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An Off-resonance Rotating Frame Relaxation Experiment for the Investigation of Macromolecular Dynamics Using Adiabatic Rotations

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^{15}N off-resonance rotating frame relaxation can be applied to the study of internal dynamics in proteins in the millisecond to microsecond regime. We show that the performance of existing methods can be improved by application of simultaneous amplitude and phase-modulated adiabatic RF pulses to align the nuclear spin magnetization with the off-resonance spin-lock field for all the spins under investigation. Application of this technique to the 269-residue serine protease PB92 allowed the measurement of ^{15}N off-resonance rotating frame relaxation rates for all nonoverlapping residues in the protein, including the arginine side chains, encompassing a chemical shift range of 50 ppm. Simulations indicate that by use of the proposed adiabatic RF pulses rotating frame relaxation rates can be obtained for magnetization vectors aligned at arbitrary angles with the static field.

Key Words: **NMR; protein dynamics; off-resonance relaxation; adiabatic pulses; chemical exchange.**

INTRODUCTION

With the advent of isotopic enrichment and the development of proton detected heteronuclear NMR the measurement of ^{15}N nuclear spin relaxation times has provided a wealth of information on intramolecular dynamics in proteins (*1, 2*). In particular, ^{15}N R_1, R_2 and ^{15}N{^{1}H} heteronuclear NOE have been measured to provide information about fast (picosecond to nanosecond) internal dynamics of the protein backbone. In contrast, relatively few experiments for measuring motions on intermediate (microsecond to millisecond) time scales have been developed or applied to proteins. Research in this area has mainly focused on chemical exchange which constitutes a mechanism of relaxation as the chemical shift is rendered time dependent (*3*). Exchange on the microsecond to millisecond time scale manifests itself by a contribution R_{ex} to the spin–spin relaxation rate, which can in principle be derived from an anomalous increase in values of R_2 or $R_{1\rho}$ (*4*). When data are obtained at multiple static field strengths it is possible to detect systematic deviations of R_2, and the relaxation contribution due to chemical exchange can be estimated from its proportionality to the square of the static field strength (*2*). A disadvantage of this method is obviously the collection of data from different NMR spectrometers, which may not be accessible.

Other methods exploit the dependence of relaxation due to chemical exchange in the presence of a continuously applied RF field (*5, 6*). This makes it possible to study molecular motions as a function of more easily accessible and more reproducible experimental parameters and to sample a larger number of points on the spectral density of the intermediate time scale motion. The theoretical framework devised by Deverell *et al.* (*6*) is often used, which applies to data obtained from spin-lock measurements performed under on-resonance conditions for each individual spin. Szyperski *et al.* (*7*), for example, applied such an approach to study a millisecond time scale event in the protein BPTI, attributed to the isomerization of a disulphide bridge. However, it is clearly not possible to simultaneously satisfy the on-resonance condition for all ^{15}N nuclei in a protein. Furthermore, probe and sample overheating limits the spin-lock field strength that can be used in practice and thereby poses a limit on the time scale that can be assessed by on-resonance rotating frame relaxation. The above limitations can be alleviated, however, by spin-locking the magnetization off-resonance. First, the requirement that the RF field is applied on-resonance is no longer relevant. Second, the increase in the effective field strength allows the investigation of faster processes. In practice, off-resonance relaxation rates $R_{1\rho}$ can be determined at multiple values of the effective field by variation of offset and/or amplitude of the off-resonance spin-lock field. From the field dependence of the relaxation rate, information about motions at frequencies on the order of $\omega_e = \sqrt{(\omega_1^2 + \Delta\omega^2)}$ is obtained (*8–10*).

Application of ^{15}N off-resonance rotating frame relaxation to the study of internal dynamics in proteins has recently been proposed by Akke and Palmer (*10*). In their method, part of which is reproduced in Fig.1A, the relaxation is measured after alignment of the nuclear spin magnetization with

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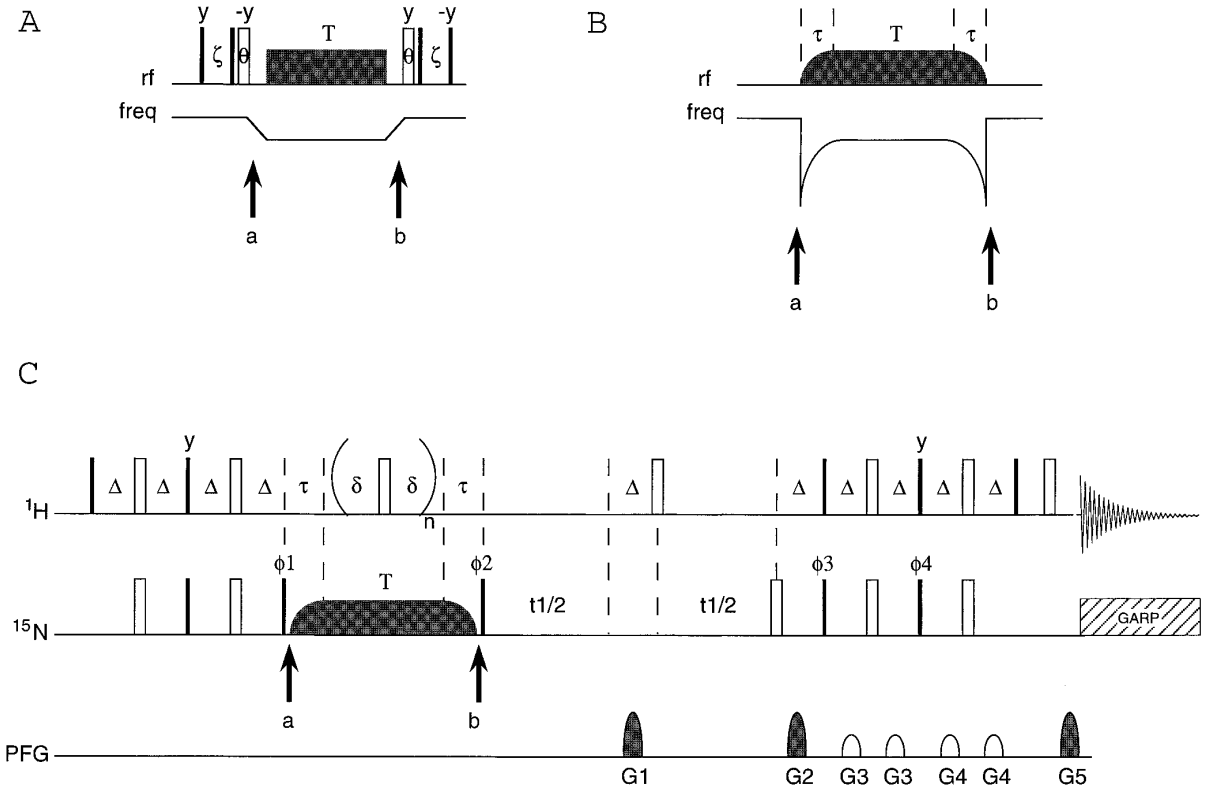

FIG. 1. Pulse sequences for measuring off-resonance rotating frame relaxation. Narrow (filled) bars and wide (open) rectangles represent 90° and 180° pulses, respectively. Unless indicated, the pulses are applied along the *x* axis. The large shaded bar represents the low power off-resonance spin-lock pulse. Panels (A) and (B) represent building blocks using chemical shift precession and adiabatic pulses, respectively. The upper traces, labeled ''rf,'' represent high power (filled and empty bars) and low power (shaded bar) RF pulses. The lower trace, labeled ''freq,'' represents the variation of offset frequency of the RF pulses. At points a and b in the pulse sequence the RF power and frequency are switched to apply the off-resonance spin-lock field. (A) For chemical shift precession the delay ζ and pulse angle θ are optimized as outlined in (*10*). Frequency switching needs to be executed phase coherently or, alternatively, the off-resonance spin-lock pulse may be frequency modulated. (B) In the case of adiabatic rotations the delay τ is chosen to satisfy the adiabatic condition. The frequency sweep is executed during the RF pulse and frequency switching does not need to be carried out phase coherently. (C) Pulse scheme of the off-resonance rotating frame relaxation experiment using adiabatic rotations. The delay Δ was chosen $(4J_{NH})^{-1}$, compensated for relaxation losses where appropriate. To eliminate relaxation pathways due to cross-correlation (*17, 18*) the delay δ was chosen to center the proton 180° pulse for mixing times less than 10 ms, and 2.5 ms for longer relaxation delays. The delay τ for the adiabatic rotations was 4 ms, and the relaxation delay T varied from 2 to 200 ms. The following phase cycling was used: $\phi1 = 4(y), 4(-y)$; $\phi2 = (x,-x)$; $\phi3 = 2(y), 2(-y)$; $\phi4 = 2(-x), 2(x)$; $\phi_{rec} = (x,-x,-x,x,-x,x,x,-x)$. Heteronuclear decoupling during acquisition was achieved using GARP. For each t_1 increment axial peaks were shifted to the sides of the spectrum by inversion of $\phi2$ in concert with the receiver phase, similar as in States–TPPI. All sequences employ sine-bell shaped pulsed field gradients (PFG) along the *z* axis. To yield absorption mode spectra P- and N-type coherence selection is achieved by inversion of gradients G1 and G2 in concert with inversion of $\phi4$ for each t_1 increment. Sensitivity enhancement is achieved by preservation of equivalent pathways in conjunction with pulsed field gradients (*19, 20*). The duration of all gradients was 800 μs, followed by a 400-μs recovery delay. Gradient strengths were G1 = -7.5 G cm^{-1}; G2 = 75.0 G cm^{-1}; G3 = 0.075 G cm^{-1}; G4 = -0.1125 G cm^{-1}; G5 = 8.36055 G cm^{-1}. Phase $\phi1$ is alternated to ensure exponential decay to zero intensity, as the steady-state value due to ^{1}H–^{15}N NOE is subtracted in successive scans. Adiabatic-shaped pulses typically contained 4k to 8k complex points and were generated by a small C-program. The modulation functions employed are described in the legend to Fig. 2. The frequency sweep was 25 kHz.

the effective field by a combination of high power RF pulses alternated with periods of evolution due to chemical shift offset. In the past such ''chemical shift precession methods'' have been reported to be successful in alignment of magnetization with the effective field in compensated ROESY (*11*) and in improved on-resonance spin-lock measurements (*12*). However, the presence of large chemical shift dispersion hampers the applicability of such preparation schemes to off-resonance rotating frame relaxation experiments (*vide infra*). Mismatch of the magnetization with the effective field for resonances that are not close to the carrier frequency will lead to oscillatory behavior of the magnetization during the relaxation delay. Although the magnetization not aligned with the effective field will eventually be destroyed by the inhomogeneity of the B_1 field, oscillations may persist for considerable time, precluding the use of short relaxation

delays. Moreover, only the projection of the magnetization onto the effective field and the back projection onto the *z* axis will be measured with concomitant loss of sensitivity.

We propose here to use simultaneous amplitude- and phase-modulated RF pulses to adiabatically rotate nuclear spin magnetization to the appropriate effective field in off-resonance rotating frame relaxation experiments. The adiabatic off-resonance spin-lock pulse is presented in Fig. 1B and consists of three components: an adiabatic passage to tilt the magnetization from the *z* axis to the effective field, an off-resonance spin lock period of variable length, and a second adiabatic passage to return the magnetization to the *z* axis. Similar approaches have been described previously for off-resonance ROESY (*13*) and for the measurement of rotating frame relaxation times on-resonance, using amplitude and phase modulation to achieve an adiabatic half-passage (*14*). For the desired adiabatic rotation we advocate the use of RF pulses with simultaneous hyperbolic tangent amplitude and tangent frequency modulation as presented in Fig. 2A. This particular set of modulation functions closely approximate numerically optimized ones (*15, 16*). Figure 2B shows simulated excitation characteristics of a 4-ms tanh/tan pulse of 2000 Hz field strength, indicating that satisfactory adiabatic rotation can be achieved for all spins resonating at frequencies higher than −1000 Hz from the position of the off-resonance field. Alternatively, a change in the sign of the frequency-modulation will result in the mirror image of Fig. 2B, allowing spins resonating at frequencies lower than 1000 Hz to be adiabatically aligned with the off-resonance field. Thus, by use of the proposed adiabatic RF pulses the nuclear spin magnetization can be aligned at arbitrary angles with the static field.

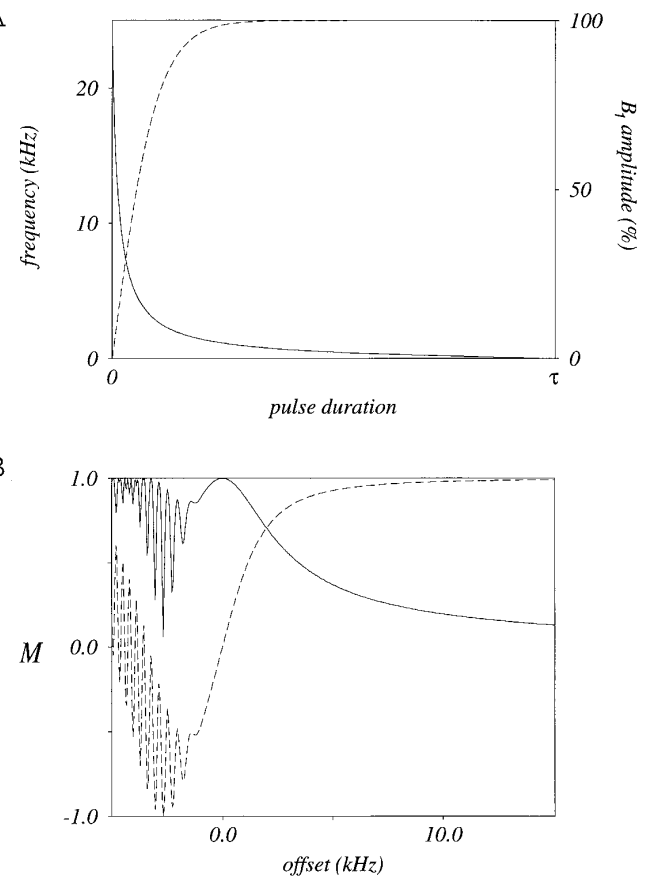

FIG. 2. (A) Profiles of amplitude (dashed) and offset frequency (full) of the tanh/tan adiabatic pulse as a function of pulse duration t. The employed modulation functions were $\omega_1(t) = \omega_1^0 \tanh(10t/\tau)$ and $\Delta\omega(t) = \Delta\omega^0[\tan(\text{atan}(50)[1 - t/\tau])]/50$, for amplitude and frequency, respectively, where τ is the adiabatic pulse length, ω_1^0 is the spin lock field strength, and $\Delta\omega^0$ is the initial frequency offset. (B) Offset excitation profile for the adiabatic pulse (A) of 4 ms duration and 2000 Hz field strength. Dashed and full curves represent projections M_z and $M_{xy} = \sqrt{(M_x^2 + M_y^2)}$, respectively, of the nuclear spin magnetization after adiabatic rotation. Good adiabaticity is achieved for all spins resonating at frequencies higher than −1000 Hz from the position of the off-resonance RF field. Simulations were performed with Pulsetool (Varian, Palo Alto).

RESULTS AND DISCUSSION

Numerical simulations of the Bloch equations were carried out to compare the performance of chemical shift precession and adiabatic alignment schemes. Figure 3 shows projections M_z and $M_{xy} = \sqrt{(M_x^2 + M_y^2)}$ of a simulation of the trajectory of the nuclear spin magnetization of 2 ms in the presence of an off-resonance RF field, applied 2000 Hz from the center of the ^{15}N spectrum, after chemical shift precession (3a–3c) or, adiabatic rotation (3d–3f) to align the magnetization with the effective field. Preparation by chemical shift precession was performed as described by Akke and Palmer (*10*). Adiabatic pulses were as described in the legend to Fig. 2. Panels 3a/3d represent spins resonating 1000 Hz from the carrier frequency—taken as the center of the ^{15}N spectrum—(θ = 33.7°), panels 3b/3e represent spins resonating exactly at the carrier frequency (θ = 45.0°), and panels 3c/3f represent spins resonating −1000 Hz from the carrier frequency (θ = 63.4°). Any oscillations are a result of misalignment of the magnetization with the effective field, with an amplitude determined by the degree of angular mismatch of the two vectors and the precessional frequency around the effective field given by ω_e. It is shown that by adiabatic alignment the amplitude of the oscillations is greatly reduced for spins that are off-resonant with respect to the nitrogen carrier frequency.

The pulse sequence to measure ^{15}N off-resonance relaxation using adiabatic rotations is presented in Fig.1C. Similar schemes can be derived from any pulse sequence used to measure ^{15}N R_1, with an off-resonance spin-lock period replacing the relaxation delay. The adiabatic off-resonance spin-lock pulse is programmed as an adiabatic half passage (AHP), an on-resonance spin-lock, and and a time-reversed AHP. Offset switching (points a and b in the pulse sequence)

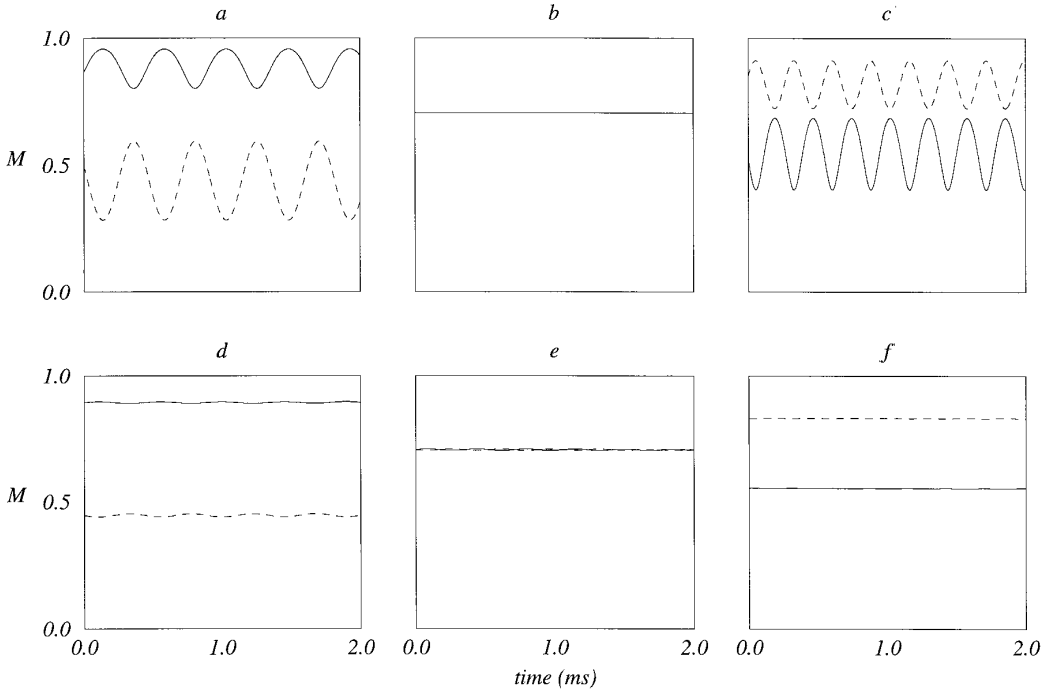

FIG. 3. Trajectories of the nuclear spin magnetization subject to an RF field applied off-resonance after different preparation schemes to align the nuclear spin magnetization with the off-resonance spin-lock field. Projections M_z (dashed) and $M_{xy} = \sqrt{(M_x^2 + M_y^2)}$ (full) of the nuclear spin magnetization are shown after (a–c) alignment by chemical shift precession (d–f) alignment by adiabatic rotation. The field strength of low and high power RF pulses was 2 and 10 kHz, repectively. The off-resonance field was applied 2000 Hz from the center of the nitrogen spectrum. Panels (a) and (d) represent spins resonating −1000 Hz from the center of the spectrum, panels (b) and (e) represent spins resonating exactly at the center of the spectrum, panels (c) and (f) represent spins resonating 1000 Hz from the center of the spectrum. Simulations (a–c) are based on the pulse scheme devised by Akke and Palmer (*10*) (cf. Fig. 1A), simulations (d–f) are based on the tanh/tan adiabatic pulse (cf. Figs. 1B and 2). Simulations were performed with Pulsetool (Varian, Palo Alto).

may be executed by changing the nitrogen carrier frequency or by additional frequency modulation. Frequency coherent offset switching or calibration of relative phases between high power and lower power RF pulses is not required since the relevant nitrogen magnetization is longitudinal at the start and finish of the off-resonance spin-lock period. The use of laminar shaped pulses should be feasible on commercially available spectrometers, provided enough RF wave form memory is available.

Adiabatic off-resonance and chemical shift precession alignment strategies were compared experimentally by placing the building blocks of Figs. 1a and 1b between the ^{15}N pulses with phases ϕ_1 and ϕ_2 of the sequence presented in Fig. 1c. With the element of Fig.1a this essentially yields the pulse scheme of Akke and Palmer (*10*), elaborated to include sensitivity enhancement in conjunction with pulse field gradients for coherence selection (*20*). However, instead of phase-coherent frequency switching a phase-modulated off-resonance spin-lock pulse was used. Two-dimensional $^1H-^{15}N$ correlation spectra obtained with the two off-resonance rotating frame relaxation experiments are shown in Fig. 4. In accordance with the simulations of Fig. 3 amides resonating close to the ^{15}N carrier frequency are equally intense in both spectra but in Fig. 4A the intensity falls with increasing offset due to successive projections of the magnetization. On the other hand, in Fig. 4B the intensity is retained over the entire spectral width. The difference is particularly manifest for the folded arginine side chain N^{ϵ} (indicated with a box), which resonates approximately 30 ppm from the nitrogen carrier frequency.

Application of the adiabatic off-resonance rotating frame relaxation experiment to the 269-residue serine protease PB92 allowed relaxation rates to be obtained for almost all ^{15}N nuclei in the protein. This represents a demanding example as the ^{15}N resonances are spread over a chemical shift range of 50 ppm, from the side chain N^{ϵ} of Arg 19 at 87.1 ppm to the backbone amide of Leu 31 at 137.1 ppm. Representative ^{15}N off-resonance relaxation curves are presented in Fig. 5. The off-resonance RF field was applied at 87.3 ppm, −2000 Hz from the center of the ^{15}N spectrum (120.2 ppm) with a field strength of 1830 Hz. The relaxation curves show no detectable oscillations and low residuals in nonlinear least-squares fitting to a single exponential, indicating good alignment with the effective field. The ^{15}N off-reso-

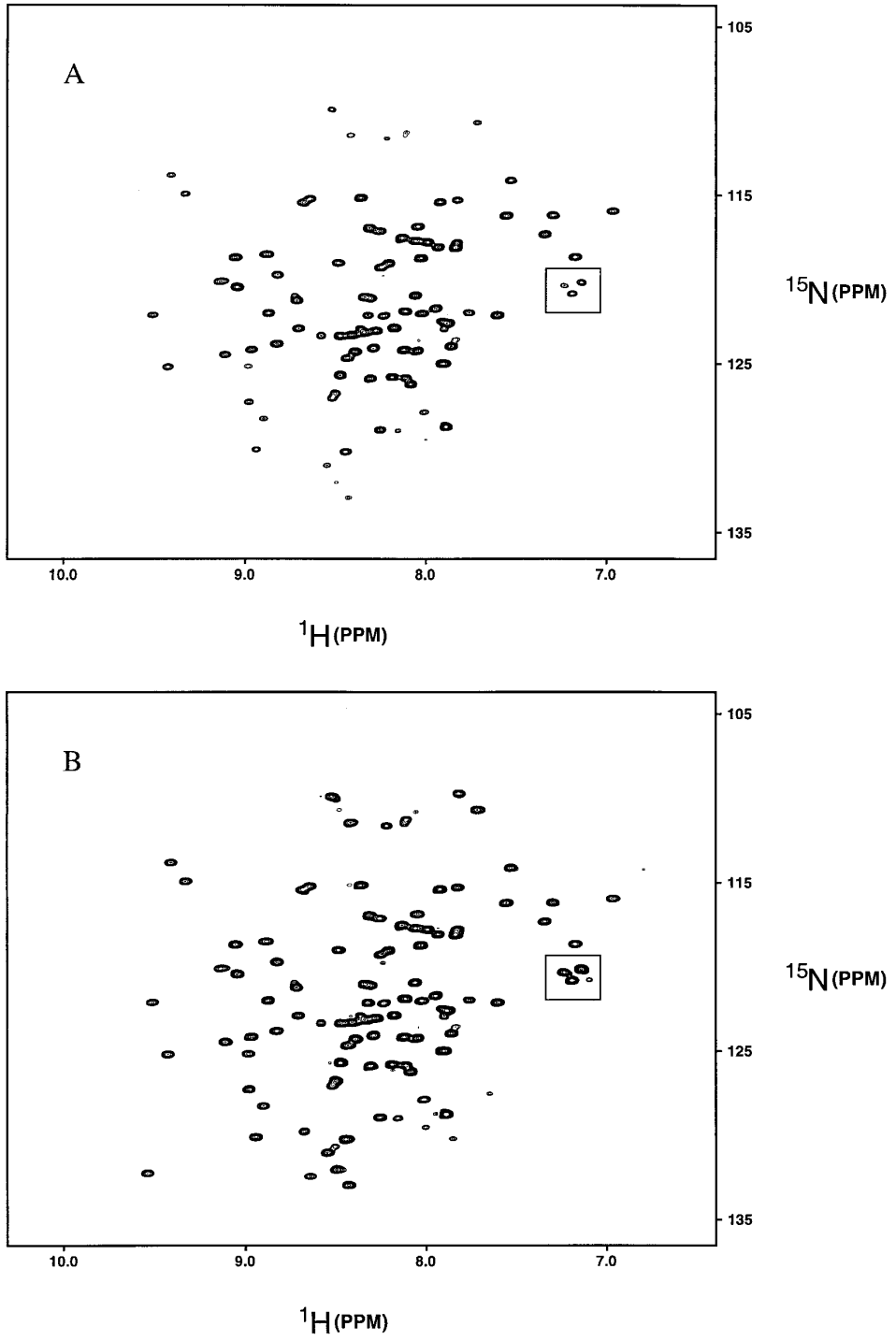

FIG. 4. Two-dimensional $^{1}H-^{15}N$ correlation spectra obtained with off-resonance rotating frame relaxation measurements. The folded arginine side chain N^{ϵ} are indicated with boxes. Spectrum (A) was obtained using the building block Fig.1A, replacing the adiabatic off-resonance spin-lock pulse in pulse scheme Fig.1C. Spectrum (B) was obtained using pulse scheme Fig.1C. NMR experiments were performed on a Varian500 UnityPlus spectrometer, operating at 37°C on a 1 mM sample of uniformly ^{15}N-labeled human colipase pH 4.65 in 93/7 H_2O/D_2O v/v. The spin-lock field strength employed was 1300 ± 30 Hz, and the off-resonance RF field was placed −500 Hz from the carrier frequency. The relaxation delay was 10 ms. Spectra were processed using the in-house developed software package TRITON and NMRPipe (*21*).

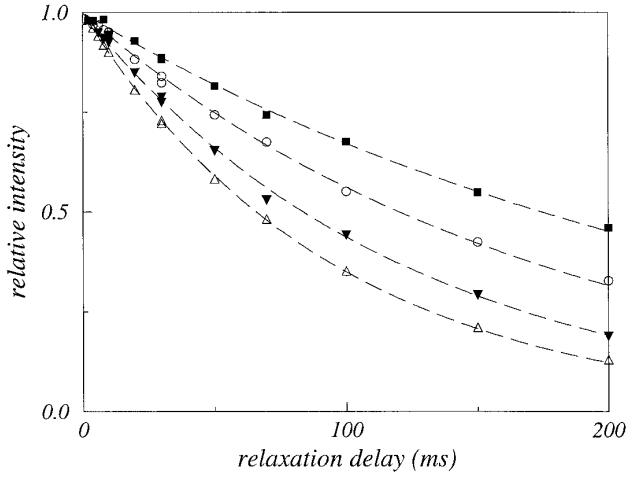

FIG. 5. Representative off-resonance relaxation curves of ^{15}N nuclei of serine protease PB92. NMR experiments were performed on a Bruker AMX600 spectrometer, operating at 42°C on a 2 mM sample of uniformly ^{15}N-labeled serine protease PB92 pH 5.0 in 90/10 H_2O/D_2O v/v. The spin-lock field strength employed was 1830 ± 30 Hz, and the off-resonance RF field was placed at 87.3 ppm, −2000 Hz from the center of the nitrogen spectrum (120.2 ppm). Relaxation analysis was done using in-house developed software to fit the relaxation curves by nonlinear least-squares fitting using the Marquardt–Levenberg algorithm. Curves represent the most upfield resonating backbone amide (■, L31, 137.1 ppm, $\theta = 31.1°$, $T_{1\rho} = 252.0 \pm 5.4$ ms), a backbone amide resonating close to the carrier frequency (○, V119, 121.6 ppm, $\theta = 41.3°$, $T_{1\rho} = 173.5 \pm 2.7$ ms), the most downfield resonating backbone amide (△, G223, 103.3 ppm, $\theta = 62.0°$, $T_{1\rho} = 95.2 \pm 0.6$ ms) and an arginine side chain N^{ϵ} close to the offset frequency of the off-resonance RF field (▼, R19, 87.1 ppm, $\theta = 90.4°$, $T_{1\rho} = 120.9 \pm 1.0$ ms). Error bars are on the order of the sizes of the symbols used and are not displayed for clarity. Relaxation delays were 2, 4, 6, 8, 10, 20, 30, 50, 70, 100, 150, and 200 ms. The measurement at 30 ms was repeated to assess the reproducibility of the measurement.

nance relaxation rates measured for residues of the well-defined core of the protein (which are unlikely to exhibit chemical exchange on the basis of the very homogeneous values of ^{15}N R_2 throughout the secondary structure elements) agree well with those calculated from ^{15}N R_1 and R_2 values.

In conclusion, a technique is presented and applied to measure protein ^{15}N off-resonance rotating frame relaxation for the study of internal dynamics in the millisecond to microsecond regime. The use of simultaneous amplitude and frequency modulated RF pulses offers improved alignment of the nuclear spin magnetization with the off-resonance spin-lock field in the presence of large chemical shift dispersion compared to previously reported methods. The improved alignment allows the use of short mixing times, is devoid of oscillatory behavior of the decaying magnetization, offers improved sensitivity, and is experimentally convenient.

The pulse sequence will be deposited with the BioMagResBank at the University of Wisconsin (URL: http://www.bmrb.wisc.edu).

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