BBA 76081

PHASE CHANGES IN THE LIPID MOIETIES OF SARCOPLASMIC RETICULUM MEMBRANES INDUCED BY TEMPERATURE AND PROTEIN CONFORMATIONAL CHANGES

S. ELETR* AND G. INESI

Laboratory of Physiology and Biophysics, University of the Pacific, San Francisco, Calif 94115 (U.S.A.)

(Received June 12th, 1972)

SUMMARY

Lipid alkyl regions in sarcoplasmic membranes were probed with hydrophobic spin labels in the temperature range between 5 and 50 °C. Results indicate that biochemically active preparations undergo transitions at 22 and 40 °C. The first one is associated with a melt of lipid alkyl chains and does not depend on membrane proteins. Evidence is presented that the second transition reflects hydrophobic lipid–protein interactions and the effect of protein conformational changes on the dynamic state of membrane lipids. In addition, proteolytic digestion of surface granular structures does not affect the behavior of lipid-soluble hydrophobic spin labels or the detected lipid–protein interactions. The partitioning of an amphiphilic spin label between polar and apolar regions is affected, however, indicating that some surface proteins interact with the lipids *via* polar forces.

INTRODUCTION

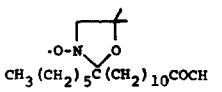
The lamellar phase of lipids in aqueous dispersions exhibits thermotropic transitions associated with the ordering of methylene groups in the hydrophobic regions[1,2]. The importance of these transitions, which are observed also in biological membrane systems[1–4], is demonstrated by the temperature behavior of membrane-bound enzymes which can be correlated with the dynamic states of membrane lipids[3–5] In fragmented sarcoplasmic reticulum membranes, which display a very high and specific ability for Ca^{2+} accumulation coupled to ATP hydrolysis, the fluidity of the lipids is a critical parameter insofar as ATPase activity and the ability to retain accumulated Ca^{2+} are concerned[6,7]. It is of great interest to characterize the mechanisms involved in determining lipid fluidity and to investigate whether membrane proteins are involved in these mechanisms We report, in this paper, the results of an investigation of protein–lipid interactions in sarcoplasmic reticulum membranes using nitroxide spin labels. Hydrophobic labels are used to probe the dynamics of lipid moieties. Also, nitroxides covalently bound to protein

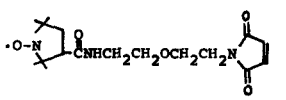
* Special Fellow of the National Institute of General Medical Sciences, U S Public Health Service

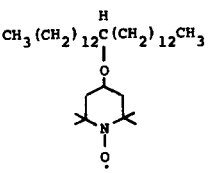
SH groups are used to probe membrane fluidity in the vicinity of these groups. Lipid dynamics are shown then to depend upon the conformation of protein regions about the labeled sites.

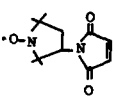
EXPERIMENTAL

Sarcoplasmic reticulum vesicles were obtained from white skeletal muscle of rabbit hind legs, using the method we have described elsewhere[9], and stored at 2–4 °C in a 10 mM histidine, 30 % sucrose, solution. Under these conditions, the Ca^{2+} pumping activity remained stable for about one week. A fraction of the sarcoplasmic reticulum preparation was subjected to partial trypsin digestion at 25 °C. This was carried out in a reaction mixture containing approximately 1 mg of sarcoplasmic reticulum membrane protein per ml, 10 μg trypsin per ml, 10 mM histidine (pH 7) and 80 mM KCl. The reaction was interrupted after 10 min by the addition of 2 mg of Soya bean trypsin inhibitor per ml. The reaction mixture was then centrifuged at 40000 × g for 30 min; the supernatant was discarded, and the pellet resuspended in 10 mM histidine (pH 7). A control sample was subjected to the same treatment except that the trypsin was omitted. The following four spin labels were used:

I 

III 

II 

IV 

The syntheses of Labels I and II have already been described[9,10]. Samples of these were provided by Professor A. D. Keith. Labels III and IV were obtained from Synvar. For each experiment with labels I and II, 2 μl of the probes in ethanol ($1 \cdot 10^{-3}$M) were added to 0.5 ml of vesicles resuspended in a histidine buffer (pH 7) at 30 mg of protein/ml. Labels III and IV, which covalently attach to protein SH groups, were added to sarcoplasmic reticulum samples at approx. $2 \cdot 10^{-4}$ M and incubated overnight at 4 °C. The vesicles then were washed and resuspended several times in histidine buffer to remove unreacted labels. All resonance spectra were recorded on a Varian E-3 EPR spectrometer equipped with a variable temperature accessory. Temperature of the sample is estimated accurate to within ± 1 °C.

Ca^{2+} accumulation by sarcoplasmic reticulum vesicles was studied by incubating sarcoplasmic reticulum at a concentration of 300 μg protein/ml in a solution of 20 mM Tris–maleate (pH 6.8), 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*, *N'*-tetraacetic acid, 0.1 mM $^{45}CaCl_2$ and 1 mM ATP. The incubation was stopped by millipore filtration and the residual radioactivity in the filtrate was measured by scintillation spectrometry.

RESULTS AND DISCUSSION

Lipid–protein interactions

Probe I is insoluble in aqueous solutions and the polarity of the N-oxyl group is not high enough to provide an effective anchoring of the molecule to the amphiphilic interface. Spectra of this spin probe in membrane systems exhibit the same solvent and local polarity effects as do spectra obtained in solvents such as octadecane. It is inferred from this, and from studies in aqueous lipid dispersions, that the probe is incorporated in the hydrophobic regions of membrane lipids[11]. Probe I is far from spherical; however, the measured spectra are very nearly the same as those measured in the case of isotropic motion of a quasi spherical nitroxide whose reorientation may be described by a correlation time[12] of the order of 10^{-9} s. This rotational correlation time may be derived from spectral parameters using the formula[12]:

$$\tau_c = K(W_{-1} - W_0) \quad (1)$$

where K is obtained from the spin label crystal parameters; W denotes a first derivative linewidth, and the subscripts —1 and 0 refer to the high- and mid-field lines, respectively. This formula is valid as long as $\tau_c < 10^{-9}$ s. In the range of temperatures used in this work, τ_c, as given by Eqn 1, is as high as $2 \cdot 10^{-9}$ s and the equation no longer holds. The values given by Eqn 1 may still be used to define an empirical motion parameter τ_0 however. Assuming that the line shapes are Lorentzian, the equation for this parameter becomes:

$$\tau_0 = 6.5 \cdot 10^{-10} \cdot W_0[(h_0/h_{-1})^{\frac{1}{2}} - 1] \quad (2)$$

where h refers to first derivative line heights.

Use of the equation in this form is more convenient as the line width W_{-1} is difficult to measure accurately when τ_c becomes greater than $1 \cdot 10^{-9}$ s. Two points must be emphasized here. The empirical motion parameter τ_0 is not an exact correlation time, even in the limit when values given by Eqn 2 are less than $1 \cdot 10^{-9}$ s. This is because the quasi-isotropic nature of the spectra does not necessarily imply isotropic motion of the probe. In the case of probes such as I, enhanced motion about the long molecular axis averages the anisotropic contribution of the three nitroxide principal axes in the same manner as would strictly isotropic motion[10]. While the empirical motion parameter may serve, in this limit, as an approximate measure of the probe's motional correlation time, it should be emphasized also that it does not describe quantitatively the motion of molecules belonging to the solvent matrix, *i.e.* the membrane lipids.

Arrhenius displays of the temperature dependence of this empirical motion parameter, measured in a variety of membrane systems[5,11], have been used to detect discontinuities in the temperature dependence of the dynamic properties of lipid moieties labeled with Probe I. The temperatures at which these discontinuities occur correlate with transition temperatures detected by other means[5]. It is believed that they are associated with transitions in the ordering or packing of methylene groups in alkyl chains in the lamellar phase of membrane lipids[1–5,11].

Spectra from the pyrrolidinyloxyl radical in spin Label III also display a large quasi-isotropic component (Fig. 1). This is because of the flexibility of the chain

anchoring it to SH sites and, presumably, because of the intrinsic mobility of a fraction of these SH groups. This is confirmed by the shape of spectra obtained from sarcoplasmic reticulum labeled with Label IV (Fig. 1). In this case the mobility of the label directly reflects that of the anchoring sites. However, some uncertainty remains in this respect as it is impossible to determine whether both III and IV label the same SH groups.

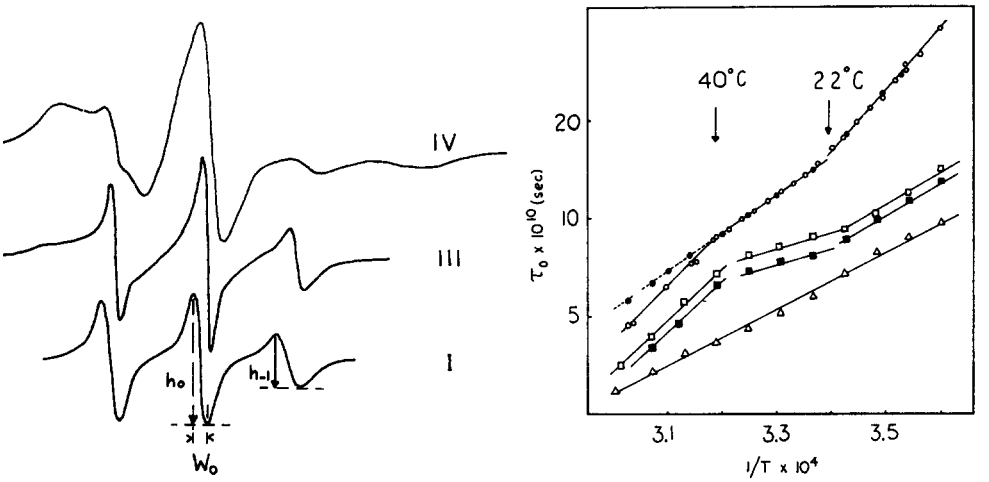


Fig 1 Resonance spectra of Probe I and Labels III and IV in sarcoplasmic reticulum vesicles. These were measured at room temperature Note the isotropic components (narrow lines) in Spectrum III Spectrum IV shows that a fraction of the labeled sites are in a relatively fluid region of the protein It is not known whether Labels III and IV attach to the same sites

Fig 2 Temperature dependence of the empirical motion parameters obtained from the spectra of Label I in native sarcoplasmic reticulum (○) and in heat denatured sarcoplasmic reticulum (●). Data from native and partially trypsinated sarcoplasmic reticulum were indistinguishable (○). The three other curves are the temperature dependences of the isotropic components' motion parameters obtained from spectra of label III in native sarcoplasmic reticulum (□), partially heat denatured sarcoplasmic reticulum (■) and totally heat denatured sarcoplasmic reticulum (△).

The empirical motional parameter τ_0 was obtained from spectra of Label I and the isotropic components of the spectra of Label III in the range between 5 and 50 °C. The results are displayed in Fig. 2. The data are reproducible provided samples are not kept above 50 °C for extended periods of time. Thermal denaturation of the proteins occurs otherwise and the Arrhenius plots of τ_0 are irreversibly modified as shown also in Fig. 2.

Several conclusions may be drawn from these results. The transition at 22 °C, detected from the motion parameter of Probe I, is unaffected by thermal protein denaturation and must be intrinsic to the lipids. We have previously shown that the majority of lipids in sarcoplasmic reticulum are organized in a layered arrangement consistent with the possible existence of a lamellar phase[8]. Therefore, it is reasonable to assign this transition to a temperature-dependent reordering of the methylene groups in lipid alkyl regions. Similar transitions have been observed in aqueous lipid dispersions as well as in other biological membranes[11]. The transition detected at 40 °C by the motion of Label I must arise, however, from a temperature-dependent protein conformational change which interferes with lipid fluidity as

a result of lipid–protein interactions, since it is destroyed by heat denaturation. NMR studies had indicated that such interactions may be taking place[13]. This interpretation is substantiated by the results obtained from spin Label III. The pyrrolidinyloxyl radical, in this label, is constrained to remain in the immediate vicinity of protein SH sites. Yet, its motional parameter is sensitive to the transition at 22 °C which is intrinsic to lipid alkyl chains. It is clear that some type of interaction must exist between these chains and the protein neighborhood of the SH sites. This interaction is destroyed by heat denaturation as the motion of Label III no longer reflects the lipid transition Finally, the transition detected at 40 °C by the motion of label III is also destroyed by heat denaturation

Correlation with membrane structure and function

It is of interest to relate these findings to known morphological features of sarcoplasmic reticulum vesicles. Negative staining of non-fixed vesicles allows electron-microscopic visualization of a granular layer on the outer surface of the membrane[14–16]. The small granules (30–40 Å diameter) disappear after short trypsin digestion, while a parallel increase in membrane permeability results, and the vesicles become unable to retain accumulated Ca^{2+} (ref. 14). Control and trypsin-treated samples were prepared as previously described and were studied also with Probe I. No differences could be detected in the temperature dependences of the motional parameters measured in the two samples (Fig. 2) Thus, the removal of these granular structures has no effect on the lipid–protein interactions for which we have presented evidence. We tentatively conclude that these interactions are hydrophobic and involve membrane regions removed from the outer surface. Probe II was used then to determine whether the disruption of polar regions could be detected. Probe II, though hydrophobic, yields a spectrum which results from the partitioning of the somewhat polar 2,2,6,6-tetramethyl piperidine-1-oxyl ring between aqueous and hydrophobic environments as shown in Fig. 3. Since the heptacosane chain cannot possibly be water soluble, the hydrophilic component of the spectrum must arise from a molecular configuration of the probing molecule which allows the chain to fold and the 2,2,6,6-tetramethyl piperidine-1-oxyl ring to "rise" to the amphiphilic interface. The partitioning coefficient, defined in Fig. 3, describes the equilibrium between the population of probes dissolved in hydrophobic regions and that of folded probes, mimicking a lipid molecule. Therefore, it is expected to be sensitive to both the fluidity of the hydrophobic regions and the polar interactions at the amphiphilic interface which may promote or quench the binding of 2,2,6,6-tetramethyl piperidine-1-oxyl rings. We know from the results with Probe I that partial trypsin digestion of sarcoplasmic reticulum does not affect hydrophobic regions. Thus, we expect any changes displayed by the partitioning of Probe II to reflect changes in the membrane polar regions. The high field lines obtained from Probe II in both control and trypsin-treated sarcoplasmic reticulum preparations are given in Fig. 4. Results imply that the granular structures on the vesicular surfaces promote the folded probe configuration and the attraction of 2,2,6,6-tetramethyl piperidine-1-oxyl rings to polar surfaces. This, in turn, implies that these structures interact with membrane lipid moieties *via* predominantly polar forces.

It is interesting also to correlate the temperature dependence of the partitioning of Probe II with the temperature dependence of the motion parameters

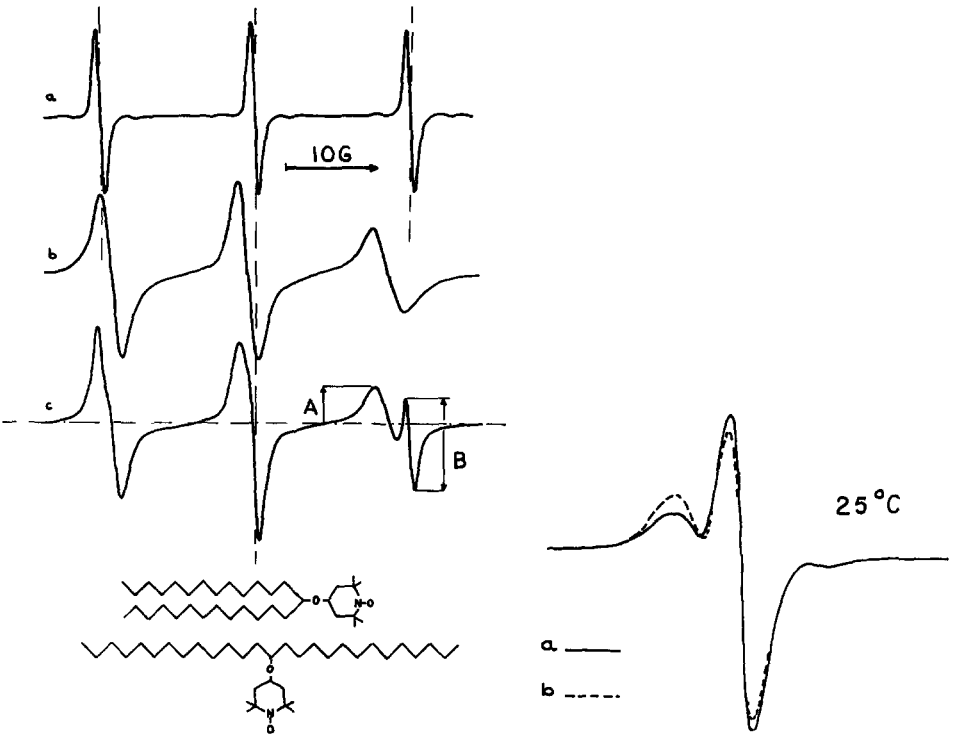


Fig. 3 Interpretation of a spectrum of Probe II. (a) Spectrum of 2,2,6,6-tetramethyl piperidine-1-oxyl in water, (b) isotropic spectrum of a nitroxide in octadecane and (c) spectrum resulting from the superposition of (a) and (b) The folded configuration of the probe allows the 2,2,6,6-tetramethyl piperidine-1-oxyl ring to penetrate the amphiphilic interface. The stretched configuration forces 2,2,6,6-tetramethyl piperidine-1-oxyl into the hydrophobic regions. Spectra from this configuration resemble isotropic spectra because of the way in which anisotropic contributions are averaged by molecular reorientations (ref 10) The partioning parameter is defined as 100 A/B.

Fig 4 Spectra of Probe II in native sarcoplasmic reticulum (a) and partially trypsinated sarcoplasmic reticulum (b)

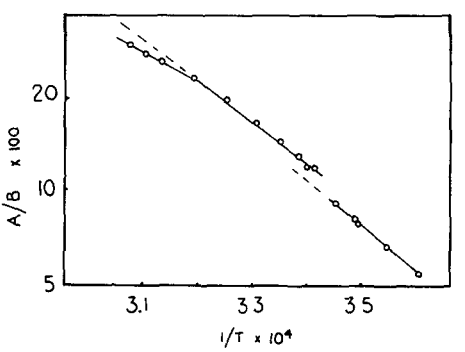


Fig 5 Temperature dependence of the partitioning parameter of Probe II in sarcoplasmic reticulum membranes

of Probe I and Label III. The partitioning parameter was measured in the range between 5 and 50 °C and the results are displayed in Fig. 5. The transition at 22 °C is detected as an abrupt jump in the partitioning parameter. This may be inter-

preted in one of two ways, either as an abrupt change in the bulk fluidity of the lipids or as a sudden increase in the population of fluid lipids. The latter interpretation implies that the transition is due to the melt of only a fraction of the lipid alkyl chains, the other fraction being fluid both above and below 22 °C. The solubility of the probe in the hydrophobic phase is expected to increase in both cases. Only the first one, however, is consistent with the results obtained from Probe I, if one assumes that the Arrhenius plots of the motional parameter are to be interpreted in terms of an activation energy for the motion of the probe in a fluid matrix[5]. The transition at 40 °C is also detected with Probe II. The change in the partitioning parameter implies an increase in the viscosity of the hydrophobic region and, therefore, agrees with the results obtained with Probe I It is difficult to draw any further conclusions from the behavior of Probe II as the parameters governing the partitioning process are not known in detail. It suffices to note the internal consistency of all the results and the apparent independence of the phenomena from the choice of spin labels.

That these transitions are of functional importance is indicated by the following considerations. It has recently been observed that the rates of diffusion of Ca^{2+} across sarcoplasmic reticulum membranes, as mediated by the ionophore X-537A, display different temperature dependences[17] below and above 22 °C. This diffusion

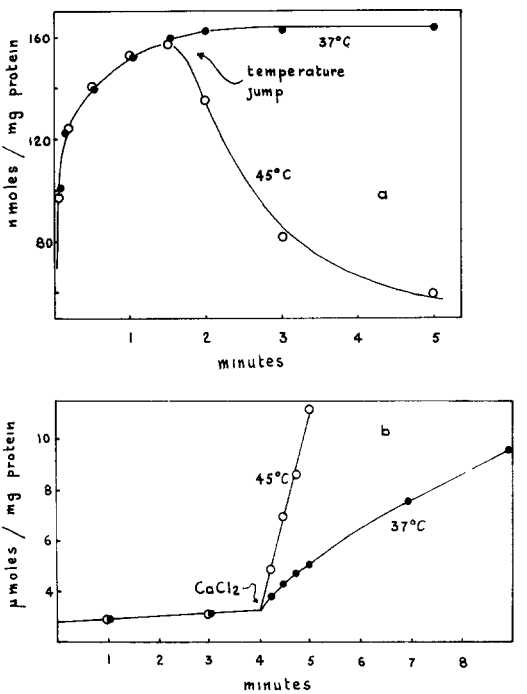


Fig. 6 (a) Ca^{2+} accumulation by sarcoplasmic reticulum Incubation as described in the experimental section. The reaction was started by the addition of ATP at 35 °C After 80 s, the experimental mixture (○) was transferred to a 45 °C bath while the control (●) was kept at 35 °C (b) ATP hydrolysis by sarcoplasmic reticulum Incubation mixture as for Ca^{2+} uptake, with the omission of $CaCl_2$ Reaction was started by the addition of ATP at 37 °C (●) or 45 °C (○) 0 1 mM of $CaCl_2$ were added after 4 min The activity was followed by determining the release of inorganic phosphate from ATP (ref 19)

is dependent upon the hydrophobic properties of the Ca^{2+}–ionophore complex, hence upon its solubility in the lipid moieties of sarcoplasmic reticulum membranes. It is thus clear that the 22 °C transition, assigned by us to a reordering of lipid methylene groups, would alter this solubility and is therefore responsible for the temperature behavior of this diffusion process.

The functional significance of the 40 °C transition, suggested by previous experiments[18], is clearly shown in Fig. 6. If ATP-dependent Ca^{2+} accumulation by sarcoplasmic reticulum is allowed to reach the steady state at 35 °C and if the temperature is subsequently raised to 45 °C prompt release of Ca^{2+} is obtained (Fig. 6a). Furthermore, the burst of ATP hydrolysis associated with Ca^{2+} uptake is prolonged and maintained at constant rates if the temperature of incubation is raised to 45 °C (Fig. 6b). These results are consistent with a temperature-induced leak of accumulated Ca^{2+} at about 40 °C which, as this work indicates, may be due to a thermotropic transition affecting membrane permeability.

ACKNOWLEDGEMENTS

This work was supported by a grant from the American Heart Association (72-847), the U S. Public Health Service (HE 14134) and the Muscular Dystrophy Association.

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