \geq 0.8$) motions, probably corresponding to large-scale internal protein motions, since the low-field line-width (Δ_L, the low-field line width, half-width at half-maximum; Mason & Freed, 1974) is temperature-independent, having a value of 3.2 ± 0.2 G over the entire temperature range. Only the spectral contribution from weakly immobilized ($\tau_r < 10^{-8}$ s) probes, observable as the second peak in the low-field region of the V_1 spectrum, changes as the temperature increases. Although this weakly immobilized component remains a very small percentage of the total spin probes, it is a potential problem for the analysis of ST-EPR spectra (discussed below).

In contrast to small changes in the V_1 spectra, the V_2' spectra show substantial changes with changing temperature. As the temperature increases, the decrease in spectral intensity and the change in spectral shape are both characteristic of increased microsecond protein mobility, probably corresponding to rotation of the entire protein with respect to the membrane (Thomas & Hidalgo, 1978; Squier et al., 1985). The Ar-

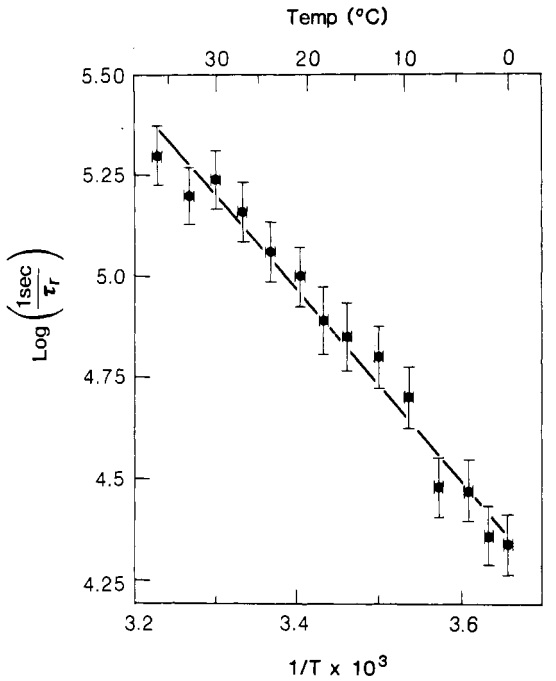

FIGURE 6: Arrhenius plot of overall (microsecond) protein rotational mobility. Rates of motion were plotted as $1/\tau_r$. The τ_r was obtained through the comparison of reference spectra corresponding to isotropic motion. The Arrhenius activation energy was computed to be 10.7 ± 0.5 kcal mol^{-1} deg^{-1} for this data set, with a correlation coefficient of 0.988. When this plot was fit to two lines, the best fit was for a break point near 20 °C but consisted of two slopes with apparent activation energies of 8 ± 1 and 12.2 ± 0.8 kcal mol^{-1} deg^{-1} above and below 20 °C, respectively. The mean correlation coefficient for the two-line fit was 0.973, indicating that the single-line fit was superior. Similar results were obtained on six other data sets with a mean activation energy for protein rotational motion of 11.2 ± 0.5 kcal mol^{-1} deg^{-1}.

rhenius plot of protein mobility was linear for each of the seven preparations studied (example shown in Figure 6). The mean apparent activation energy from these seven preparations was 11.2 ± 0.5 kcal mol^{-1} deg^{-1}.

Discussion

Summary of Results. The primary question in this study is whether the Arrhenius break in enzymatic activity correlates with a temperature-dependent change in the dynamic properties of the lipid and/or the Ca-ATPase in SR. Arrhenius plots of lipid hydrocarbon chain mobility, as measured by spin-labeled fatty acids either near the lipid's polar head region or at the center of the bilayer, show no breaks (Figure 3), regardless of whether this mobility is measured in the presence or the absence of the Ca-ATPase. In addition, the rate of overall rotational mobility of the protein increases with temperature and lipid chain fluidity, yielding a linear Arrhenius plot with an activation energy that matches that of the ATPase activity above 20 °C (Figure 6).

Relationship to Other Studies of Lipid Chain Dynamics and Protein Mobility in SR. By comparing the lipid hydrocarbon chain dynamics of native SR to those of extracted SR lipids, we have been able to assess the influence of the protein on lipid dynamics, avoiding the difficult data interpretation inherent in studies of the Ca-ATPase reconstituted into exogenous lipids. Although many studies in reconstituted and/or partially delipidated systems derived from SR have attempted to resolve intrinsic lipid properties from those of protein (Lee et al., 1974; Nakamura et al., 1976; Hidalgo et al., 1976, 1978; Anzai et al., 1978; Kaizu et al., 1980; Higashi & Kirino, 1983), they suffer complications arising from the use of detergents in their preparation, which can cause inversion of transmembrane proteins with respect to their native orientation in the bilayer. In addition, variability in the extent of endogenous lipid replacement and protein aggregation is a common characteristic of these preparations. Freeze–fracture electron micrographs of the Ca-ATPase reconstituted into DMPC and DPPC (Kleeman & McConnell, 1976; Gomez-Fernandez et al., 1980) show protein-rich patches at temperatures below the lipid phase transition and a uniform distribution of protein at temperatures above the lipid phase transition, indicating that the protein is excluded by bulk lipids in the gel state and suggesting that exogenous lipids often do not accommodate the protein in the same manner as do endogenous SR lipids, limiting the usefulness of reconstituted systems for the study of lipid–protein interactions.

In our measurements of lipid dynamics, we have chosen spin-labels that probe various bilayer depths and have well-defined positions in the bilayer (Seelig & Hasselbach, 1971; Moules et al., 1982). This is in contrast with some of the fluorescent probes of lipid dynamics, frequently used in temperature-dependence studies of the Ca-ATPase, which lack specificity in probing well-defined positions with respect to the bilayer normal (Yguerabide & Yguerabide, 1985; Jones & Lee, 1985), thereby complicating analysis. In order to avoid artifactual breaks in our Arrhenius plots of lipid fluidity, we have only included data from spin-labels whose line shapes can be reliably analyzed with one spectral parameter and motional model over the entire temperature range studied. This is not always the case for stearic acid spin-labels (e.g., 12-SASL in this system), and this may account for a number of Arrhenius breaks reported for fatty acid spin-labels in SR (Hidalgo et al., 1976; Eletr & Inesi, 1972; Kirino et al., 1981).

Another potential problem preventing a reliable Arrhenius analysis in SR is the presence of two distinguishable motional populations of fatty acid spin-labels, one similar to that obtained from extracted lipid and another less mobile component that is presumably due to contact with the protein (Thomas et al., 1982). Thus, temperature-dependent spectral changes can arise from changes in the fractions of these two components, their exchange rate, changes in the mobility of either component, or artifacts due to the erroneous assumption of a single component. In contrast, vesicles of extracted SR lipids, having a lipid composition identical with that of intact SR membranes (see Table I and Materials and Methods), provide a more homogeneous environment for the spin-label, making the analysis in terms of a single correlation time or order parameter much more valid and resulting in a less ambiguous Arrhenius analysis. Thus, the most reliable characterization of the temperature-dependence of SR lipid chain dynamics comes from the Arrhenius plots of 5-SASL and 16-SASL in extracted SR lipids (Figure 4).

As in previous studies by us and others, the overall rotational mobility of the protein was measured with MSL attached covalently and specifically to the Ca-ATPase (Hidalgo & Thomas, 1977), maintaining enzymatic function. Unlike previous studies, we used a 37 °C preincubation treatment after spin-labeling, thus avoiding the hysteresis observed in EPR spectra due to loss of spins at elevated temperatures (King & Quinn, 1983), which has been a problem in previous studies of the temperature dependence of MSL-labeled Ca-ATPase (Kirino et al., 1978; Kaizu et al., 1980; Higashi & Kirino, 1983), probably rendering those results unreliable (Thomas, 1985a). We have analyzed protein rotational mobility by using the V_2' integral, an intensity parameter that eliminates errors due to the line-shape distortion caused by

weakly immobilized probes. The artifactual decrease in the temperature dependence of line-shape parameters above 20 °C has probably been the cause of artifactual Arrhenius breaks in apparent protein mobility (Kirino et al., 1978; Kaizu et al., 1980; Thomas, 1985a).

The linear Arrhenius plot of protein mobility (Figure 6) supports the lipid spin-label result that there is no abrupt change in the temperature dependence of SR lipid chain mobility over the temperature range from 0 to 37 °C. If the lipid fluidity were to change abruptly at a particular temperature, whether due to an increase in the overall bulk fluidity of the bilayer or due to a change in the fraction of fluid lipid, then the overall protein mobility would be expected to reflect this change, since overall protein mobility is predicted to be proportional to lipid fluidity (Saffman & Delbrück, 1975). An Arrhenius plot of protein mobility would, under such conditions, be expected to be nonlinear. The correlation between enzyme activity and protein mobility, whenever we have perturbed protein mobility (Thomas & Hidalgo, 1978; Hidalgo et al., 1978; Thomas et al., 1982; Bigelow & Thomas, 1985; Squier et al., 1985), suggests that overall protein mobility may be essential to the enzymatic mechanism. The agreement between the activation energies of protein mobility and of ATPase activity above 20 °C (Figures 2 and 6) further supports this hypothesis.

The rotational mobility of a cylindrical membrane protein is predicted to be proportional to both the lipid fluidity and the surface area of protein in contact with lipid (Saffman & Delbrück, 1975). Although this theory refers to a perfect cylindrically shaped protein, irregularly shaped membrane proteins have been shown to obey it reasonably well (Peters & Cherry, 1982), and the Ca-ATPase rotational mobility increases with cross-linking as predicted by this theory (Squier et al., 1985). Thus, the observation of linear Arrhenius plots for both lipid and protein mobility suggests that there is no abrupt temperature-dependent change in the shape or size of the rotating unit. Thus, our results argue against models in which the resting enzyme (no ATP) undergoes an abrupt temperature-dependent change in either the protein's large-scale conformation or the extent of protein–protein association (Hoffman et al., 1979; Kirino et al., 1978; Kaizu et al., 1980; King & Quinn, 1983).

Changes in the apparent activation energies from Arrhenius plots of protein rotational mobility have been observed in transient optical anisotropy studies (Hoffman et al., 1979; Burkli & Cherry, 1981; Spiers et al., 1983). However, all of these studies employed nonaqueous solvents (e.g., glycerol) to slow vesicle tumbling (Hoffman et al., 1979; Burkli & Cherry, 1981; Spiers et al., 1983). These solvents have been shown to inactivate the Ca-ATPase (deMeis et al., 1980; Squier et al., 1985) and produce protein aggregates in electron micrographs (Van Winkle et al., 1985).

Alternative Interpretations. Although our results, obtained with both lipid and protein probes, rule out an Arrhenius break in bulk lipid hydrocarbon mobility and argue against a large-scale change in protein structure or association, we cannot exclude the possibility that more subtle changes in lipid and protein occur at 20 °C that are not detected by our probes, e.g., a change localized primarily to lipid head groups, a change in a small fraction of the lipids, and/or a very localized change in the protein.

In particular, some lipid head group structures have been related to the efficiency of Ca transport, which changes markedly around 20 °C (Ikemoto, 1974; Sumida & Tonomura, 1974). The presence of phosphatidylethanolamine (PE) and other lipids that readily form the nonlamellar, reverse hexagonal (H_{II}) phase, have been reported to improve coupling efficiency (ratio of calcium transported/ATP hydrolyzed) of the Ca-ATPase (Hidalgo et al., 1982; Navarro et al., 1984). A structural change restricted primarily to PE might be involved in the observed change in slope in Arrhenius plots of enzymatic activity. Such a change would be difficult to detect with our probes because (a) EPR spectra of lipid spin-labels display only small line-shape changes that correspond to H_{II} formation of pure PE bilayers (Hardman, 1982) and (b) PE represents less than 20% of the total SR lipids.

Even if changes do occur in hydrocarbon chain mobility, they may be localized primarily to the boundary lipids, i.e., the lipids interacting directly with the Ca-ATPase. In this case, too, our measurements may be insensitive to such changes, not only because they are a small fraction of the total SR lipids (35%; Thomas et al., 1982) but also because the boundary lipid hydrocarbon chains are less mobile, rendering V_1 spectra less sensitive to changes in mobility (Thomas et al., 1982). Nevertheless, it seems unlikely that such a change could occur without a detectable effect on overall protein mobility (Figure 6).

Local conformational changes within the protein might not be detected by our protein spin-labels. Changes in the temperature dependence of several properties have been reported to occur near 20 °C, as measured by nanosecond mobilities of spin-labels attached to sulfhydryl groups (Inesi et al., 1973), hydrogen–deuterium exchange kinetics (Kirino et al., 1977; Anzai et al., 1978), and laser Raman spectroscopy (Lippert et al., 1981). However, until these internal motions are more clearly related to the enzyme's structure, their role in the enzyme mechanism remains unclear.

Relationship to Enzyme Kinetics. Transient enzyme kinetic studies of the Ca-ATPase have demonstrated that there is no change near 20 °C in the activation energy of any single step in the ATPase cycle (de Meis et al., 1982; Pierce et al., 1983). This underscores the present conclusion that the nonlinearity of Figure 2 is probably not due to an abrupt change in protein or lipid physical state near 20 °C. It is beyond the scope of the present work to determine the molecular or mechanistic basis of the nonlinearity in Figure 2, but a plausible speculation is that it corresponds to a change in the enzyme mechanism, from one rate-limiting step below 20 °C to another above 20 °C. In that case, an abrupt change in slope in Figure 2 would not be predicted, given the lack of an abrupt change in any single step. It is clear that a number of functional forms could fit the data in Figure 2 besides the arbitrarily chosen pair of intersecting lines, including the kind of curvilinear graph that can result when more than one kinetic step contributes to the overall reaction rate (Han, 1972; Silvius & McElhaney, 1981).

Our results do not directly address the possibility of temperature-dependent changes of the protein dynamics during enzyme action, since all EPR studies were conducted on the resting enzyme, with no substrate present. Recent experiments suggest that phosphoenzyme formation may increase protein–protein interactions and that under phosphorylating conditions this association is temperature-dependent (Squier et al., 1985). Thus, the temperature-dependent change in the activation energy (Figure 2) could involve a change in the kinetic step involving phosphoenzyme decomposition.

The agreement between the apparent activation energies of enzymatic activity and overall protein mobility above 20 °C could be coincidental, but in combination with the other evidence cited above for the correlation between activity and protein mobility, this agreement suggests that protein mobility

may be essential to the rate-limiting step at physiological temperatures. Of course, a detailed description of essential protein motions is not yet possible. Although the protein mobility measured in this study is rotational, lateral protein mobility is likely to have the same temperature dependence (Saffman & Delbrück, 1975), so the possible importance of both rotational and lateral motion should be considered. For example, both types of protein motion would probably be important to a reaction mechanism involving specific protein–protein associations and dissociations: lateral mobility modulates the proximity of interacting proteins, and rotational mobility decreases orientational constraints that might have to be overcome to allow successful (functional) associations.

Summary and Future Prospects. The present study adds to the evidence that protein mobility is important to enzymatic activity in SR; the agreement in activation energies for enzymatic activity and protein rotational mobility, for temperatures above 20 °C, suggests a requirement for dynamic protein–protein interactions in the rate-limiting step of active Ca transport. However, the change in the apparent activation energy for Ca-ATPase activity near 20 °C is not accompanied by a comparable change for either the bulk lipid chain mobility or overall protein rotational mobility, at least in the absence of ATP. Thus, future attempts to explain the Arrhenius break should focus on the possibilities of either (a) a localized structural change, e.g., at the enzyme's active site or the protein–lipid interface, or (b) a change in protein structure or protein–protein associations that occurs only in the presence of substrate and/or other ligands. Physical studies on different biochemical states will be important in understanding this temperature-dependent change in the activation energy of the Ca-ATPase.

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