ROTATIONAL DYNAMICS OF PROTEIN AND BOUNDARY LIPID IN SARCOPLASMIC RETICULUM MEMBRANE

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ABSTRACT We have used spin labels and electron paramagnetic resonance (EPR) to study the correlation between the rotational dynamics of protein and lipid in sarcoplasmic reticulum (SR) membranes. A short-chain maleimide spin label was used to monitor the submillisecond rotational mobility of the Ca-ATPase enzyme (using saturation transfer EPR); a free fatty acid spin label was used to monitor the submicrosecond rotational mobility of the bulk lipid hydrocarbon chains (using conventional EPR); and a fatty acid spin label derivative (long-chain maleimide) attached to the enzyme was used to monitor the mobility of hydrocarbon chains adjacent to the protein (i.e., boundary lipid). In the native SR membranes, the protein was highly mobile (effective correlation time 50 μs). The spectra of the hydrocarbon probes both contained at least two components. For the unattached probe, the major component indicated nearly as much mobility as in the absence of protein (effective rotational correlation time 3 ns), while a minor component, corresponding to 25–30% of the total signal, indicated strong immobilization (effective correlation time $\geq$ 10 ns). For the attached hydrocarbon probe, the major component ($\sim$ 70% of the total) was strongly immobilized, and the mobile component was less mobile than that of the unattached probe. When the lipid-to-protein ratio was reduced 55% by treatment with deoxycholate, protein mobility decreased considerably, suggesting protein aggregation. A concomitant increase was observed in the fraction of immobilized spin labels for both the free and attached hydrocarbon probes. The observed hydrocarbon immobilization probably arises in part from immobilization at the protein-lipid boundary, but protein-protein interactions that trap hydrocarbon chains may also contribute. When protein aggregation was induced by glutaraldehyde crosslinking, submillisecond protein mobility was eliminated, but there was no effect on either hydrocarbon probe. Thus protein aggregation does not necessarily cause hydrocarbon chain immobilization.

INTRODUCTION

The crucial role of phospholipids in the function of the Ca-ATPase (calcium pump) of sarcoplasmic reticulum (SR) has been intensively investigated for the past several years (reviewed by Bennett et al., 1980). For optimal function, the enzyme appears to require a minimum of ~30 phospholipids/protein molecule (Warren et al., 1974), and a fluid lipid environment (Hidalgo et al., 1976; Nakamura et al., 1976; Hesketh et al., 1976).

In SR and other membrane systems, it is clear that the direct measurement of molecular motions is essential for understanding protein-lipid interactions. Accordingly, motion-sensitive spectroscopic probes, especially lipid spin labels, have played a major role in this field. Jost et al. (1973), in a study on lipid spin labels in cytochrome oxidase-containing membranes, reported a protein-dependent strongly immobilized component (effective correlation time $\geq$ 10 ns) in EPR spectra, in addition to the highly mobile component observed in the absence of protein. They proposed that a single boundary layer of relatively immobile lipid surrounds each cytochrome oxidase molecule. Similar data were obtained by Hesketh et al. (1976) on a preparation in which the Ca-ATPase was inserted into a membrane of dipalmitoylphosphatidylcholine (DPPC). These workers proposed that the Ca-ATPase is coated with a single annular layer of lipid that determines the activity. However, an alternative explanation for the observed lipid immobilization is that lipids could be immobilized due to trapping between associated protein molecules (Chapman et al., 1977; Favre et al., 1979; Davoust et al., 1980; Swanson et al., 1980). In the case of rhodopsin, this controversy appears to have been resolved in favor of the immobilized boundary layer model (Watts et al., 1981).

In the study of lipid-protein interactions, it is essential to probe the protein motions as well as the lipid motions. In

addition to providing information about the relationship between protein mobility and function, such measurements are useful as a probe of the aggregation state of proteins, which would be expected to correlate with lipid trapping.

We have previously carried out studies on SR, using spin labels to probe either lipid or protein motions (Thomas and Hidalgo, 1978; Hidalgo et al., 1978). A key aspect of those studies was the use of saturation transfer EPR, a technique that permits the detection of rotational motions in the microsecond time range, where membrane protein motions often fall (Thomas, 1981). In those studies, we found that lipid fluidity, protein mobility, and enzymatic activity correlated well as temperature, lipid type, and detergent concentration were varied. In the present study, we have investigated the effects of varying the lipid-to-protein ratio on the dynamics of proteins and lipids. In addition to a free fatty acid spin label (to probe lipid hydrocarbon chain motions) and a short-chain maleimide spin label (to probe protein motions), we have used a fatty acid spin label derivative that attaches covalently to the protein through a maleimide linkage. This attached hydrocarbon probe provides a means of selectively probing the hydrocarbon region adjacent to the protein, i.e., the boundary lipid (Favre et al., 1979).

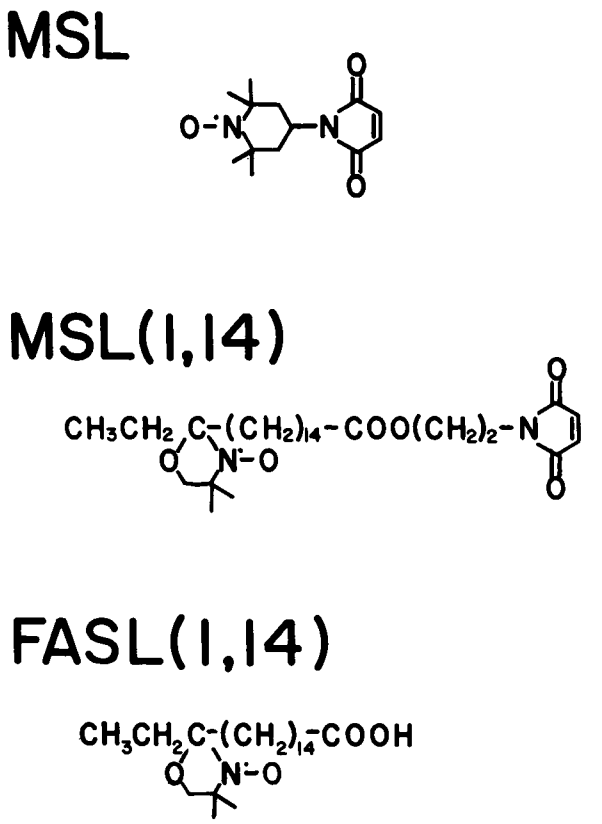

FIGURE 1 Spin labels used in this study (see Methods).

METHODS

SR Preparations

Unless otherwise noted, all preparations were carried out at 0–4°C. Fragmented SR was prepared from rabbit skeletal white (fast) muscle as described previously (Fernandez et al., 1980). The membrane vesicles were suspended in SR buffer (0.3 M sucrose, 1 mM NaN_3, 20 mM MOPS, pH 7.0), frozen in liquid nitrogen, and stored at −70°C. Two types of modifications of the membrane lipids were performed, either before or after spin-labeling. In one preparation, designated DPPC-SR, the fluid endogenous lipids were replaced with the fully saturated dipalmitoylphosphatidylcholine (DPPC) by incubating a mixture of SR, DPPC (Calbiochem. Corp., San Diego, CA), and recrystallized deoxycholate (Sigma Chemical Co., St. Louis, MO), and collecting the DPPC-replaced membranes after centrifugation on a sucrose gradient (for details, see Hidalgo et al., 1976, 1978). The weight ratio of protein:DPPC:deoxycholate during the incubation was 1:1.33:0.67. In a second preparation, designated del-SR, the lipid:protein ratio of SR was decreased by ~ 50%. The same procedure used in the DPPC-SR preparation was followed, except that no exogenous lipid was added, and the weight ratio of protein:deoxycholate was 1:0.4 (see Hidalgo et al., 1976).

SR proteins were cross-linked with glutaraldehyde (Polysciences Inc., Warrington, PA) essentially as described previously (Thomas and Hidalgo, 1978). SR was suspended at a protein concentration of 2 mg/ml in SR buffer plus 0.1 M MOPS, pH 7.0, at 25°C and incubated with 40 mM glutaraldehyde for 20 min. The reaction was quenched with an excess of glycine, and the membranes were washed with SR buffer. Cross-linking was verified by polyacrylamide gel electrophoresis, under conditions where cross-linked Ca-ATPase complexes with molecular weights as high as 10^6 are resolvable (Louis and Holroyd, 1978).

Spin Labeling

The spin labels used in this study are shown in Fig. 1. To monitor the rotational motion of the Ca^{2+}-ATPase protein, SR was labeled with a short-chain maleimide spin label (MSL), *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide (Syva Co., Palo Alto, CA), as described previously (Thomas and Hidalgo, 1978). This preparation is designated MSL-SR.

To monitor the rotational mobility of hydrocarbon chains near the center of the bilayer in the bulk lipid phase, SR was labeled with a fatty acid spin label [FASL(1,14)], 2-(14-carboxytetradecyl)-2-ethyl-4-4-dimethyl-3-oxazolidinyloxyl (Syva). This probe is also sometimes referred to as 16NS, indicating that the nitroxide spin label is attached to the number 16 carbon atom of stearic acid. The label was dissolved in ethanol and added to SR at a ratio of less than one spin label:200 phospholipids. The final ethanol concentration was < 1% and the membrane concentration was sufficiently high (> 30 mg protein/ml) that the EPR spectrum contained a negligible contribution from unbound (i.e., aqueous) spin labels. This preparation is designated FASL(1,14)-SR.

To monitor selectively the rotational mobility of a hydrocarbon chain near the lipid-protein boundary, SR was labeled with a long-chain maleimide spin label [MSL(1,14)], 2-(14-carboxytetradecyl-*N*-ethyl maleic ester)-2-ethyl-4,4-dimethyl-3-oxazolidinyl oxyl, kindly provided by P. Devaux (Favre et al., 1979). This probe is a maleimide derivative of FASL(1,14). A suspension of SR (10 mg protein/ml) was incubated overnight at 0°C with 10^{-4}M MSL(1,14) in SR buffer. To remove noncovalently attached probes the labeled SR was washed with bovine serum albumin (BSA, Sigma) as follows. The membranes were diluted by a factor of five in SR buffer containing a final BSA concentration of 10 mg/ml. After incubating for 10 min, the membranes were pelleted by centrifugation for 30 min at 150,000 *g*, then resuspended in SR buffer. BSA decreased the intensity of the weakly immobilized EPR spectral component (correlation time < 10^{-8} s) relative to the strongly immobiil-ized component. This BSA washing was repeated, except that subsequent washes contained 2 mg BSA/ml, until the EPR spectrum showed no further change. Three to four washes usually sufficed. The membranes

were then washed once more with SR buffer to remove traces of BSA. This preparation is designated MSL(1,14)-SR. As an alternative to FASL(1,14) for the study of the hydrocarbon mobility in the bulk lipid phase, an unreactive derivative of MSL(1,14) was prepared by incubating 1 mM MSL(1,14) with 5 mM cysteine pH 7.5 overnight. This cys-MSL(1,14) probe was used to label SR by the same method used for FASL(1,14).

EPR Spectroscopy

EPR spectra were obtained with a Varian E-109 spectrometer (Varian Associates, Palo Alto, CA) as described previously (Thomas and Hidalgo, 1978; Hidalgo et al., 1978), and spectra were digitized and analyzed with a microcomputer (Northstar Co., Berkeley, CA) interfaced to the spectrometer. Submicrosecond rotational motion of spin labels was detected by conventional EPR (first harmonic absorption in phase, designated V_1), using 100 kHz field modulation with a modulation amplitude of 2 gauss and a microwave field intensity of 0.14 G. Submillisecond rotational motion was detected by saturation transfer EPR (second harmonic absorption out of phase, designated V_2') using 50 KHz field modulation with a modulation amplitude of 5 G and a microwave field intensity of 0.25 G. Spectra were interpreted in terms of rotational mobility as described previously (Thomas et al., 1976; Thomas, 1978; Hyde and Thomas, 1980; Thomas, 1981). Temperature was controlled to within 0.5°C with a Varian V4540 variable temperature controller. During data acquisition, temperature was monitored with a YSI Telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH), using a thermistor probe (Model 511) touching the outside of the sample cell in the center of the cavity. The fraction of strongly immobilized hydrocarbon probes in each sample was determined by computer analysis of the digitized spectra, using the single-component subtraction method (Jost and Griffith, 1978). A reference spectrum corresponding to strongly immobilized probes was subtracted from the multicomponent spectrum until the strongly immobilized component was removed. Two different reference spectra were used for each analysis: that of FASL(1,14) in DPPC (Fig. 3, bottom left) and the corrected spectrum of partially delipidated MSL(1,14)-SR (Fig. 4, bottom right). The results of the two determinations were averaged, and the uncertainties reported below indicate the range of the two results.

Assays

Ca^{2+}-dependent ATPase activity was measured at 25°C in a solution containing 0.2 mg protein/ml, 60 mM KCl, 5 mM $MgCl_2$, and 25 mM MOPS (pH 7.0). 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. (1977). Calcium uptake was measured at 22° as described previously (Hidalgo, 1980). Lipid extraction and phospholipid determination was carried out as described previously (Hidalgo et al., 1976). We assume that the Ca-ATPase is the only integral membrane protein present in significant amounts (Fernandez et al., 1980) and the phospholipid:protein ratio is therefore expressed as the ratio of moles phospholipid mole Ca-ATPase. The molar phospholipid concentration was determined as described previously (Hidalgo et al., 1976). The protein concentration was determined by the biuret method, using bovine serum albumin as a standard. The molar Ca-ATPase concentration was determined by dividing the protein concentration by a molecular weight of 115,000 and multiplying by the fraction of the total protein that had this mol wt. This fraction, determined from polyacrylamide gels, was usually ~ 0.8 for SR and 1.0 for del-SR.

RESULTS

Characterization of Membrane Preparations

The lipid:protein ratios (mol phospholipid:mol Ca-ATPase) were 83 ± 6 and 37 ± 3 for typical preparations of SR and del-SR, respectively. Thus the lipid depletion procedure reduces the ratio to 45 ± 7% of the original value. No significant changes in the Ca-ATPase activity of the enzyme (within the 15% experimental uncertainty) were observed due to the spin-labeling. Spin-labeling with MSL (1.5 mol MSL bound/mol Ca-ATPase) inhibited the initial rate of Ca uptake by 30–40% but had no effect on the final level of Ca accumulation.

EPR Spectra

Fig. 2 shows the effect of the Ca-ATPase on the rotational mobility of the unattached hydrocarbon probes, FASL(1,14) and cys-MSL(1,14), as measured by conventional EPR. In lipid extracted from SR, the spectra (Fig. 2, bottom) are similar to those predicted for rapid isotropic motion. Therefore, it is meaningful to assign an effective rotational correlation time τ_2, according to the theory that relates linewidths to τ_2 (Keith et al., 1970). From Fig. 2, our estimates for τ_2 in pure SR lipid are 1.8 ns for FASL(1,14) and 2.0 ns for cys-MSL(1,14). In SR, the EPR spectrum of each probe is no longer homogeneous, but must be analyzed as a sum of components. The major component has a shape similar to that in the absence of protein, but the linewidths are slightly greater, corresponding to a slight decrease in rotational mobility (effective correlation time, 3.0 ns). A minor component is also present that has significant intensity in the wings of the spectrum, characteristic of strongly immobilized spin labels ($\tau_2 \geq 10^{-8}$ s). This type of component is difficult to detect and quantify, due to its broad shape and low intensity per mole relative to the mobile component. However, the presence of a substantial fraction of this immobile component is clear not only from the intensity in

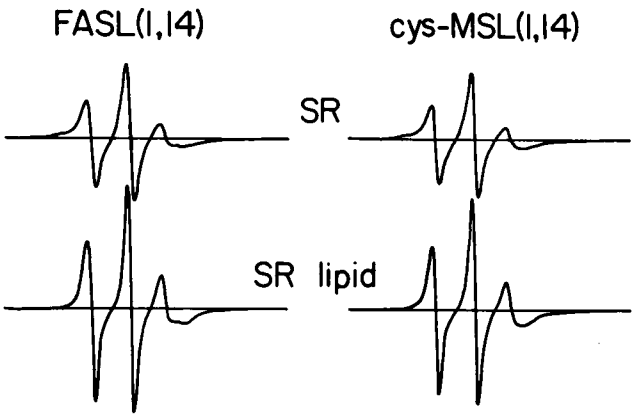

FIGURE 2 Effect of the Ca-ATPase on the conventional EPR spectra of the two unattached hydrocarbon probes, FASL(1,14) and cys-MSL(1,14), at 4°C. Top: 0.1 mM spin label in a 50 mg/ml suspension of SR in SR buffer. Bottom: 0.1 mM spin label in a 50 mM suspension of SR lipid in SR buffer. The spectral intensities have been normalized by the computer; each derivative spectrum was divided by its double integral, so that the spectra shown all correspond to the same number of spins. The baselines drawn in this and subsequent figures are 100 gauss long.

the wings of the spectra (Fig. 2, top), but also from the relative intensities of the mobile spectral components in Fig. 2. The spectra in Fig. 2 have been computer-normalized; each has been divided by a number proportional to the double integral, thus making all four spectra correspond to the same number of spins. Since the mobile spectral component is only slightly broadened by the protein, most of the decrease in its intensity must arise from the conversion of some of the mobile spins to immobile ones. A quantitative computer analysis indicates that the immobilized component corresponds to 29 ± 5% of the FASL(1,14) probes and 26 ± 5% of the cys-MSL(1,14) probes.

To investigate the correlation between lipid and protein mobilities, we varied the lipid environment of the enzyme and probed both lipid and protein dynamics (Fig. 3). Hydrocarbon chain motions in the nanosecond time range were probed with FASL(1,14), using conventional EPR (left), and protein motions in the microsecond time range were probed with MSL, using saturation transfer EPR. According to the L″/L spectral parameter (Thomas et al. 1976), the effective correlation time for MSL-SR (Fig. 3, top right) is 50 μs, confirming our previous results (Thomas et al. 1976; Thomas and Hidalgo, 1978). In contrast with the high degree of both lipid and protein mobility in SR, replacing the endogenous lipids with DPPC eliminates most of the nanosecond mobility of the hydrocarbon chains (Fig. 3, bottom left) and most of the microsecond mobility of the protein (Fig. 3, bottom right). Partial delipidation of SR, without the addition of DPPC, produces spectra (Fig. 3, center, del-SR) intermediate between those of SR and DPPC-SR. The immobilized component in the wings of the FASL(1,14) spectrum is enhanced by lipid depletion, and the mobile component is broadened. Computer analysis indicates that the immobilized component corresponds to 53 ± 8% of the FASL(1,14) probes. The saturation transfer spectrum of MSL-del-SR indicates that the protein motion is nearly as slow as in DPPC-SR.

In order to have a more direct probe of the boundary lipid chains, we labeled SR with MSL(1,14) and prepared the lipid-depleted del-MSL(1,14)-SR from this sample (Fig. 4, top). In order to obtain spectra corresponding only to probes covalently attached to the protein, excluding unreacted probes or probes attached to lipid, we corrected the spectra by subtracting the spectrum obtained from the $CHCl_3$/MeOH extract of labeled SR. The resulting corrected spectra are shown at the bottom of Fig. 4. The corrected spectrum of MSL(1,14) SR indicates the presence of two components: a strongly immobilized component (first peak, Fig. 4, bottom left) and a weakly immobilized component (second peak). Even this less immobilized component, which comprises ~ 30% of the total double-integrated signal, is broader (less mobile) than the mobile component of the FASL(1,14) or cys-MSL(1,14) spectra (Figs. 2, 3). Lipid depletion virtually eliminates the weakly immobilized component (Fig. 4, bottom right).

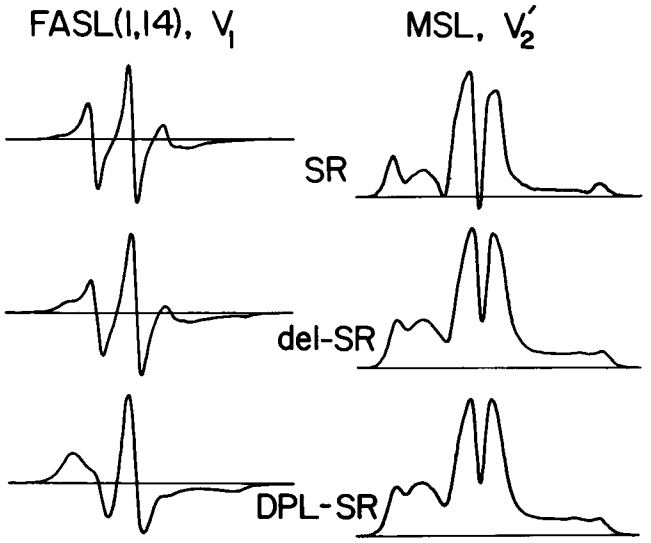

FIGURE 3 Effect of varying the SR lipid content on the bulk lipid hydrocarbon rotational mobility (conventional EPR spectra, V_1) of FASL(1,14) and on the protein rotational mobility (saturation transfer EPR spectra, V_2') of MSL attached to the Ca-ATPase, at 4°C.

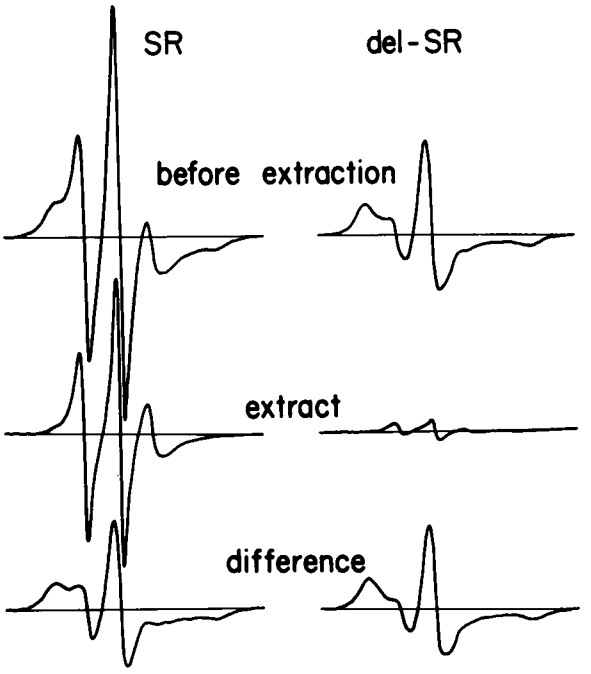

FIGURE 4 Conventional EPR spectra of MSL(1,14) in SR (left) and del-SR (right), in SR buffer at 4°C. Top: uncorrected spectra obtained before extraction with $CHCl_3$/MeOH. Center: $CHCl_3$/MeOH extract, dried and resuspended in the original volume of unlabeled SR. Bottom: corrected spectra, the result of subtracting the second row from the first. Thus the bottom spectra arise from those labels that are covalently attached to the protein. The spectra in each column are shown at the same gain, so that the relative signal amplitudes before and after correction are accurately shown.

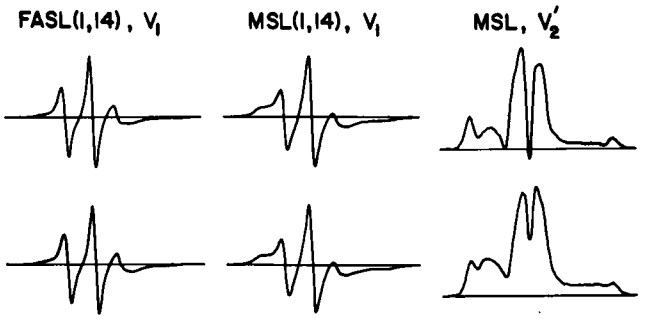

FIGURE 5 EPR spectra of SR before (top) and after (bottom) glutaraldehyde treatment. Conventional (V_1) spectra were recorded for the unattached hydrocarbon probe [FASL(1,14), left] and the attached hydrocarbon probe (MSL(1,14), center); saturation transfer (V_2') spectra were recorded for MSL (right). The temperature was 4°C.

To achieve protein aggregation without lipid depletion, we cross-linked SR with glutaraldehyde. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicated the absence of any polypeptides with a molecular weight $< 10^6$. This treatment eliminated the protein's submillisecond mobility, as indicated by the change in the saturation transfer spectrum of MSL (Fig. 5, right) but had no effect on either the unattached [FASL(1,14), Fig. 5, left)] or attached [MSL(1,14), Fig. 5, center] hydrocarbon probes. The spectra of FASL(1,14) were independent of whether the label was added before or after cross-linking.

DISCUSSION

If we assume that the fraction of immobile phospholipids is the same as the fraction of immobile FASL(1,14) molecules (determined from the spectra in Figs. 2 and 3), the product of this fraction and the lipid:protein ratio (number of phospholipids/Ca-ATPase) is the number of immobile phospholipids/Ca-ATPase. These calculations are summarized in Table I. Within experimental uncertainty, the number of immobile phospholipids/Ca-ATPase is about the same for two preparations having markedly different lipid:protein ratios. These data are in good agreement with the results of a similar spin label study in which SR was delipidated with phospholipase (Nakamura and Ohnishi, 1975) and a study on the Ca-ATPase using reconstituted membranes,[1] although in another study on the reconstituted Ca-ATPase only eight immobile lipids/Ca-ATPase were found (McIntyre et al., 1981). As in the case of cytochrome oxidase (Jost et al., 1973; Knowles et al., 1979) and rhodopsin (Watts et al., 1979), this constant number of immobile lipids/protein seems consistent with the model of an immobilized boundary layer coating each protein. As pointed out by Watts et al. (1979), the numbers of immobilized lipids/protein found for cytochrome oxidase (55) and rhodopsin (24) scale with the square root of their molecular weights. Assuming the same scale factor for the Ca-ATPase, the value predicted would be 42, almost twice the value we find. Although this suggests that there are insufficient immobile lipids to account for a boundary layer that completely surrounds the protein, too little is known about the Ca-ATPase structure to estimate accurately the surface area of the protein-lipid boundary. Since the effect of the protein on the uncharged cys-MSL(1,14) probe is not significantly different from the effect on the negatively charged FASL(1,14), it appears that there is less charge selectivity in SR than in membranes containing the Na,K-ATPase (Brotherus et al., 1980).

TABLE I
CALCULATED IMMOBILE PHOSPHOLIPIDS PER Ca-ATPase

	Phospholipids/ Ca-ATPase	Fraction Immobile	Immobile Phospholipids/ Ca-ATPase
SR	83 ± 6	0.29 ± 0.05	24 ± 5
del-SR	37 ± 3	0.53 ± 0.08	20 ± 4

The decrease in protein mobility upon lipid depletion (Fig. 3) probably results from a substantial increase in protein-protein interactions, either due to aggregate formation or to an increase in the effective viscosity of the membrane. This raises the question whether some of the observed increase in the fraction of immobile FASL(1,14) probes (Fig. 3) is due to trapping of probes (and presumably lipids) between proteins (Favre et al., 1979; Chapman et al., 1977; Swanson et al., 1980). One result that argues against this alternative explanation is that the number of immobile lipids/protein remains constant (or decreases slightly) upon lipid depletion (Table I), whereas the amount of lipid trapped/protein might be expected to increase with increased protein-protein interaction. We also observe that the mobile component of the FASL(1,14) del-SR spectrum (Fig. 2) is less mobile (broader) than that of SR, which in turn is less mobile than that of SR lipid (Fig. 1). As argued by Marsh et al. (1978) and Knowles et al. (1979) when analyzing similar results for cytochrome oxidase, these results seem consistent with a model in which increased protein-protein interactions result in (*a*) a decrease in the number of strongly immobilized lipids/protein due to the exclusion of boundary lipid from the protein-protein interface, and (*b*) a slight decrease in the mobility of the remaining relatively mobile lipid due to trapping.

The attached hydrocarbon probe MSL(1,14) offers a more direct look at the boundary lipid. The strong immobilization of a majority of these probes in SR (Fig. 4) is consistent with the model of an immobile boundary layer. However, Fellmann et al. (1980), using a photoaffinity label, and Andersen et al. (1981), using MSL(1,14), have interpreted a similar immobilization in terms of a proposed oligomeric structure for the enzyme. That is, these

[1]Silvius, J., and P. Jost. Personal communication.

strongly immobilized probes may be trapped between the subunits of an oligomeric enzyme. An oligomeric structure, which would be expected to have a lower ratio of surface area to molecular weight than a monomer, might also help explain the small value observed for the number of immobilized FASL(1,14) probes/protein.

The observation of strongly immobilized MSL(1,14) in the presence of highly mobile protein in SR contrasts with the observation, in reconstituted cytochrome oxidase, of highly mobile MSL(1,14) (Swanson et al., 1980). However, there is a qualitative similarity between the two studies: a decrease in protein mobility, presumably due to an increase in protein-protein interaction (aggregation), results in a decrease in the mobility of MSL(1,14). This apparent correlation of boundary lipid mobility with protein mobility suggests that some of the hydrocarbon chain immobilization observed at low lipid:protein ratios could arise from lipid trapping due to protein-protein interactions.

The lack of a glutaraldehyde effect on either the attached or unattached hydrocarbon probes, despite a large immobilizing effect on the protein (Fig. 5), suggests that protein aggregation does not necessarily perturb lipid chains. The lipid-trapping hypothesis would predict an increase in the immobilized fraction, while the model of an immobilized boundary layer would predict a decrease in the immobilized fraction, due to exclusion of boundary lipid at the protein-protein interface. The lack of any effect suggests that hydrocarbon chain immobilization is caused mainly by protein-protein interactions that are not affected by crosslinking, e.g., the interactions of subunits within an oligomeric enzyme (as proposed by Andersen et al., 1981). However, the actual state of aggregation of the protein cross-linked by glutaraldehyde is not known. It is possible that the extent of protein-protein surface contact is not greatly changed, in which case it would not be surprising that hydrocarbon chains are not strongly perturbed.

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DISCUSSION

Session Chairman: V. Adrian Parsegian *Scribe*: Richard A. Dluhy

McINTYRE: In Table I, how do you estimate the amount of immobilized lipid? Does that fitting give a unique solution to the problem?

THOMAS: This is always a problem with using freely-diffusing probes. It is the reason we've also used the covalently-attached probe. We've tried to subtract out various amounts of the mobile signal component (using pure lipid as our reference), but the mobile signal is somewhat less mobile in the absence of protein than in the presence of protein. Therefore we subtracted an immobilized component.

Among our models was the same fatty acid spin label in rigid DPL and the boundary layer probe, giving significantly different immobilized spectra. For these models, one seemed to underestimate the fraction of immobilized lipid while the other seemed to overestimate the fraction. Hence the range of values in Table I.

McINTYRE: What is the apparent rotational correlation time of the immobilized species as detected by the fatty acid spin label, and does the amount or the motion of that species change with temperature?

THOMAS: With respect to the attached probe, the saturation transfer spectra are difficult to interpret but do clearly show immobilization down to the microsecond time scale.

McINTYRE: As I understand it, the Devaux label can give information at higher temperature concerning the rotation of lipid around the protein, but it cannot give a quantitative number for the amount of label immobilized.

THOMAS: One of our goals is to subtract out the spectra of the mobile component and look at the saturation transfer spectrum of the immobile component. The spectrum in this case is still very difficult to interpret. One possible explanation is that exchange is significant on the microsecond time scale.

McINTYRE: How is the phospholipid content of your preparation determined? Could it indicate contamination? Your determination is lower than ours.

THOMAS: We do consistently get numbers 10–20% lower than yours. We measure phospholipid by phosphorous assay and protein by the Biuret method. Since 80–90% of the protein is Ca^{++}-ATPase and the other 10–20% is known not to be integral membrane proteins, we correct by subtraction to get the actual Ca^{++}-ATPase concentration.

McINTYRE: This may be the answer since we use the Lowry assay with which we obtain the same result as by dry weight, whereas you are using the Biuret method.

MARSH: The work by the group of Philippe Devaux on the origin of immobilization of the lipid chains by the protein comes to a conclusion different from yours. Could you comment?

THOMAS: The hypothesis of the Devaux group (Andersen et al. 1981. *Biochemistry.* 20:4928–4936) is that the strong observed immobilization of the probe might come from trapping of the probe between subunits of a possible oligomer. My feeling is that our results give no strong evidence either for or against. There certainly is a lot of circumstantial evidence to suggest oligomeric structure for the calcium-pump protein; the best measurements are the energy transfer measurements of Vanderkooi and co-workers. An additional experiment that Devaux et al. have done is to add small amounts of detergents to their membranes and show that the immobilized boundary layer probes become mobilized. This they interpret as the breakup of the oligomers of Ca^{++}-ATPase. However, I consider it only suggestive evidence, and that is how it is discussed in their paper.

MARSH: Do you think that the preparations you are using are physically different from those of Devaux?

THOMAS: Most of their experiments were done using reconstituted systems while we use the native membranes and partially *delipidated* native membranes. I think that if we added detergent to our system we would see the same result, namely increased mobility of the probe; but whether that is due to breakup of the oligomeric structure is open to question.

MARSH: In regard to rhodopsin, Devaux's interpretation now is that the long chain maleimide probe is not sampling solely boundary layer but is in exchange between the first and second shell. Do you think that adding detergent would increase this exchange rate?

THOMAS: Yes, that is an obvious alternative explanation.

MARSH: Do you think you have evidence for exchange in your spectrum of MSL(1, 14)?

THOMAS: Our data are consistent with exchange but only suggestive. In our paper we did not say there was exchange because we can't conclude it.

LENTZ: With regard to the effect of temperature on the boundary layer, our work with the fluorescent probe diphenylhexatriene has allowed us to collect data as a function of both temperature and protein-lipid content. Our results (Lentz et al., this volume) suggest that

we can not fit the data with a simple model of annular and bulk lipid. However, by assuming that there is a ring of lipid outside the annular ring that has a disruptive packing order we can fit our data over the entire temperature and composition range. Could you comment on that?

THOMAS: I am not arguing in favor of any simple models. In fact, in our so-called two-component spectra that we see in the presence of protein with freely diffusing spin label, the mobile component is not the same as that of the probe in the absence of protein. We see then that the "mobile" probe is certainly being perturbed by protein and it is certainly possible that it corresponds to more than one component.

Getting back to Derek Marsh's point about rhodopsin, we do seem to get very different results from those obtained for rhodopsin and some other systems that have been studied by this method. The immobilized signals, especially those we get with the boundary layer probe, seem to be much more strongly immobilized than in other cases. No nanosecond motions seem to be readily apparent, certainly not at 4°C. That is one result which led Devaux to conclude that motions could be stopped between proteins. But I think that is a very indirect argument.

SILVIUS: Neil Saley and I, working in the laboratory of Patricia Jost and Hayes Griffith, have studied the effect of glutaraldehyde cross-linking on the association of a spin-labeled phosphatidylcholine (PC*) with the sarcoplasmic reticulum (SR) ATPase in its native membrane and in bilayers of egg phosphatidylcholine (PC). Our findings are in good agreement with those you have reported using the single-chain amphiphilic spin labels in native SR.

In Fig. *A* we show spectra of the 14-proxylstearoyl phosphatidylcholine spin label incorporated either into native SR vesicles (by ethanolic injection) or into a sample of the ATPase reconstituted into PC bilayers by a modification of the procedure of Warren et al. (1974. *Proc. Natl. Acad. Sci. U.S.A.* 71:622–626). The reconstituted sample whose spectra are shown in the lower half of the figure has a lipid:protein ratio (78 ±3) essentially the same as that of the native SR preparation (79 ±6).

On a qualitative level, the spectra shown in the figure exhibit no major changes in line shape when the ATPase is extensively cross-linked by glutaraldehyde. The cross-linking was carried out under the conditions described by Thomas and Hidalgo (1978. *Proc. Natl. Acad. Sci. U.S.A.* 75:5488–5492) on samples already labeled with PC*. Massive cross-linking of the ATPase by glutaraldehyde was confirmed by SDS-polyacrylamide gel electrophoresis, which showed complete exclusion of the protein in glutaraldehyde-treated samples from 7.5% Laemmli gels, in which the ATPase monomer normally runs about one-half of the distance to the dye front.

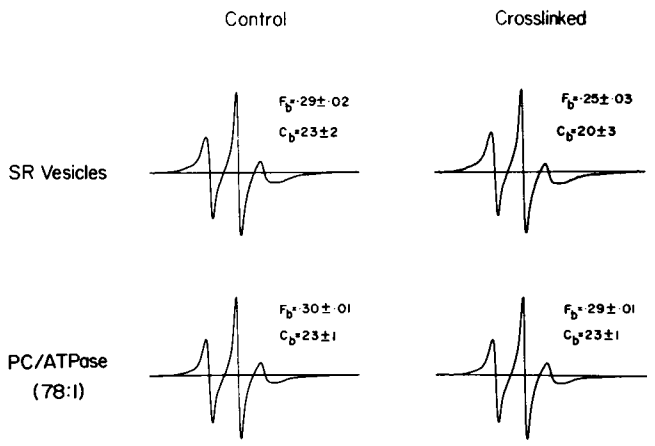

FIGURE *A* Electron spin resonance spectra of sarcoplasmic reticulum (SR) membrane before and after cross-linking by glutaraldehyde (Silvius).

The spectra shown in Fig. *A*, and those of other samples of native and reconstituted ATPase-lipid membranes, were analyzed by quantitative spectral subtraction to determine the fraction of PC* that is protein-associated (and contributes to the broad spectral component) in each sample. The broad spectral line shape used in these subtractions was obtained from the spectrum of a reconstituted ATPase sample of relatively low lipid:protein ratio, from which the residual mobile component was removed by subtracting a PC*-in-egg PC spectrum of appropriate line width.

The fraction of PC* associated with the ATPase (F_b), and the estimated number, C_b, of ATPase-associated lipids (F_b × lipid:ATPase ratio) are given for each spectrum shown in the figure. Within experimental error, C_b is the same (~23) for the ATPase in native SR vesicles and in ATPase-PC recombinants. Though the contribution of the bound component in the native SR and reconstituted ATPase samples would seem to be different in the spectra shown, this apparent difference is artificial and arises from differences in the line shapes of the mobile spectral components for the two systems. Glutaraldehyde cross-linking causes no change, within experimental error, in the fraction of PC* that is motionally restricted on the ESR time scale by the ATPase in either the SR membrane or PC bilayers. A number of similar analyses of PC* spectra in native and reconstituted samples (with varying lipid:protein ratios in the case of the reconstituted ATPase) have given results entirely comparable to those shown in Fig. *A*, both with respect to the number of ATPase-associated lipids and with respect to the lack of glutaraldehyde cross-linking on this number or on spectral line shapes.

These results, and those you report, indicate that the effect of extensive ATPase cross-linking on the spectral characteristics of diffusible lipid spin labels (i.e., those that can diffuse laterally in the bilayer) in ATPase-lipid samples is negligible.

THOMAS: The Ca^{++}-ATPase seems to be a special case. Both the immobilized component from the freely diffusing probes and the one from the attached probes are more strongly immobilized than what one sees, for example, in rhodopsin. It is true that one cannot make the assumption that the environment being probed by the attached probe is the same as the motionally-restricted environment that is being probed by the freely diffusing probes. I do think it is valid to address them together but one must realize that they do not necessarily speak to the same question.

With respect to the cross-linking experiments, the point is to ask whether protein-protein interactions are a source of the hydrocarbon chain immobilization. The cross-linking can do this without changing the lipid:protein ratio. If you think that protein-protein interactions are the main source for hydrocarbon chain immobilizations, you might expect the glutaraldehyde to have a large immobilizing effect, which it did not. However, we don't have a good model for the structure of the protein in the membrane. There is a good chance that it has a large component outside the membrane and this might be the part that is being cross-linked. That would explain why the ultra-membrane parts of the protein don't show large effects.

WOLBER: Where is the maleimide probe when it is attached to the protein? Do you know for sure that it is in the lipid?

THOMAS: Yes, qualitatively those things have been checked, insofar as these probes are not accessible to quenching agents from the aqueous solution. The problem is that so little is known about the structure of the enzyme that the hard information about the location of the probe is not available.

DAHLQUIST: Some of our experiments with cytochrome oxidase and spin-labeled fatty acids show that the electron T_1 changes with lipid:protein ratio. However, only one T_1 is needed to describe both the sharp and the broad components of the ESR spectrum. A straightforward conclusion is that exchange is fast relative to the electron T_1 time scale, in this

case a few microseconds. Given this information, could you simulate saturation transfer spectra?

THOMAS: We have a problem with computer time, but we're starting to do these simulations. It is important to ask the question because one of the best ways to study multiple components is to have a time-resolved measurement. That is why the saturation recovery experiment is a good one.

WEINSTEIN: This meeting appears divided into two camps: those interested in what goes on in the plane of the membrane and those interested (the "perpendicularists") in how things can approach from outside and enter the bilayer. How many of the lipids not in the "boundary layer" annular ring behave physicochemically like those in free bilayers? How mobile are they? Can they accommodate small molecules?

THOMAS: The most mobile component is not as mobile as in a bilayer (in the absence of protein) but is not very restricted.

LIGHT: Because the malemide protein probe will react with nucleophiles (the —SH group being among the most reactive) but the amino groups can also react, is there any information about the exact reactive sites of the maleimide with the protein?

THOMAS: There is functional information to the extent that we know that the attachment of the maleimide probes does not inhibit either the Ca^{++}-pumping or the ATPase activities of the enzyme. There is a lot of work being done now on the reactivity of various maleimide and iodoacetamide probes with the various sulfhydryls of the protein, but because the structure of the protein is still so poorly understood, the question, while important, is premature.