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Determination of slow motions in extensively isotopically labeled proteins by magic-angle-spinning ^{13}C-detected ^{15}N exchange NMR

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Abstract

A solid-state NMR exchange technique for detecting slow segmental dynamics in proteins is introduced. The technique exploits motion-induced incomplete refocusing of amide ^{15}N chemical shift anisotropy under magic angle spinning. Slow motions on the millisecond timescales are detected as reduced NMR signals. Detection of ^{13}C magnetization transferred from ^{15}N allows the identification of mobile residues with high resolution. This exchange technique is demonstrated on two proteins with opposite motional properties. Combined with extensive ^{13}C and ^{15}N labeling, this high-resolution exchange NMR technique allows for the first time the efficient determination of slow dynamics at multiple residues of proteins.

1. Introduction

Molecular motion is an important aspect of the structure and function of proteins. It plays important roles in enzyme active site chemistry, protein folding, protein–lipid interactions, and other biological functions. Nuclear magnetic resonance (NMR) is an important method for studying molecular dynamics on a wide range of timescales (10^{-11}–10 s) [1]. For biological solids such as membrane proteins, solid-state NMR has been used to characterize fast molecular dynamics (10^{-9}–10^{-5} s) through ^{2}H lineshape and relaxation studies [2–4]. However, relatively little is known about slow segmental motions (10^{-3}–10 s) in proteins. The millisecond-to-second motional regime is normally studied by exchange NMR [5–7], in which the frequencies of nuclear spins are recorded before and after a mixing period, and changes in the frequencies indicate slow motions. However, traditional exchange NMR is difficult to apply to proteins because of its poor sensitivity and resolution. The static exchange experiments spread the intensities over inhomogeneously broadened lines, while magic-angle-spinning (MAS) exchange experiments rely on accurate sideband intensities, which is possible only under slow MAS [8,9]. Both suffer from insufficient sensitivity and resolution.

To identify and characterize slow motions of multiple segments in proteins efficiently, it is desirable to employ an exchange technique suitable under fast

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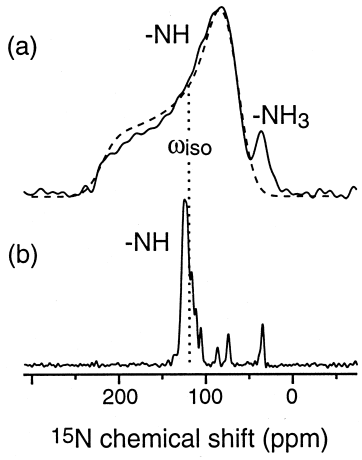

Fig. 1. ^{15}N spectra of uniformly ^{15}N-labeled ubiquitin. (a) Static spectrum (solid line), fit with a single powder pattern (dashed line) with an anisotropy of 100 ppm and asymmetry parameter of 0.2. (b) MAS spectrum, showing the limited amide ^{15}N resolution.

MAS and to combine it with extensive isotopic labeling. Recently, a 1D MAS exchange technique for determining slow dynamics with site resolution was introduced [10]. Termed CODEX for *c*enter-band-*o*nly *d*etection of *ex*change, it exploits the fact that motions interfere with the refocusing of a recoupled anisotropic spin interaction before and after a mixing period. This interference is manifested as reduced spectral intensities. Thus, the difference between a control and an exchange spectrum shows exclusively the sites that have reoriented during the mixing time. Initially applied to synthetic polymers, the CODEX technique used ^{13}C chemical shift anisotropy (CSA) to probe segmental reorientations [10].

However, in ^{13}C highly labeled proteins, this CODEX experiment fails because ^{13}C spin diffusion occurs rapidly. In selectively and extensively ^{13}C-labeled human ubiquitin (MW, 8.6 kD), ^{1}H-driven ^{13}C spin diffusion has been observed within as short as 100 ms [11]. In uniformly labeled proteins, spin diffusion is expected to be even faster. Since spin diffusion changes the orientation-dependent frequencies, it cannot be distinguished from motion-induced exchange. Therefore, an alternative experiment less prone to spin diffusion is desirable.

In this Letter, we introduce a ^{13}C-detected ^{15}N-CODEX experiment that circumvents spin diffusion and identifies slow-moving residues in ^{13}C and ^{15}N-labeled proteins with high resolution. The amide ^{15}N chemical shift anisotropy (CSA) is an ideal probe for slow motions in protein backbones. First, the ^{15}N CSA is relatively uniform in peptide bonds. For example, the ^{15}N static spectrum of uniformly ^{15}N-labeled human ubiquitin (Fig. 1a) displays an amide signal that can be fit by a single powder pattern with an anisotropy of 100 ppm and an asymmetry parameter of 0.20, even though the spectrum is the sum of signals from 75 amide ^{15}N sites. This uniformity of the chemical shift tensors permits the use of a single ^{15}N CSA recoupling time in the CODEX sequence shown in Fig. 2. Also, the near uniaxiality of the ^{15}N chemical shift tensor simplifies the determination of reorientation angles [10]. Most importantly, nitrogen is a sparser and lower-frequency nucleus in proteins than carbon: the ^{15}N–^{15}N dipolar coupling constant is 6.3 times smaller than the ^{13}C–^{13}C dipolar coupling constant. Further, the closest possible distance

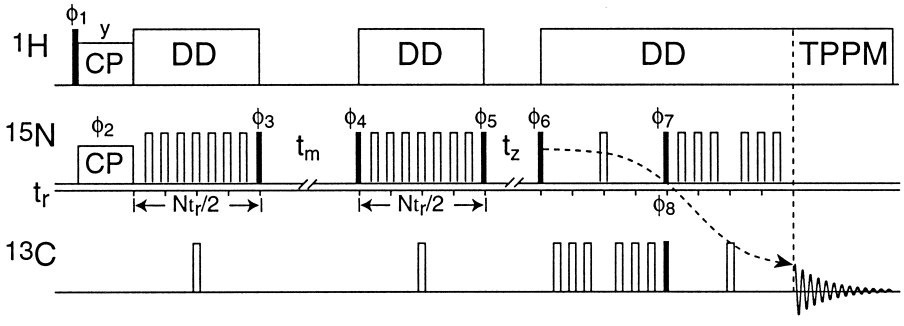

Fig. 2. Pulse sequence of ^{13}C-detected ^{15}N-CODEX experiment. Filled and open rectangles indicate 90° and 180° pulses, respectively. Phase cycles: $\phi_1 = x\bar{x}$, $\phi_2 = \overline{R2}$, $\phi_3 = R1\,R1\,R2\,R2\,\overline{R1}\,\overline{R1}\,\overline{R2}\,\overline{R2}$, $\phi_4 = \overline{R1}\,R1\,\overline{R2}\,R2$, $\phi_5 = R1$, $\phi_6 = \overline{R1}$, $\phi_7 = \overline{R2}$, and $\phi_8 = R2$, where $R1 = \bar{y}\bar{y}xxyy\bar{x}\bar{x}$, $R2 = \bar{x}\bar{x}\bar{y}\bar{y}xxyy$, and bars indicate inversion. DD, dipolar decoupling.

between two amide ^{15}N sites in proteins is much longer (about 2.8 Å, for α-helix) than the closest ^{3}C–^{13}C distance (1.45 Å). These correspond to a ^{15}N–^{15}N dipolar coupling of about 55 Hz, compared to a ^{13}C–^{13}C coupling of about 2500 Hz. Therefore, ^{15}N spin diffusion, mediated by ^{15}N–^{15}N dipolar couplings, is negligible in proteins within 500 ms, leaving a suitable window to detect motional exchange.

2. Pulse sequence

The ^{13}C-detected ^{15}N CODEX pulse sequence is shown in Fig. 2. ^{15}N magnetization is generated by cross polarization (CP) from ^{1}H and evolves under the recoupled chemical shift anisotropy for $N/2$ rotor periods (t_r) before and after a mixing period, t_m. The mixing period is an integral multiple of t_r to maintain the phase coherence of the two CSA recoupling periods. The ^{15}N CSA is recoupled by 180° pulses spaced half-a-rotor period apart. To refocus the ^{15}N–^{13}C dipolar coupling, a ^{13}C 180° pulse is applied at the center of each recoupling period. The phases of the 90° pulses sandwiching the mixing periods are set to store the ^{15}N magnetization that is modulated by $\cos\Phi_1 \cos\Phi_2$ and $\sin\Phi_1 \sin\Phi_2$. Here Φ_1 and Φ_2 are the accumulated MAS phases due to the CSA interaction during the first and second recoupling periods, respectively [12],

$$\Phi_1 = -N\int_0^{t_r/2} \omega_1^{CSA}(t)\,dt \text{ and}$$

$$\Phi_2 = -N\int_0^{t_r/2} \omega_2^{CSA}(t)\,dt, \quad (1)$$

and $\omega_1^{CSA}(t)$ and $\omega_2^{CSA}(t)$ are the instantaneous anisotropic chemical shift frequencies. The two modulation terms are added in two consecutive scans to yield

$$N_X\left(\langle\cos\Phi_1\cos\Phi_2 + \sin\Phi_1\sin\Phi_2\rangle\right) = N_X\langle\cos(\Phi_1-\Phi_2)\rangle. \quad (2)$$

If motion is absent during t_m, $\cos(\Phi_1-\Phi_2)=1$, and the ^{15}N magnetization (N_X) is completely refocused. If segmental reorientation occurs during t_m, then $\Phi_1 \neq \Phi_2$, and the ^{15}N magnetization is reduced. Pure-exchange signals from the mobile sites but not the rigid segments are detected as the difference between the exchange spectrum $S(t_m) = \langle\cos(\Phi_1-\Phi_2)\rangle$ and a control spectrum $S(0)$, which is acquired by making t_m sufficiently short to avoid exchange. The normalized exchange intensity $S(t_m)/S(0)$ as a function of the mixing time t_m and the length of the CSA-recoupling periods Nt_r contain information on the correlation time and the motional amplitudes, respectively [13].

Since the control and exchange experiments have different mixing periods, T_1 relaxation effects will be different. To account for this, a z-filter t_z is added at the end of the second CSA recoupling period. This z-filter is chosen such that $t_m + t_z$ is the same for the control and exchange experiments. The t_z in the exchange experiment is typically set to 1 ms or less, while the t_z in the control experiment equals the mixing period used in the exchange experiment.

After t_z, the ^{15}N magnetization is transferred to ^{13}C by a rotor-synchronized TEDOR [14] pulse train. The TEDOR sequence refocuses the ^{13}C and ^{15}N chemical shifts while recoupling the ^{15}N–^{13}C dipolar interaction that drives the polarization transfer. The evolution of the spin coherence during TEDOR follows the path:

$$\begin{aligned} &N_x\langle\cos(\Phi_1-\Phi_2)\rangle \\ &\xrightarrow{H_D \propto N_z C_z} N_y C_z\langle\sin\Phi_3\rangle\cdot\langle\cos(\Phi_1-\Phi_2)\rangle \\ &\xrightarrow{90^\circ\text{pulses}} N_z C_y\langle\sin\Phi_3\rangle\cdot\langle\cos(\Phi_1-\Phi_2)\rangle \\ &\xrightarrow{H_D \propto N_z C_z} C_x\langle\sin\Phi_4\sin\Phi_3\rangle\cdot\langle\cos(\Phi_1-\Phi_2)\rangle. \end{aligned} \quad (3)$$

The dipolar phases $\Phi_3 = \Phi_4$ are fixed by the constant TEDOR period, thus the final ^{13}C intensities are modulated by the same cosine factor $\langle\cos(\Phi_1-\Phi_2)\rangle$ as the ^{15}N magnetization.

Detection of ^{13}C spectra has the advantage of enhanced site resolution. Measured by the ratio of the isotropic chemical shift range to linewidth, the ^{13}Cα resolution is about 30 ppm: 1 ppm, while the amide ^{15}N resolution is only about 40 ppm: 3 ppm. The limited resolution of the amide ^{15}N is verified by the ^{15}N cross-polarization MAS spectrum of hydrated ubiquitin (Fig. 1b), which exhibits one broad peak with three poorly resolved shoulders in the amide region (~ 120 ppm).

In the ^{13}C-detected ^{15}N CODEX experiment, *xy*-16 phase cycling was used for the 180° pulses to minimize the effects of pulse and phase imperfections. Potential T_1 artifacts were eliminated by inverting the phases of the read-out pulse ϕ_8 and the corresponding receiver phase. In measuring the motional correlation time, the control and exchange experiments for various mixing times were interleaved and block-averaged to minimize the effects of long-term drifts of the radio frequency power levels.

The sensitivity of this ^{13}C-detected ^{15}N CODEX experiment is about 12% that of the ^{13}C CP experiment. This is a product of the one-bond ^{15}N–^{13}C transfer efficiency (25%) and the intrinsic amplitude modulation (50%) of the technique. Nevertheless, due to fast MAS, the experiment is far more sensitive than any other exchange experiments so far available. This exchange experiment is also similarly robust and sensitive as REDOR [15], an important triple-resonance experiment routinely carried out under fast MAS.

3. Experimental

Selectively and extensively ^{13}C-labeled and uniformly ^{15}N-labeled human ubiquitin was obtained from VLI-Research (Malvern, PA) and hydrated to 30% water by weight to reduce conformational heterogeneity. Several 2D and 3D MAS spectra of the same ubiquitin sample have been shown to exhibit nearly identical isotropic shifts as those in solution [16,17], indicating that the solid protein is in the native state. The ^{13}C and ^{15}N-labeled triblock protein, termed ACA, was expressed from an artificial gene in the *Escherichia coli* strain SG13009 containing the repressor plasmid pREP4 [18]. The gene was constructed in the expression plasmid PQE9 as previously reported [19]. The purified protein was hydrated with an equal amount of water and formed a concentrated gel.

Both proteins were selectively and extensively ^{13}C-labeled. We recently developed this biosynthetic ^{13}C-labeling approach [16,20] in order to enrich multiple carbon sites in proteins without the resolution degradation associated with uniform ^{13}C labeling. This enables multiple structural or dynamical constraints to be measured in each NMR experiment. With [2-^{13}C] glycerol as the sole carbon source in the minimal media, most Cα sites of the ten amino acid products of glycolysis and the pentose phosphate pathway are labeled at 100%, while various carbons of the ten amino acids produced from the citric-acid cycle are labeled at 50% or lower [16]. The ubiquitin sample was labeled by the [2-^{13}C] glycerol scheme, while the triblock protein hydrogel was labeled by the modified TEASE (*te*n-*a*mino-acid *s*elective and *e*xtensive) protocol [20]. The latter labeled the ten amino acids from the linear biosynthetic pathways and kept the amino acids from the citric-acid cycle unlabeled to simplify the NMR spectra. Both proteins were uniformly labeled in ^{15}N at 100% using $^{15}NH_4Cl$ as the nitrogen source in the minimal media.

NMR spectra were acquired at 30.4 MHz for ^{15}N and 75.5 MHz for ^{13}C using a Bruker DSX-300 spectrometer (7.0 Tesla magnet). A 4-mm ^{1}H/^{13}C/^{15}N MAS probe was used. Proton decoupling fields were 105–125 kHz, and typical ^{13}C and ^{15}N 90° pulse lengths were 4 μs and 5.5 μs. The two-pulse phase modulation (TPPM) scheme [21] was used for ^{1}H decoupling during the detection period.

4. Results and discussions

We use the ^{13}C and ^{15}N-labeled ubiquitin and a triblock protein hydrogel to demonstrate the ^{13}C-detected ^{15}N CODEX experiment for site-resolved determination of slow motions. Ubiquitin is a globular protein with 87% of its residues in hydrogen-bonded secondary structures [22]; therefore, large-amplitude slow internal motions are not expected. The triblock protein ACA (MW, 22 kD) is a novel genetically engineered protein that gels reversibly under appropriate pH and temperature conditions [19]. The hydrogel has been characterized by diffusing-wave spectroscopy, which suggested the presence of large-amplitude motions from 10^{-2} to 5^{-1} s. The protein consists of a central random-coil domain (C) with the sequence $[(AG)_3PEG]_{10}$, flanked by two terminal domains (A) designed after the leucine zipper motif, which tends to oligomerize into α-helical coiled coils [23].

Fig. 3 displays the CODEX spectra of ubiquitin with 100 ms mixing. Only Cα and CO signals are

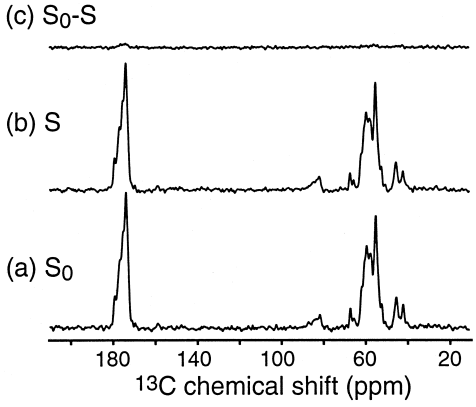

Fig. 3. ^{13}C-detected ^{15}N-CODEX spectra of ubiquitin. (a) Control, $t_m = 0.2$ ms, $t_z = 100$ ms. (b) Exchange, $t_m = 100$ ms, $t_z = 0.2$ ms. (c) Difference, showing negligible intensities. $N = 4$ for CSA recoupling, spinning speed $\nu_r = 7$ kHz, 4096 scans each for (a) and (b).

observed due to the short TEDOR period (571 μs) used. The difference between the control (S_0) and the exchange (S) spectra is negligible, indicating little motion within 100 ms. The C-terminus of ubiquitin is known to undergo fast motions on a much shorter timescale than the exchange mixing time [22,24], thus it does not contribute slow-motion difference signals. Correlation of ^{15}N T_1/T_2 ratios with residual ^{15}N–^{1}H dipolar couplings in solution suggested that residues I23 and N25 undergo conformational exchange with rates of 0.8 ± 0.3 s^{-1} and 2.1 ± 0.4 s^{-1}, respectively [25]. Since these timescales are much longer than the mixing time used here, and only two out of 76 residues are involved, very little exchange from these sites is expected in the CODEX spectra.

In contrast, the CODEX spectra of the triblock protein at 150 ms mixing (Fig. 4) exhibit substantial exchange intensities amounting to nearly 50% of the control spectrum. The exchange intensities resulted only from the A domains, since ^{1}H/^{15}N cross polarization removed most of the signals of the liquid-like C domain in both the control and exchange spectra. The signals were assigned based on 2D ^{15}N–^{13}C and ^{13}C–^{13}C correlation experiments [18]. The clear presence of the difference intensities demonstrates a significant amount of slow motions in the leucine zipper domains. Moreover, the fact that almost all residues exhibited exchange suggests that the A domain moves as a whole. Gelation of the protein has been proposed to be caused by aggregating A domains flanking the central random coil C-domain [19]. The reversibility of gelation implies that the helical blocks dissociate and reassociate. Our NMR results may be related to this dissociation and reassociation dynamics.

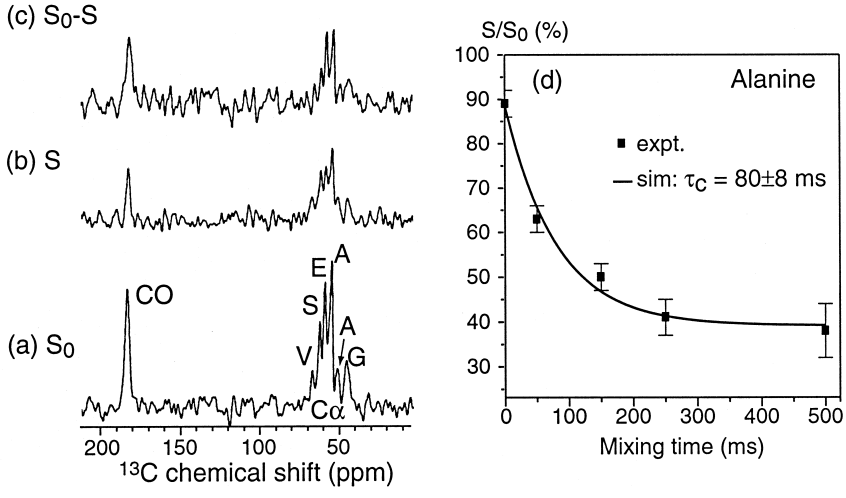

Fig. 4. ^{13}C-detected ^{15}N CODEX spectra of the protein hydrogel. (a) Control, $t_m = 0.2$ ms, $t_z = 150$ ms. (b) Exchange, $t_m = 150$ ms, $t_z = 0.2$ ms. (c) Difference, indicating the presence of motion. (d) Mixing-time dependence of the normalized exchange intensity (S/S_0) for the 55-ppm Ala peak. Spinning speed $\nu_r = 5.5$ kHz, 8192 scans (5 h) each for (a) and (b). The 55-ppm Ala results from the A domains while the 52-ppm Ala is a residual signal from the C domain [18].

We measured the correlation times of the leucine-zipper motion by monitoring the normalized exchange signals $S(t_m)/S(0)$ as a function of t_m. Fig. 4d summarizes the t_m-dependence of the 55-ppm Ala peak. The data were obtained from several pairs of CODEX spectra with mixing times ranging from 1 ms to 500 ms. The Ala data are best fit by a simple exponential decay with a time constant of 80 ± 8 ms. This correlation time is similar to that of other residues of the A domain and is consistent with the diffusing-wave spectroscopy measurements. Further results on the triblock protein hydrogel and their implications on the molecular basis of gelation will be presented in a full paper [18].

5. Conclusion

The ^{13}C-detected ^{15}N CODEX experiment is a robust, sensitive and high-resolution technique for studying slow motions of multiple residues in isotopically labeled solid proteins. We have demonstrated this technique on a protein without motions and a protein hydrogel with substantial motions on the timescale of hundreds of milliseconds. The experiment readily distinguished the two opposite motional behaviors. The upper limit of the motional timescales detectable by this ^{15}N MAS exchange technique is defined by the competing exchange mechanism of ^{15}N spin diffusion, which becomes non-negligible above 500 ms. This, however, is a general limitation of all exchange techniques available so far. The current experiment can be applied to uniformly ^{15}N-labeled proteins that are also labeled in ^{13}C. The ^{13}C labeling can be either uniform or selective. The combination of this high-resolution ^{15}N exchange technique with extensive ^{13}C and ^{15}N labeling allows for the first time the site-resolved and efficient determination of slow dynamics of multiple residues of a protein.

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