^{2}H NMR Determination of the Global Correlation Time of the Gramicidin Channel in a Lipid Bilayer

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ABSTRACT A detailed experimental description of molecular dynamics requires an accurate description of the global correlation time. For the gramicidin cation-selective channel the local dynamics of the polypeptide backbone are thought to play a very significant role in the functional process of this channel which occurs on the 10–100-ns time scale. By solid-state NMR spectroscopy an experimental description of the local dynamics including a description of the axis about which the motions occur, the amplitude of the motions, whether they are diffusional or discontinuous and the frequency of the motions is possible. Initial interpretations of the NMR data for this polypeptide backbone in fully hydrated lipid bilayers suggests that the time scale for the local motions is the same as the kinetic time scale (North and Cross, 1993). Here the global correlation time is found from d_4-Ala_3 and d_4-Ala_5-gramicidin A powder pattern spectra to be 36 μs at 309 K. This surprisingly long correlation time may reflect the high viscosity of the peptide environment or possibly the higher molecular weight of a peptide-lipid aggregate. Furthermore, this reassessment of the global correlation time supports the initial interpretation of relaxation data for the local motions.

INTRODUCTION

One of the greatest challenges in the spectroscopy of macromolecules is the experimental determination of molecular dynamics. Without such determinations correlations between structure and function represent only qualitative models of life processes. In the channel formed by gramicidin A the role of backbone dynamics has been the subject of much interest, because it may play a direct role in the conductance of cations that occurs on the 10–100-ns time scale (North and Cross, 1993). Consequently, if a detailed dynamics characterization could be achieved for this molecular system, it would provide a unique opportunity to study the general problem of relating functional rate processes in the form of kinetics with molecular dynamics.

Great use of NMR and fluorescence anisotropy has been made for the experimental determination of molecular dynamics. The difficulty in achieving such a goal is harbored in the complexity of the answer that is desired. In the molecular frame, the axis about which the dynamic process is occurring, whether the process is discontinuous as in jump motions or diffusional, and what the amplitude of the motion is, are all important and interesting questions. Such a characterization leads to a structural model of the molecular motion. In order to complete the characterization it is also necessary to determine the motional frequency. Multiple motions affecting a given site, the degree of correlation among motions, all contribute to a labyrinth of complexity. To achieve meaningful solutions the problem needs to be dissected so that the number of variables is minimal for each experimental fact. NMR offers a variety of approaches for characterizing dynamics. Depending upon the molecular preparation a range of different relaxation parameters can be assessed (Peng and Wagner, 1992). Sensitivity to the dynamic frequencies varies within the picosecond to millisecond time scales depending on the specific relaxation parameter being studied. In solid state NMR it is possible to analyze dynamics not only by relaxation measurements, but by lineshape analyses. The spin interactions are incompletely averaged by molecular motions in anisotropic samples. Such motions that have a frequency much greater than the measured interaction magnitude result in a characteristic lineshape that can yield characteristic information on the structural model for the molecular dynamics. Furthermore, small amplitude, high frequency motions result in a mere scaling of the spectral lineshape. In contrast, motional frequencies on the order of the spectral anisotropy result in complex lineshape dependencies on the pulse sequence timing (Spiess and Sillescu, 1981; Spiess, 1985; Vega and Luz, 1987; Wittebort et al., 1987).

Gramicidin A is a 15-amino acid polypeptide with an alternating pattern of L and D residues. All of the residues are hydrophobic with the single exception of a glycine residue. Both termini are blocked, the amino terminus with a formyl group and the carboxyl terminus with an ethanolamine group. As a dimer this polypeptide forms a channel with a 4-Å pore lined with the polypeptide backbone and having the sidechains radiating away from the pore and interacting with the fatty-acyl chains of the lipid environment. The folding motif for this channel was first proposed by Urry in 1971, when he suggested that a strand of β-sheet was rolled into a helix with 6.3 residues per turn. This was possible only because the unique alternating pattern of D/L stereochemistry results in all of the C_α-C_β bonds radiating to the same side of the strand, thereby forcing the strand to turn and hence form a helix. The helical sense has been determined to be right-handed (Nicholson and Cross, 1989). The pore, so formed, is only wide enough to permit a single file of water molecules. Consequently, for cations to enter the channel they must be stripped of all but two water molecules in their primary hydration sphere. Once in the channel, the cations

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can only proceed from one side of the bilayer to the other by a concerted motion of all the water molecules in the channel. Therefore, the kinetic properties of this channel include correlated motions of the water molecules.

Computationally, the polypeptide backbone motions have been the subject of many studies. MacKay et al. (1984) were the first to perform molecular dynamics calculations on the entire molecular structure of the channel. Their results suggested, that in the presence of cations that the peptide planes would rotate by as much as 50° to move the carbonyl oxygens toward the channel axis. More recent molecular dynamics calculations indicate far less distortion of the polypeptide backbone (Chiu et al., 1991). Computational efforts with molecular mechanics has suggested helical librations, in which local motions of the peptide planes are correlated over the entire length of the helix (Venkatachalam and Urry, 1983). Efforts with a normal mode analysis suggests that correlations are only with adjacent peptide planes (Roux and Karplus, 1988).

To make conclusive progress with NMR on the problem of characterizing local motions, the global correlation time needs to be evaluated. From a ^2H NMR relaxation study of indole-deuterated gramicidin in DMPC bilayers at a temperature of 325 K a correlation time of 200 ns has been published (Macdonald and Seelig, 1988). These authors assumed that the local motions of the indole rings would not contribute significantly to the relaxation of these sites. Recent T_1 relaxation studies of the polypeptide backbone performed at different field strengths have utilized this correlation time and a structural model developed for the local motions of the polypeptide backbone (Nicholson et al., 1991) to determine a time constant for rotational diffusion about the C_α-C_α axis (North and Cross, 1993). These relaxation times were performed at approximately 309 K, and therefore it was important on two accounts to redetermine the global correlation time; because the temperature is different and because of the potential contribution of the indole rings to the relaxation rate. Here, deuterated Ala_3 and Ala_5 sidechain sites have been chosen for a determination of the global correlation time and instead of using relaxation parameters that are sensitive to a wide range of motional frequencies, we have used powder pattern lineshapes. Fortuitously, the global correlation time at 309 K is of such a frequency that the ^2H lineshape is sensitive to the global motions. The alanine sidechains are uniquely suited for this purpose. The sidechain dynamics (methyl three-site jumps or diffusion) are very rapid compared to the quadrupole interaction magnitude. Similarly, the correlation time for local backbone dynamics, tentatively determined by North and Cross (1993) to be 36 ns represents a rapid motion on the ^2H time scale. Consequently, the only large amplitude motions that potentially have a correlation time on the time scale of the ^2H interaction magnitude is the global correlation time. Therefore, the observed anisotropic T_2 relaxation is justifiably considered to result from the global motion. The dynamics of any other sidechains in gramicidin will be much more complicated and would yield a more ambiguous result in that additional variables would be involved in the analysis of the data. Alanine is an optimal residue for the study of global motions.

MATERIALS AND METHODS

d_4-Ala_3- and d_4-Ala_5-gramicidin A were synthesized as described previously (Fields et al., 1988, 1989) using Fmoc chemistry so that acid conditions could be completely avoided. In so doing sites that are somewhat labile to exchange such as C_α deuterons could be fully retained. The effectiveness of this approach has been clearly demonstrated with the spectral observation of d_5-indole-labeled gramicidin A (Cross et al., 1992), which cannot be incorporated using tBoc chemistry, because of the acid cleavage steps. Unoriented channel preparations of gramicidin were prepared by first codissolving a 1:8 molar ratio of gramicidin to lipid in methanol doped with 5% water. The samples were dried under vacuum before hydrating with 40% by weight deuterium-depleted water. Samples were allowed to hydrate without agitation for at least 14 days at 45°C.

Spectra were recorded on a spectrometer assembled around a Chemagnetics data acquisition system and an Oxford Instruments 400/89 magnet. The ^2H resonant frequency was 61.5 MHz and the spectra were recorded at 309 K with the quadrupole echo pulse sequence using a 2.8-μs 90° pulse width, a 0.7-s recycle delay and a 1-μs dwell. An eight-step phase cycling routine was used to minimize artifacts. The interpulse echo delay was varied as noted in the figure legends.

The double precision Fortran program, MXQET, operated on a Silicon Graphics Personal Iris 4D/25TG workstation was employed to simulate the experimental results (Greenfield et al., 1987; Vold and Vold, 1991). To model the global rotation of the channel about the bilayer normal, the rotational diffusion was modeled as an exchange between 36 nearest neighbor exchange sites (i.e., 10° steps). Because of clockwise and counterclockwise directions for each jump, the statistics of a random walk of molecules requires 36^2 displacements to generate an rms average to model the diffusion about the entire bilayer normal axis. The finite pulse width of 2.8 μs for a $\pi/2$ pulse was accounted for in the spectral simulations.

RESULTS AND DISCUSSION

Fig. 1 and Fig. 2 show the ^2H quadrupole powder pattern spectra of d_4-Ala_3- and d_4-Ala_5-gramicidin A, respectively, as a function of the interpulse delay in the quadrupole echo experiment. A static methyl C-D quadrupole coupling constant of 171 ± 5 kHz has been reported for alanine at −135°C (Keniry et al., 1984). These methyl sites exposed to a hydrated lipid bilayer environment will undoubtedly be undergoing rapid rotational motions about C_α-C_β bond characterized by τ_{Me} (See Fig. 3; Jelinski et al., 1980; Kinsey et al., 1981; Keniry et al., 1984; Beshah et al., 1987; Beshah and Griffin, 1989). The fast averaging of methyl reorientation produces the same averaged lineshapes via a three-site jump or via a diffusional rotation model (Torchia and Szabo, 1982). The experiments here cannot discriminate between these models and neither is it important for the conclusions of this paper to define the methyl group motional model. The averaging of the electric field gradient tensor by this motion in the fast exchange limit will result in a quadrupole coupling constant of one third its static value (57 kHz) assuming perfect tetrahedral geometry. Furthermore, librational averaging of the polypeptide backbone either in the form of a local motion about the C_α-C_α axis (Nicholson et al., 1991; North and Cross, 1993) or wobbling in a cone for the C_α-C_β axis (Cross and Opella, 1982; Batchelder et al., 1982; 1983) will

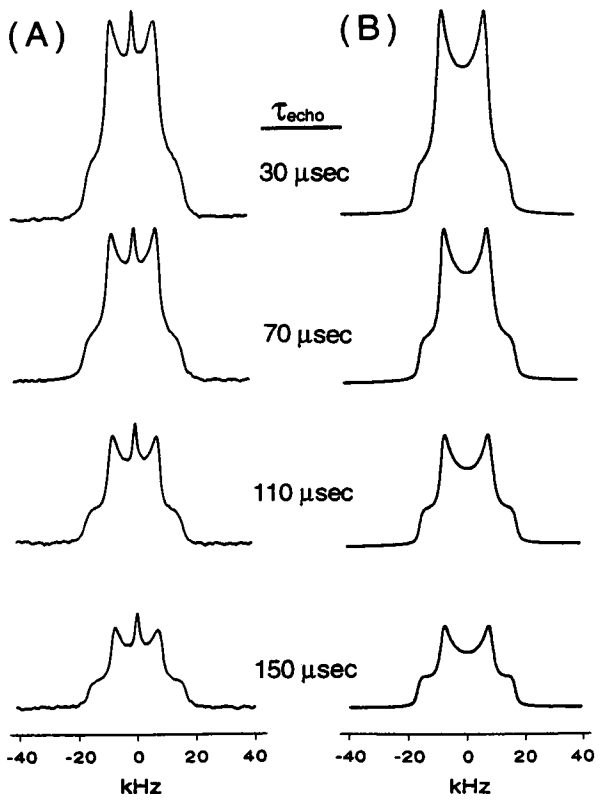

FIGURE 1 ^{2}H NMR powder pattern spectra of Ala_3-d_4 gramicidin A in hydrated DMPC bilayers. (*A*) Experimental spectra obtained with 60,000 acquisitions using the quadrupole echo experiment with the various echo delays shown. (*B*) Spectral simulations for these delay times. Simulations of the global motion were achieved by 10° jumps between 36 sites for the $C\text{-}_\alpha\text{-}C_\beta$ axis in a cone of semi-angle 102°. A quadrupole coupling constant of 51 kHz was used and a jump rate of 4.5×10^7 Hz (τ_p = 29 μs) resulted in the best fit to the experimental data.

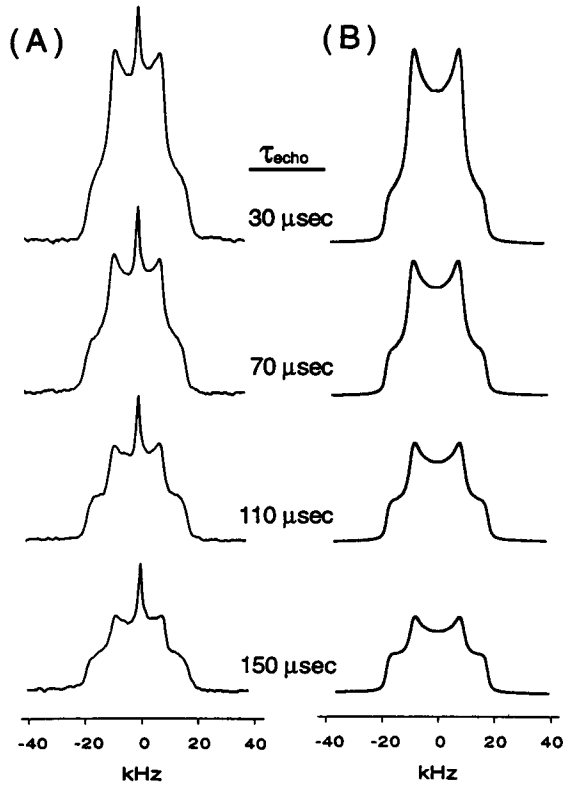

FIGURE 2 ^{2}H NMR powder pattern spectra of Ala_5-d_4 gramicidin A in hydrated DMPC bilayers. (*A*) Experimental spectra obtained with 60,000 acquisitions using the quadrupole echo experiment with the various echo delays shown. (*B*) Spectral simulations for these delay times. Simulations of the global motion were achieved by 10° jumps between 36 sites for the $C\text{-}_\alpha\text{-}C_\beta$ axis in a cone of semi-angle 98°. A quadrupole coupling constant of 51 kHz was used and a jump rate of 3.0×10^7 Hz (τ_p = 43 μs) resulted in the best fit to the experimental data.

simply scale the magnitude of the residual quadrupole interaction, because such motions, characterized by τ_i, are also very rapid. The observed ^{2}H quadrupole coupling constant in lyophilized channel preparations is 51 kHz. Consequently, either the covalent geometry of the methyl group is nonideal (Lehmann et al., 1972; Batchelder et al., 1983) or there is a significant degree of librational averaging. Since neither of these explanations affect the lineshape dependence of the interpulse delay our conclusions are independent of resolving this question.

In the hydrated samples there is an additional degree of motional freedom, that of a uniaxial global motion, characterized by τ_p, occurring about an axis parallel with respect to the bilayer normal (Smith and Cornell, 1986; Fields et al., 1988). The definitions of these various dynamic processes is shown in Fig. 3. The orientation of the C_α-C_β axis to the motional axis is accurately known from structural studies of the polypeptide backbone; for Ala_3 this angle is 102° (Teng et al., 1991) and for Ala_5 it is 98° (Cross et al., 1992). This final degree of motional freedom, τ_p, results in the anisotropic T_2 of the observed spectra displayed in Figs. 1, 2, and 4, and in the simulated spectra of Figs. 1, 2, 4, and 5. In particular, the relative magnitudes of the $\nu_\parallel$ and $\nu_\perp$ shoulders change significantly as a function of this inter-pulse delay in Figs. 1, 2, and 5.

Supporting evidence that the global axial motion is responsible for the anisotropic T_2 is provided in Fig. 4 where the temperature dependence of the Ala_3 powder pattern is shown. The spectra have been simulated with a temperature-dependent correlation time that clearly shows the rigid (for this motion) limit at 6°C (well below the 28°C phase transition temperature (Nicholson et al., 1987)) and in the fast exchange limit at 52°C. The motional model that fits this data is severely constrained by the known structural details at this site in the gramicidin channel (Teng et al., 1991). While the success of this model to fit the data does not rigorously preclude other motional models we have been unable to rationalize all of this data with any other model. Therefore, we conclude that the motion that causes the anisotropic T_2 is, indeed, the global axial rotation.

Due to the large magnitude of the quadrupole coupling constant of the ^{2}H electric field gradient tensor, motions must have a frequency greater than 10^5 Hz in order for the powder pattern spectra to represent the fast exchange limit. The sensitivity of the spectra to the rotational diffusion of the channel is shown in Fig. 5 where both the quadrupole echo delay time

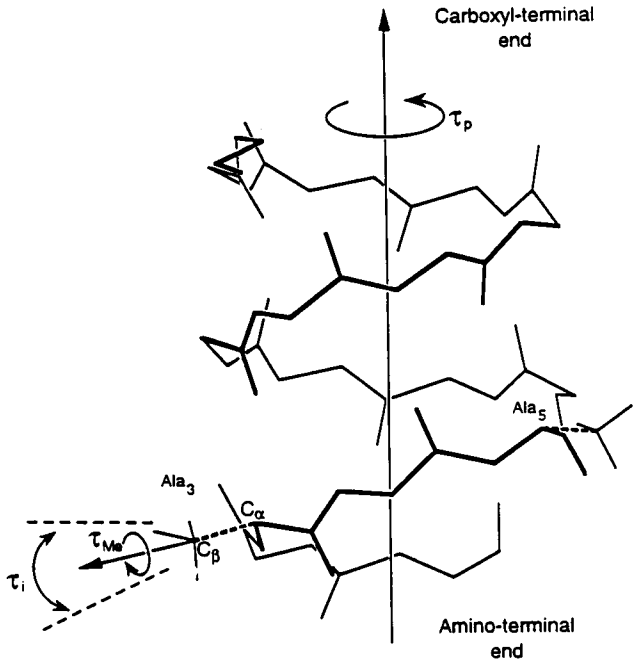

FIGURE 3 A structural model of the polypeptide backbone of the gramicidin channel monomer is shown along with an illustrated definition of the three pertinent correlation times. τ_p is the global correlation time for the channel and it is known to occur about the bilayer normal. τ_{Me} represents the motion about the C_α-C_β axis of the methyl group and it is shown to be a rapid motion on the ^2H NMR time scale. τ_i represents the librational motions affecting the C_α-C_β axis and these motions have also been shown to be rapid on the ^2H NMR time scale.

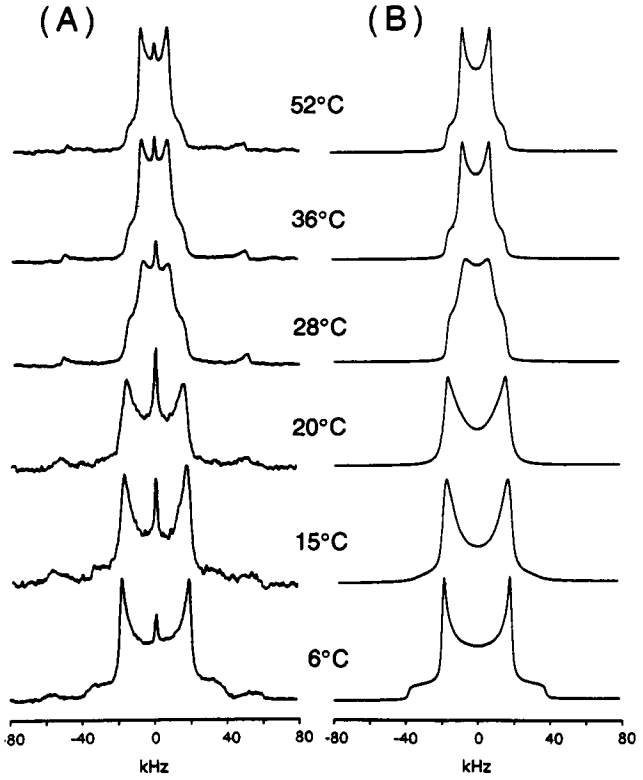

FIGURE 4 Temperature dependence of the Ala_3-d_4 gramicidin A ^2H powder pattern spectra in hydrated DMPC bilayers. (*A*) Experimental spectra obtained with 60,000 acquisitions using the quadrupole echo experiment with a 30-μs echo delay. (*B*) Spectral simulations for the various temperatures using the following correlation times: 52°C (325 K), $\leq$ 13 μs; 36°C (309 K), 29 μs; 28°C (301 K), 130 μs; 20°C (293 K), 1.3 ms; 15°C (288 K), 4.3 ms; 6°C (279 K), $\geq$ 1.3 s.

and the motional frequency are varied in an array of simulated powder pattern spectra. From these figures it is concluded that the global correlation time at 309 K for rotation about the bilayer normal is approximately 43 μs from the Ala_5 results and 29 μs from the Ala_3 results. These values are within experimental error of each other, and the average value of 36 μs represents the numerical conclusion of this study. This is more than two orders of magnitude slower than previous estimates at a higher temperature (Macdonald and Seelig, 1988). In the results of Fig. 4 spectra obtained at 325 K show no such lineshape dependence, and therefore the correlation time is less than 13 μs at this elevated temperature. 309 K is well above the center of the gel to liquid-crystalline phase transition temperature (301 K). However, at this molar ratio of gramicidin to lipid the phase transition has a low enthalpy, and it is spread over a broad temperature range (width at half height of the transition is $\pm$ 5°C). Therefore, at 309 K the sample is on the upper edge of the phase transition and may have a significantly increased viscosity in the bilayer that results in a slowing of the rotational rate. The rate determined by Macdonald and Seelig may indeed be accurate or at least not as inaccurate as would be suggested by a direct comparison of the determined correlation times. Both the rate determined here and that of Macdonald and Seelig are slow compared to the correlation times determined for a polypeptide of such size in an aqueous environment. There are numerous possible explanations including the viscosity argument just mentioned. Lipid bilayers are rich in low-frequency motions (Cornell et al., 1982, 1983; Peng et al., 1988, 1989), and there may be coherences between the global peptide motions and the lipid motions. Experimental evidence for specific interactions between the peptide and lipid have recently been demonstrated (Lazo et al., 1992), and consequently, the correlation time may be that of a higher molecular weight aggregate than just a gramicidin dimer.

The longer global correlation times obtained here strengthens the conclusions of the local dynamics study (North and Cross, 1993), in which the local motional frequencies were shown to have a surprisingly long (36 ns) correlation time. Interestingly this time frame for the local motions is almost identical with the period of time required for a cation to move from one carbonyl oxygen binding site to another along the channel axis.

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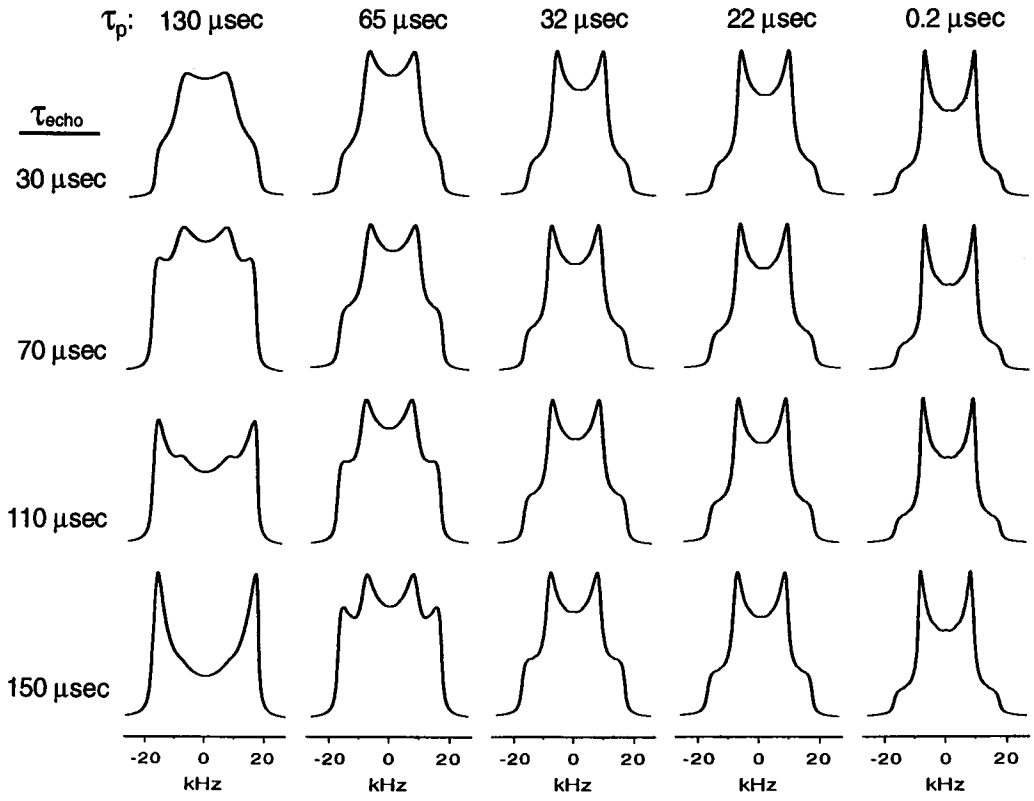

FIGURE 5 Sensitivity of the spectral simulations to the quadrupole echo delay time and the global correlation time, τ_p, for the channel. Simulations of the global motion were achieved by 10° jumps between 36 sites for the C_α-C_β axis in a cone of semi-angle 100°. A quadrupole coupling constant of 51 kHz was used. Simulations show that the lineshapes are sensitive to motional frequencies up to 10^5 Hz. The lineshape changes as a function of echo delay time shown in Figs. 1 and 2 demonstrate that the global frequency is less than 10^5 Hz.

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