A FLUORINE NMR STUDY OF MODEL MEMBRANES CONTAINING ^{19}F-LABELED PHOSPHOLIPIDS AND AN INTRINSIC MEMBRANE PROTEIN

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Received 28 July 1981

1. Introduction

To study lipid–protein interaction in a model membrane we have incorporated a protein from bovine brain myelin, lipophilin isolated from the proteolipid, into bilayers of fluorine-labeled phospholipids. This protein has no known enzymatic function but is a so-called structural protein [1]. Its precise function is not known, but it is supposed to be mainly embedded in the bilayer, because this protein is characterized by its large content of apolar amino acid residues and because it has the rather uncommon property for proteins of being soluble in organic solvents [2].

We describe how magnetic resonance measurements, using a specific pulse sequence, can be used for the study of the behaviour of a specifically fluorinated phospholipid, i.e., 1,2-4,4-difluoro-myristoyl-*sn*-glycero-3-phosphocholine(F-DMPC), in complex model membranes. Three systems were investigated: (*i*) F-DMPC/D_2O (70:30, wt %); (*ii*) F-DMPC/cholesterol/D_2O (62:8:30, wt %); and (*iii*) F-DMPC/lipophilin/D_2O (63:7:30, wt %). In [3] it was shown that the pulse sequence used $[90^\circ-(\tau-180^\circ_{90}-\tau)_n]$ selects the F–F dipolar coupling while cancelling all other interactions (F–H coupling, chemical shift anisotropy and magnetic field inhomogeneity). Thus the order parameter [4] $|S_{FF}|$, which varies between 0 (no order) and 1 (perfect alignment) can be determined from the observed line splitting.

We were especially interested in the effects of the presence of cholesterol or protein on the ordering of the phospholipids, and if such effects, if present, could easily be detected by ^{19}F-NMR of fluorinated lipid probes. From ^{2}H-NMR experiments it is known that the phospholipid/water system gives rise to sharp spectra, while the addition of cholesterol or proteins broadens these spectra considerably [5]. It is still a matter of discussion if this line-broadening is due to a distribution of order parameters, caused by the incorporation of cholesterol or protein, or to extra relaxation processes like exchange or other slow motions. As a contribution to this discussion we will present some arguments, obtained from our ^{19}F-NMR experiments, that support the opinion that an exchange process between 'free' and 'perturbed' or 'boundary' lipid, surrounding the protein, is responsible for the observed ^{19}F-NMR spectra.

These ^{19}F-NMR results are in good agreement with ^{2}H-NMR results with similar membrane systems [6]. We find that cholesterol increases the order parameter $|S_{FF}|$, while the protein decreases the order parameter. Also the spectra are broadened by addition of cholesterol or lipophilin. Furthermore we find for the pure phospholipid/water system that both chains have slightly different order parameters, also in agreement with ^{2}H-NMR data [7]. Our work illustrates the applicability of ^{19}F-NMR to complex membrane systems.

2. Experimental

The 4,4-difluoro-tetradecanoic acid was synthesized according to [8], starting from 4-keto-tetradecanoic acid [9]. The corresponding phosphatidylcholine (F-DMPC) was obtained using the procedure in [10]. The raw product was purified on a Sephadex LH-20

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Published by Elsevier/North-Holland Biomedical Press

column (3 × 100 cm) using ethanol/H_2O (95:5, v/v) as solvent [11]. Fractions of 7 ml were collected at 30 ml/h. Each fraction was analyzed by thin-layer chromatography on silica gel, using chloroform/methanol/H_2O (65:25:4, v/v/v) as solvent. Molybdenum phosphate was used for visualisation of the spots [12]. As a reference dimyristoyl phosphatidylcholine (Fluka) was included on each plate. The fractions containing F-DMPC were pooled and F-DMPC was obtained by evaporation of ethanol. F-DMPC was dissolved again in chloroform and precipitated with diethylether. This procedure was repeated and F-DMPC was dried under vacuum. The final product was found to be pure by thin-layer chromatography. A fluorine analysis showed it to contain 98% of the theoretical fluorine content.

Myelin was obtained from bovine brain white matter as in [13]. Lipophilin was prepared by the method in [2] and stored in the lyophilized form. SDS gel electrophoresis showed a single band of 32 000 M_r. The phosphorous content of the protein was 0.04 ± 0.01%. To incorporate lipophilin into F-DMPC bilayers, lipophilin and F-DMPC were dissolved in dichloro-ethanol, after brief sonication, and a 100 mM NaCl solution was slowly added under gentle stirring, until recombined phospholipid protein vesicles precipitated. This procedure was used in [14], except that there dialysis was against 100 mM NaCl instead of slowly mixing, which results in a homogeneous incorporation of lipophilin into phospholipid bilayers. The recombined lipid–protein material was centrifuged at 4500 × g, washed with distilled water to remove NaCl and centrifuged again. Then the lipid–protein material was lyophilized.

Cholesterol was obtained from Baker and used without further purification.

The samples were prepared in a tube with a small constriction. The required amounts of dry material and D_2O were mixed by centrifugation back-and-forth through this constriction until a homogeneous sample was obtained.

All NMR measurements were carried out with a Bruker SXP/4-100 high power pulse NMR spectrometer. The ^{19}F resonance frequency was 65.5 MHz. The 90° pulse length was 1.2 μs and the detection bandwidth was 100 kHz. A $90^\circ-(\tau-180^\circ_{90}-\tau)_n$ pulse sequence was used with $\tau = 50$ μs and $n = 100$. The repetition time for a cycle was 5 s; 200 scans were averaged with a Digital-MINC computer, interfaced with the instrument. The averaged NMR signal was sampled once for each interval between two 180° pulses. These data points were Fourier transformed using a fast Fourier transform routine.

3. Results and discussion

^{19}F Dipolar spectra were obtained between 25–50°C, where the F-DMPC/D_2O system is in the lamellar L_α phase. At 25°C this system transforms to the so-called $P_{\beta'}$ phase, whereas for the unfluorinated DMPC/H_2O system this phase transition is found at 23°C [15]. From this small difference we may conclude that the perturbing effect of ^{19}F labeling at the C-4 position of the myristoyl chains is very weak. This finding is in reasonable agreement with the results of a DSC study [16] on 4,4-, 8,8- and 12, 12-^{19}F-labeled dimyristoyl phosphotidylcholine/H_2O mixtures. These authors found that the perturbing effect of the introduction of CF_2 groups on both chains was smallest for the C-4 position, while the perturbation was stronger for labeling the C-8 and C-12 atoms.

In fig.1, ^{19}F dipolar spectra are shown for the three investigated systems, obtained at 40°C. The F-DMPC/D_2O system gives rise to spectra which clearly

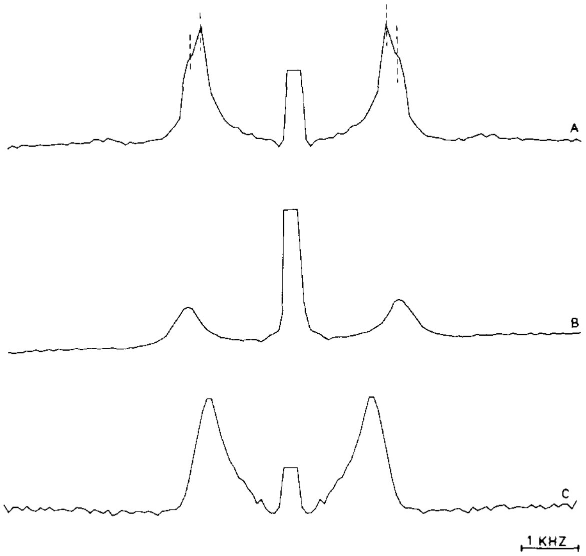

Fig.1. ^{19}F Dipolar spectra obtained at 40°C by Fourier transforming spin echo amplitudes, using a $90^\circ-(\tau-180^\circ_{90}-\tau)_n$ pulse sequence: (A) F-DMPC/D_2O; (B) F-DMPC/cholesterol/D_2O; (C) F-DMPC/lipophilin/D_2O. The central signal is due to baseline distortions and has no physical significance.

exhibit two splittings as indicated in the figure. This means that the order parameter $|S_{FF}|$ is different for the CF_2 groups of both chains. This observation is in agreement with ^{2}H-NMR experiments of the system dipalmitoyl phosphatidylcholine/H_2O with both palmitoyl chains ^{2}H-labeled at the C-2 atom [7]. There it was found that the order parameter $|S_{CD}|$ for the C-2 atom of the *sn*-1 chains is roughly twice that of the *sn*-2 C-2 order parameter. This result was interpreted in terms of a model where the *sn*-1 chain is oriented perpendicular to the bilayer, whereas the first two methylene groups of the *sn*-2 chain are oriented parallel to the bilayer. From the C-2 segment the rest of the *sn*-2 chain is also oriented perpendicular to the bilayer. This model, which has been confirmed by neutron diffraction studies [17] and X-ray studies [18], explains why there is a difference between the $|S_{FF}|$ values for both chains. The difference is not so dramatic as the difference between the $|S_{CD}|$ order parameters of the C-2 segments, because the kink in the *sn*-2 chain is located at the C-2 atom. The difference in depth between the two CF_2 groups will be ~3 methylene segments.

The temperature dependence of $|S_{FF}|$ is shown in fig.2. There is a regular decrease of $|S_{FF}|$ with increasing temperature for all samples. The effect of the addition of cholesterol is clearly an increase in order parameter, in agreement with ^{2}H experiments [6,19]. In this case it is not possible to distinguish the *sn*-1 and *sn*-2 chain fluorine pairs. The effect of the addition of protein is to decrease the average order of the acyl chains. Only one spectral component, consisting of a spectrum considerably broadened compared to the F-DMPC/D_2O system, is observed in agreement with ^{2}H-NMR results [6]. An estimate from the spectra in fig.1 shows that the spectrum is broadened by ~500 Hz compared to the linewidth for the protein-free system. An explanation for this broadening might be the existence of a distribution of order parameters $|S_{FF}|$, caused by the presence of proteins. In that case $|S_{FF}|$ would vary from ~0.18 to ~0.22. On the other hand, if the shape of the spectrum is caused by an exchange process between an unperturbed lipid fraction and a fraction of lipids surrounding the protein, then the resonance line should be a time average of the two resonance lines of these two fractions, if these were measurable separately. The resulting splitting is then the weighted average of the two splittings. For our sample, we may assume that roughly 50% of the lamellar structure is perturbed, since it is known from X-ray diffraction that above a protein/lipid weight ratio of 20% all phospholipid bilayer structure is perturbed by the protein [20]. We estimate then an order parameter $|S_{FF}|$ of 0.16 for the lipids surrounding the protein compared to 0.22 for unperturbed lipids. This difference in S_{FF} corresponds to a frequency difference $\Delta\nu$ of ~1000 Hz in the dipolar coupling of both species. From simple NMR theory of molecular rate processes [21] we calculate the exchange time from:

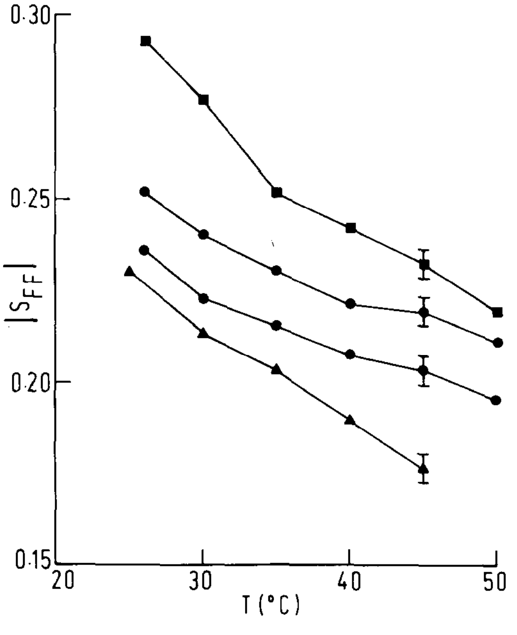

Fig.2. Order parameter $|S_{FF}|$ as a function of temperature: (■) F-DMPC/cholesterol/D_2O; (●) F-DMPC/D_2O, *sn*-1 chain (upper) and *sn*-2 chain (lower); (▲) F-DMPC/lipophilin/D_2O.

$$\tau_{ex} = 1/(2\pi\Delta\nu)^2 T_2$$

where $1/\pi T_2$ is the residual broadening of 500 Hz. This yields $\tau_{ex} \approx 50\ \mu s$.

If we assume that the lateral diffusion constant D of the phospholipid molecules is not dramatically altered by the presence of proteins, and take a value of $D = 10^{-8}\ cm^2 . s^{-1}$ [22], then the r.m.s. displacement in 50 s is $4Dt \approx 140$ Å. Considering the molecular dimensions, where we assume that the lipophilin molecules are spheres of ~50 Å diameter, this value will be more than enough for the phospholipid molecule to diffuse from the boundary lipid fraction to the free lipid fraction and vice versa.

The above exchange model and its numerical estimates also explains why with ESR spin labels in lipid protein systems in general two fractions are observed [23,24]. For an effective averaging between these two fractions exchange rates $\gg 10^8 s^{-1}$ would be required [25], which is impossible in view of the rather slow diffusion in these systems. So we see that all numerical estimates can be brought into agreement with the exchange model. Therefore we feel that this model is more likely to give a correct description of the phospholipid behaviour in lipid–protein systems than a model of order parameter distribution.

In conclusion we may say that the use of ^{19}F as a label is a useful method to study complex model membrane systems. For example it would be very interesting to use the multipulse method described here for the study of lipid polymorphism [26], for it is expected that the ^{19}F dipolar spectra will clearly show the different phases in these systems, like hexagonal phase, lamellar phase and others. The advantage of ^{19}F-NMR above ^{31}P-NMR and ^{2}H-NMR, which are often used for such studies, is that ^{19}F is much more sensitive, while the perturbing effect of a CF_2 group do not appear to be very strong.

Acknowledgement

This research was supported in part by the Foundation for Biophysics under the auspices of the Netherlands Organization for Pure Research (ZWO).

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