Rotational Dynamics of Lipid and the Ca-ATPase in Sarcoplasmic Reticulum

THE MOLECULAR BASIS OF ACTIVATION BY DIETHYL ETHER*

Diana J. Bigelow and David D. Thomas

From the Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455

We have investigated the role of lipid and protein dynamics in the activation of the Ca**+**-dependent ATPase in sarcoplasmic reticulum (SR) by diethyl ether. Conventional and saturation-transfer electron paramagnetic resonance (EPR) were used to probe rotational motions of spin labels attached either to fatty acid hydrocarbon chains or to the Ca-ATPase in SR. We confirm previous studies (Salama, G., and Scarpa, A. (1980) J. Biol. Chem. 255, 6525-6528; Salama, G., and Scarpa, A. (1983) Biochem. Pharmacol. 32, 3465-3477; Kidd, A., Scales, D., and Inesi, G. (1981) Biochem. Biophys. Acta 65, 124-131) reporting that addition of diethyl ether to SR results in an approximately 2-fold enzymatic activation, without loss of coupling. Diethyl ether progressively fluidizes the SR membrane with respect to lipid hydrocarbon chain dynamics probed at several depths in the bilayer. Digital subtractions, used to analyze two-component lipid spin label spectra, reveal that a 2-fold mobilization occurs in the population of lipid probes motionally restricted by the protein, while the remaining more mobile population is less affected. The microwave saturation properties of lipid probes also indicate that restricted motions of these probes are mobilized in maximally activated SR membranes. Saturation-transfer EPR, applied to maleimide spin-labeled Ca-ATPase, demonstrates that a 2-fold increase in microsecond rotational motion of the Ca-ATPase correlates with the maximal enzymatic activity. Effects of diethyl ether on both the enzymatic activity and molecular dynamics are completely reversible by dilution with buffer. We propose that ether activates by selectively mobilizing lipid chains adjacent to the enzyme, thus facilitating protein motions that are essential for calcium transport.

In an effort to understand the role of molecular motions in enzyme mechanisms, we perturb the enzyme physically, correlating change in molecular dynamics with changes in enzymatic activity. An especially intriguing means of perturbing the function, and presumably the molecular dynamics, of the Ca-ATPase of sarcoplasmic reticulum (SR), is via the addition of diethyl ether. Two laboratories have reported that the addition of 5-7% diethyl ether (v/v) to dilute SR (0.1-2.0 mg of SR protein/ml) results in a 2-fold enzymatic activation without loss of coupling (Salama and Scarpa, 1980, 1983; Kidd et al., 1981). Ether also stimulates Ca**+** efflux coupled to ATP synthesis through reversal of the Ca**+** pump. Rapid quench experiments show that ether treatment of SR results in an increased enzyme turnover without affecting the concentration of the phosphorylated enzyme intermediate, indicating that ether does not activate by recruitment of ATPase pump units. No ultrastructural damage to SR vesicles from ether treatment is observed by ether negatively stained or freeze-fracture electron micrographs (Kidd et al., 1981). Ether activation was observed to be fully reversible by a gentle centrifugation procedure, after which enzymatic activity could be stimulated again upon readdition of diethyl ether (Salama and Scarpa, 1983). In addition, this effect seemed to be specific to diethyl ether, since several other ether compounds tested showed no significant activation.

The present study is an investigation of the underlying molecular dynamics of diethyl ether's activation of the Ca-ATPase. We have used conventional and saturation-transfer EPR (ST-EPR) of nitroxide spin labels, which provide a sensitive measure of the rotational dynamics of both the Ca-ATPase and lipids in SR. Previous studies of molecular dynamics in SR, under various conditions that inhibit the enzyme's function, have shown a consistent correlation of protein mobility (modulated by lipid fluidity) with enzymatic activity (Hidalgo et al., 1978; Thomas and Hidalgo, 1978; Thomas et al., 1982). Therefore, we have extended these studies by investigating this relationship under conditions that activate the Ca-ATPase.

**EXPERIMENTAL PROCEDURES**

**Biochemical 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 lipids were stored in chloroform/methanol (2:1) and stored at -20°C. Liposomes were prepared by drying an aliquot of extracted lipid under nitrogen, then vortex mixing in 0.3 M sucrose, 20 mM MOPS (pH 7.0).

The molar ratio of phospholipids per Ca-ATPase was determined by dividing the molar concentration of total lipids, determined from phosphorus assays (Chen et al., 1956), by that of the Ca-ATPase. The latter was determined by dividing the protein concentration by a molecular weight of 115 kilodaltons 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

*This work was supported by National Institutes of Health Grant GM 27906 and by an Established Investigatorship (to D. D. T.) from the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: SR, sarcoplasmic reticulum; \( \Delta \), half-width at half-height of the low-field peak; MOPS, 3-(N-morpholino)propanesulfonic acid; MSL, maleimide spin label; PESL, phosphatidylethanolamine spin label; S, order parameter; SASL, stearic acid spin label; \( S_i \), in-phase saturation parameter; \( \tau_r \), effective correlation time; \( V_i \), conventional EPR spectrum; \( V_s \), saturation-transfer EPR (ST-EPR) spectrum; \( T_{11} \), outer extrema of conventional EPR spectrum.
typically has about 80 mol of phospholipid/mol of Ca-ATPase. Cholesterol was assayed by the method of Alain et al. (1979).

**Enzymatic Assays—Ca\(^{2+}\)-dependent ATPase activity was measured in a solution containing 0.05 mg of protein/ml, 60 mM KCl, 6 mM MgCl\(_2\), 25 mM MOPS (pH 7.0), and either 0.1 mM CaCl\(_2\) or 2 mM EGTA; 2 mM A23187 or 5 mM oxalate were added in some cases. The reaction was stopped 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). Activity assayed in the presence of EGTA (fossil activity) was subtracted from that assayed in the presence of CaCl\(_2\) (total Ca-ATPase activity) in order to obtain Ca\(^{2+}\)-dependent ATPase activity. Protein concentrations were determined by the biuret method, using bovine serum albumin as a standard.

Ca\(^{2+}\) transport was measured spectrophotometrically, using the differential absorbance of Arsenazo III, a calcium-sensitive dye, as an indicator of extravesicular calcium concentration (Salama and Scarpas, 1983). Reaction conditions were 0.7 mg of protein/ml, 0.1 mM KCl, 10 mM MgCl\(_2\), 10 mM MOPS (pH 7.0), 0.1 mM CaCl\(_2\), and 0.1 mM Arsenazo III, in the presence or absence of 5% (v/v) diethyl ether, at 22°C. 330 mM ATP was added to start the reaction. Spectra were recorded sequentially each second, with 1.0 s integration time on a Hewlett Packard 8451A diode array spectrophotometer. Time-dependent absorbance changes of Arsenazo III at 675 and 685 nm were calculated from stored spectra.

**Gel Electrophoresis—** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970), using both 7.5% acrylamide gels with 3% stacking gels and 2.2% gels without a stacking gel. The former were standardized to resolve the Ca-ATPase of the sarcoplasmic reticulum protein, presumably phosphorylase b (Pickart and 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. Such gels indicated that 80 ± 5% of the proteins in our SR preparation migrated as a 100-kilodalton band.

**Ether Treatment—** Ether was pipetted underneath the surface of the membrane suspension and immediately mixed by inverting the covered reaction vessel five times. Assays were performed in open vials; it has been shown that no significant changes in ether concentration occur within the time (5 min) required for the assay (Salama et al., 1979). Ether was centrifuged for 90 min at 18,500 × g, at 22°C. 330 PM ATP was added to start the reaction. Spectra of the two slopes was greater than the sum of their standard deviations, indicating the presence of lipids, cholesterol, or protein, while Ca\(^{2+}\) transport and ATPase activity were restored to control values.

**Arrhenius Analysis—** Lines and breakpoints in Arrhenius plots were fit using linear regression least-squares analyses. The data was fit to a straight line giving 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 plot is \(\frac{-E}{R}\) for the activation energy and \(R\) is the gas constant.

**EPR Spectroscopy**

Spin Labeling—** Hydrocarbon chain rotational mobility was measured with fatty acid spin labels, N-oxyl-4'-4',4'-dimethylxazolidine derivatives of stearic acid (Fig. 1), which are designated 5- and 16-SASL (Aldrich). A similar derivative of phosphatidylethanolamine, labeled at the 14 position of the fatty acyl chain (stearic acid) in the \(n\)2 position, 5- and 16-SASL (stearic acid spin label) are N-oxyl-4'-4',4'-dimethylxazolidine derivatives of stearic acid labeled at the 5 and 16 positions, respectively, of the fatty acyl chain.

![MSL](image)

**Fig. 1. Spin labels used in this study.** MSL (maleimide spin label) = N-1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmaleimide. 14-PESL (phosphatidylethanolamine spin label) is an N-oxyl-4'-4',4'-dimethylxazolidine derivative of phosphatidylethanolamine, labeled at the 14 position of the fatty acyl chain (stearic acid) in the \(n\)2 position. 5- and 16-SASL (stearic acid spin label) are N-oxyl-4'-4',4'-dimethylxazolidine derivatives of stearic acid labeled at the 5 and 16 positions, respectively, of the fatty acyl chain.

(pH 7.0). Both Ca\(^{2+}\) uptake and ATPase activity were unaffected by this procedure. To monitor the rotational motion of the Ca-ATPase protein, SR was labeled with a maleimide spin label (MSL) (Fig. 1), N-1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl maleimide (Aldrich), as described previously (Bigelow et al., 1986).

**EPR Spectroscopy—** EPR spectra were obtained with a Varian E-109 spectrometer (Varian Associates) as described previously (Thomas and Hidalgo, 1978; Squire and Thomas, 1986a) and spectra were digitized and analyzed with a microcomputer (Northstar Co.) interfaced to the spectrometer (Lipscomb and Salo, 1983). Submicrowave saturation-transfer EPR (second harmonic absorption out of phase, designated Vi) by 50-kHz field modulation (with a modulation amplitude of 5 G) and a microwave field intensity of 0.25 G (Hyde and Thomas, 1980). Submillisecond rotational motion of the protein was detected by saturation-transfer EPR (second harmonic absorption in-phase, designated V1) by using 100-kHz field modulation (with a modulation amplitude of 2 G) and a microwave field intensity of 0.14 G. Submillisecond rotational motions of lipids were detected by saturation-transfer EPR from the first harmonic absorption in-phase, designated V2, using 50-kHz field modulation (with a modulation amplitude of 5 G) and a microwave field intensity of 0.25 G (Squire and Thomas, 1986a). The accurate and reproducible setting of the microwave intensity incident on the sample (\(V_1\)) required a correction for the dielectric loss of the sample. This was done by measuring and comparing the cavity 
Q for the sample with that of a standard of known saturation properties (Fajer and Marsh, 1982). All saturation studies were performed in the absence of oxygen. Oxygen was removed from reference and experimental samples using gas-permeable sample cells purged with \(N_2\) (Popp and Hyde, 1981). Ether did not evaporate from these sample holders over the time course of a ST-EPR experiment, as ascertained by the lack of changes in conventional EPR spectra of 16-SASL in ether-treated SR recorded sequentially over a 4-h time period.

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 \(V_1\) spectra with the number obtained for a 0.1 mM MSL standard, whose \(V_1\) spectrum had been digitized and double integrated in the same manner. Conventional and ST-EPR (\(V_1\)) spectra were all normalized to the same number of spins, by dividing each spectrum by a number proportional to the double integral of the \(V_1\) spectrum.

**Spectral Analysis—** Conventional EPR—Fatty acid spin label spectra were evaluated by several methods. For \(V_1\) 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, \(r\), (effective correlation time) is calculated from the following formula (Keith et al., 1970):

\[
r = 6.5 \times 10^{-10} W_0/(h_0h_1/n) - 1
\]

where \(W_0\) is the line width of the midfield line and \(h_0\) and \(h_1\) are the peak-to-peak line heights in the mid- and high-field regions of the spectrum, respectively. Spectra having sufficiently resolved extremes were analyzed by measuring the effective order parameter \(S\) (Gaffney,
were obtained from a calibration plot of the effective order parameter solvents of known viscosity (Squier et al., 1986).

phospholipid spin labels in SR membranes; they are: Measurements of membrane viscosity for the calculation of effective correlation times with the case of two-site exchange in magnetic resonance. Two methods were used to estimate the rotational correlation time of the restricted motional component in the spectrum of fatty acid and phospholipid spin labels in SR membranes; they are:

\[ \tau_r = 5.4 \times 10^{-10} \text{s} \left(1 - \frac{T_1}{T_2}\right)^{-1.30}, \]

(2)

\[ \tau_r = 1.15 \times 10^{-8} \text{s} \left(\Delta T/\Delta T_0 - 1\right)^{-0.85}, \]

(3)

where, \( T_1 \) and \( \Delta T \) are outer extrema and half-width at half-height of the low-field peak, respectively, of the experimental spectrum; \( T_2 \) and \( \Delta T_0 \) are outer extrema and half-width at half-height of the low-field peak, respectively, of a rigid limit powder spectrum. The rigid limit powder spectrum was obtained from 14-PESL in a solution of ether/pentane/ethanol (1:1:3 v/v/v) –120 °C, a solvent mixture found to have the same polarity as biological membranes (Griffith et al., 1974; Ellena et al., 1983). Values of \( T_1 \) and \( \Delta T_0 \) are 33 and 3.0 G, respectively.

**Spectral Analysis: Saturation-transfer EPR**—The effective rotational correlation time for the experimental ST-EPR spectrum of maleimide spin-labeled Ca-ATPase is determined by comparing experimental spectra with reference spectra obtained from isotropically tumbling spin-labeled hemoglobin in solutions of known viscosity (\( \eta \)) and temperature (\( T \)). These spectra are commonly interpreted using either the line-shape (ratio of line heights) or total intensity. In the case of spectra having a weakly immobilized component, it is important to remove the contribution of this interfering signal, which can best be done by using spectral intensity parameters (Evans, 1981; Horvath and Marsh, 1983; Fajer and Marsh, 1983; Squier and Thomas, 1986a, 1986b).

Protein motions (probed by MSL) were measured from the \( V_{II} \) integral, since this parameter provides high signal-to-noise, while adequately suppressing the small weakly immobilized components that are present in \( V_{II} \) spectra of MSL-SR (Squier and Thomas, 1986a, 1986b). Although large amplitude nanosecond motions make only a small contribution to the \( V_{II} \) spectrum of maleimide spin-labeled Ca-ATPase, they are the primary component in the \( V_{II} \) spectrum of spin-labeled phospholipids, making accurate measurement of slower or more restricted motions difficult. Therefore, motions of the less mobile lipid chains were detected by measuring the in-phase intensity parameter derived from \( V_{II} \), designated \( S_{II} \) (Squier and Thomas, 1986a). This parameter is more effective than the \( V_{II} \) integral in rejecting signals from lipid chains undergoing large amplitude nanosecond motions, thus making \( S_{II} \) selective for the lipid hydrocarbon chains restricted by contact with protein (Squier and Thomas, 1986a).

**Spectral Subtractions**—Subtractions were done by computer analysis of digitized spectra using the single component subtraction method of Jost and Griffith (1978a). The bilayer component was approximated by the spin label in aqueous dispersions of extracted SR lipid at a slightly lower temperature in order to match the splittings of the composite spectrum.

**Results**

**Enzymatic Activation by Diethyl Ether**—When Ca\(^{2+}\)-dependent ATPase activity was assayed in dilute solutions of SR, i.e. at protein concentrations of 0.1 mg/ml in the presence of diethyl ether, a progressive increase in activity was observed up to 5% (v/v) ether, followed by a further decrease in activity (Fig. 2A). Maximal activation was approximately 2-fold, in agreement with previous studies (Salama and Scarpa, 1980, 1983; Kidd et al., 1981). Ca\(^{2+}\)-dependent ATPase (basal) activity progressively decreased with increasing amounts of ether in this concentration range but constituted less than 5% of the total ATPase activity thus resulting in no significant effect on Ca\(^{2+}\)-dependent ATPase activity.

Ca-ATPase activity was measured in the presence and absence of the calcium ionophore A23187, in order to assess the effect of ether on vesicle permeability (Fig. 2A). In the absence of ether and the presence of A23187, SR shows a 3-fold activation of ATPase activity due to increased vesicle permeability to calcium. This activation is maintained when increasing amounts of ether are added. Conversely, the activation due to ether is maintained in the presence of A23187, indicating independent mechanisms for each effector, i.e. that ether does not activate the enzyme by increasing vesicle permeability. Similarly, ether-induced activation is maintained in the presence of oxalate (data not shown).

These results are further confirmed by the observation that Ca\(^{2+}\) transport, assayed by the differential absorption of the calcium dyes Arsenazo III, shows a 2-fold activation by 5% diethyl ether over the control sample (data not shown), in agreement with previous studies (Salama and Scarpa, 1980, 1983; Kidd et al., 1981). These results confirm that, under our experimental conditions, ether activation is not a result of increased vehicle permeability.

**EPR measurements require higher protein concentrations than those previously characterized. At concentrations of 40 mg of protein/ml, maximal activation of SR requires the addition of 8% (v/v) ether (Fig. 2B), both in the presence and absence of A23187. Maleimide spin-labeled Ca-ATPase exhibited an ether activation profile identical to that of the unlabeled ATPase.**

**Lipid Dynamics in Ether-activated SR**—Lipid hydrocarbon chain mobility (submicrosecond motions) was measured at two depths in the bilayer from the conventional EPR spectra of fatty acid spin labels (5- and 16-SASL) incorporated into SR membranes in the presence of increasing amounts of ether (Fig. 3). 5-SASL probes near the lipid head groups, while 16-SASL probes near the bilayer center. Spectra of 5-SASL in SR were analyzed by determining effective order parameters (\( S \)), while those of 16-SASL were analyzed by determining effective correlation times (\( \tau_r \)). In the case of each spin label (Fig. 3), increasing amounts of ether resulted in progressive lipid chain mobilization (decreased order parameter or decreased correlation time). However, direct comparisons of the extent of ether’s effect on each spin label are not possible because of differences in spectral anisotropy and consequently in the spectral parameter used to analyze the motion.
order to better assess the influence of the Ca-ATPase protein of SR lipid dynamics, the \( r \), of lipid hydrocarbon chain mobility, as measured by 14-PESL in SR, was compared to that in vesicles of extracted SR lipids. The only difference in composition of extracted lipids from that of SR is the presence of protein in SR (Bigelow et al., 1986). Spectra of lipid hydrocarbon chain mobility in SR contain contributions from two motional populations; the less mobile population probably corresponds to lipids interacting directly with the protein's surface (boundary lipid), whereas the remaining (bulk) lipids are very similar in mobility to extracted lipids (Thomas et al., 1982). Therefore, the measured value of \( r \) in SR is affected by both components (bulk and boundary lipid). Vesicles of extracted SR lipids have only one motional population whose mobility is slightly greater than that of the bulk lipid in intact SR membranes (Thomas et al., 1982). In the case of 14-PESL in both SR and extracted SR lipids (Fig. 5), the \( r \) values indicate progressive mobilization of the lipid hydrocarbon chains with increasing ether. The \( r \) of 14-PESL in maximally activated SR coincides with that in SR lipids in the absence of ether, suggesting that maximal activation by ether correlates with a decrease of the protein's restricting effect on lipid chain dynamics.

This hypothesis is supported by a more detailed examination of spectral line shapes (Fig. 6). The spectrum of 14-PESL in SR at \( 4 \, ^\circ \text{C} \) (Fig. 6A, top) contains two components, as has been shown previously for 16-SASL in SR (Thomas et al., 1982). The primary effect of ether addition is the mobilization of the motionally restricted population of probes; this population is evident in the wings (especially the low-field region) of the spectrum obtained from SR in the absence of ether (Fig. 6A, top). In contrast, the spectrum obtained from ether-activated membranes (Fig. 6B, top) appears quite similar to the one-component spectrum from vesicles of extracted lipids (Fig. 6A, L).

In order to quantitatively evaluate the effects of ether on the two motional populations, these two components were deconvoluted by means of computer subtractions of digitized spectra. Fig. 6A shows this method applied to quantitate the two motional components of 14-PESL in SR. Subtraction was performed using a suitable mobile spectrum obtained from the membrane preparation. Error bars represent the S.E. from triplicate samples.
14-PESL in vesicles of extracted SR lipids at -1 °C (L in Fig. 6A). Subtraction of 60 ± 2% of this spectrum from the composite spectrum (14-PESL in SR at 4 °C) yielded a difference spectrum (D in Fig. 6A) having a broad line shape and an outer splitting (2Tll) of 59 G (Table 1). All spectra are displayed with 100-G scan width. B, spectral subtraction of mobile component of 14-PESL in ether-activated SR. SRem = spectrum of 14-PESL in ether-activated SR at 4 °C. Lm = spectrum of 14-PESL in ether-treated (8%) SR lipids at 0 °C and serves as the mobile component. D = the resulting difference spectrum obtained by subtracting 60 ± 4% of spectrum L from spectrum SRem. This spectrum represents 40% of the total spin concentration and is scaled accordingly with respect to the height of the spectrum SR. D × 4 = the difference spectrum enlarged 4-fold for better resolution of spectral line shape. Lm,4°C = model spectrum for restricted motion obtained from 14-PESL in SR lipids at -17°C. All spectra are displayed with 100-G scan width. L, spectral subtraction of mobile component of 14-PESL in ether-activated SR. SRem = spectrum of 14-PESL in ether-activated SR at 4 °C. Lm = spectrum of 14-PESL in ether-treated (8%) SR lipids at 0 °C and serves as the mobile component. D = the resulting difference spectrum obtained by subtracting 60 ± 6% of spectrum Lm from spectrum SRem. This spectrum represents 42% of the total spin concentration and is scaled accordingly with respect to the height of the spectrum SR. D × 4 = the difference spectrum enlarged 4-fold for better resolution of spectral line shape. L,4°C = model spectrum for restricted motion obtained from 14-PESL in SR lipids at -12 °C. All spectra are displayed with 100-G scan width. SRE, composite spectrum (14-PESL in SR at 4 °C). Subtraction of 60% of this spectrum from the spectrum of 14-PESL in ether-treated (8%) SR lipids at 0 °C (4°C) yielded a difference spectrum with 2Tll of 54 G, matching a model for restricted motion obtained from 14-PESL in SR lipids at -12 °C (Fig. 6B, bottom). Thus, the restricted component represents 42 ± 6% of the composite spectrum, corresponding to 32 ± 2 mol of restricted lipids/mol of Ca-ATPase.

As was observed for 5- and 16-SASL in SR (Fig. 3), increasing the temperature by 10 °C (i.e. to 14 °C) of 14-PESL in SR results in a spectrum (not shown) that is virtually identical to that of ether-activated SR at 4 °C. Spectral subtraction of 60 ± 5% of a suitable mobile spectrum (14-PESL in vesicles of extracted SR lipids at 9 °C) results in a broadly shaped difference spectrum with 2Tll of 54 G, matching the same model for restricted motion used in subtractions of 14-PESL in ether-activated SR at 4 °C (Table I).

Temperatures that yield spectra serving as models for mobile or restricted motion can be taken as a measure of the degree of interaction of the enzyme with the two lipid populations. The interaction of the enzyme with the adjacent (boundary) lipids is equivalent to a temperature decrease in SR lipids of 21 and 16 °C, for control and ether-activated membranes, respectively. The enzyme's interaction with bulk lipids is less, i.e. equivalent to a temperature decrease in SR lipids of 5 and 4 °C, respectively.

Estimations of τr (Table I) for the restricted lipids are most often made by comparison of their spectral parameters, (either 2Tll or Δν) to those from a rigid limit (no motion) spectrum, assuming isotropic motion (Freed, 1976). Estimations from 2Tll yields values for τr of 11.5 and 5.5 ns for immobilized lipids in SR and in ether-activated SR, respectively. Estimation from Δν yields correlation times of 41 and 17 ns, respectively. Both estimates are consistent with a 2-fold mobilization by ether of the lipids restricted at the surface of the protein, correlating well with the enzymatic activation. The disparity in τr values obtained from these two methods of estimation is an indication that the spin label's motion does not entirely fit the model of isotropic motion, as might be expected of lipids in the anisotropic bilayer. Alternatively, analysis of the restricted component by an order parameter (S) takes into consideration this anisotropy by measuring the amplitude of hydrocarbon chain motion. As shown in Table I, restricted lipids in ether-activated SR display a decreased order parameter (increased amplitude) compared with those in untreated membranes. The rate of protein motion in a bilayer is predicted to be inversely proportional to the viscosity (Saffman and Delbruck, 1975), so that these order parameters were converted to viscosity values (Table I), using the method of Squier et al. (1986). Ether activation results in an almost 2-fold decrease in viscosity of the restricted lipid population, again correlating well with the 2-fold activation of enzymatic activity.

The hydrocarbon chains of the mobile component are mobilized in ether-activated membranes, but the effect is less than for the restricted component: correlation times for the mobile components for SR and ether-activated SR are 3.2 ± 0.4 and 2.3 ± 0.3 ns, respectively. These results suggest that ether binds preferentially to the lipid-protein interface. Selective Detection of Restricted Lipid Motion Using Saturation-Transfer EPR—As an alternative to spectral subtractions, in order to selectively probe the motion of the restricted population of lipids, to the exclusion of the more mobile population, the in-phase saturation parameter (Sr) was meas-
ured for 14-PESL in intact SR, ether-activated SR, and vesicles of extracted SR lipids. This parameter selectively detects probe populations having their motion restricted on the microsecond time scale or slower (Squier and Thomas, 1986a). Comparison of the values obtained to calibration curves obtained from spin probes undergoing isotropic motion indicates that 14-PESL in ether-activated SR and vesicles of extracted SR lipids shows no restricted motion, in contrast to 14-PESL in intact SR membranes (Table II) which exhibits motion on the order of microseconds. This result, using a technique that does not require spectral subtraction, confirms that ether mobilizes the restricted lipid component.

**Microsecond Protein Mobility in Ether-activated SR**—Fig. 7 shows conventional ($V_i$) and saturation-transfer ($V_f$) EPR spectra of the maleimide spin-labeled Ca-ATPase of control and ether-activated SR. Conventional EPR spectra, sensitive only to nanosecond probe motion (Thomas, 1978), show little or no evidence for nanosecond motion, and show no effect of ether (Fig. 7, left). The parameter $S_k$ (Mason and Freed, 1974), a sensitive measure of changes of moderately slow ($10^{-9}$ ≤ $\tau$ ≤ $10^{-6}$ s) or highly restricted ($S$ ≤ 0.8) motion, remains the same, i.e. 3.3 ± 0.2 G. Thus, any changes in $V_f$ spectra (discussed below) must be due to changes in slower (microsecond) motion. In contrast ether activation has a significant effect on the $V_f$ spectra (Fig. 7), resulting in both decreased spectral intensity and line shape changes that are characteristic of increased microsecond protein mobility, probably corresponding to rotation of the entire protein with respect to the membrane (Thomas and Hidalgo, 1978; Squier et al., 1985). These effects of ether on microsecond protein mobility were completely reversible by dilution of ether. Quantitation of the effect of progressive amounts of ether on the microsecond rotational motion of the Ca-ATPase (Fig. 8) demonstrates that the enzyme's mobility increases progressively with increasing ether concentration. Maximal activation coincides with a 2-fold stimulation of enzyme mobility, which correlates with the 2-fold stimulation of enzymatic activity. Enzymatic activity does not correlate with protein mobility at higher ether concentrations (Fig. 2), suggesting that the enzyme requires an optimal fluidity of the surrounding lipids.

**Arrhenius Analysis of Ether-activated SR**—In order to compare the effects of temperature and ether on enzymatic activation, Ca-dependent ATPase activity of control and ether-activated SR was analyzed with an Arrhenius plot (Fig. 9). Control SR shows a change in slope at 20 °C, with apparent activation energies of 11.8 ± 0.4 kcal/mol degree and 23 ± 1 kcal/mol degree, above and below 20 °C, respectively. Enzymatic activity of ether-activated SR, plotted as an Arrhenius plot is linear, with an apparent activation energy of 22 ± 1 kcal/mol degree, suggesting that ether alters the rate-limiting step at physiological temperatures. Below 20 °C ether activates without changing the apparent activation energy.

**Table II**

<table>
<thead>
<tr>
<th>Membrane Preparation</th>
<th>$S_i$</th>
<th>$\tau_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact SR (4 °C)</td>
<td>0.445</td>
<td>7.7 μs</td>
</tr>
<tr>
<td>Ether-activated SR (4 °C) (8%)</td>
<td>0.274</td>
<td>≤0.7 μs</td>
</tr>
<tr>
<td>SR lipids (4 °C)</td>
<td>0.270</td>
<td>≤0.7 μs</td>
</tr>
</tbody>
</table>

**Fig. 7.** Conventional ($V_i$) and saturation-transfer ($V_f$) EPR spectra of (A) MSL-labeled Ca-ATPase in SR and in (B) ether-activated SR (8% ether). $V_i$ spectra were scaled to the same center line heights; $V_f$ spectra were normalized to the same spin concentration. Spectra are displayed with 100-G scan widths.

**Fig. 8.** Effect of diethyl ether on microsecond protein mobility in SR, as measured by saturation-transfer EPR ($V_f$) spectra of MSL-Ca-ATPase. Correlation times obtained (see “Experimental Procedures”) were normalized to the control, i.e. no added ether.

**Fig. 9.** Arrhenius plot of Ca$^{2+}$-dependent ATPase activity in SR and in maximally ether-activated (8%) SR. The lines and breakpoint were determined by linear regression least-squares analyses of the experimental data. The correlation coefficients for data from untreated SR are 0.998 and 0.990, above and below 20 °C, respectively. The correlation coefficient for data from ether-activated SR is 0.993.

**DISCUSSION**

**Summary of Results**—In this study, we have explored the molecular mechanism underlying the enzymatic activation of the Ca-ATPase of SR by diethyl ether, using spin labels that provide a sensitive measurement of nanosecond lipid hydrocarbon chain dynamics and microsecond protein mobility. Two-component spectra of lipid chain motion were deconvoluted by spectral subtractions (Fig. 6), permitting analysis of the motional population corresponding to those lipids interacting with the protein surface. These physical measurements were correlated with functional properties. The 2-fold stimulation of Ca-ATPase and transport activity occurs without increased vesicle permeability and is reversible by dilution with buffer, as are the changes in molecular dynamics. The maximal extent of enzymatic activation correlates with a 2-
fold increase in protein mobility, as measured by saturation-transfer EPR (Fig. 8), and in boundary lipid mobility, as measured by analysis of spin-labeled lipid spectra after digital subtraction of a more mobile component (Table I). The temperature dependence of ATPase activity in ether-activated SR demonstrates, by Arrhenius analysis, that the apparent activation energy above treated membranes display a change in slope at 20 °C.

Activation Profile—Results from our enzymatic studies of ether-activated SR agree with previous studies (Kidd et al., 1981; Salama and Scarpa, 1983); activation is not a product of increased vesicle permeability but rather represents an increased turnover rate of the Ca-ATPase. Ether is unusual in its ability to fluidize SR membranes without increasing vesicle permeability or denaturing the enzyme, unlike some other general anesthetics (Diamond and Berman, 1980), local anesthetics (Nash-Adier et al., 1980), n-alcohols (Kondo and Kasai, 1973; Hara and Kasai, 1977), or various detergents (Dean and Tanford, 1978, Murphy et al., 1982). In this respect, ether may prove useful as an exclusive effector of membrane fluidity, in other systems.

The plot of enzyme activity as a function of ether concentration shows a decrease in ATPase activity at ether concentrations greater than 8% (at 40 mg of protein/ml), as shown in Fig. 2B, while protein and lipid hydrocarbon chain mobility (Figs. 3 and 8) continue to increase. These results are unique compared with previous studies on SR in which protein mobility correlates with enzyme activity under many conditions, whenever the molecular dynamics have been perturbed (Hidalgo et al., 1978; Thomas et al., 1982; Squier et al., 1985). Therefore, the findings of this study are consistent with the hypothesis that SR, like several other membrane proteins (Yuli et al., 1981; Saless et al., 1982; Fong and MacNamee, 1985), has an optimal fluidity for optimal functioning. Nevertheless, we cannot rule out a direct solvent effect on the protein that is responsible for decreased activity at high ether concentrations.

Site of Ether's Action—The concentration of ether in maximally activated membranes has been previously determined by gas chromatography to be about 0.5 mol of ether/mol of lipid, equivalent to 40 mol of ether/mol of ATPase (Salama and Scarpa, 1983). Reversibility, by dilution with buffer, of ether's effects on both enzymatic activity and molecular dynamics, indicates that activation does not involve irreversible protein denaturation or the formation of covalent bonds, and ether's solubility properties suggest that it associates with the lipid or the hydrophobic portions of the protein. The extent of fluidization of the bilayer by ether, as monitored by 5- and 16-SASL, was shown to be the same for each spin label (Fig. 3), indicating that ether does not show any localization with respect to bilayer depth, as measured by parameters representing averages from two motional populations of lipids, i.e. boundary and bulk lipids.

However, the observation that ether mobilizes the lipid chains at the lipid-protein interface by a factor of at least two (from estimations of r, from both Δα and 2T0 and of viscosity from S; see Table I), while its effect on bulk lipids is 40% less, argues for ether's preferred binding at the lipid-protein interface. Our results are consistent with reports of lipid spin label motions in mitochondrial ATPase membranes (Lenaz et al., 1978), where fluidizing anesthetics show larger effects when the ATPase protein is present.

Results from Spectral Subtractions—The effects of perturbants on the lipid-protein interface can be measured qualitatively by comparing measurements of lipid spin label motion in SR membranes with those in aqueous dispersions of extracted SR lipids, but these measurements in SR only yield average correlation times. Spectral subtractions (Fig. 6) allow more precise characterization, i.e. quantitation of the proportions of each motional population contributing to the spectrum, and its motional characteristics, i.e. correlation times. Despite the subjectivity of this technique, it has provided consistent results in a variety of systems (Jost and Griffith, 1978b; Andersen et al., 1981; Marsh et al., 1982; Thomas et al., 1982; Silvius et al., 1984).

Fraction of the Restricted Component—The value (30 ± 2) of immobilized lipids/Ca-ATPase, as calculated from the spectrum of 14-PESL in SR, is slightly higher than previous findings in this laboratory (24; Thomas et al., 1982), but agrees with other reported results in SR (East et al., 1985). In the case of other membrane enzymes (Jost et al., 1973; Knowles et al., 1979; Watts et al., 1979), the number of immobilized lipids/enzyme is consistent with the model that these lipids coat each protein, i.e. the number of immobilized lipids/protein scales with the square root of the protein's molecular weight. Assuming the same scale factor for the Ca-ATPase, this predicted value would be 42, considerably greater than the value obtained in any of these studies. The smaller apparent ratio of protein surface to molecular weight may be due to an oligomeric protein structure (Thomas et al., 1982a), but not enough is known about the structure of the Ca-ATPase to accurately estimate the surface area of the lipid-protein interface.

No alteration in the percentage of the restricted component occurs in the presence of maximally activating concentrations of ether, indicating that the surface area of the protein in contact with the lipid is not affected. Thus, ether does not induce a large-scale conformational change in that portion of the resting enzyme that is in contact with the lipid bilayer.

Subtractions done at 4 °C and 14 °C demonstrate a lack of temperature dependence in the amount of restricted component, in agreement with findings from similar lipid spectral subtractions in other systems (Watts et al., 1979). Such subtractions could not be extended to temperatures above 15 °C due to the difficulty of matching sharp spectra, a common limitation in spectral subtractions (Jost and Griffith, 1978a).

Motional Characteristics of the Restricted Component—As previously reported for lipid spin labels in contact with other membrane enzymes, the motionally restricted component in the spectrum of 14-PESL in SR exhibits both a sharper line shape and a smaller splitting than that of the rigid limit spectrum (66 G), indicating that the 14-PESL in contact with the Ca-ATPase is not rigidly immobilized, but exhibits some motion on the nanosecond time scale and some disorder (Brotherus et al., 1980; Silvius et al., 1984; Marsh, 1986). Analysis of the motional characteristics of the restricted component shows that ether activation correlates with a 2-fold increase in rate or decrease in order (or a combination of the two; Table I).

Arrhenius Analysis—Previous work in this laboratory (Bigelow et al., 1986; Squier et al., 1986) has shown that overall protein rotational mobility and lipid fluidity have the same activation energies as does enzymatic activity at physiological temperatures (>20 °C), suggesting that the mobility of the Ca-ATPase, modulated by lipid fluidity, is essential to the rate-limiting step. Ether activation, which increases lipid and protein mobility, alters the apparent activation energy of enzymatic activity at physiological temperatures (Fig. 9), probably because the rate-limiting step changes. Below 20 °C, ether stimulates the enzymatic activity but does not change.
the apparent activation energy, (i.e. the rate-limiting step). These results suggest that the step that is rate limiting above 20 °C, in the absence of ether, requires lipid and protein mobility, while ether accelerates this step to the point that another step becomes rate limiting, probably the same step that is rate limiting below 20 °C.

Implications for the Molecular Mechanism of Calcium Transport—The ether-enhanced mobility of lipids immediately surrounding the Ca-ATPase probably facilitates rotational and translational protein motions necessary for calcium transport. These may be large-scale protein motions affecting the entire protein (and detected by our ST-EPR measurements) or more localized motions affecting the internal conformation of the protein. Other studies of protein dynamics in SR have provided evidence that changes in protein-protein association may be essential for the reaction cycle (Squier et al., 1985, 1986). Therefore, ether may activate by decreasing the strength of protein-lipid interactions, thus decreasing the tendency of lipids to act as steric constraints to protein-protein contacts. However, some other conditions that alter protein-protein interactions, i.e. cross-linking of ATPase polypeptide chains (Squier et al., 1985) and vanadate-induced crystallization (Lewis and Thomas, 1986), exhibit no effect on the boundary lipids. A more precise description of the mechanism of ether's activation of the Ca-ATPase will depend on higher resolution structural information, coupled with further probes of molecular dynamics and enzyme kinetics.

Acknowledgments—We are grateful to Cecilia Hidalgo and to Carl Polnasek for many helpful discussions and to Dani Vinh and Scott Lewis for technical assistance. We thank John Lipscomb for making the spectrophotometer and EPR spectrometer available. We thank Anthony Watts for the generous gift of phospholipid spin labels.

REFERENCES

13, 510–518