Communications to the Editor

^{15}N $R_{1\rho}$ Measurements Allow the Determination of Ultrafast Protein Folding Rates

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Single domain proteins can fold in vitro at rates in excess of 1×10^4 s^{-1}.[1−7] Measurement of folding rates of this magnitude poses a considerable technical challenge. Dynamic ^{1}H NMR line shape analysis has been successfully applied to study the folding of several proteins on this time scale.[1−3] However, significant populations of both folded and unfolded protein species must be present under suitable experimental conditions for line shape analysis to be utilized. In addition, this method also relies on the observation of well-resolved ^{1}H resonances in the one-dimensional NMR spectrum. This contribution describes the application of off-resonance ^{15}N rotating frame relaxation measurements to protein folding studies. The rotating frame relaxation rate constant, $R_{1\rho}$, is significantly affected by chemical exchange between folded and unfolded states even if the population of either state is very low; consequently, this technique allows characterization of folding kinetics under otherwise inaccessible conditions. The ^{15}N labeling also overcomes the requirement for resolved ^{1}H resonances.

The method was applied to a small helical protein, the peripheral subunit-binding domain (PSBD) of the dihydrolopoamide acetyltransferase component of the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus*. The structure of this 41-residue protein is illustrated in Figure 1.[8] The protein was prepared by solid-phase synthesis, as previously described,[9] and was specifically labeled with ^{15}N at Ala 11 in the first helix. PSBD has been shown by thermodynamic measurements to fold by a two-state mechanism with a melting temperature of 52 °C and $\Delta G°$ of 2.0 kcal/mol at 25 °C.[4,5] Folding and unfolding rates have been determined by dynamic NMR line shape analysis between 41 and 54 °C in D_2O at pD 8.0; however, folding kinetics at lower temperatures are not accessible by line shape analysis.[3]

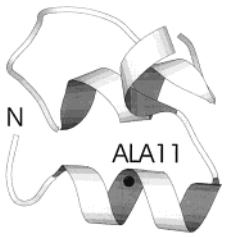

Figure 1. Molscript[20] diagram of the structure of the peripheral subunit binding domain prepared from the Brookhaven Protein Data Bank file, 2PDD. The position of the Ala 11 ^{15}N isotope label is shown as a sphere, and the N-terminus is indicated.

Off-resonance ^{15}N $R_{1\rho}$ relaxation rate constants were measured using the method developed by Akke and co-workers[11] modified to incorporate adiabatic pulses and gradient coherence selection.[12,13] Briefly, $R_{1\rho}$ is measured for ^{15}N magnetization spin-locked along the direction of the effective field in the off-resonance rotating frame. For fast chemical exchange, the relaxation rate constant, R_{ex} is given by:[11]

$$R_{ex} = R_{1\rho}/\sin^2\theta - R_2{}^0 - R_1/\tan^2\theta = \Phi_{ex}k_{ex}/(k_{ex}{}^2 + \omega_e{}^2) \quad (1)$$

in which $R_2{}^0$ is the spin−spin relaxation rate constant in the absence of chemical exchange effects; R_1 is the spin−lattice relaxation rate constant; θ is the tilt angle between the direction of the reduced static field, $\Delta\omega = \omega - \omega_0$, and the effective field, $\omega_e = (\omega_1{}^2 + \Delta\omega^2)^{1/2}$; ω_0 is the population average Larmor frequency, ω and ω_1 are the frequency and amplitude of the spin-locking radio frequency field, respectively; $\Phi_{ex} = p_F p_U \delta\omega^2$; p_F and p_U are the fractions of folded and unfolded conformations, respectively; $\delta\omega$ is the change in the ^{15}N chemical shift between folded and unfolded conformations; $k_{ex} = k_F + k_U$ is the exchange rate constant; and k_F and k_U are the rate constants for folding and unfolding, respectively.

In the present application, ω_1 was held constant, and ω_e was varied by changing $\Delta\omega$. Values of Φ_{ex} and k_{ex} were obtained from $1/R_{ex} = k_{ex}/\Phi_{ex} + \omega_e{}^2/(k_{ex}\Phi_{ex})$. The amplitude of the spin-locking field was determined from independent measurements of θ. $R_2{}^0$ was calculated from the transverse dipole−dipole/chemical shift anisotropy relaxation interference rate constant, η_{xy}, using the relationship $R_2{}^0 = 3^{1/2}(4c^2 + 3d^2)/(12cdP_2[\cos\beta])\,\eta_{xy}$, in which $c = \gamma_N B_0 \Delta\sigma/\sqrt{3}$, $d = -\mu_0 h\gamma_H\gamma_N/(8\pi^2 r_{NH}{}^3)$, μ_0 is the permeability of free space, h is Planck's constant, γ_H and γ_N are the gyromagnetic ratios for ^{1}H and ^{15}N, respectively, $r_{NH} = 1.02$ Å is the N−H bond length, B_0 is the static magnetic field strength,

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Table 1. Off-Resonance ^{15}N $R_{1\rho}$ Measurements of Folding of PSBD

T (°C)	δ_N (ppm)	p_U	Φ_{ex} (10^5 s^{-2})	k_{ex} (10^4 s^{-1})	$\delta\omega$ (ppm)	k_F (10^4 s^{-1})	k_U (10^3 s^{-1})
31	119.1	0.057 ± 0.008	3.39 ± 0.01	2.2 ± 0.4	6.6 ± 0.5	2.1 ± 0.3	1.3 ± 0.3
40	119.7	0.150 ± 0.006	9.82 ± 0.87	3.9 ± 0.8	7.2 ± 0.4	3.3 ± 0.7	5.8 ± 1.2
50	121.5	0.420 ± 0.007	19.9 ± 1.9	4.1 ± 1.0	7.5 ± 0.1	2.4 ± 0.6	17.1 ± 4.1

$\Delta\sigma = -172 \pm 5$ ppm is the ^{15}N chemical shift anisotropy, $P_2[x] = (3x^2 - 1)/2$, and $\beta = 18.5 \pm 2.8°$ is the angle between the N−H bond vector and symmetry axis of the ^{15}N chemical shift tensor.[15−17] R_1 and, η_{xy} were measured using published methods.[14,15,18] Systematic errors in determining R_2^0 from η_{xy} due to contributions from the unfolded state have only a small effect on the fitted parameters because p_U approaches 0 at low temperature and $R_{ex} \gg R_2^0$ at high temperature. The values of p_F and p_U were measured from equilibrium temperature denaturation experiments monitored by CD spectroscopy.[9]

Figure 2 shows $R_{1\rho}$ dispersion curves obtained at temperatures of 31, 40, and 50 °C. Values of R_1 and R_2^0 are given in the caption to Figure 2. The values of R_2^0 vary linearly with η/T, in which η is the viscosity of water. The rotational correlation time determined from the R_2^0/R_1 ratio at 31 °C is 2.82 ± 0.02 ns and agrees well with an estimate of 2.7 ns derived from the buildup curve for the $^1H^N-^1H^N$ NOE cross-peak between Tyr 10 and Ala 11. Fitted exchange parameters are given in Table 1. The values of p_U and $\delta\omega$ predict that the population-averaged isotropic ^{15}N chemical shifts at 40 and 50 °C will differ from the shift at 31 °C by 0.69 ± 0.14 and 2.72 ± 0.13 ppm, respectively. These estimates are in good agreement with the observed shifts (Table 1).

To confirm the present results, the chemical exchange rate constant also was estimated by line shape analysis of the resolved ring current shifted methyl ^1H resonances of V16 and V21 using methods described previously,[3] but under the same solution conditions as for $R_{1\rho}$ measurements. At 50 °C, the value of $k_{ex} = 1.8 \times 10^4$ s^{-1} differs from the results of the $R_{1\rho}$ method by less than a single *ln* unit, which is smaller than the uncertainty of the line shape analysis.[3] The excellent agreement both validates the ^{15}N $R_{1\rho}$ methodology and provides further evidence for the two-state folding of PSBD. Below 41 °C, the population of the denatured state is too low to permit line shape analysis;[3] in contrast, ^{15}N $R_{1\rho}$ measurements conducted at 31 °C allow the determination of the folding rate even though $p_U \approx 6\%$.

In conclusion, ^{15}N off-resonance $R_{1\rho}$ experiments allow the determination of the folding and unfolding rate constants for rapidly folding proteins over a wider range of populations than can be approached by dynamic NMR line shape analysis. The method also alleviates the need for resolved ^1H resonances. Applied to fully ^{15}N-labeled proteins, the technique provides means to determine folding rate constants on a residue-specific basis.[19] The results of such experiments serve as an additional test of the two-state folding mechanism.

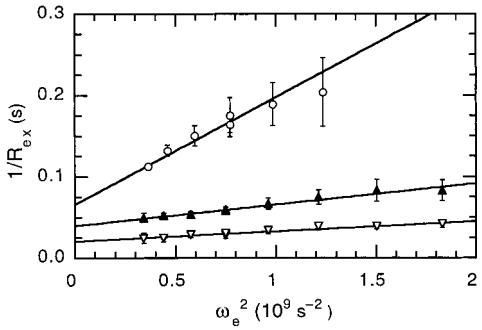

Figure 2. ^{15}N R_{ex} data for Ala 11 obtained at eight effective field strengths at 31 °C (○), 40 °C (▲), and 50 °C (▽). The values of ω_1 were 2.7 ± 0.1, 2.6 ± 0.1, and 2.6 ± 0.1 kHz at 31, 40, and 50 °C, respectively. The values of offset, $\Delta\omega/2\pi$, were 1.4, 2.1, 2.8, 3.5, 4.2, 5.1, 5.6, and 6.4 kHz; a duplicate set was obtained for $\Delta\omega/2\pi = 3.5$ kHz. At 31 °C, uncertainties in R_{ex} obtained at $\Delta\omega/2\pi = 5.6$ and 6.4 kHz were >25%, and data for these points were not included in the analysis. Values of R_1 were measured to be 2.14 ± 0.02, 2.09 ± 0.06, and 1.64 ± 0.15 s^{-1} at 31, 40, and 50 °C, respectively. Values of R_2^0 obtained from η_{xy} were 4.04 ± 0.22, 3.48 ± 0.21, and 2.33 ± 0.35 s^{-1} at 31, 40, and 50 °C, respectively. The best least-squares fitted line is drawn for each data set. Values of $k_{ex} = (b/m)^{1/2}$ and $\Phi_{ex} = (mb)^{-1/2}$, in which m and b are the slope and intercept of the fitted lines, are reported in Table 1. Uncertainty estimates were obtained by Monte Carlo and jackknife simulations.[21,22] The sample was 3 mM PSBD (90%/10% H_2O/D_2O, pH = 5.4). NMR spectra were recorded on Bruker DRX600 and Varian INOVA spectrometers operating at ^1H Larmor frequencies of 600.13 and 599.73 MHz, respectively. Sample temperatures were calibrated using a 100% ethylene glycol standard.

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