Elucidation of Deceptively Slow Magnetization Exchange between Protein Labile Protons and Water by Dilution-Enhanced Exchange Spectroscopy

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Nuclear magnetic resonance spectroscopy (NMR) plays a prominent role in the study of protein−water interactions because of its ability to provide a picture at atomic resolution.[1−4] Most of the relevant NMR methods are based on incoherent magnetization exchange, which encompasses chemical exchange[5] and cross-relaxation.[6] The labile protein protons can exchange magnetization with water protons by both mechanisms, and for an accurate picture, their separation and identification is essential. One possible way to distinguish chemical exchange from cross-relaxation is to compare the exchange experiments in the laboratory and rotating frame.[7,8] Whereas the chemical exchange is invariant to the change of reference frame, the cross-relaxation rate depends on the reference frame and the motional regime. In the spin diffusion regime, cross-relaxation can be distinguished from chemical exchange by its sign reversal in the two frames. Unfortunately, in the extreme narrowing limit, cross-relaxation in the two frames has the same sign. Furthermore, in this regime, the sign of magnetization transferred by cross-relaxation is opposite to that transferred by chemical exchange. Thus, it is possible that the two processes can cancel each other when they take place concurrently. In other words, it is possible to have negligible resulting magnetization transfer, even if the two processes take place at considerable rates.

A likely system in which this could happen is magnetization exchange between the amide protons of a protein and water. It is well-known that most of the water molecules around proteins are in the extreme narrowing regime (exhibiting negative cross-peaks in exchange experiments) and that amide protons readily exchange with water protons. For example, in calcineurin B, exchange rates for amide protons have been reported to range from 0 to 20 s^{-1}.[9] Of the 60 amide protons, 21 were reported to have values of less than 1 s^{-1}, however, it is not known how many of these small values are due to the absence of exchange and how many to the cancellation of the two exchange processes. Thus, a method is critically needed that separates the two processes.

Recently, we proposed such a method based on the different sensitivities of chemical exchange and cross-relaxation to isotope dilution.[10] We call it dilution-enhanced exchange spectroscopy (DEEXSY) because the dilution of a proton reservoir reveals chemical exchange cross-peaks in the exchange spectrum. Our previous analysis showed that, in random fractionally deuterated molecules, full matrix analysis of magnetization exchange fails and quantitative relations can be obtained only in the form of a Taylor series.[11] Also, we showed that in the presence of chemical exchange, an effective magnetization exchange rate L^D is related to the chemical exchange rate constant, k, and cross-relaxation rate constant, σ, by linear approximation[10]

$$L^D = k - p\sigma \tag{1}$$

where p ($0 < p \leq 1$) is the degree of protonation of the spin sites. Linear approximation assumes that the cross-peak volumes are in the initial build-up rate regime where normalized peak volume is directly proportional to the effective magnetization exchange rate constant, $a \approx L^D\tau_m$. If the same exchange experiment with the same mixing time is performed at two different proton dilutions, p_A and p_B (protons are diluted by deuterons), then both magnetization exchange rate constants can be obtained from the normalized peak volumes at two dilutions, a_A and a_B[10]

$$\sigma = \frac{1}{\tau_m}\frac{a_B - a_A}{p_A - p_B}$$

$$k = \frac{1}{\tau_m}\frac{p_A a_B - p_B a_A}{p_A - p_B} \tag{2}$$

Because eq 2 requires peak volumes at a high degree of deuteration (low proton concentration) where the signal-to-noise ratio is not favorable, its use is limited to semiquantitative analysis. More reliable results can be obtained from a series of exchange experiments recorded at different mixing times and different degrees of protonation. Then, the actual k and σ for each spin pair H^N-H^w can be obtained by a linear fit to eq 1.[10]

The important consequence of eq 1 is that if $\sigma \geq k$ cross-relaxation and chemical exchange cancel each other at a certain proton concentration, $p = k/\sigma$. To test whether the mutual cancellation of cross-relaxation and chemical exchange between protein amide protons and water is an exception or the rule, we investigated ^{15}N-labeled human ubiquitin at 90% and 20% H_2O concentration ($p = 0.9$ and 0.2, respectively). DEEXSY specifies only that the exchange experiment needs to be performed in the initial build-up rate approximation (where eqs 1 and 2 are exact) and that the experiment is repeated at two (or more) isotope dilutions. Thus, the actual experiment can be any exchange experiment designed to monitor magnetization transfer between amide and water protons.[9,12,13]

In the present example, we chose to record the 3D NOESY [^{1}H−^{15}N] HSQC[14] experiment, which gives cross-peaks between H^N and all other protons. We used a somewhat longer mixing time ($\tau_m = 200$ ms) to make intensity changes more obvious. Figure 1 shows cross-sections along the Gln41−H^N at $p = 0.9$ and $p = 0.2$. Cross-sections are plotted with the same scale for the diagonals (at 7.7 ppm) and, thus, apart from the difference in signal-to-noise ratio, are in all respects comparable. The most prominent difference between the two cross-sections is the emergence of a prominent H^N-H^W line in the isotope-diluted

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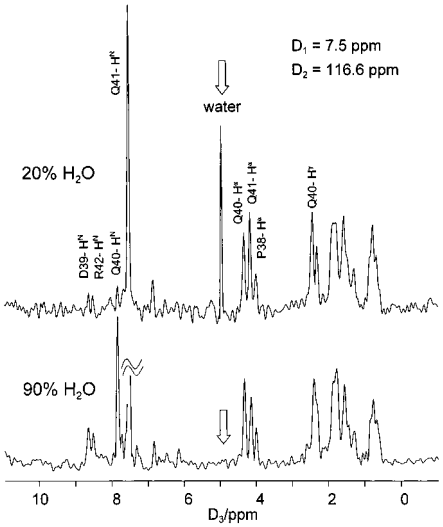

Figure 1. 1D sections from 3D NOESY [^{1}H−^{15}N] HSQC spectrum at the resonance of Gln41−H^N in human ubiquitin, 2 mM protein in 25 mM acetic acid in H_2O/D_2O mixture, pH = 4.1. Data were recorded under identical conditions (T = 275 K, τ_m = 200 ms, t_1(H) = 43 ms, t_2(N) = 10 ms, t_3(H) = 45 ms) from samples that differ only by the H_2O/D_2O ratio 90/10 and 20/80 (i.e., with $p_A = 0.9$ and $p_B = 0.2$). In the proton-diluted sample, the number of scans was doubled from 8 to 16. Note the appearance of a cross-peak at the water resonance (4.8 ppm) in the spectrum of the sample with reduced proton concentration. To keep all H^N diagonals commensurate, the protein sample in each H_2O/D_2O mixture was equilibrated overnight at 55 °C.

spectrum. The line is in phase with the diagonal, which indicates its chemical-exchange origin. This is in full agreement with eq 1 which says that if at certain proton concentration (here p = 0.9) cross-relaxation and chemical exchange cancel each other, lowering the proton concentration (here to p = 0.2) quenches cross-relaxation, and chemical exchange prevails. Using eq 2, we readily find for Gln41 H^N−H^W $\sigma \approx k = 0.5$ s^{-1} instead of $k = \sigma = 0$, which otherwise might be deduced from the exchange experiment in a fully protonated sample. Comparison of other cross-peaks in Figure 1 reveals that cross-peaks toward aliphatic and aromatic protons are mainly unchanged (some changes occur as a result of the change in the spin diffusion network due to dilution), whereas cross-peaks with other labile protons are greatly diminished with isotope dilution. Peaks with H−C protons are independent of proton concentration because diluted H^N protons have an unchanged H−C proton environment. Peaks to other amide protons are reduced because both protons in the magnetic exchange have been diluted.[11] This simple description does not take into account possible indirect interactions. For instance, cross-relaxation between water and amide protons may be mediated by NH−CH interactions. However, an analysis of the cross-peaks buildup curves shall reveal these effects.

By analyzing the peaks in the water plane of a 3D spectrum (Figure 2), we found that, in 38 of 76 amide protons, the cross-peak with water changes intensity with isotope dilutions. This indicates that mutual cancellation of cross-relaxation and chemical exchange between the amide and water protons is the rule rather than an exception. Figure 3 shows the correlation plot of the chemical exchange rate constant and the cross-relaxation rate constant for the protons susceptible to isotope dilution with $\sigma >$ 0.2 s^{-1}. A strong correlation between the two exchange rate constants should be expected because the two protons must approach each other to exchange. Whereas there is no upper limit for the chemical exchange rate constant (amide exchange spans the range of several orders of magnitude), the upper limit of the cross-relaxation rate constant is determined by the distance of the closest approach between the H^N and H^W protons, the residence time of the water molecule on the protein surface, and the number of water protons interacting with each amide proton.

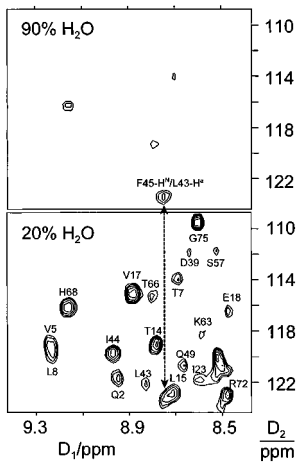

Figure 2. Region of amide proton resonances in the water plane of 3D NOESY [^{1}H−^{15}N] HSQC spectra from samples at two proton dilutions. Contours are drawn in an exponential mode with the increment of 1.41. In both spectra, levels are scaled in the same manner with respect to the H^N diagonals. The magnitude of the F45-H^N/L43-H$^\alpha$ cross-peak is the same in both spectra. The peak originates from cross-relaxation exclusively, and its intensity, when normalized to the H^N diagonal, is independent of dilution.

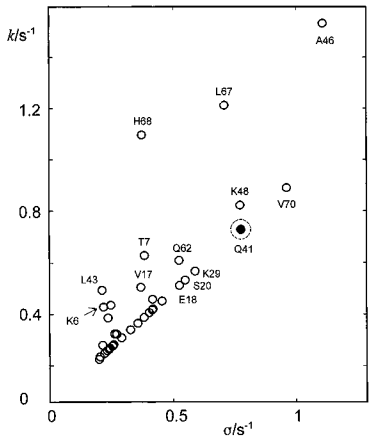

Figure 3. Correlation diagram of chemical exchange rate constants, k, and cross-relaxation rate constants, σ, between indicated amide protons and water. Of 76 amide protons, 38 exhibit observable concurrent cross-relaxation and chemical exchange with water. Shown are only the pairs with $\sigma >$ 0.2 s^{-1}.

It is interesting that in Figure 3, most points lie above the line $k = \sigma$, signifying a dominant contribution of chemical exchange. Whether this is generally valid could be determined only after systematic study of samples at different conditions (temperature, pH).

Using a conservative estimate of r = 1.75 Å, $\omega_0\tau_c \approx 0.386$ (when the cross-relaxation rate in the rigid body isotropic motion has a maximum) and n is 2 or 3, one can find that the upper limit of the cross-relaxation rate H^N−H^W is in the range of 1 to 1.5 s^{-1}. Thus, the mutual cancellation of cross-relaxation and chemical exchange between the amide and water protons can happen only for moderate chemical exchange rates in the range of 0.1 to 1 s^{-1}. The smaller exchange rates hardly can be measured by standard exchange experiments, and the larger ones will overwhelm any cross-relaxation rate. However, it is important to perform the DEEXSY analysis to extract the otherwise inaccessible cross-relaxation rates with water and obtain a more complete description of protein−water interactions.

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