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Phospholipid exchange between restricted and nonrestricted domains in sarcoplasmic reticulum vesicles

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Phosphorus nuclear magnetic resonance spectra of rabbit muscle light sarcoplasmic reticulum membranes consist of two overlapping resonances, one much broader than the other. The broad resonance arises from phospholipids motionally restricted, probably by association with the Ca^{2+}-ATPase, while the narrow resonance arises from phospholipid only slightly perturbed by the presence of the protein. (Selinsky, B.S. and Yeagle, P.L. (1984) Biochemistry 23, 2281–2288). The rate of exchange between the two phospholipid domains represented by the resonances was determined by measuring the transfer of magnetization from the broad resonance to the narrow resonance. The rate of exchange of phospholipids from the restricted domain to the nonrestricted domain was determined to be 1 s^{-1}.

The interaction between lipids and the Ca^{2+}-ATPase of sarcoplasmic reticulum has been intensively studied. Early work indicated that an 'annulus' of approx. 30 phospholipids are necessary to maintain full biological activity of the ATPase [1]. This work was complemented by early magnetic resonance studies [2,3], which indicated that 20–30 phospholipids per Ca^{2+}-ATPase were restricted by the protein. Later studies, primarily using deuterium nuclear magnetic resonance (^{2}H-NMR), did not detect phospholipids closely interacting with the Ca^{2+}-ATPase, but detected instead a general disordering of the phospholipid acyl chains promoted by the protein [4,5]. Recently, this laboratory has presented strong evidence for the existence of at least two phospholipid domains, one of which is motionally restricted by the Ca^{2+}-ATPase of sarcoplasmic reticulum at the phospholipid headgroup and exhibits a dramatically broadened ^{31}P-NMR resonance [6,7]. The other domain is characterized by a ^{31}P-NMR resonance which is only modestly perturbed compared to phospholipids in the absence of membrane protein. This latter observation is remarkably similar to that seen in ^{2}H-NMR spectra of protein-containing membranes, in which the ^{2}H-NMR resonance is also only slightly perturbed by the presence of protein.

In this communication, exchange between the motionally restricted phospholipid domain and the normal bilayer domain detected by ^{31}P-NMR is examined by measuring the transfer of magnetization between the two resonances reflecting the two phospholipid domains. Saturating the broad feature of the two-component ^{31}P-NMR spectrum of light sarcoplasmic reticulum totally eliminates the broad feature. At the same time, resonance intensity is lost from the 'normal bilayer' component. The amount of intensity lost is dependent upon the length of the saturation pulse applied. These

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Abbreviation: Ca^{2+}-ATPase, calcium-dependent, magnesium-stimulated adenosine-5′-triphosphatase.

reuslts are interpreted with respect to phospholipid exchange between motionally restricted and normal phospholipid domains in sarcoplasmic reticulum vesicles.

Materials and Methods

Egg phosphatidylcholine was purchased from Avanti Biochemicals, Inc., Birmingham, AL. Lactate dehydrogenase, pyruvate kinase, adenosine 5'-triphosphate, adenosine 5'-diphosphate, phospho*enol*pyruvate, and nicotinamide adenine dinucleotide were purchased from Sigma Chemical Co., St. Louis, MO.

Preparation and characterization of light sarcoplasmic reticulum. Sarcoplasmic reticulum was prepared from white hind leg mucles of New Zealand White rabbits as previously described [8]. This material was further purified into light sarcoplasmic reticulum by discontinuous sucrose gradient centrifugation [9]. The turbid band at the 25%/32% interface was harvested and washed twice in 20 mM Hepes/100 mM KCl (pH 7.0) by centrifugation at $200\,000 \times g$ at 4°C for 30 min. The final pellet was resuspended in 20 mM Hepes/100 mM KCl/25% sucrose (pH 7.0) and stored under argon at 4°C. This material is referred to as light sarcoplasmic reticulum and is derived from the longitudinal tubules of the sarcoplasmic reticulum. This material represents a subfraction of the material usually referred to as sarcoplasmic reticulum. The characterization of the final pellet was performed as previously described [7]. SDS-polyacrylamide gel electrophoresis of light sarcoplasmic reticulum shows a major band at 105 kDa, corresponding to the Ca^{2+}-ATPase, with minor bands at 70, 160, and 53 kDa. Negative stain electron micrographs of the preparation show unilamellar sealed vesicles.

The viability of the sarcoplasmic reticulum samples used in this study was monitored by the continuous ATP hydrolysis assay [1], substituting 125 mM KH_2PO_4 for triethanolamine and KCl. The ATP hydrolysis activities of light sarcoplasmic reticulum were 2.0–3.5 μmol/mg per min, depending upon the preparation, similar to earlier measurements [7,10]. Calcium transport was measured using 50 μM arsenazo III, a calcium sensitive dye [11]. In the presence of 125 mM KH_2PO_4 as a precipitating agent for calcium inside the vesicles, the calcium transported/ATP hydrolyzed ratio for light sarcoplasmic reticulum was 1.2 to 1.6, which is comparable to other preparations [10].

All NMR and activity measurements were performed within 24 h of isolation. We measured ATP hydrolysis activity before and after the NMR measurements. In all cases, at least 90% of the enzymatic activity was retained after the NMR measurements.

Electron microscopy. Electron micrographs were prepared on a Siemmons 101 electron microscope at 80 kV. Sarcoplasmic reticulum samples were stained with 2% ammonium molybdate directly on Formvar carbon-coated grids.

Phosphorus nuclear magnetic resonance. ^{31}P-NMR spectra were obtained at 109 MHz on a JEOL FX-270 spectrometer. Spectra were acquired using a 32 pulse fully phase cycled Hahn echo pulse sequence described in Ref. 12. This pulse sequence eliminates instrumental distortions and enables data acquisition immediately after refocusing of the echo. Saturation transfer experiments were performed using the DANTE pulse sequence [13] to saturate selected regions of the spectrum at discrete intervals from the excitation frequency. Spectra with pre-saturation were obtained by linking the saturation sequence with the echo sequence described above. A schematic diagram of the linked sequence is shown in Fig. 1. Gated broadband decoupling of 9 kHz (on only during acquisition) was used to minimize sample heating. This compares favorably with the static dipolar ^{1}H-^{31}P interaction of 6 kHz [14]. In all cases 1000 transients were collected. The only ^{31}P containing compounds in the NMR samples are the phospholipids, so that the ^{31}P NMR spectra reflect only the membrane.

Measurement of exchange rate. The rate of exchange between the two domains of phospholipids was determined as shown previously [15–17]. The relationship used is:

$$\frac{I_{B\infty}}{I_{B0}} = \frac{\rho_B}{(\rho_B + k_B)} \tag{1}$$

where $\rho_B = 1/T_1$ of resonance B, I_{B0} = intensity of resonance B without saturation at A, $I_{B\infty}$ = intensity of resonance B after A is totally saturated,

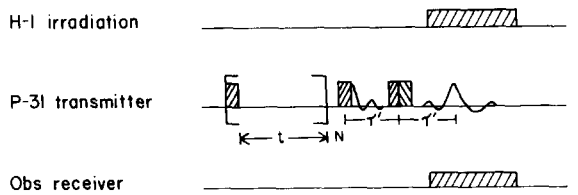

Fig. 1. A schematic diagram of the selective saturation-Hahn echo linked pulse sequence. The frequency of saturation is determined by varying t, while the time of saturation time is determined as the product (pulse width + t) × N. The $\pi/2$ and π pulses are phase cycled as described by Rance and Byrd (1983) [12].

and k_B = the rate constant describing exchange from resonance B to resonance A.

For this analysis, resonance B represents the normal bilayer resonance, while resonance A represents the broad resonance corresponding to motionally restricted phospholipid [7].

Spectral simulations. Spectral simulations were calculated on a microcomputer using the equations of Seelig [18]. In this procedure the transition probability for an axially symmetric powder pattern is convoluted with a Lorentzian lineshape to produce the partially motionally averaged ^{31}P-NMR powder pattern observed for normal phospholipid bilayers. In the cases where a second broad component was required to satisfactorily simulate the observed spectra, either a broad Lorentzian or a broad trapezoidal lineshape was used with similar results. The available experimental definition of the broad resonance is inadequate to produce an accurate description of the exact lineshape. This limitation in the simulations does not materially affect any of the conclusions in this paper.

Results

We have recently shown [7] the existence of two phospholipid domains in sarcoplasmic reticulum membranes. The basis for such a conclusion can be seen in Fig. 2 in the absence of any presaturation. The bottom left shows the ^{31}P-NMR spectrum of an unsonicated dispersion of egg phosphatidylcholine. The same resonance shape is obtained from unilamellar egg phosphatidylcholine vesicles of the same size as the unilamellar light

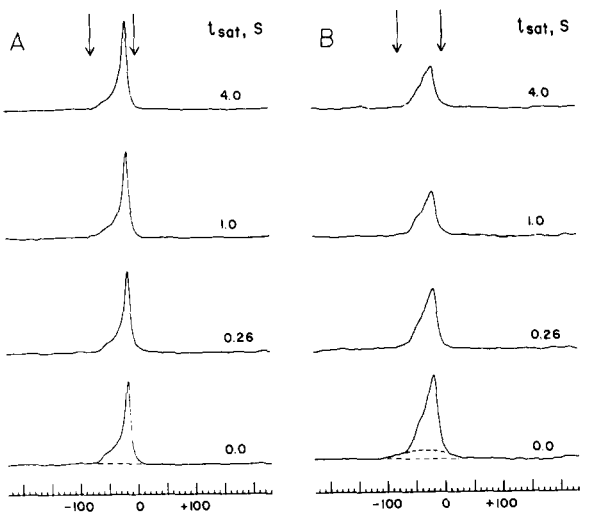

Fig. 2. 109 MHz ^{31}P-NMR spectra of egg phosphatidylcholine (A) and light sarcoplasmic reticulum (B). Selective saturation was applied to both A and B as marked by the arrows. Saturation was applied for the times indicated in the figure. The scale values are in parts per million.

sarcoplasmic reticulum vesicles studied here [7]. The spectrum in the lower right of Fig. 2 of the light sarcoplasmic reticulum shows as the dominant feature a pseudo-axially symmetric powder pattern similar to the pure phospholipids. However, closer inspection indicates that there is a significant difference between the two. The biological membrane exhibits spectral intensity in 'wings' well beyond the region occupied by the normal bilayer resonance that is not seen in the pure lipid spectrum. Similar results are obtained if comparison is made with unsonicated dispersions of the total lipid extract of the light sarcoplasmic reticulum rather than with egg phosphatidylcholine [7].

Spectral simulations demonstrate that this is not explainable by a single resonance. Incorporation of the measured T_2 into the simulation allows the excellent simulation of the normal bilayer portion of the ^{31}P-NMR spectrum of light sarcoplasmic reticulum. However, no simulation using a single resonance is capable of describing simultaneously the normal bilayer portion and the broad portion of the spectrum. However, introduction of a second, broad component into the simulation produces a simulated spectrum with all features of the observed spectrum [7].

On the basis of these results, we suggested that the phospholipid headgroups in light sarcoplasmic reticulum must be in at least two different domains. One domain of phospholipid headgroups exhibits a broad ^{31}P-NMR resonance apparently due to restricted motion of this population caused by the Ca^{2+}-ATPase of sarcoplasmic reticulum. This conclusion results from the observation of a broad component in reconstituted membranes containing only the Ca^{2+}-ATPase and phosphatidylcholine whose intensity is proportional to the protein content of the membrane [7]. The other phospholipid headgroup domain in the membrane exhibits a bilayer resonance similar in lineshape to that obtained with phospholipid vesicles containing no protein.

It was of interest, therefore, to determine the exchange rate of phospholipids between the two domains. We selectively presaturated the broad feature of our ^{31}P-NMR spectra using the DANTE pulse sequence of Morris and Freeman [13], before obtaining the ^{31}P free induction decay using the Hahn echo sequence described in Methods. Upon obtaining the Fourier transformation of the free induction decay, we looked for evidence of transfer of magnetization into the normal bilayer component.

This method has been used in ^{31}P-NMR to study the rotational characteristics of pure phospholipid vesicles [19]. Several important characteristics of the application of the DANTE sequence to ^{31}P-NMR resonances from membranes were demonstrated in this study. The pulse sequence can be constructed to irradiate only a narrow region of the spectrum. De Kruijff et al. [19] demonstrated that for a relatively short period of saturation (about 100 ms), with the irradiation located in the region of the ^{31}P powder pattern from large phospholipid vesicles, a 'hole' is punched in the powder pattern, corresponding to the frequency of the presaturation irradiation. The narrow region of the saturation effect is expected from the DANTE sequence. If the presaturation time is increased to the range of 1 s, then a very different phenomenon occurs. Now the whole powder pattern is eliminated. In fact, De Kruijff et al. [19] used the technique to selectively eliminate the pseudo-axially symmetric powder pattern from the ^{31}P-NMR spectrum containing a narrow resonance. The reason the technique works is that lateral diffusion of the phospholipids among all the possible orientations of the director (describing the axial motion of the phospholipid headgroup with respect to the external magnetic field) occurs effectively on the 1 s timescale, but not effectively on the timescale of 100 ms. Therefore by exchange of the phospholipids among all the sites on the vesicle surface, the magnetization induced at one orientation is transferred to all other orientations, if the saturation is applied for a long enough time.

Thus this technique is suitable for the question of interest here. Saturating irradiation was applied upfield and downfield of, but not overlapping, the resonance which describes the normal phospholipid bilayer in light sarcoplasmic reticulum (see Fig. 2). As a control, the same pattern of saturation was applied to a multilamellar dispersion of egg phosphatidylcholine of approximately the same phospholipid concentration as the sarcoplasmic reticulum sample. This is a suitable control, since the width of the bilayer resonance of egg phosphatidylcholine is greater than the width of the same feature of sarcoplasmic reticulum spectra [6,20].

One concern with this control is that the intrinsic linewidths in the biological membrane are greater than the linewidths in the pure phospholipid dispersions. As described previously, spectral simulation has been used to simulate the lineshapes observed. Using the measured T_2 the normal bilayer component of the ^{31}P-NMR spectrum from light sarcoplasmic reticulum can be well simulated [7]. This simulation demonstrates that there is no significant resonance intensity in the region in which the presaturation irradiation is applied. Therefore there is no opportunity for direct transfer of magnetization from one part of the normal bilayer resonance to another. Furthermore, we have observed that if one applies the same presaturation sequence used here in such a way that the edge of the normal bilayer resonance is irradiated, then the whole bilayer spectrum is affected and effectively disappears (data not shown). This is in agreement with previously published results [19].

When this sequence is applied to the ^{31}P-NMR resonance of unsonicated egg phosphatidylcholine liposomes, with irradiation at the positions indi-

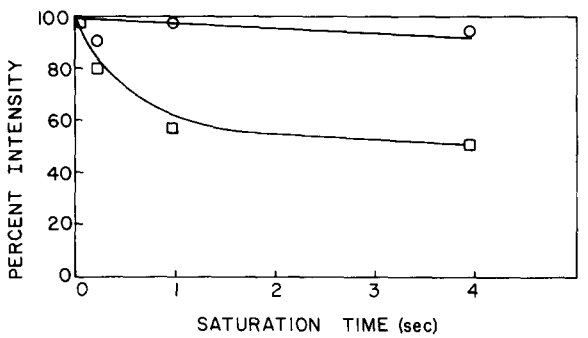

Fig. 3. A plot of percent intensity of the normal bilayer component of ^{31}P-NMR spectra of light sarcoplasmic reticulum as a function of saturation time. The intensity values were determined by integration from the spectra in Fig. 2, with the intensity of the spectra obtained with no saturation taken to be 100%. ○, egg phosphatidylcholine; □, light sarcoplasmic reticulum.

cated in Fig. 2 by arrows, there is no difference in intensity or lineshape of the resonance compared to the absence of the saturation pulse (Fig. 2A). Therefore the presaturation sequence does not affect the normal bilayer resonance under these conditions.

As shown in Fig. 2B, with no saturation, the spectrum of light sarcoplasmic reticulum consists of two overlapping resonances, one with a width of approx. 40 ppm, and the other approx. 150 ppm wide [7]. Applying a saturating pulse at the frequencies indicated by the arrows eliminates the broad resonance. Also seen is a significant reduction in the measured intensity of the normal bilayer component of light sarcoplasmic reticulum. The longer the saturating pulse is applied, the greater the degree of saturation of the normal bilayer resonance. The intensities of the resonances were determined at each level of saturation. Fig. 3 displays the relative intensity of the normal bilayer resonance as a function of saturation time. As shown, the intensity decreases exponentially to an equilibrium value. In contrast, De Kruijff et al. [19] found no transfer of saturation between bilayer resonances arising from large liposomes and isotropic ^{31}P-NMR resonances arising from an isotropic lipid phase. One final control was to change the irradiation frequency. When that frequency was outside the powder pattern, no saturation occurred.

Using Eqn. 1, the rate of exchange between the environments characterized by the broad and narrow components of the spectra can be calculated. Two assumptions need to be made in using this relationship: (1) The broad feature must be entirely saturated. A comparison of the spectra obtained with 0 and 1 seconds saturation time in Fig. 2B shows that most, if not all, the broad component is saturated at short saturation time. (2) The exchange rate must be within one order of magnitude of the T_1 of the narrow component [17]. The T_1 of the normal bilayer spectrum is 1.4 s [7].

Two independently prepared samples were measured using the selective saturation technique described above. Using Eqn. 1, we calculate an average rate of exchange between nonrestricted and restricted phospholipids of approximately 1.0 s^{-1}. Both of the assumptions are therefore satisfied.

We have measured the exchange rate as a function of temperature, and find no significant change between 14°C and 37°C (data not shown). This probably does not mean that the exchange rate is insensitive to temperature, but rather that the changes in the exchange rate are smaller than the sensitivity limit of the method of measurement.

Discussion

In our previous report, we concluded that the ^{31}P-NMR spectra obtained from subfractions of sarcoplasmic reticulum could only be explained by the summation of two different overlapping resonances. This led to the conclusion that at least two domains of phospholipid headgroups must exist in this biological membrane. Here we briefly summarize the results from the previous work.

The ^{31}P-NMR spectra from the light sarcoplasmic reticulum show a pseudo-axially symmetric powder pattern characteristic of phospholipids in a bilayer configuration. The majority of the phospholipids of the membrane inhabit such an environment. However, a second much broader resonance also is observed, indicating that some of the phospholipid headgroups are in a motionally restricted environment. The nature of the two resonances has been considered in more detail.

Whether the broad component is indeed a separate resonance component must first be ex-

amined. ^{31}P-NMR spectra which contain broad features cannot be simulated assuming a single phospholipid population [7]. Only the addition of a second much broader component can explain the results from this viewpoint. The results from a determination of the broad component as a function of decoupler field strength do not support the concept of a poorly decoupled spectrum as the explanation for the resonance shape [7]. Furthermore, the static dipolar coupling is not very strong (6 kHz [14]), because there are no directly bonded protons. It must also be considered whether the broad feature arises because at some orientation of the director, the H-P dipolar interactions are too strong to decouple effectively. However, the region of the pseudo-axially symmetric powder pattern corresponding to that orientation would have intensity missing, intensity which would then show up as the broad component. This is not observed in any of the biological membrane spectra.

Finally the magnetization transfer data recorded here also support the presence of a broad component. For one, the observation that presaturation outside the region of the bilayer resonance for the light sarcoplasmic reticulum produces a perturbation on the bilayer resonance can only be explained by a resonance that extends well beyond that for the normal phospholipid bilayer. Furthermore, previous measurements [19] as well as our own, show that using the conditions we use here, lateral diffusion of the phospholipids would effectively transfer the magnetization to all orientations of the bilayer resonance on the timescale of the saturation. Therefore, if the resonance were all one, the total resonance would be wiped out in our experiment. That it is not can only be explained by the presence of a second component. Therefore the broad component must be a true separate resonance and reflect a separate phospholipid domain in the membrane.

In our earlier work [7] we state that the exchange rate for phospholipids between the two environments represented by the two resonances must be lower than the time resolution of the NMR experiment (10^4 s^{-1}). If faster, a single resonance corresponding to the time average of the two environments would be observed. This selective saturation experiment indicates that the rate of exchange is several orders of magnitude lower than the observation limit of ^{31}P-NMR. These data are consistent with existing calorimetric [21], ^{13}C-NMR [3], and phospholipase data [22] which indirectly indicated two slowly exchanging populations of phospholipids.

Other investigators hypothesized (though they did not measure) an exchange rate 5 to 6 orders of magnitude higher than the rate reported herein [23,24]. Clearly their hypothesis is not applicable in the case of the Ca^{2+}-ATPase and phospholipid headgroups. The reason for this discrepancy needs to be examined in more detail.

The suggestion of such fast exchange arose from an apparent discrepancy between data from electron spin resonance of spin labels in the presence of membrane proteins and data from ^{2}H-NMR spectra of specifically deuterated phospholipids (in the hydrocarbon chains) in the presence of the same membrane proteins. In a combined electron spin resonance and ^{2}H-NMR study of cytochrome oxidase recombined membranes, it was shown that two spectral components are seen in the electron spin resonance spectra, while only one component is seen in the ^{2}H-NMR data [24]. This result was rationalized in terms of an exchange between two environments, one perturbed by the protein and the other bulk lipid bilayer, that was rapid on the ^{2}H-NMR timescale and slow on the electron spin resonance timescale [24]. This would put the exchange rate in the range of 10^5 s^{-1} to 10^7 s^{-1}.

It is very important to note, however, that this is not in any way a direct measure of the exchange rate. It is simply a means which, in the absence of any other data, was satisfactory in rationalizing the electron spin resonance and ^{2}H-NMR data. This rationalization is incapable of encompassing the results in the present report, for here we have a direct measure of the exchange rate which requires an exchange that is much slower than in the hypothesis just described. Therefore an important question is whether a model can be described which is consistent with all the results.

It should be considered whether ^{2}H-NMR can see a deuterium labelled lipid bound to a membrane protein. If ^{2}H-NMR is insensitive to a molecule in such an environment then one would not expect to see two components in the ^{2}H-NMR spectra, regardless of the exchange rate. It would simply be a case where the technique is sensitive to

some but not all the environments in the system. In fact there is a difficulty. Because of the rotational correlation time of the Ca^{2+}-ATPase (60 to 200 μs [25,26]), a lipid bound to this protein in the membrane would be expected to experience intense motional fluctuations at frequencies in the 10 to 100 kHz range. This is of the order of the ^2H quadrupole splitting and would lead to a loss of signal intensity from deuterated lipids in such an environment [27]. A similar problem occurs in the cross polarization ^{13}C magic angle spinning NMR spectra of protein containing membranes which show no resonance attributable to protein [28]. Here the problem is that fluctuations in the same frequency range cause very short proton $T_{1\rho}$ which defeats the cross polarization phenomenon. Thus even though the carbons in the protein are obviously there in the membrane, they cannot be seen by this technique.

There are then two ways to model all the results noted above. In one, the ^2H-NMR cannot detect lipids which are bound to the protein on the timescale determined here using ^{31}P-NMR. Therefore, there is a two component system, with some of the phospholipids bound to the ATPase on a long time scale. However, this does not explain all the results. The ^{31}P normal bilayer resonance in the sarcoplasmic reticulum is not identical to the pure phospholipids, in the absence of the protein. The resonance is somewhat broader reflecting a shorter T_2 [7]. Neither are the ^2H-NMR resonances identical to pure lipids. Again there is a shortening of the T_2 and a broadening of the resonance in the presence of the protein [29]. Therefore, the membrane protein is affecting the normal bilayer phospholipid through some mechanism.

Therefore another model should be considered. In this model, the phospholipids one layer removed from the protein may inhabit a slightly perturbed environment, which is in fast exchange with normal bilayer phospholipids. Alternatively, some of the surface of the membrane protein in contact with the phospholipid bilayer of the membrane may be characterized by weak phospholipid binding sites, which allow rapid exchange with normal bilayer phospholipids. Via this exchange, the resonance arising from normal bilayer lipid is not normal bilayer, but is a weighted mixture of the properties of normal bilayer phospholipid and phospholipid interacting either directly or indirectly with the protein. By this mechanism, the perturbations in the ^2H-NMR spectra and the ^{31}P-NMR spectra (of the normal bilayer component) can be explained. This model would then describe at least three phospholipid environments. One is normal bilayer, which constitutes the most highly populated environment in biological membranes. Another consists of sites in a protein-perturbed environment characterized by fast exchange with normal bilayer phospholipid on the NMR timescale. A third environment consists of sites on the protein with high affinity for phospholipid headgroups and characterized by an exchange rate of 1.0 s^{-1}.

One important perturbation on this model which at present has equal validity is that only the phospholipid headgroups may be tightly bound to the protein. The hydrocarbon chains could be sampling different environments rapidly. This has been observed for a spin label covalently bound to the Ca^{2+}-ATPase [30]. In this regard, it should be recalled that the ^{31}P-NMR measurements primarily measure the behavior of the phospholipid headgroups, while because of the labelling, the ^2H-NMR measurements primarily measure the behavior of the hydrocarbon chains of the phospholipids. This model would also result in an apparent single component ^2H-NMR spectrum.

With an exchange rate of 1.0 s^{-1}, phospholipids closely associated with the Ca^{2+}-ATPase of sarcoplasmic reticulum remain associated with the enzyme through many turnover cycles. This associated phospholipid may influence the physiological activity of this enzyme. We are presently pursuing experiments which test the influence of membrane phospholipids on the Ca^{2+}-ATPase.

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