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In Situ Modification of the Phospholipid Environment of Native Rabbit Sarcoplasmic Reticulum Membranes

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A water soluble hydrogenation catalyst (palladium di(sodium alizarine monosulphonate)) in a deuterium-containing environment has been used for the *in situ* insertion of deuterium atoms into the fatty acyl chains of biological membranes. The thermotropic response of the stretching vibrations of the formed C-D bonds, as detected by Fourier transform IR spectroscopy, was used as a selective probe of biological membrane structure. Partial deuteration of unsaturated fatty acyl chains coupled with IR detection potentially provides a means for detecting specific biological roles of particular lipid classes. In the current study of sarcoplasmic reticulum membranes and purified phospholipid/CaATPase vesicles, it is also shown that νC-D monitors change at particular membrane locations which may remain undetected through the CH_2 symmetric stretching frequency, a widely used IR spectral parameter. The latter reflects the average environment of the acyl chains. The approach described here may be suitable for wide applications to the study of biomembranes.

In isolated native membranes and in intact cells, palladium di(sodium alizarine monosulphonate), $Pd(QS)_2$, the homogeneous hydrogenation catalyst, has been successfully used to reduce fatty acyl chain double bonds, and the consequences of the reduced lipid flexibility on various cell functions has been demonstrated [1-7]. This catalyst is able to reduce isolated cis double bonds of acyl chains but leaves conjugated systems (chlorophylls, carotenoids, plastoquinone) unaltered [8].

Recently it has been shown that the same catalyst in a gaseous D_2 atmosphere aids the insertion of deuterium labels into the saturated chains that form upon reduction of C=C bonds [9]. The particular locations of C=C bonds in the fatty acyl chains of membranes suggest that the CHD groups that are formed should be selective structural probes of

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membrane organization. The utility of deuterated methylenes as structural probes arises because the CD stretching frequencies occur in a spectral region (2050-2200 cm^{-1}) free from interference from other vibrational modes, except for the water association band. Indeed, the thermotropic response of νCD revealed phase transitions/ separations in mixed lipid model systems which were undetected *via* changes in $\nu_{sym}CH_2$ [9]. The greater sensitivity of νCD may arise from the uniformity of their positions in the fatty acyl chains while $\nu_{sym}CH_2$ reports on the average environment of all CH_2 groups.

The current study describes an attempt to use the CD stretching modes of phospholipid acyl chains deuterated *in situ* as structural probes in native biological membranes. Experiments on active sarcoplasmic reticulum (SR) demonstrate that the homogeneous hydrogenation catalyst can yield valuable insight into structural changes taking place at particular depths within the bilayers of complex biological membranes. We also demonstrate the potential of this approach for the study of lipid/protein interaction in experiments involving reconstitution of purified Ca^{2+}-ATPase from rabbit skeletal muscle with partially deuterated phospholipids.

Materials and Methods

SR membranes were prepared and fully characterized as previously described [10]. About 10-20% of the original ATPase activity remained upon C=C bond reduction. Ca^{2+}-ATPase was reconstituted with partially deuterated 1,2-dilinolenoyl-sn-glycero-3-phosphocholine (di18:3 PC, Avanti Polar Lipids, Alabaster, Al., USA) as described [11], except that precautions were taken to exclude oxygen.

Deuteration experiments: $Pd(QS)_2$, was purchased from Molecular Probes, Eugene, OR., USA. Catalytic saturation of lipid dispersions were conducted exactly as in [5] except that D_2 gas (from Matheson) and D_2O (from Sigma) were used instead of H_2 gas and H_2O. SR membranes were deuterated as follows: Membrane suspensions were centrifuged and re-suspended to be 0.2 mg/ml in protein concentration in a 20 mM TRIS buffer (pD 8.0) in the presence of 0.25 mM sucrose. The catalyst at 5mg/ml was activated under D_2 gas and was used at a catalyst concentration of 0.05 mg/ml in the reaction mixture. Technical details of the deuteration experiments were exactly the same as in [12]. The reaction was stopped by introducing air into the reaction vessel at selected time intervals between 5 and 30 minutes to achieve different levels of deuteration. Membranes were collected by centrifugation and washed twice in H_2O-based TRIS buffer (pH 7.6) in the presence of 0.25 mM sucrose. Gas chromatographic analysis of the fatty acids was carried out as described in [10].

Prior to FTIR measurements, the membranes were further centrifuged (Beckmann TL100 ultracentrifuge, rotor TLA 100.3, 75000 RPM, 30 min) to produce a pellet that minimized the water content of the membrane fragments, since the broad association band of water (~ 2140 cm^{-1}) overlaps νCD. Samples for FTIR measurements were placed between CaF_2 windows using 10μm thick "Teflon" spacers. To retard evaporation during the temperature scans, the edges of the windows were sealed with silicone grease. FTIR spectra were recorded with 4 cm^{-1} resolution on a Polaris spectrophotometer (Mattson, Instruments, Madison, WI, USA). Individual background spectra with the same number of scans (128) were recorded with the aid of a shuttle sample holder. Temperature was controlled to an accuracy +/- 0.1 °C by flow of thermostated water around the sample holder. For thermotropic studies, temperature was increased in 2-3 °C steps. After each step, samples were equilibrated for 10 minutes.

Data processing (baseline subtraction, fitting of Lorentzian bands, etc.) was carried out with a spectrum handling program package (SPSERV). The frequencies of the bands

were determined after baseline subtraction by fitting Lorentzian curves to the given bands (vide infra). The frequencies of the fitted Lorentzians are used in to monitor shifts in band positions as temperature is raised.

Results

Fig. 1a shows the FTIR spectrum of a partially deuterated SR membrane. The CD and CH stretching regions used to characterize lipid structures are marked. It is evident that baseline subtraction is required for the precise determination of band positions. The C-H stretching region is shown in Fig. 1b after subtracting a second order polynomial baseline. For fitting the baseline, only points outside of the C-H stretching range were utilized.

Fig. 1c shows the C-D stretching region of the spectrum. Here the subtraction of the entire water association band contour is needed. This was accomplished by representing the band as a single Lorentzian with an appropriate linewidth. Subtraction of the fitted band from the infrared spectrum revealed the narrower and weaker C-D stretching modes. It is

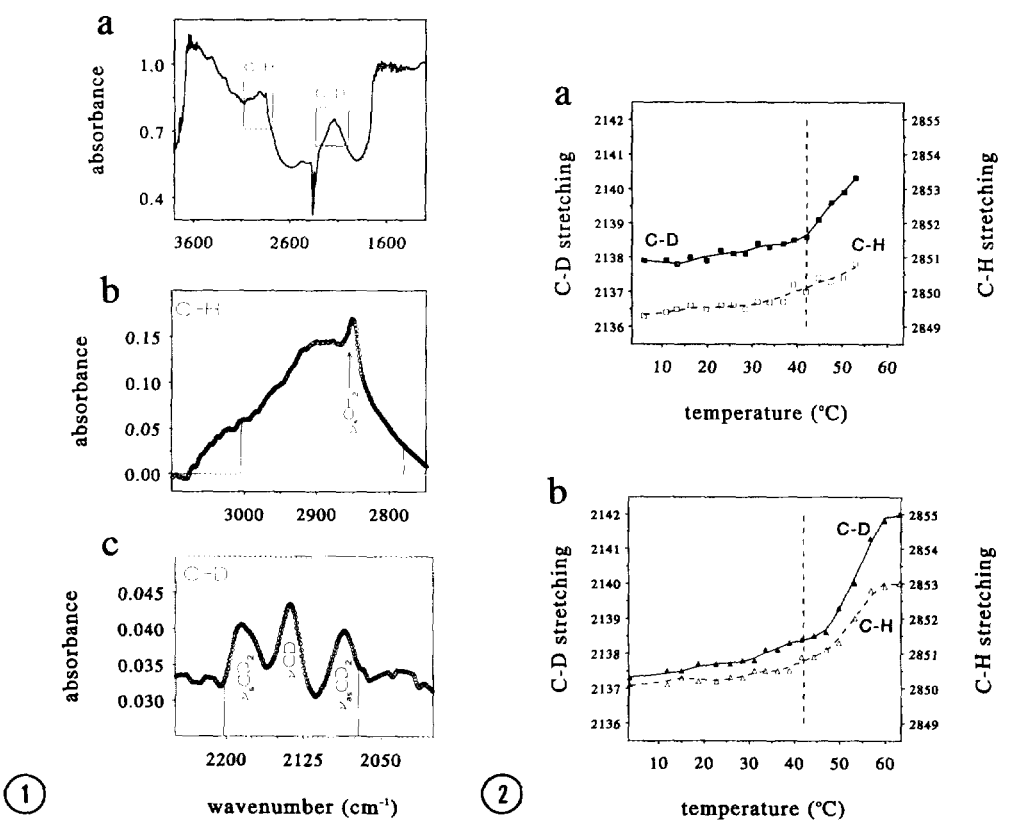

Fig. 1. **a** - FTIR spectrum of partially deuterated SR membranes from rabbit skeletal muscle, (fatty acyl chain distribution: 18:0=57%, 18:1=33%, 18:2=4%, 18:3=6%). Boxes indicate C-H and C-D stretching regions, respectively. **b** - C-H stretching region; **c** - C-D stretching region. Circles indicate experimental spectra, continuous line shows fitted curves.

Fig. 2. Temperature dependence of νCD (solid line) and $\nu_{sym}CH_2$ (dashed line) stretching in SR membranes at different levels of deuteration: **a** - mild deuteration (18:0=57% 18:1=33% 18:2=4% 18:3=6%); **b** - strong deuteration (18:0=76% 18:1=17% 18:2=1% 18:3=6%).

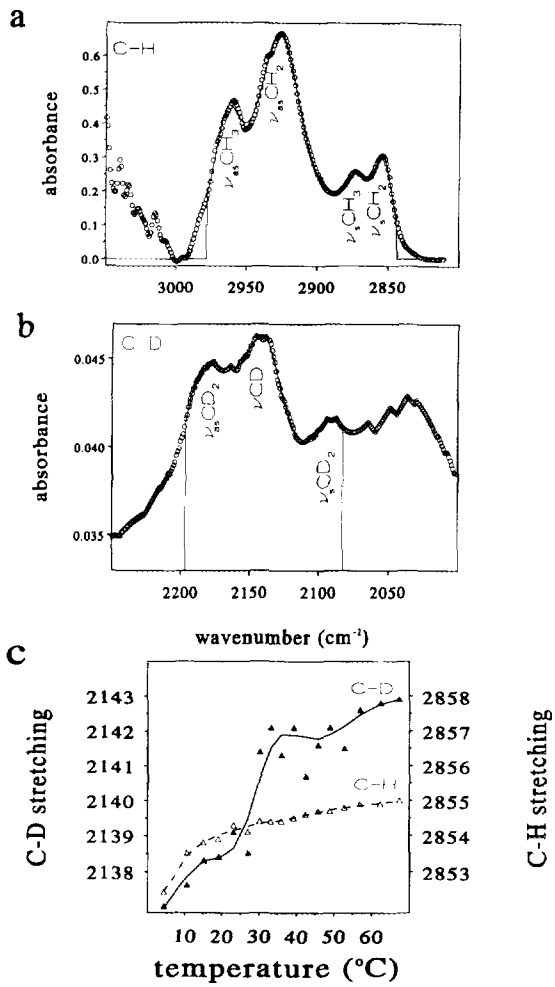

Fig. 3. FTIR spectra of C-H (**a**) and C-D (**b**) stretching regions of Ca^{2+}-ATPase reconstituted with a mixture of (18:0=26% 18:1=16% 18:2=1% 18:3=57%) fatty acyl chains obtained from partial deuteration of di18:3 PC. The solid line shows fitted curves, circles indicate experimental spectrum. (**c**) Temperature dependence of $\nu_{sym}CH_2$ and νCD vibrational frequencies.

noted that according to the mechanism of catalyst action, deuteration should produce CHD groups from reduction of C=C bonds. But, as has been observed for lipid dispersions [9], a significant number of -CD_2- groups are also present in the SR membrane. Thus in Figure 1c, three bands are noted, two of which arise from the symmetric and asymmetric CD_2 stretching modes. Only the central CD stretching mode has been monitored to follow changes in the structure of the biological membrane. Detailed studies have been carried out to determine the origin of the CD_2 stretching bands. The results (to be published elsewhere) do not influence structural conclusions deduced from current experiments.

The level of saturation induced in the membrane is controlled by the deuteration time. The results of two such experiments for SR membranes are shown in Fig 2. The curves show frequency increases with increasing temperatures both for $\nu_{sym}CH_2$ and νCD. SR was

mildly deuterated in the sample for Fig. 2a; the original acyl chain distribution for the C18 chains that contain the bulk of the unsaturated species (18:0=25%, 18:1=41%, 18:2=30%, 18:3=5%) has been altered (final values: 18:0=57%, 18:1=33%, 18:2=4%, 18:3=6%). According to the mechanism of the catalyst action [5], increased levels of 18:0 and 18:1 chains in the reduced membrane are derived primarily from saturation of the 18:1 and 18:2 chains respectively, in the original membrane. The results from a more extensive deuteration (final values: 18:0=76%, 18:1=17%, 18:2=1%, 18:3=6%) are shown in Fig. 2b.

Reconstitution experiments involving Ca^{2+}-ATPase from rabbit skeletal muscle with previously partially deuterated di18:3 PC (final lipid composition: 18:0=26%, 18:1=16%, 18:2=1%, 18:3=57%) also exhibited C-D stretching bands in the FTIR spectra of the lipid-protein complexes (Figures 3a and 3b). Due to the low proportion of deuterated lipid in this system, the signal to noise ratio is poorer than for the native SR. Nevertheless, the CD stretching bands are easily detected. The thermotropic responses of νCD and $\nu_{sym}CH_2$ are shown in Figure 3c.

Discussion

The detection of CD stretching bands in the FTIR spectra of both (reduced) native SR membranes and reconstituted Ca^{2+}ATPase demonstrate the feasibility of introducing and detecting deuterium atoms in the unsaturated acyl chains through the use of the water soluble catalyst, D_2 gas and D_2O. Thus, two different IR spectral parameters, with different sensitivities to thermotropic events, are available. First, all CH_2 groups situated either on inherently saturated fatty acyl chains or on saturated segments of unsaturated lipid molecules, contribute to CH stretching region of the spectrum. Their frequencies monitor an average CH_2 group conformational order, weighted according to the different molecular species and the different positions of these methylene moieties along the fatty acyl chains. Using these frequencies as markers of the lipid phase states [13], information about the bulk conformational properties of the lipid molecules can be deduced.

In contrast, νCD emphasizes two other aspects of membrane lipid structure. First, this spectral marker probes a population of lipid molecules which possess on average a different level of saturation than that sampled by the CH_2 stretching modes. The function, location, conformation, and interactions of this population may be quite different from the entire lipid population. Secondly, since the reduced C=C bonds in the unsaturated fatty acyl chains are located primarily at the C9, C12, and C15 positions, deuteration of these bonds (in the case of partial deuteration, the C15 and C12 double bonds [5] are preferentially attacked) provides a structural probe of the central regions of the bilayer. It is obvious that low levels of deuteration which produce species that retain a substantial level of unsaturation are favorable for this experiment since they best preserve the native membrane character. The resultant CD stretching signals are very weak.

For more saturated membranes (high levels of deuteration), the acyl chains of the deuterium-containing lipids should behave the same way as the inherently saturated lipid molecules. That is, the thermotropic responses should be the same for $\nu_{sym}CH_2$ and νCD.

This simple rationale is sufficient to explain the data in Figure 2. In Figure 2b, data are shown for an extensively deuterated SR sample with a high level of acyl chain saturation. In this instance, both vibrations show the same thermotropic behavior, with a cooperative phase transition/separation characterized by a change of slope in the frequency *vs* temperature curve commencing at 47°C, with a midpoint at 53°C, and a completion temperature of about 59°C.

In Figure 2a, thermotropic data for a sample deuterated to a lesser extent are shown. Minor differences between the response to temperature of the CH_2 and CD stretching frequencies are suggested. The former seems to show the onset of a cooperative process at a somewhat lower temperature (37°C *vs* 42°C) than the former. In addition, the rate of the frequency increase above the onset of the transition appears higher for vCD. The reliability of these observations is limited by scatter in the data. It seems premature to speculate on possible structural origins.

The interaction of purified Ca^{2+}-ATPase with particular lipid classes has been investigated in a series of papers [10,12,14] from the Rutgers group. The current approach provides a means to extend these studies via evaluation of lipid conformational order at specific positions in the bilayer. In the current experiments, di18:3 PC was used for reconstitution and a final composition of 18:0=26%, 18:1=16%, 18:2=1%, 18:3=57% was achieved. Although the signal-to-noise ratio is less than that in native SR, a widely differing response for the CH_2 and CD stretching frequencies is readily noted (Figure 3c). The CD stretching frequency shows a cooperative transition centered at about 30°C which is absent in the CH_2 melting data. This is interpreted as arising from the melting of (mostly) saturated chains which are segregated into domains excluded from the vicinity of the protein. The observed transition is consistent with the phase behavior expected of the lipid population sampled by this spectral parameter, namely a mixture of 18:0 and 18:1-containing PC's. This interpretation is also consistent with the observation [12] that the saturated chains of DPPC reconstituted with Ca-ATPase and native SR lipids, are excluded from the vicinity of the protein and have phase transition properties only slightly altered from pure lipid dispersions.

The major advance of the current study lies in the demonstration of the suitability of the homogeneous hydrogenation catalyst for studies of intact biological membranes as well as reconstituted systems. Although more precise structural interpretations will require more elaborate experiments, the principle of the method is now well established.

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