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Dynamic properties of the backbone of an integral membrane polypeptide measured by ^{2}H-NMR

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Abstract. The ^{2}H-NMR spectrum of the exchangeable hydrogens of the synthetic amphiphilic polypeptide, lys_2-gly-leu_{24}-lys_2-ala-amide, was measured for the solid peptide at room temperature and, as a function of temperature, for the peptide incorporated into hydrated dipalmitoylphosphatidylcholine (DPPC) bilayers. This study is a prototype of a similar class of experiments which can be carried out on integral membrane proteins to characterize, quantitatively, the dynamic properties of integral membrane proteins. At temperatures below the DPPC gel-liquid crystalline phase transition, the ^{2}H NMR spectrum was very similar to that of the solid peptide indicating that the peptide was immobilized in the lipid bilayer on the time scale ($\approx 10^{-5}$ s) of the ^{2}H-NMR measurements. The ^{2}H-NMR spectrum above the phase transition corresponded to that expected from a peptide in the α-helical conformation reorienting rapidly about the symmetry axis of the α-helix. Measurements of the quadrupolar echo relaxation time, T_{2e}, gave a quantitative measure of the correlation time, τ_c, for this motion. The value of τ_c decreased rapidly with increasing temperature as the fraction of DPPC molecules in the liquid crystalline phase increased, reaching a value of 2×10^{-7} s above the phase transition. The observation of a characteristic minimum in T_{2e} as the temperature was raised provided a definitive, quantitative interpretation of the T_{2e} measurements. Using the known geometry of the peptide and the theory of uniaxial rotational diffusion, a value of $\eta = 1.1$ poise was obtained for the effective viscosity of the membrane in close agreement with values obtained previously from transient linear dichroism measurements.

Key words: Deuterium NMR, peptide dynamics, lipid-peptide interaction, integral membrane polypeptide

Introduction

A large number of studies have indicated that the physical properties of biological membranes have an important influence on their function (Kimelberg 1977; McElhaney 1982). For this reason, many experimental techniques have been used to characterize the physical interactions between lipids and proteins in intact membranes and model membranes (Quinn and Chapman 1980; Quinn 1981). The nuclear magnetic resonance (NMR) technique has been especially useful in describing the orientational order and the dynamic aspects of different portions of phospholipid molecules in model membranes and in determining how the proximity of proteins influences these microscopic properties (Seelig and Seelig 1980; Jacobs and Oldfield 1981; Davis 1983; Devaux 1983).

The papers cited above review results obtained thus far on the effects of lipid-protein interactions on the phospholipid molecules. The first use of deuterium (^{2}H)-NMR to study integral membrane proteins directly was carried out recently with different types of membranes which had been labelled biosynthetically with specifically labelled amino acids (Kinsey et al. 1981; Rice et al. 1981; Keniry et al. 1984). These innovative studies have given information on the dynamic properties of the side chains of integral membrane proteins.

In this paper, we report a prototype experiment using ^{2}H-NMR to obtain information about backbone motions of integral membrane proteins. Our system consists of the synthetic amphiphilic polypeptide

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Abbreviations: NMR, nuclear magnetic resonance; DPPC, dipalmitoylphosphatidylcholine; $K_2GL_{24}K_2A$-amide, lys_2-gly-leu_{24}-lsy_2-ala-amide

lys_2-gly-leu_{24}-lys_2-ala-amide ($K_2GL_{24}K_2A$-amide) incorporated into dipalmitoylphosphatidylcholine (DPPC) phospholipid bilayers. Deuterium (^2H) NMR spectra and quadrupolar echo relaxation times were acquired for the exchangeable hydrogen sites on the polypeptide molecule. A comparison of NMR results obtained from the pure solid peptide with those from the peptide incorporated into DPPC bilayers constitute the main results of this paper. An analogous study of backbone dynamics has been carried out by means of ^{15}N NMR for ^{15}N labelled backbone sites of *fd* bacteriophage (Cross and Opella 1982).

In order to avoid complications due to hydrogen exchange, the sample containing the peptide in DPPC bilayers was prepared in ^2H_2O. The accurate determination of the relatively weak and broad ^2H-NMR spectrum from the incorporated peptide in the presence of such an enormous background signal presented a formidable challenge. A solution to this problem was recently published as a separate report by Callaghan et al. (1984).

The perturbation of the thermodynamic and orientational properties of the DPPC molecules by the peptide molecules has been determined previously using calorimetry and ^2H-NMR (Davis et al. 1983). Circular dichroism measurements made at the same time indicate that the peptide assumes an alpha (α)-helical conformation both inside the bilayer and in solution (Davis et al. 1983). Since such amphiphilic α-helices may be considered as the "building blocks" of integral membrane proteins (Henderson 1981; Engelman et al. 1982; Ovchinnikov 1982), the information we present here on the dynamic properties of individual, α-helical polypeptides enable us to make general comments on the utility of ^2H-NMR in studying integral membrane proteins and peptides. Our experimental results show that the characteristic "time-scale" of ^2H-NMR of a few microseconds is neatly tuned to the onset of rotational motions of the peptides about their long axes as the phospholipid bilayer is warmed through the gel-liquid crystalline transition region. The effects of the onset of rotation are manifest in the shape and width of the ^2H-NMR spectrum and in the characteristic variation with temperature of the relaxation time of the quadrupolar spin echo.

Materials and methods

Peptide synthesis

The amphiphilic peptide ($K_2GL_{24}K_2A$-amide) was synthesized using the general procedures for solid-phase peptide synthesis on a Beckman peptide synthesizer model 900 as described in detail elsewhere (Davis et al. 1983).

Sample preparation

Exchangeable protons on the peptide were replaced with deuterons by dissolving the peptide in MeO^2H. High resolution NMR of the peptide in MeO^2H at 23° C indicated that approximately 40% of the amide hydrogens exchanged rapidly for deuterons (in less than $^1/_2$ h) but that the spectrum changed very little over a subsequent period of weeks. The extent of exchange was calculated from the relative intensity of the peaks in the amide region of the spectrum as compared to the intensity of the leucine methyl hydrogens. In order to catalyze the exchange, deuterated ammonia gas (N^2H_3) was used to make the MeO^2H solution alkaline (Mathew and Richard 1983). Virtually complete exchange of the amide protons was achieved after treating the solution with N^2H_3 and allowing it to stand at 23° C for 24 h.

Mixtures of the exchanged peptide and DPPC were prepared by codissolving the components at a lipid to peptide molar ratio of 50 : 1 in MeO^2H and chloroform. After removing the bulk of the solvent with a stream of N_2, and the residual solvent under vacuum, the mixture was used to prepare multilamellar liposomes. This was done by adding ^2H_2O, heating the sample to 50° C, and mixing with a stirring rod until the sample was homogeneous. The resulting bilayers containing peptide were examined by ^2H-NMR.

NMR spectroscopy

Deuterium NMR spectra were obtained with a Bruker SXP4-100 or a ^2H-NMR spectrometer constructed in the electronics workshop of the UBC Physics department. Both were operated at 35 MHz as described in detail elsewhere (Davis et al. 1976; Davis 1983). All spectra were taken on resonance using a quadrupolar echo technique (Davis et al. 1976) and using a cycle of four pairs of pulses [90_0-τ-90_{90} (add); 90_{180}-τ-90_{90} (subtract); 90_0-τ-90_{270} (add); 90_{180}-τ-90_{270} (subtract)]. The width of a 90° pulse was not greater than 4 μs. Echoes arising from alternate pairs of pulses were added and subtracted from the computer memory as indicated. The spectra were recorded in quadrature and the spectrometer was adjusted so that the signal occurred in only one channel. The out-of-phase channel, which contained only noise, was zeroed, giving perfectly symmetrical spectra on Fourier transformation and an increase in

signal/noise by a factor of $\sqrt{2}$ (Davis 1983). No filters were used and no phase corrections were required. Details about number of scans, repetition rate and spectral width are given in the figure captions.

Spectra arising from the peptide in the DPPC/peptide/^2H_2O sample were obtained by the following procedure which has been described in detail elsewhere (Callaghan et al. 1984). The problem is to separate with high fidelity a very weak short lived free induction NMR signal superimposed on a very strong long lived signal.

1. A low order polynomial was fit to the long time portion of the NMR signal which arises mainly from the ^2H_2O.

2. The polynomial fit was extrapolated back to zero time which corresponds to the peak of the quadrupolar echo.

3. This extrapolated curve was subtracted from the whole ^{2}H-NMR signal to yield the peptide signal. The Fourier Transform of this signal corresponds to the peptide ^{2}H-NMR powder spectrum.

The solid spectrum was "de-Paked" following the numerical procedure described by Sternin et al. (1983) and Bloom et al. (1981). For powder spectra arising from nuclei with local axial symmetry corresponding to the case $\eta = 0$ in the theoretical section of this paper, the de-Paked spectrum is the true oriented sample spectrum. For $\eta \neq 0$, the singularities of the spectrum correspond to the frequency values $(\omega_Q/2\pi)$ $(1 \pm \eta)$ for $\theta = \pi/2$, $\varphi = 0$ and $\pi/2$ [see Eq. (1)], thus yielding values of ω_Q and η. In general, the quadrupolar coupling constants can be measured more accurately from the de-Paked spectrum than from the original powder spectrum.

The time constant, T_{2e}, for the decay of the quadrupolar echo amplitude as a function of τ was measured for the deuterons in the solid peptide sample and, again, after incorporation in the DPPC bilayer.

The sample temperature was regulated by an oven enclosing the sample and radio frequency coil. At least 30 min were allowed for the sample to reach equilibrium after each temperature change. A sensitive measure of the approach to equilibrium was found to be the variation with time of the dielectric properties of the sample as detected by the tuning bridge across the coil.

Calorimetry

Calorimetry was performed on a few milligrams of the peptide/lipid mixture suspended in ^2H_2O at a concentration of 5 mg/ml using a Microcal MC-I high-sensitivity differential scanning calorimeter. A scan rate of 30° C/h was used.

Review of relevant NMR theory

Effect of molecular reorientation on ^{2}H-NMR properties

Spectrum. The quadrupolar splitting of the two members of a ^{2}H-NMR doublet is given by $2\,\omega(\theta, \phi)$, where (Seelig 1977; Abragam 1961)

$$\omega(\theta,\phi) = \omega_Q \left[P_2(\cos\theta) + \frac{1}{2}\eta \sin^2\theta \cos 2\phi \right], \quad (1)$$

where $\omega_Q = \frac{3}{4}\left(\frac{e^2 qQ}{h}\right)$ is the principal value and η the asymmetry parameter of the quadrupolar interaction, (θ, ϕ) are the spherical polar angles of the magnetic field $\mathbf{B}_0$ in the electric field gradient principal axis coordinate system and $P_2(x) = \frac{1}{2}(3x^2 - 1)$.

If rapid rotation of the molecule-fixed principal axes takes place about an axis having spherical polar angles (β, α) in the principal axis coordinate system, the splitting is motionally averaged to $2\,\omega(\beta, \alpha; \theta)$, where

$$\omega(\beta,\alpha;\theta) = \omega_Q \left[P_2(\cos\beta) + \frac{1}{2}\eta \sin^2\beta \cos 2\alpha \right] P(\cos\theta), \quad (2)$$

where θ is the angle between $\mathbf{B}_0$ and the rotation axis. Additional motions would reduce the quadrupolar splitting further. This reduction could be represented formally by an average of $\omega(\beta, \alpha; \theta)$ over an appropriate statistical distribution of values of β and α.

Relaxation. The fluctuations in the secular part of the quadrupolar interaction which reduce the quadrupolar frequency from $\omega(\theta, \varphi)$ to $\omega(\beta, \alpha; \theta)$ give rise to a random accumulation of phase which results in a decay of the quadrupolar echo. Though the angular dependence of the decay rate T_{2e}^{-1} should give rise to non-exponential relaxation, the signal-to-noise ratio of the measurements reported in this paper was insufficient to detect the departure from exponential behaviour. Thus, the measured T_{2e}^{-1} corresponds to the powder averaged relaxation rate which may be interpreted, using the general theory of NMR relaxation (Abragam 1961), in terms of the change in the apparent second moment of the powder due to

rotation. Using Eqs. (1) and (2), this may be shown to be

$$\Delta M_2 = \langle[\omega(\theta,\phi)]^2\rangle - \langle[\omega(\beta,\alpha;\theta)]^2\rangle$$
$$= \frac{1}{5}\omega_Q^2\left\{\left[1+\frac{\eta^2}{15}\right] - [P_2(\cos\beta) + \frac{1}{2}\eta\sin^2\beta\cos 2\alpha]^2\right\}, \quad (3)$$

where the brackets $\langle\ \rangle$ denote a powder average.

For systems, such as the one studied in this paper, in which the terms involving η are all negligible,

$$\Delta M_2 \approx \frac{1}{5}\omega_Q^2\{1 - [P_2(\cos\beta)]^2\}. \quad (4)$$

A quantitative analysis of T_{2e} measurements can be made for the limiting cases of fast and slow motion which are defined in terms of the relationship between M_2 and the correlation time, τ_c, for the motion.

Fast motion: $\Delta M_2 \cdot \tau_c^2 \ll 1$

In this short correlation time limit, the general theory of motional averaging leads to a result for isotropic motions which is essentially independent of the details of the motion for a large class of systems (Abragam 1961; Pines and Slichter 1955; Bloom et al. 1965), namely

$$\frac{1}{T_{2e}} = \Delta M_2 \cdot \tau_c. \quad (5)$$

In this paper, we shall be particularly concerned with motional narrowing due to reorientation about a single molecular axis. For this purpose, we model the molecule as a cylinder of radius a and length h immersed in a membrane of viscosity η (Saffman and Delbruck 1975). Then the result of Eq. (5) is only valid when the random rotational angular jumps of the molecule about its long axis are very large. In the opposite limit of very small rotational jumps, which is described by the rotational diffusion equation, the theory of motional narrowing gives the result

$$\frac{1}{T_{2e}} = \frac{1}{5}\omega_Q^2\left\{1 - [P_2(\cos\beta)]^2 - \frac{9}{16}\sin^4\beta\right\}\tau_c, \quad (6)$$

where τ_c is expressed in terms of the diffusion constant for axial rotation as

$$\tau_c = D_R^{-1} = \frac{4\pi a^2 h\eta}{kT}. \quad (7)$$

We have not provided the details of the calculation leading to Eq. (6), but remark that it follows from a calculation of the correlation function of $P_2[\cos\theta(t)]$ which yields a result identical in form to that used in linear dichroism [see e.g., Cherry 1979, Eq. (4)]. In that case, β is the angle between the transition dipole moment and the axis of rotation.

Slow motion: $\Delta M_2 \cdot \tau_c^2 \gg 1$

The result obtained in this limit depends on the nature of the motion and also on how the measurements are made. In a two-pulse echo experiment in which the separation, τ, of the pulses is sufficiently large to satisfy $\Delta M_2\tau^2 \gg 1$, and in which the random rotational jumps of the molecule are large, the result is of the form

$$T_{2e} = p\tau_c, \quad (8)$$

where $p \geq 1$. The interpretation of this result is very simple. Whenever a molecular jump occurs which changes the frequency by an amount $\gtrsim (\Delta M_2)^{1/2}$ in a random manner, the ^{2}H-nucleus acquires random phase in the time 2τ characteristic of the echo experiment, i.e., as a result of the jump, the second pulse at time τ does not result in a refocussing of the magnetization at time 2τ. Thus, only the spins which undergo no large jumps in time 2τ, i.e., the fraction $\exp[-2\tau/(p\tau_c)]$, contribute to the echo signal. The factor p is a measure of the "memory" of the original quadrupolar frequency following the jump. In the case of a symmetric two-site problem (Bloom et al. 1965), for example, it was found that $p = 2$. The relaxation behaviour is different in the slow motion limit in the case of very small rotational steps, i.e., rotational diffusion, but theory (Woessner et al. 1969), still predicts that T_{2e} increases with increasing τ_c.

Intermediate motion: $\Delta M_2 \cdot \tau_c^2 \approx 1$

Since $T_{2e} \propto \tau_c^{-1}$ for fast motions while $T_{2e} \propto \tau_c$ for slow motions, the value of T_{2e} must go through a minimum for intermediate values of τ_c. Such T_{2e} minima have been discussed elsewhere in a different context (Bendel et al. 1983). In general, the relaxation is predicted to be non-exponential. The form of the relaxation curve is sensitive to the nature of the motion responsible for the relaxation and is capable of providing an independent check on the absolute values of ΔM_2 and τ_c. For systems satisfying Eqs. (5) and (8), we propose the following interpolation

formula for the intermediate regime.

$$\frac{1}{T_{2e}} = \frac{\Delta M_2 \tau_c}{1 + p\Delta M_2 \tau_c^2}. \quad (9)$$

Note that Eq. (9) gives correct values for the fast motion and slow motion limits corresponding to Eqs. (5) and (8), respectively. The numerical values of T_{2e} and τ_c at the minimum are given by

$$T_{2e}(\min) \approx 2\left(\frac{p}{\Delta M_2}\right)^{1/2}; \quad \tau_c \approx (p\Delta M_2)^{-1/2}. \quad (10)$$

As a final remark to this theoretical section, we wish to emphasize that the formulae presented here represent crude approximations to the relaxation behaviour of many systems. As mentioned above, the relaxation of the quadrupolar echo should, in general, be non-exponential for at least two reasons. Firstly, the relaxation rate is expected to be orientation dependent. Secondly, even for an oriented sample, the relaxation is expected to be non-exponential except for the short correlation time limit, $\Delta M_2 \tau_c^2 \ll 1$. Nevertheless, we are obliged to use formulae for the average relaxation rate in this study because all our measurements for the peptide dissolved in lipid bilayers were adequately fitted by a single exponential function of the pulse spacing. (Some representative relaxation curves are shown in Fig. 1). With better signal/noise, which we hope to achieve in future studies, it may be possible to define more precisely the nature of the molecular reorientation when the short correlation time approximation is not satisfied by studying the variation in the shape of the quadrupolar echo Fourier transform spectrum with τ as described by Spiess and Sillescu (1980) and Griffin (1981).

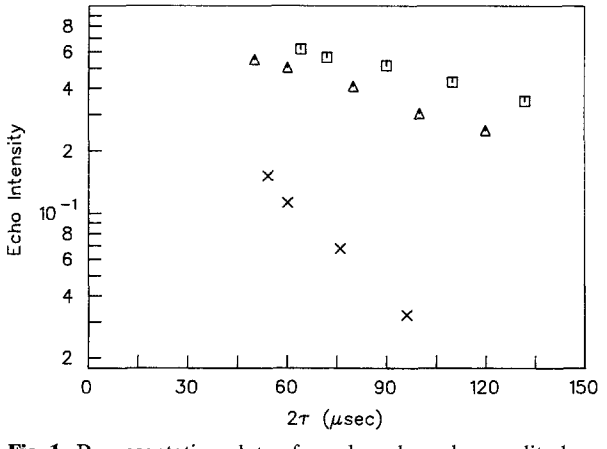


Fig. 1. Representative plots of quadrupolar echo amplitude as a function of 2τ in the measurement of T_{2e}. The data shown correspond to △ 26° C, × 34° C and □ 40° C

Results

The ^{2}H-NMR spectrum of the exchangeable hydrogens in the solid peptide is shown in Fig. 2a. Three components can be identified: a narrow, isotropic central peak, a powder pattern approximately 36 kHz wide of the type obtained for $\eta = 0$ and a second powder pattern with $\eta \neq 0$, approximately 150 kHz wide and containing most of the spectral intensity. These components are clearly separated in the "de-Paked" spectrum shown in Fig. 3. The singularities in the de-Paked spectrum of the broad component occur at $(\omega_Q/2\pi)$ $(1 \pm \eta)$ which gives the

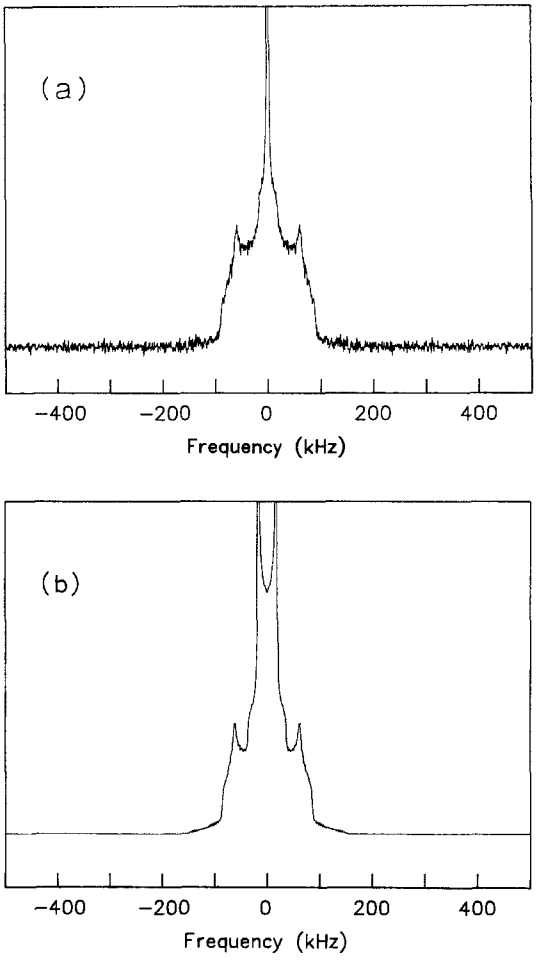


Fig. 2. a ^{2}H-NMR spectrum of the hydrogen-exchanged polypeptide $K_2GL_{24}K_2A$-amide in solid powder form at room temperature. The number of scans was N_S = 150,000, time between repetitions of the pulse sequence was T_R = 2.5 s and the time between 90° pulses in the quadrupolar echo sequence was τ = 40 μs. **b** Simulated spectrum of the solid using the quadrupolar coupling constants from the de-Paked spectrum shown in Fig. 3 for a ratio of intensities of broad ($\omega_Q/2\pi$ = 150 kHz, η = 0.16) to narrow ($\omega_Q/2\pi$ = 36 kHz, η = 0) powder spectra corresponding to 31/15 as discussed in the text and using Lorentzian broadening corresponding to a value of $T_{2e} \approx 160$ μs. The distortion due to finite r.f. pulse length (Bloom et al. 1980) has been included in calculating the simulated spectrum

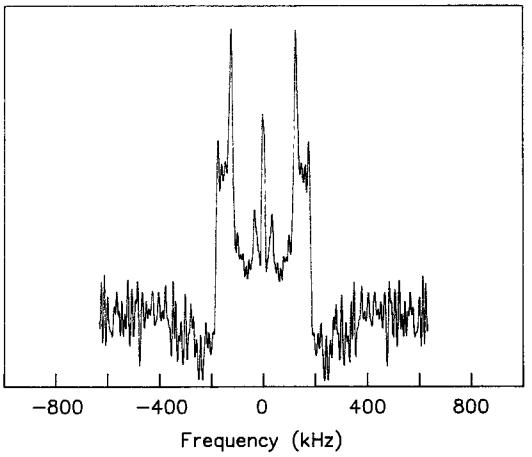


Fig. 3. The "de-Paked" spectrum (Bloom et al. 1981; Sternin et al. 1983) derived from the solid peptide powder pattern of Fig. 2a

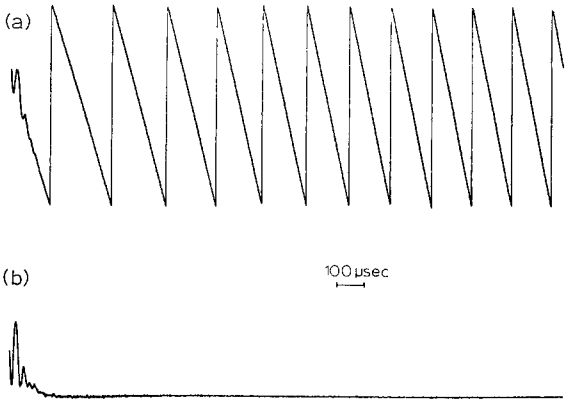
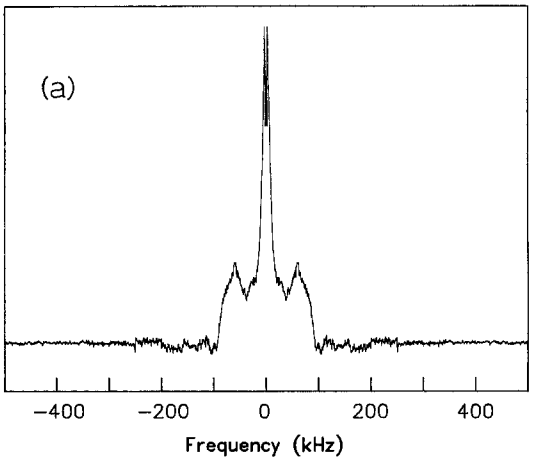


Fig. 4a and b. Illustration of the use of the polynomial fit method (Callaghan et al. 1984) to remove a large solvent peak. (Note that the similar figure (Fig. 5) in the paper by Callaghan et al. (1984) was interchanged with their Fig. 2). In **a**, it is seen that the peptide signal appears only during the first 100 μs shown while the much larger solvent peak overflows the computer memory 12 times during the 2,048 μs shown in the figure. Subtraction of the solvent peak, as described in the text, leaves the peptide signal shown in **b**. **a** Quadrupolar echo arising from the sample containing dispersions of DPPC and peptide (lipid to peptide molar ratio = 50 : 1) plus ^2H_2O (40% by weight). **b** Signal remaining after subtraction of a polynomial fit to data of a starting at time 100 μs after the echo peak

values $\omega_Q/2\pi$ = 150 kHz and η = 0.16, while the doublet arising from the narrower powder pattern indicates that η = 0 and $\omega_Q/2\omega$ = 36 kHz.

The quadrupolar echo decay in the solid peptide was found to be an exponential function of time with a time constant T_{2e} = 160 μs. The spin-lattice relaxation (T_1) curve was non-exponential, however, and had characteristic slopes in the limit of short and long times corresponding to relaxation times of 0.26 s and 0.90 s, respectively.

The values of ω_Q, η and T_{2e} were used to simulate the powder spectrum as a superposition of Lorentzian-broadened doublets of half-width $(\pi T_{2e})^{-1}$ and



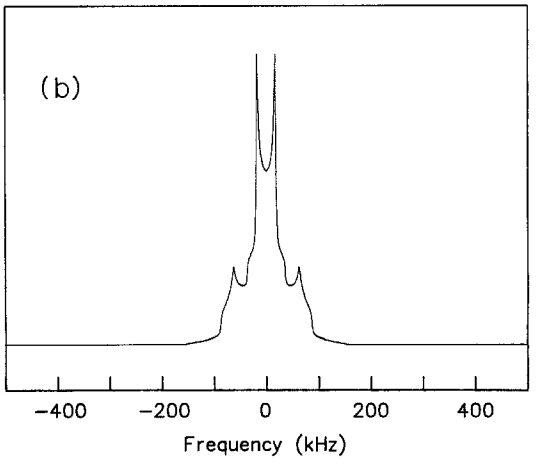


Fig. 5. a ^{2}H-NMR spectrum at 10° C of the hydrogen-exchanged peptide incorporated into liposomes of DPPC. N_S = 160,000, T_R = 0.5 s, τ = 34 μs. **b** Simulated spectrum identical to that of Fig. 2 b except that a value of T_{2e} = 116 μs was used

separation (in angular frequency units) 2 $\omega(\theta, \phi)$ [see Eq. (1)] with values of (θ, φ) uniformly distributed in solid angle for each of the broad and narrow powder patterns. For reasons to be discussed later, the ratio of the integrated intensities of the broad to narrow spectra was taken to be 31 : 15. The simulated spectrum shown in Fig. 2b has been scaled vertically to match the amplitude of the broad powder pattern peak. A comparison of Figs. 2a and b shows that this procedure results in an excellent fit for the broad spectrum. However, the relative intensity of the narrower powder pattern is too large in the simulated spectrum. The significance of this observation will be discussed later.

Because of chemical exchange, the liposomes containing peptide had to be formed in ^2H_2O. Since the ratio of the number of deuterons, in ^2H_2O to those associated with the peptide was approximately 300 : 1, the ^{2}H-NMR signals were dominated

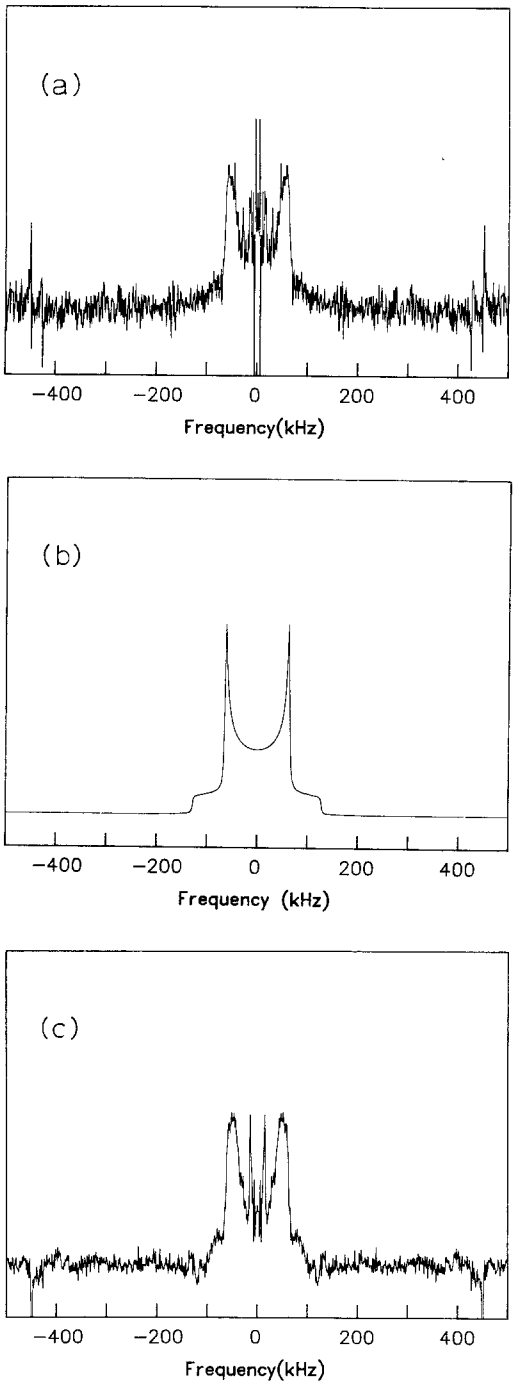


Fig. 6. a ^{2}H-NMR spectrum at 42° C of the hydrogen-exchanged peptide incorporated into liposomes of DPPC. N_S = 416,000, T_R = 0.5 s, τ = 40 μs. **b** Simulated powder spectrum of the broad component using $\omega_Q/2\pi$ = 127 kHz, η = 0 and T_2 = 100 μs. **c** ^{2}H-NMR spectrum at 50° C of the same sample as in **a** with Ns = 690,000, T = 0.5 s, τ = 40 μs

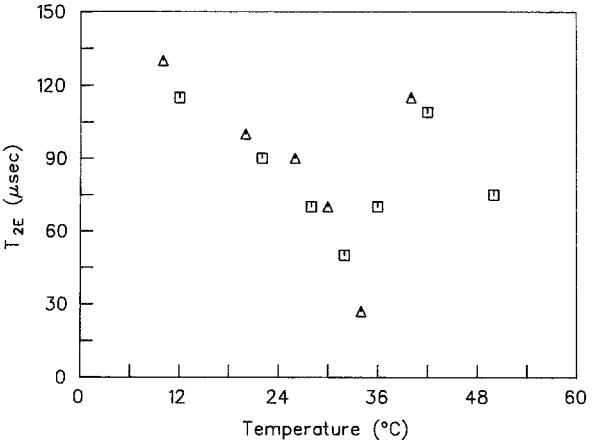


Fig. 7. Plot of measured values of T_{2e} of the ^{2}H-NMR quadrupolar echoes of the hydrogen-exchanged peptide as a function of temperature in the peptide/DPPC/^2H_2O samples. The different symbols represent measurements on two different samples

by the solvent signal as seen from Fig. 4a. After applying the polynomial fit and solvent signal subtraction technique described in the Methods section (Callaghan et al. 1984), only the quadrupolar echo arising from the peptide remains (Fig. 4b). The Fourier transform of the echo gave the 10° C spectrum shown in Fig. 5. At this temperature, the DPPC bilayer in which the peptide is imbedded is in the gel state (Davis et al. 1983). The spectrum observed for the peptide-lipid mixture, at this temperature, is similar to that shown in Fig. 2a for the peptide alone. Indeed, an excellent fit of the broad powder pattern in the observed spectrum was obtained with the same values of ω_Q and η as were used in Fig. 2b and the value of T_{2e} = 116 μs determined in a quadrupolar echo decay experiment. The T_1 values were about 30% shorter than those measured for the solid peptide.

Figure 6a shows the spectrum obtained from the protein/lipid mixture at 42° C. Most of the lipid bilayer is in the liquid crystalline phase at this temperature (Davis et al. 1983) and the spectrum is somewhat narrower than that obtained at 10° C. The 42° C spectrum is best fit by a powder pattern with η = 0, and the experimentally measured values of $\omega_Q/2\pi$ = 127 kHz and T_{2e} = 100 μs (Fig. 6b). The spectrum obtained at 50° C (Fig. 6c) was characterized by the same maximum quadrupolar splitting value, but had more intensity at frequencies slightly smaller than the edges of the simulated spectrum.

Figure 7 is a plot of the temperature dependence of T_{2e} from 10° C to 50° C. A pronounced minimum occurs between 30° and 40° C so that the T_{2e} measured at 35° C is one fifth of the 10° C value. The temperature at which the T_{2e} minimum occurs is close to the onset temperature of the endothermic peak observed using differential scanning calorimetry. The peak was found to have a maximum at 41° C and extended from approximately 35° to 46° C (data not shown) in agreement with previous studies by Davis et al. (1983).

Discussion

Interpretation of the NMR spectra

The aim of this study was to obtain information on the motions and conformational states of the polypeptide $K_2GL_{24}K_2A$-amide in a DPPC phospholipid bilayer membrane by studying the ^2H-NMR spectrum of exchangeable hydrogen sites in the polypeptide. Our starting point is the spectrum of the solid peptide shown in Fig. 2a. The broad component of this spectrum is characterized by the quadrupolar coupling parameters $\omega_Q/2\pi$ = 150 kHz and η = 0.16. These values are typical of rigid amide deuterons which are hydrogen-bonded to oxygen atoms in crystalline solids (Hunt and MacKay 1976). Since this peptide is presumably in an α-helical conformation in the solid (Davis et al. 1983), the CO group of each amino acid is hydrogen bonded to the NH group of the residue four residues ahead in the linear sequence (see, e.g., Stryer 1981, p 30). The N-^2H vector, which should be close to the main principal axis of the electric field gradient at the ^2H nucleus, is inclined at a small angle to the symmetry axis of the α-helix.

The narrower powder pattern in Fig. 2a, having $\omega_Q/2\pi$ = 36 kHz and η = 0 is attributable to rotating N^2H_3 groups of the four lysine side chains and the terminal N^2H_3 (Hunt and MacKay 1974, 1976).

It remains to interpret the relative intensities of the broad and narrow pattern powder spectra. Assuming that the two terminal amide hydrogens contribute to the broad spectrum and assuming complete hydrogen exchange, the ratio of the number of amide deuterons to rotating amino group deuterons is 31 : 15. Figure 2b is a simulation of the solid peptide NMR spectrum in which 31/46 of the intensity was assigned to the broad component and 15/46 to the narrow component. The low intensity in the wings of the peptide bond amide experimental spectrum can be accounted for in the simulated spectrum by including the distortion associated with a 4 μs r.f. pulse (Bloom et al. 1980), though the actual length of the pulse used was somewhat shorter. The simulation matches the broad spectrum well but the rotating amino group spectrum seems to be much more intense in the simulated spectrum than in the experimental spectrum. A possible reason for this is that some of the lysine amino groups were not rotating in the solid peptide. The non-rotating amino groups would then contribute to a broad spectrum (Hunt and MacKay 1974, 1976).

The ^2H-NMR spectrum of the peptide in the gel phase of DPPC (Fig. 5a) is similar to that obtained from the solid peptide. The broad component is fitted quite well by a simulated spectrum (Fig. 5b) with the same ω_Q and η as for the solid peptide spectral simulation (Fig. 2b) but with a value of T_{2e} = 116 μs as measured for the DPPC gel phase sample at 10° C. This result means that the peptide is almost immobilized in the gel phase of the phospholipid. As we shall discuss below, the value of T_{2e} reflects reorientational motions of the peptide about its long axis but these motions are too slow at 10° C to affect the spectral characteristics. The excellent fit of the broad component to a single set of quadrupolar parameters indicates that all of the peptide bonds are equivalent and that the measured value of T_{2e} represents well the broadening of the individual members of the doublets.

The excellent fit of the broad component to a single set of quadrupolar parameters has important implications. It has been found empirically that for such hydrogen bonds, the quadrupolar splitting scales as the inverse cube of the hydrogen bond distance (Hunt and MacKay 1976). Since the width of the distribution of quadrupolar splittings has been found to be $\lesssim (\pi T_{2e})^{-1} \simeq 3$ kHz, it follows that the variation in the bond distances associated with different hydrogen bonding sites in the α-helix must be $\lesssim 0.05$ Å (Hunt and MacKay 1976). Furthermore, the bond distances in the crystalline solid must be almost identical to those for the peptide in the gel phase of the lipid bilayer. Thus, the α-helices associated with poly-leucine in these two environments must be very regular and similar to each other. In general, it has been found that the separation of the N and O atoms in N-H−O bonds is sensitive to the environment (Blundell et al. 1983). The high stability we have established for the $K_2GL_{24}K_2A$-amide α-helix using the ^2H-NMR spectra of the exchangeable hydrogens is consistent with the absence of any temperature dependence of the circular dichroism spectrum of the same peptide in phopholipid vesicles (Davis et al. 1983; see discussion on page 5303).

The distortion of the central region (± 5 kHz) of the spectrum by the method used to eliminate the background ^2H_2O signal (Callaghan et al. 1984) precludes a complete quantitative treatment of the rotating $N^2H_3^+$ spectrum in the samples in which the peptide was incorporated into the lipid bilayer. Nevertheless, it can be said that the intensity of the narrow powder spectrum for the peptide in DPPC below the gel-liquid crystalline transition is, within experimental error, consistent with rotation of all of the amino groups. Furthermore, the $N^2H_3^+$ spectrum is significantly narrower than for the solid peptide sample indicating a greater degree of side chain flexibility and/or chemical exchange with the ^2H_2O. This is not surprising since the lysines are close to the boundary between the inside and outside regions of the bilayer (Keniry et al. 1984).

Examination of the peptide spectra obtained for the peptide dispersed in liquid crystalline DPPC at 42° C (Fig. 5a) and 50° C (Fig. 5c) indicates that most of the amide ^{2}H-NMR spectrum can be represented by a powder pattern with $\omega_Q/2\pi = 127$ kHz and $\eta = 0$ as may be seen from the simulated spectrum of Fig. 6b. The separation of the edges of the N-^{2}H spectrum at 50° C is almost indentical to that at 42° C indicating that ω_Q does not change appreciably with temperature. If the value of 127 kHz is identified with $2\omega(\beta, \alpha; \theta)/2\pi$ in Eq. (2), we have

$$P_2(\cos\beta) + \frac{1}{2}\sin^2\beta\cos 2\alpha = \frac{127}{150} = 0.84\,. \qquad (11)$$

This is satisfied by $\beta = 19°$. It should be noted that the term involving η in Eq. (11) is negligible in this case since $\eta = 0.16$ and $\sin^2\beta = 0.11$. The value of $\beta = 19°$ is close to the angle between the N-^{2}H vector and the symmetry axis of the α-helix (Pauling and Cory 1951). This result provides additional evidence in support of the interpretation of circular dichroism measurements (Davis et al. 1983) that the integrity of the α-helical conformation of the peptide is maintained in the liquid crystalline phase of the DPPC bilayer. A slight weakening of the α-helical conformation may be indicated by the increased width of spectral edges of the 50° C spectrum. A quantitative investigation of this point will require an improvement in signal/noise.

Interpretation of the T_{2e} measurements

The analysis given above of the amide ^{2}H-NMR spectrum provides striking evidence that the $K_2GL_{24}K_2A$-amide peptide molecules dispersed in DPPC bilayers undergo rapid rotation about their long axes as the temperature of the system is increased through the DPPC gel-liquid crystalline phase transition. More precisely, the correlation time for peptide reorientation, τ_c, satisfies the limiting condition $\tau_c \gg (\Delta M_2)^{-1/2}$ in the gel phase and $\tau_c \ll (\Delta M_2)^{-1/2}$ in the liquid crystalline phase, where ΔM_2 is the change in the *apparent* second moment of the ^{2}H-NMR spectrum due to the motion, as given by Eqs. (3) and (4). From the results of the spectral analysis and Eq. (4), the time scale factor for the change in the spectrum is found to be $(\Delta M_2)^{-1/2} = 4.4$ μs.

As shown in the Theory section, the T_{2e} measurements can yield quantitative estimates of τ_c over the entire range of values possible for this parameter. If the conditions leading to Eq. (8) were satisfied for our system, the decrease of T_{2e} observed as the temperature was increased from 10° to 30° C (see Fig. 7) implies that τ_c decreases by approximately a factor of about two in this temperature range and that it has a value greater than 100 μs. We tentatively propose that the decrease of τ_c is associated with the onset of peptide rotation due to the presence of fluid phase DPPC that makes up an increasing proportion of the total lipid as the temperature is raised through this range (Davis et al. 1983). Near 35° C, the value of T_{2e} goes through a minimum corresponding to $\tau_c \approx 9\ \mathrm{p}^{1/2}$ μs according to Eq. (10). The *measured* minimum value for $T_{2e} \approx 27$ μs. This sets an upper limit for T_{2e} (min) since it represents just about the smallest value of T_{2e} which our apparatus was capable of measuring due to receiver dead-time limitations (Davis 1983) and the weakness of the ^{2}H-NMR signal associated with the exchangeable hydrogens. It is interesting that while the ^{2}H-NMR spectra of bilayers prepared from chain perdeuterated DPPC plus unlabelled peptide consist of a superimposed gel and liquid crystalline spectra in the phase transition region, the T_{2e} measurements of the deuterated peptide are not sensitive to this heterogeneity.

At temperatures above 40° C where almost all of the DPPC molecules are in the fluid phase, the signal was again relatively easy to detect and the measured values of $T_{2e} \simeq 100$ μs. Using this value, Eq. (5) and $\Delta M_2 \approx (4.4\ \mu\mathrm{s})^{-2}$ give the value of $\tau_c \simeq 2 \times 10^{-7}$ s. This can be identified with the time constant for rotational diffuson of the peptide molecules about their long axes in DPPC bilayers in liquid crystalline DPPC bilayers, as given by Eq. (7), since the term involving $\sin^4\beta$ in Eq. (6) makes a negligible contribution for $\beta = 19°$. Using the values a = 4 Å and h = 40 Å, estimated from a model of our α-helix, and $\tau_c = 2 \times 10^{-7}$ s in Eq. (7), we obtain the value $\eta = 1.1$ poise for the membrane viscosity. This value is in remarkable agreement with the viscosity determined from transient dichroism studies of dilute concentration of bacteriorhodopsin in the fluid phase of DMPC bilayer membranes (Peters and Cherry 1982).

Concluding remarks

These results provide a basis for future studies of other, less well characterized polypeptides in membranes. For small peptides such as gramicidin A or proteins which are believed to have a single α-helix spanning the membrane such as glycophorin or *fd* phage coat protein (Henderson 1981), it is anticipated that the values of τ_c in the liquid crystalline phase of membranes will also be sufficiently short to satisfy the short correlation time limit, $\Delta M_2\tau_c^2 \ll 1$. It should thus be possible to obtain new information on the dynamic local structure of the hydrogen bonding sites in these membranes and proteins. When combined with

similar measurements on ^{2}H-labelled amino acid residues (Kinsey et al. 1981; Rice et al. 1981; Keniry et al. 1984), it should be possible to separate side chain motions of the residues from backbone motions in these systems.

The moderately large protein, rhodopsin, is known to have a rotational correlation time of $\tau_c \simeq 20\,\mu s$ (Cherry 1979), which satisfies the long correlation time limit, $\Delta M_2 \tau_c^2 \gg 1$, while the larger band three protein has a still larger value of $\tau_c \simeq 200\,\mu s$ (Cherry 1979). If protein reorientation represents the only important backbone motion for such larger proteins, the ^{2}H-NMR spectrum of the hydrogen bonding sites of the membrane-spanning α-helices should correspond to the broad component of Fig. 5 even in the liquid crystalline phase of membranes. It is not surprising that this type of spectrum was, indeed, observed for a lyophilized sample of bacteriorhodopsin in its native "purple membrane" at room temperature (Spohn and Kimmich 1983). If, however, the α-helical secondary structural units in these proteins undergo additional faster motions such as large amplitude oscillations or rotations in fluid membranes, the measurements we have described should be capable of detecting them. It thus seems worthwhile to search systematically, using the ^{2}H-NMR techniques described in this paper, for internal backbone motions which may be related to protein action.

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