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Evidence for Slow Motion in Proteins by Multiple Refocusing of Heteronuclear Nitrogen/Proton Multiple Quantum Coherences in NMR

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A novel NMR cross-correlation experiment for characterizing protein dynamics is based on the application of a Carr-Purcell-Meiboom-Gill (CPMG) multiple refocusing sequence[1] to two-spin coherence $2N_yH_y$ involving a backbone amide proton and the neighboring nitrogen.[2] The experiment provides new insight into internal dynamics of proteins and may thus help to improve our understanding of protein function. The relaxation rates of double- and zero-quantum coherences DQC $= (^1/_2)(N^+H^+ + N^-H^-)$ and ZQC $= (^1/_2)(N^+H^- + N^-H^+)$ depend on the pulse repetition rate if there are local motions on slow timescales (μs-ms). This *dispersion* effect occurs when the two nuclei experience *slow correlated modulations* of their isotropic chemical shifts. Compared to the triple-quantum method recently developed by Wist et al.[3] for $C'NH^N$ subsystems, which is sensitive to concerted fluctuations of C' and N shifts, the new experiment is sensitive to modulations of the N and H^N shifts. In our new experiment, the sensitivity of either DQC or ZQC signals for the first point ($T \approx 0$) is half as good as ordinary HSQC. In ubiquitin, the new method reveals dynamic features that could not be identified with conventional nitrogen-15 T_2 CPMG dispersion experiments.[3]

The difference of the decay rates of DQC and ZQC is given by the sum of chemical shift anisotropy cross-correlation (CSA/CSA) and isotropic chemical shift modulation effects (CSM/CSM) as well as additional dipole/dipole cross-correlation contributions due to couplings to neighboring ^{13}C nuclei:

$$R_{cc} = (^1/_2)(R^{DQC} - R^{ZQC}) = R_{N,H}^{CSA/CSA} + R_{N,H}^{CSM/CSM} + \sum R_{CiN,CjH}^{DD/DD} \quad (1)$$

The CSA/CSA rate does not depend on the frequency $\nu_{CPMG} = 1/(4\tau)$ of a Carr-Purcell-Meiboom-Gill sequence (2τ is defined as the interval between the centers of two consecutive π pulses) while the CSM/CSM rate is attenuated with increasing ν_{CPMG}, like the R_{ex} contribution in a conventional nitrogen-15 T_2 CPMG dispersion experiment. The new DQC/ZQC experiment has been applied to triply labeled ^{15}N, ^{13}C, ^{2}H ubiquitin. Deuteration is necessary to avoid effects due to homonuclear dipole−dipole ^{1}H−^{1}H couplings. Most couplings to $^{13}C'$ and $^{13}C^\alpha$ nuclei enhance autorelaxation of both DQC and ZQC. However, four dipole/dipole cross-correlations, $R_{C'N,C'H}^{DD/DD}$, $R_{C\alpha N,C\alpha H}^{DD/DD}$, $R_{C'N,C\alpha H}^{DD/DD}$, and $R_{C\alpha N,C'H}^{DD/DD}$ have a similar effect as $R^{CS/CS}$. Although each of these four rates is estimated to contribute only about -0.2 s^{-1}, their combined effect cannot be neglected. The experimental rates should therefore be more accurate in the absence of ^{13}C labeling. The pulse sequence (see Supporting Information) generates two-spin coherence $2N_yH_y$ via longitudinal two-spin order $2N_zH_z$. The $2N_yH_y$ term is properly refocused even if the refocusing pulses are imperfectly calibrated since both N_y and H_y components are parallel to the phases of the refocusing pulses, in accordance with the Meiboom-Gill idea. If the DQC and ZQC components have unequal decay rates, however, $2N_yH_y$ will be partly converted into $2N_xH_x$. If the RF pulses are not ideal, either because of miscalibrations or because of offset effects, and if the J_{NH} coupling were negligible, $2N_xH_x$ would not be refocused properly, preventing its build-up. However the presence of the scalar coupling restores proper refocusing. This effect will be discussed elsewhere.[4] The DQC or ZQC components are selected by phase cycling of the receiver.[3] By way of example, Figure 1 shows ZQC and DQC decays of isoleucine 23 in ubiquitin. The cross-correlation rate is negative ($R_{cc} < 0$) in this residue since the ZQC decays faster than the DQC.

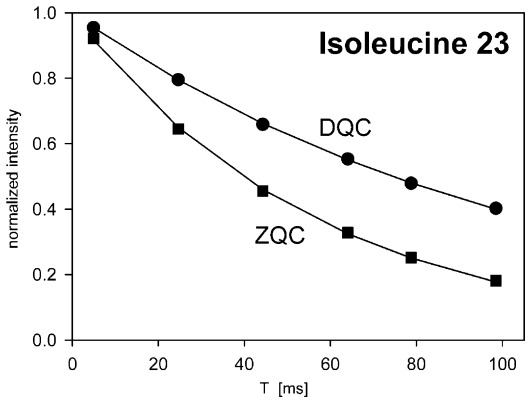

Figure 1. Decays of DQC and ZQC of isoleucine 23 in ubiquitin for $\nu_{CPMG} = 1/(4\tau) = 200$ Hz. A 300 μL sample of a 1.5 mM solution of triply labeled ^{15}N, ^{13}C, ^{2}H ubiquitin (VLI) in H_2O:D_2O = 9:1 with phosphate buffer at pH 6.8 was investigated in a Shigemi tube at 600 MHz and 296 K using the pulse sequence shown in the Supporting Information with 40 and 20 μs 180° pulses for ^{15}N and ^{1}H.

Figure 2 shows cross-correlation rates R_{cc} measured at three different fields. Most residues show slightly negative rates at 300 MHz. The rates increase by about $+0.2$ s^{-1} at 400 MHz and again by about $+0.5$ s^{-1} at 600 MHz. The general pattern is maintained for most residues. There is a negative field-independent contribution (the dipolar terms in eq 1), and a positive contribution $R_{N,H}^{CSA/CSA}$ which increases with B_0^2. We predict the $R_{N,H}^{CSA/CSA}$ rate to be about 1 s^{-1} at 600 MHz depending on the proton CSA.[5] The $R_{N,H}^{CSM/CSM}$ contribution can be either positive or negative and also scales with B_0^2. At 600 MHz the residues I23, L43, F45, and T55 deviate significantly from the average, indicating the presence of a $R_{N,H}^{CSM/CSM}$ contribution. I23 stands out in the new DQC/ZQC experiment, although its ^{15}N R_2 ($= 1/T_2$) relaxation rate is only slightly above average.[6] Asparagine 25 however shows a rate R_{cc} that is not higher than average, although this residue is the only

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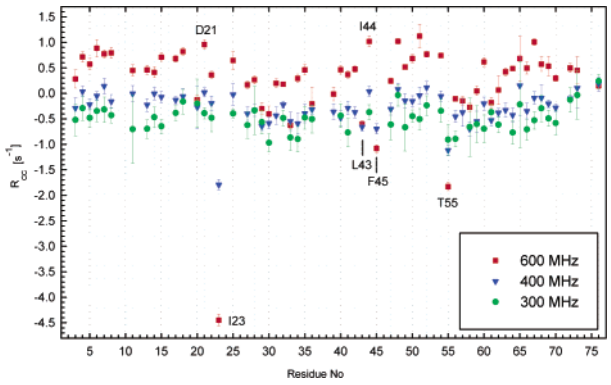

Figure 2. Cross-correlation rates R_{cc} in ubiquitin, determined at different fields with $\nu_{CPMG} = 100$ Hz.

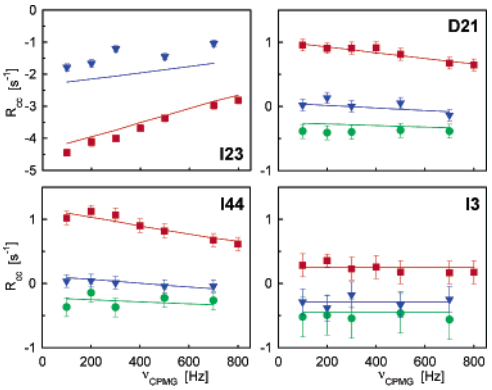

Figure 3. Relaxation dispersion of cross-correlation rates R_{cc} of a few typical residues in ubiquitin as a function of the pulse repetition frequency $\nu_{CPMG} = 1/(4\tau_{CPMG})$ measured at 300 (circles), 400 (triangles), and 600 (squares) MHz. The lines show the curve fit to eqs 1 and 2.

one known[6] to have a distinctly enhanced ^{15}N R_2 relaxation rate due to R_{ex}. It also has been shown[3] to have a very rapid cross-correlation rate $R_{N,C'}^{CSM/CSM}$.

The rates in Figure 2 were measured at 300, 400, and 600 MHz with Bruker Avance spectrometers, using a CPMG sequence with $\nu_{CPMG} = 1/(4\tau) = 100$ Hz. The time requirement at 600 MHz was 2 h (1 h each for DQC and ZQC) for each interval T using 16 scans, a recovery delay of 2 s, and 50 t_1 increments. Differences in decay rates as small as $R_{cc} = 0.15$ s^{-1} are significant. To explore 6 T intervals and 5 ν_{CPMG} rates, one needs 60 experiments (about 2 $^1/_2$ days.)

For a few selected residues, the dependence of the cross-correlation rates R_{cc} on ν_{CPMG} is shown in Figure 3. Isoleucine 23, the residue with the strongest CSM/CSM contribution, shows a strong relaxation dispersion at 600 MHz, which is still detectable at 400 MHz. (At 300 MHz spectral overlap prevents reliable quantification.) At the opposite end of the scale, isoleucine 3 provides an example where relaxation dispersion is not detectable. Aspartate 21 and isoleucine 44 provide intermediate examples: there is a slight dependence on ν_{CPMG} at 600 MHz, but it is barely detectable at 300 or 400 MHz. With increasing ν_{CPMG} the relaxation rates R_{cc} approach $R_{N,H}^{CSA/CSA} + \sum R_{CiN,CjH}^{DD/DD}$, since the second term in eq 1 is averaged out.

For a two-site system in fast exchange, the dependence of the cross-correlation rate on ν_{CPMG} is given by[3]

$$R_{N,H}^{CSM/CSM} = 2p_A p_B \Delta\omega_N \Delta\omega_H \frac{1}{k_{ex}}\left(1 - \frac{\tanh(k_{ex}\tau_{CPMG})}{k_{ex}\tau_{CPMG}}\right) \quad (2)$$

with the populations p_A and p_B of the two sites A and B, the exchange rate k_{ex}, and the isotropic chemical shift differences $\Delta\omega_N = \omega_N^A - \omega_N^B$ and $\Delta\omega_H = \omega_H^A - \omega_H^B$.

By simultaneously fitting the rates observed for isoleucine 23 at 400 and 600 MHz to eq 2, assuming $p_A = p_B = 0.5$, $R_{N,H}^{CSA/CSA} = 1.0$ s^{-1} at 600 MHz and $\sum R_{CiN,CjH}^{DD/DD} = -0.8$ s^{-1}, we obtained $k_{ex}(I23) = 4200$ s^{-1} and $\xi(I23) = \Delta\omega_N\Delta\omega_H/(\omega_{0,N}\omega_{0,H}) = 0.027$ ppm^2. For D21 and I44 we tentatively obtained similar exchange rates $k_{ex}(D21) = 3300$ s^{-1}, $k_{ex}(I44) = 2700$ s^{-1} but much smaller chemical shift differences $\xi(D21) = -0.0036$ ppm^2, $\xi(I44) = -0.0034$ ppm^2.

The average of the DQC and ZQC decay rates gives the autocorrelated relaxation rate R_{ac}. However, this rate is more sensitive to dissipative effects such as exchange with water protons.

It is noteworthy that the residues I23 and T55 which show the strongest CSM/CSM effect are quite close in space in different loops of the protein.

In conclusion, we propose a new DQC/ZQC experiment which complements the widely used nitrogen-15 T_2 CPMG experiments. The new method reveals some residues that are subject to slow motion that could not be detected by conventional methods.

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Supporting Information Available: Pulse sequence and details of the acquisition. This material is available free of charge via the Internet at http://pubs.acs.org.

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