NMR STUDIES OF THE MOTIONAL CHARACTERISTICS OF THE PHOSPHOLIPID IN RECONSTITUTED SARCOPLASMIC RETICULUM MEMBRANE VESICLES

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PREPARATION OF RECONSTITUTED SARCOPLASMIC RETICULUM MEMBRANE VESICLES (RSR)

The major protein component of sarcoplasmic reticulum (SR) is the calcium pump protein (CPP) comprising 90% of the membrane protein of purified SR. The CPP has all the known characteristics of the pumping molecule including energized calcium pumping when reconstituted to form membrane vesicles. The subunit molecular weight has been estimated to be 119,000 daltons. The functional pump appears to be an oligomer, possibly a trimer (1).

Functional RSR vesicles were prepared containing either dioleoylphosphatidylcholine (DOPC) or dielaidoylphosphatidylcholine (DEPC). For some studies, the SR phospholipid (PL) was exchanged to 99% with a single PL. CPP accounted for >90% of the protein in these membranes and the lipid:protein molar ratio (L/P) was varied from 50 to 130 mol PL/mol CPP (2, 4). Such a high protein content, comparable to that in normal SR (L/P ~ 110), is a favorable range to study the influence of the protein on the motional characteristics of the PL.

RSR is functionally similar to normal SR in the rate of phosphoenzyme formation, phosphoenzyme hydrolysis, calcium-stimulated ATPase activity and phosphoenzyme levels formed from ATP or inorganic phospate. The calcium pumping rate in the presence of oxalate, a calcium precipitating agent, is somewhat less than half that of normal SR. RSR is also capable of energized calcium uptake (in the absence of oxalate) and can synthesize ATP from a calcium gradient (1, 2).[1]

The CPP is bidirectionally aligned in the RSR as compared with the unidirectional alignment in normal SR (1, 2).[1] Nonetheless, RSR is capable of unidirectional Ca^{++} pumping because added substrates, Ca^{++} and ATP, react only with the correctly oriented pumps whose substrate-binding sites are exposed to the external medium.

NMR STUDIES (3, 4)

Deuterium (D) NMR studies of the motion of the hydrophobic domain of the PL in RSR were carried out using deuterium labels located at specific positions along the hydrocarbon chains of the PL. Spectra for PL were compared with those obtained in the presence of the CPP in functional RSR (Fig. 1). The spectra were obtained for DEPC liposomes deuterated at both 9,10 *trans* double bonds, i.e., 1,2[9,10 D_4] DEPC (Fig. 1 *A*) and RSR with the same lipid (Fig. 1 *B*). The symmetry of all four deuterons in the two double bonds gives rise to a single quadrupole splitting: 21.4 kHz in the lipid membrane and 18.8 kHz in the RSR at 25°C. Thus, both spectra have qualitatively the same appearance and are characteristic of one single homogeneous lipid phase. There is no evidence for two discrete lipid environments. Less than 5–10% of the total lipid could escape detection, given the signal-to-noise level of the spectra (4). Any possible undetected lipid is insufficient to form an immobilized boundary (5) around the CPP. The reduction in the quadrupole splitting by 12% in the presence of the CPP and the line broadening indicate increased disorder of the DEPC lipid environment in RSR membrane.

D-NMR studies of PL in the absence and presence of

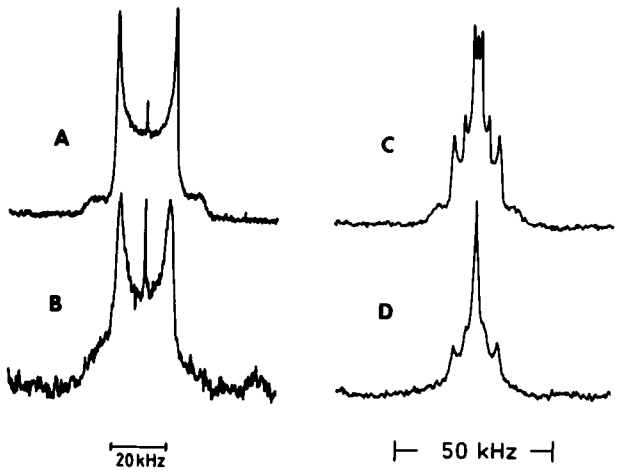

FIGURE 1 D-NMR spectra (46.1 MHz) of PC and functional RSR exchanged with PC. *A*, 1,2-di[9,10 D_2] DEPC (25°C); *B*, RSR exchanged 99% with DEPC (25°C); *C*, 1,2-di[9,10 D_2] DOPC (4°C); *D*, RSR exchanged 99% with DOPC (4°C).

[1]Inesi, G., R. Nakamoto, L. Hymel, and S. Fleischer. Functional characterization of reconstituted sarcoplasmic reticulum vesicles. Submitted for publication.

CPP were also carried out using 1,2[9,10 D_4] DOPC (Fig. 1 *C* and *D*). The D-NMR spectrum of DOPC liposomes consists of three overlapping "powder type" spectra with three observed quadrupole splittings (*cf.* Fig. 1 *C*). This results from a tilting of the *cis* double bond by 5–10° with respect to the bilayer normal, which makes the two deuterons magnetically inequivalent. In addition, the two fatty acyl chains assume different average conformations in the membrane. The smallest splitting comes from the C-10 deuteron in the *sn*-2 oleoyl chain (Fig. 1 *C*); the intermediate from the corresponding deuteron in the *sn*-1 chain; while the largest is a superposition of the two remaining C-9 deuterons (4). Fig. 1 *D* shows a D-NMR spectrum of RSR exchanged with [9,10-D_2]DOPC. As with DEPC, this spectrum qualitatively resembles the pure PL spectrum, with small differences. The lines are broader and the quadrupole splittings are smaller than those observed for pure DOPC. Most conspicuous is the innermost splitting, which is well-resolved in pure DOPC but collapsed to a single broad line in RSR. Again, there is no spectral evidence for a second lipid class, i.e., long-lived boundary lipids. Essentially the same result was obtained with RSR containing [2,2-D_2] DOPC.

The quadrupole splittings, hence order parameters, of pure DOPC liposomes and RSR are found to decrease monotonically with increasing temperature (Fig. 2 *A*). Thus, the lipid is slightly (10–15%) more disordered in the presence of CPP.

Deuterium T_1 relaxation times were measured in order to determine the rate of fatty acyl chain motion. The recovery of the magnetization after a 180° pulse followed a single exponential for both pure DOPC and in RSR containing DOPC (not shown). The temperature dependence of the T_1 relaxation times of [9,10-D_2]DOPC is represented as Arrhenius plots in Fig. 3. Since the considered segmental flexing motions are fast compared to the spectrometer frequency used (46 MHz), $1/T_1$ is directly proportional to the rotational correlation time τ_c. The slopes of these lines represent an activation energy for the motion of the *cis* double-bonded segment. The activation barrier is higher for the pure lipid system (4.7 kcal/mol) than for the RSR (2.9 kcal/mol). At temperatures > 5°C, the τ_c of the label in pure PC is always shorter than that for RSR. Therefore, in the presence of the CPP the fatty acyl chain is moving more slowly by ~ 20% (4).

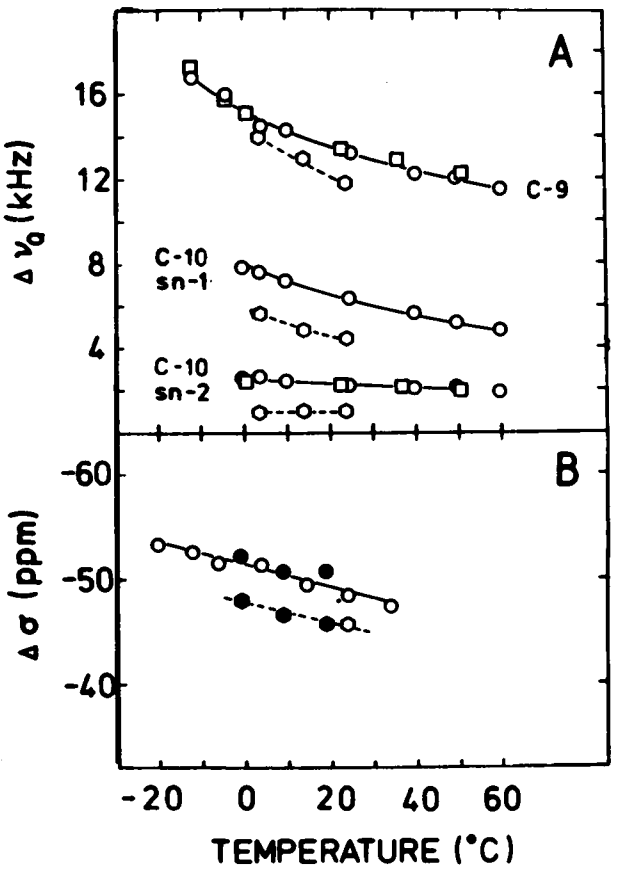

FIGURE 2 Temperature dependence of D and ^{31}P-NMR order parameters. RSR was exchanged with 1,2[9,10-D_2] DOPC. *A*, D-NMR (46.1 MHz). Temperature dependence of the residual quadrupole coupling constant $\Delta\nu_Q$. O, 1,2-di[9,10-D_2]DOPC, liposomes in excess water; □,1-oleoyl-2-[9,10-D_2] DOPC; ○ RSR. The smallest splittings in RSR are estimated to be < 1 kHz (same sample as Fig. 1 *D*). *B*, ^{31}P-NMR. Variation of the chemical shielding anisotropy, $\Delta\sigma$, with temperature. Measurements were made at 121.4 MHz (open symbols) and at 36.4 MHz (solid symbols). O, ●, DOPC in excess water (same sample as Fig. 1 *C*); ○, ●, RSR (same sample as in Fig. 1 *D*).

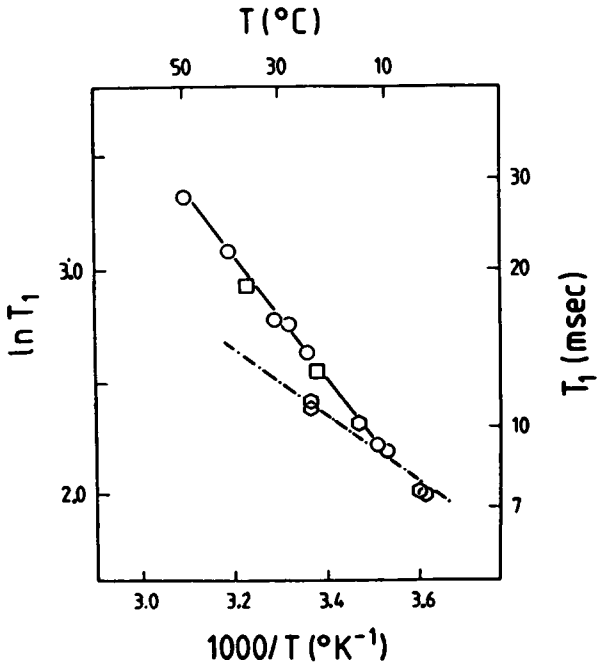

FIGURE 3 Arrhenius plots of the deuterium T_1 relaxation times, at 46.1 MHz. O,1,2-di[9,10-D_2] DOPC dispersed in excess water; □,1-oleoyl-2-[9,10-D_2] DOPC; ○, RSR with 1,2-di[9,10-D_2] DOPC (same sample as Fig. 1 *D*).

Similar studies have been carried out using ^{31}P-NMR which probes the polar region of the PL molecule. In a membrane where the PL molecules are not able to diffuse isotropically, the phosphorus signal is not a sharp line but exhibits a characteristic asymmetric lineshape which can be calculated theoretically. In presence and absence of the CPP, the ^{31}P NMR spectra (proton decoupled) exhibit the same lineshape; no broad component indicative of a strongly bound phospholipid class can be detected. The distance between the two edges of the spectrum, called the chemical shift anisotropy, $\Delta\sigma$, is a measure of the structural order of the phosphate segment. In the presence of protein, a small but consistent decrease in order was observed; the headgroup order decreased with increasing temperature (Fig. 2 *B*) (4, 6). ^{31}P-NMR T_1 relaxation measurements lead to the conclusion that the motion of the polar moiety of the PL is slowed by 10–20% in the presence of the CPP.

In summary, the D-NMR and ^{31}P-NMR measurements detect motional parameters of the hydrophobic and polar moieties of the PL, respectively, and lead to similar conclusions: (*a*) in the presence of the CPP, there is in RSR, to a first approximation and with respect to the NMR time domain, a single homogeneous PL environment typical of a PL bilayer; (*b*) the ordering of the PL is decreased (10–15%) (more orientational disorder) in the presence of CPP as evidenced by the decrease in quadrupole splitting (deuterium NMR) and decrease in chemical shift anisotropy (^{31}P NMR); and (*c*) the motion of the PL is decreased (10–20%) by the presence of CPP in RSR as detected by the changes in T_1 times (4).

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EPR STUDIES OF THE MOTIONAL CHARACTERISTICS OF THE PHOSPHOLIPID IN FUNCTIONAL RECONSTITUTED SARCOPLASMIC RETICULUM MEMBRANE VESICLES

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Functional reconstituted sarcoplasmic reticulum membrane vesicles (RSR) were prepared containing spin-labeled lecithins. The spin probe (doxyl) was located at either carbon 5 (SL-I) or carbon 16 (SL-II) of the *sn*-2 stearoyl moiety to monitor the motional parameters at different locations within the phospholipid (PL) bilayer. Membranes were reconstituted with protein content greater than or similar to that of normal SR (L/P [molar ratio PL:CPP] 110) to facilitate the study of the influence of the protein on the motion of the PL.

RESULTS AND DISCUSSION

The EPR spectra of SL-I (5-doxyl) in SR PL and RSR were similar. There was no indication of a signal referable to very slow motion ($\tau_c < 10^{-7}$ s) in the presence of calcium