Effects of Membrane Peptide Dynamics on High-Resolution Magic-Angle Spinning NMR

Effets de la dynamique d'un peptide membranaire sur un spectre RMN haute résolution en rotation a l'angle magique

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RÉSUMÉ

Depuis une quinzaine d'années, des cas d'interférence entre la dynamique de molécules et la manipulation cohérente de l'aimantation nucléaire réalisée dans le cadre d'expériences de résonance magnétique nucléaire (RMN) ont été identifiés et étudiés en détail, qu'il s'agisse du découplage de spins, de la polarisation croisée ou de la rotation à l'angle magique (magic-angle spinning, ou MAS). Des expériences récentes réalisées dans notre laboratoire nous apportent des détails concernant la nature d'une perturbation responsable de l'élargissement des raies ^{13}C et ^{15}N dans le cas de molécules modèles, celle de l'interférence entre un mouvement moléculaire et le découplage des protons. Le même effet est démontré ici pour la première fois dans le cas d'un peptide membranaire, la *gramicidine A* (gA), dans une bicouche lipidique hydratée. Les expériences présentées ici constituent la première observation d'un spectre RMN solide ^{13}C à haute résolution d'un C_α de peptide membranaire en bicouche lipidique. De plus, le développement de stratégies destinées à contourner l'effet d'élargissement des raies nous permet d'extraire des informations concernant la dynamique de gA dans des conditions physiologiques.

mots-clés : Gramicidine, membrane, dynamique, découplage des ^{1}H

ABSTRACT

In the past fifteen years, interference between molecular dynamics and coherent manipulation of nuclear magnetization in nuclear magnetic resonance (NMR) experiments such as spin decoupling, cross-polarization or magic-angle spinning (MAS) have been identified and studied carefully. Recent experiments performed in our laboratory on model compounds have provided insight into the nature of a perturbation responsible for the broadening of ^{13}C and ^{15}N signals, namely the interference of some molecular motion with ^{1}H-decoupling. The same effect is demonstrated here for the first time in the case of a membrane peptide, *gramicidin A* (gA), in an hydrated lipid bilayer. The present experiment provides

the first successful attempt to observe high-resolution solid-state ^{13}C NMR spectra of a C_α moiety in membrane peptide contained in a lipid bilayer. Furthermore, the development of strategies to circumvent the broadening effect allows the extraction of relevant data concerning the dynamics of gA under physiological conditions.

key words : Gramicidin, membrane, dynamics, ^{1}H-decoupling

INTRODUCTION

Solid-state nuclear magnetic resonance (NMR) presents a powerful approach for structural and dynamical investigations of membrane peptides. Within this framework, these systems may be studied in their natural environment: hydrated bilayers in the liquid-crystalline L_α phase. Line narrowing can be performed by macroscopic sample orientation, if possible, or by magic-angle spinning (MAS). For example, Cross and coworkers have used NMR of oriented hydrated lipid bilayers to provide an atomic structure of Gramicidin A (gA) in its native state [1]. One can notice that the *structure* and, more dramatically, the *dynamics* of the peptide obtained with single crystals, solutions of peptides in organic solvents or micellar systems are affected by changes in environment.

Gramicidin A is the major form of a linear peptide produced by the bacteria *Bacillus brevis*. It has a sequence of 15 alternating L- and D-amino acid residues and consists of a right-handed β-helix having 6.3 residues per turn that dimerizes, N-terminus to N-terminus, to form a monovalent cation selective channel in model membranes. The development of synthetic procedures which provide large quantities of this peptide and its relatively small size make gA an ideal candidate for spectroscopic studies of ion channels and membrane peptides. Although the general features of membrane peptide molecular dynamics are understood, accurate rates associated with various motional processes are lacking. We report a line broadening effect that has implications for both dynamical and structural studies of membrane/peptide systems: ^{1}H-decoupled ^{13}C-MAS NMR spectra of gA incorporated into hydrated lipid bilayers present a dramatic line width dependence on temperature.

Broadening of MAS-NMR lines related to dynamics of membrane peptides and proteins in liquid-crystalline membranes have been reported previously [2-5]. In the case of gA, this broadening is maximised at room temperature and is attributed to an interference between molecular motion and high power proton decoupling. This interference effect has been observed in model compounds [6-11] and is described by Rothwell and Waugh [7]. We believe that we demonstrate here for the first time the occurrence of such an interference in the case of a membrane peptide.

MATERIAL AND METHODS

(^{15}N,^{13}CO,$^{13}CH_2$-Gly$_{-2}$; $^{15}N_1$-Trp$_{-9}$) gA and (^{15}N,^{13}CO,$^{13}CD_2$-Gly$_{-2}$) gA have been synthesised and purified to over 98% purity following the procedure of Fields *et al.* [12,13] and will be designated as NCCH$_2$-Gly gA and NCCD$_2$-Gly gA respectively. Glycine was dideuterated at the alpha position following the procedure of Cable *et al.* [14]. Each peptide was mixed together with dimyristoyl phosphatidylcholine (DMPC), in a molar ratio of 10:1 lipid/peptide, in a solution of trifluoroethanol which was then removed by rotoevaporation followed by high-vacuum lyophilization overnight. After adding an equal weight of D_2O, the dispersion was kept frozen for 24 hours, incubated at 70°C for an additional 24 hours and subjected to several freeze-thaw cycles to ensure sample homogeneity. Thin layer chromatography was performed on the samples before and after the NMR experiments. Only minor traces of lysolipids were found in samples that were heated for extended periods of time.

NMR experiments were performed on approximately 150 mg of sample, in a 5 mm high-speed Doty rotor, using a custom designed double resonance probe with a Doty spinning assembly. NMR spectra were acquired with a custom designed spectrometer where ^{13}C frequency was 79.9 MHz and ^{1}H frequency was 317 MHz. Each experiment consists of a simple ^{13}C Bloch decay and two-pulse phase modulation scheme (TPPM [15]) for decoupling, with a pulse length of 5.4 μs, and a phase angle ϕ of 15° (radio frequency field strength is 90 kHz). At each temperature, careful optimisation of the relaxation delay has been performed to avoid any loss of magnetization due to incomplete relaxation.

RESULTS AND DISCUSSION

The bottom trace of Figure 1 shows the ^{1}H-decoupled ^{13}C-MAS NMR spectra of NCCH$_2$-Gly gA/DMPC/D_2O at 25°C. One can see, besides the highly resolved natural abundance ^{13}C lipid peaks, a strong resonance at 171 ppm attributed to the labelled ^{13}CO of the glycine and a much broader resonance at 45 ppm attributed to the $^{13}CH_2$ of the labelled glycine in the peptide. In the case where where NCCH$_2$-Gly has been replaced by NCCD$_2$-Gly, none of the two carbons are protonated, *two* strong resonances are observed (Fig. 1a).

When varying the temperature of the sample away from 25°C, keeping all other conditions identical, the $^{13}CH_2$ peptide resonance of NCCH$_2$-Gly gA/DMPC/D_2O presents a dramatic line narrowing (Fig. 2). One may note that this line width dependence on temperature between -10°C and 60°C is very different

and much more dramatic than the one affecting the ^{13}CO peptide resonance or the lipid resonances in the same conditions.

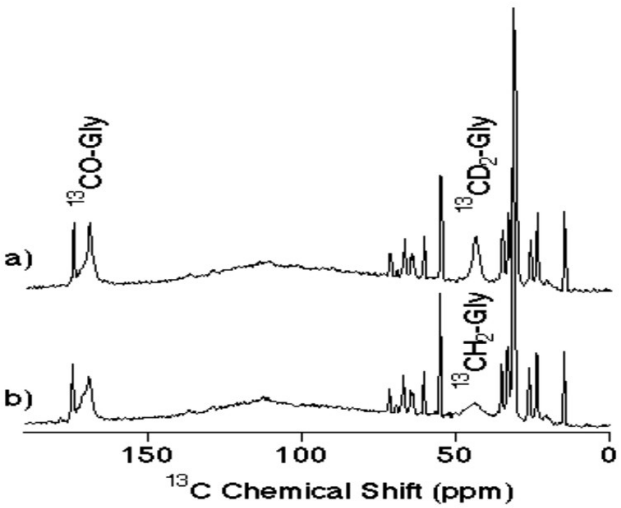

Figure 1 : 79.9 MHz ^{13}C-MAS-NMR spectra of: a) $NCCD_2$-Gly gA/DMPC/D_2O with 90 kHz ^1H-decoupling. T = 25°C, ω_r = 4.7 kHz, r. d. = 15 s, 2048 scans; b) $NCCH_2$-Gly gA/DMPC/D_2O with 90 kHz ^1H-decoupling. T = 25°C, ω_r = 6.5 kHz, r. d. = 10 s, 2048 scans.

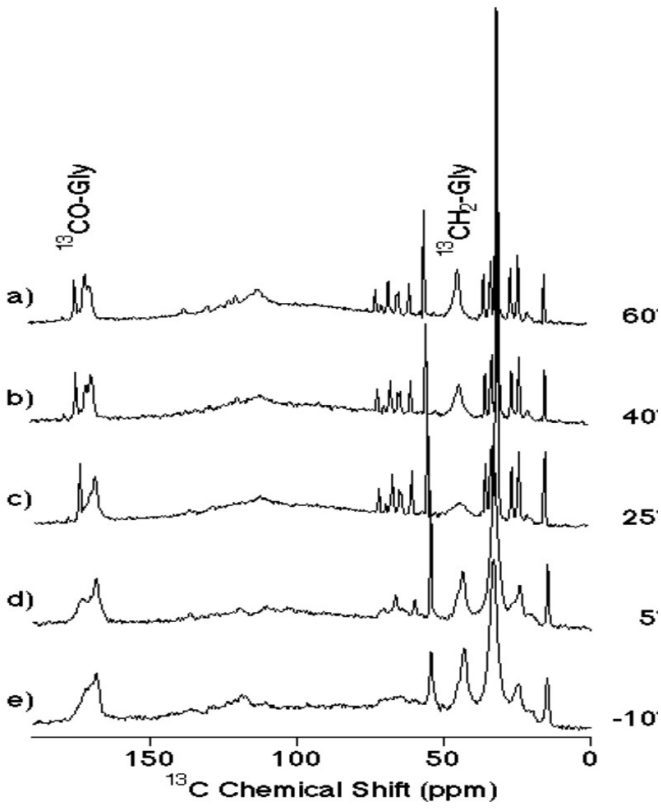

Figure 2 : 79.9 MHz ^{13}C-MAS-NMR spectra of $NCCH_2$-Gly gA/DMPC/D_2O with 90 kHz ^1H-decoupling at different temperatures. a) T = 60°C, ω_r = 6.7 kHz, r. d. = 5 s, 2048 scans; b) T = 40°C, ω_r = 6.2 kHz, r. d. = 5 s, 1024 scans; c) T = 25°C, ω_r = 6.5 kHz, r. d. = 10 s, 2048 scans; d) T = 5°C, ω_r = 5.25 kHz, r. d. = 60 s, 1024 scans; e) T = -10°C, ω_r = 4.1 kHz, r. d. = 120 s, 960 scans.

Since $NCCH_2$-Gly gA contains two directly bound ^{13}C, the 50 Hz J-coupling between them is not removed by MAS, and therefore contributes to the residual line width. In the case of $NCCD_2$-Gly gA, in addition to the aforementioned 50 Hz CC J-coupling, the 25 Hz CD J-coupling combines into two quintets that contribute to the observed line width of the $^{13}CD_2$ resonance by approximately 75 Hz at half

height. It may be possible to remove this additional broadening by ^{2}H-decoupling. Contributions from sample heterogeneity or peptide/peptide interactions are probably present but should affect equally the $^{13}CH_2$ and the ^{13}CO resonances, unlike what can be seen in Figure 1. The major difference between these two spin systems is clearly the presence of protons and a careful analysis of the effect of the residual ^{13}C-^{1}H dipolar coupling is in order.

The ^{13}C-^{1}H dipolar coupling is on the order of 20 kHz for a static CH bond and is generally suppressed by MAS and ^{1}H-decoupling at an r.f. field of 90 kHz using TPPM. In the case of $NCCH_2$-Gly gA, this coupling is suppressed both *below* and *above* 25°C. The spectra of Figure 2 demonstrate that there is a regime where ^{1}H-decoupling is rendered inefficient by a temperature dependant process. We postulate here that this process is an interference between the r.f. decoupling field and a temperature dependant molecular motion of the peptide, a phenomenon that had been previously observed with hexamethylbenzene and described by Rothwell and Waugh for the case of isotropic motion [7]. A similar interference effect has also been observed when comparing $^{13}CH_3$-Ala gA and $^{13}CD_3$-Ala gA. The difference in broadening between the protonated and the deuterated methyls is not as dramatic as for the Cα of glycine showed in Figure 1, presumably due to a reduced ^{13}C-^{1}H dipolar coupling generated by the additional three-site hop at the methyl group.

The variation of the $^{13}CH_2$-Gly resonance line width as function of 1/T presents a Lorentzian shape predicted by Rothwell and Waugh [7]. In analogy to the T_1 being minimized when the correlation time is on the order of the inverse Larmor frequency, the T_2 minimum occurs when the correlation time is on the same time scale as the inverse decoupling frequency. While r.f. decoupling field strength stays constant, peptide dynamics in the membrane is greatly affected by temperature variation and, as a first approximation, the present experiments therefore demonstrate the existence of motions in gA whose rates are on the order of $10^5 s^{-1}$ at 25°C in hydrated DMPC. More accurate rates may be extracted and assigned to models of molecular dynamics through numerical line shape simulations. Motional models of gA have been discussed in previous studies performed at temperatures exceeding 30°C [16-29] and include (1) small amplitude and very fast helical librations ($\tau = 10^{-9}$ s); (2) slow lateral diffusion on the surface of the vesicle ($\tau = 10^{-2}$ s to 10 s); (3) axial diffusion (values between 10^{-5} s to 10^{-9} s have been proposed) and (4) wobble within a cone.

In addition to understanding the interference effect and extracting motional rates, one may want to circumvent it in order to get highly resolved ^{13}C-MAS NMR spectra of peptides in physiological conditions. Since it is impossible with the

present technology to either spin or to decouple at a rate a few orders of magnitude higher than used here, *two* other solutions have been proposed: (1) performing the experiments at 40°C or 60°C, as in Figure 2a and 2b or (2) removing the CH interactions by replacing the relevant protons with deuterons so that dynamical information can be obtained at any given temperature, as in Figure 1a.

CONCLUSIONS AND PERSPECTIVES

We have observed the temperature dependence of the line broadening of a protonated carbon peptide resonance. We have demonstrated that this broadening was due to an interference between coherent averaging and incoherent motion of the peptide, a phenomenon that had been previously identified in model compounds only. Nevertheless, we believe that this effect is general and will occur in many other biological systems, particularly other peptides of similar size in biomembranes.

We have extracted rates associated with molecular motion and we are currently performing numerical line shape simulations in order to characterise better the dynamics of the peptide. It should be pointed out that motional rates obtained here are only approximate and that more accurate rates may be extracted with a more appropriate model. In addition, we have devised ways to circumvent the interference effect and to obtain highly resolved ^{13}C-MAS NMR spectra of peptides in hydrated lipid bilayers, at any temperature. Similar results are expected in ^{15}N-NMR spectra where the coupling to protons is reduced by half.

Accurate information about peptide dynamics will be required to allow the interpretation of motionally averaged dipolar couplings in terms of structural restraints. Furthermore, solid-state MAS NMR techniques devised to measure internuclear distances (Rotational Resonance, DIPSHIFT, REDOR...) can be combined with high-resolution chemical-shift correlation spectroscopy, which is routinely employed in solution NMR. Since high-resolution ^{1}H MAS NMR of membrane peptides can be obtained at high temperature (60°C) and high spinning-speed, as shown by Davis *et al.*, [30] it provides an exciting opportunity to probe structure and dynamics of membrane/peptide systems using a variety of ^{1}H, ^{13}C and ^{15}N-MAS NMR experiments under physiologically relevant temperatures.

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