Application of Phase-Modulated CLEAN Chemical EXchange Spectroscopy (CLEANEX-PM) to Detect Water−Protein Proton Exchange and Intermolecular NOEs

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Water plays a crucial role in the structure formation and function of macromolecules in aqueous solution. Hence, considerable research efforts have been made to understand macromolecule−water interactions.[1] NOESY[2] and ROESY[3] experiments are rich sources of such information because they allow one to study chemical exchange between the solvent and labile protons as well as intermolecular NOEs between hydration water and macromolecules. However, these experiments are prone to various sources of artifacts such as exchange-relayed NOE/ROE from rapidly exchanging protons (hydroxyl or amine groups) in the macromolecules, intramolecular NOE/ROE peaks from protein $C_\alpha H$ protons which have chemical shifts coincident with water, or TOCSY-type interactions. It is not trivial to distinguish the contributions from different magnetization transfer pathways. Water-selective 1D NOESY and ROESY experiments can be expanded to include a spin−echo exchange filter[1g] between the selective pulse and mixing period to eliminate the intramolecular NOE or ROE peaks from C_αHs. However, exchange-relayed peaks can still survive and complicate the spectrum. In this Communication, we introduce the application of phase-modulated CLEAN chemical exchange spectroscopy (CLEANEX-PM) to the mixing period of a water-selective 1D sequence, which effectively suppresses all mentioned artifacts, thereby facilitating detection of the actual water−macromolecule interactions.

The 1D CLEANEX-PM sequence is outlined in Figure 1. To specifically detect water−macromolecule interactions, the 90° (hard)−G_1−180° (selective)−G_1 combination[1g,1h,4] excites water, while other spins outside the 180° excitation profile are dephased by gradients. The CLEANEX-PM sequence is a repetitive windowless multiple-pulse sequence 135°(x) 120°($-x$) 110°(x) 110°($-x$) 120°(x) 135°($-x$) of radio-frequency (rf) pulses and has the following characteristics within the ±0.5 γB_1 chemical shift range: (1) Magnetization trajectories cancel intramolecular NOE and ROE contributions due to the opposite sign of the longitudinal and transverse cross-relaxation rates when the ratio reaches −1 to 2 for molecules tumbling in the slow motion limit.[5] By using the invariant trajectory calculation,[6] the observed effective cross-relaxation rates are within the range of ±(1/11) of the NOE rates and therefore are small enough to suppress the NOE contributions to within the noise level in the experimental data. (2) Under the action of CLEANEX-PM, the final positions of spins are locked in the yz plane toward the y axis (>90%), similar to the 180°(x) 180°($-x$) rf series in Tr-ROESY,[7] and the chemical shift difference of two coupled spins is scaled down only by a factor of around 82%−76%, depending on the chemical shifts of the spins. Thus, in most cases, the reduced chemical shift difference would still be much greater than the scalar coupling, precluding the occurrence of TOCSY effects.[7] Previously reported chemical exchange sequences consist of pulses interleaved with delays in the mixing period to keep spins along the z axis longer,[5a] or slow down the spins whenever they move toward the z axis by switching to weaker rf fields (CLEANEX-AM),[5b] to compensate ROE contributions and suppress TOCSY in varying degrees. CLEANEX-PM is more efficient than these two approaches in suppressing the NOE/ROE contributions and prohibiting TOCSY transfer in the working bandwidth.

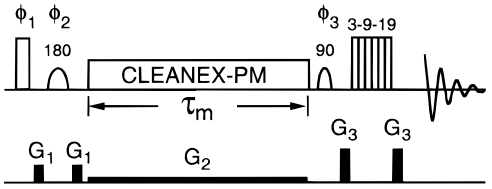

Figure 1. Timing diagram of the water-selective 1D CLEANEX-PM sequence. At 500 MHz, the selective 180° pulse is a 7.5 ms Gaussian. G_1, G_2, and G_3 are 8.5, 0.1, and 23 G/cm, respectively. The small gradient pulse G_2 prevents radiation damping during spin locking. A 5 ms Gaussian 90° pulse after mixing flips water back to the z axis. The 3-9-19 WATERGATE pulse combination suppresses residual water in the xy plane. Difference spectroscopy is necessary to eliminate peaks that relax during mixing.[4] Phase cycle: ϕ_1 {x}, ϕ_2 {$x,y,-x,-y$}, ϕ_3, and rec. {$x,-x,x,-x$}. The pulse train in CLEANEX-PM and 3-9-19 modules are started from the x direction.

To prevent radiation damping,[8] a small gradient is applied through the whole mixing period.[9] We found a gradient strength of 0.1 G/cm to be strong enough to suppress radiation damping yet weak enough that spin locking and cancellation of ROE/NOE contributions are still valid. This is because the rf field strength used for spin locking is still much greater than the spatial field variation caused by the small gradient. Thus, magnetizations across the sample are still approximately aligned along the same spin-locking direction and no signal is lost. At the end of mixing in the CLEANEX-PM, a water flip-back pulse[10] is applied to allow increasing the repetition rate and residual water is suppressed by the 3-9-19 WATERGATE[11]

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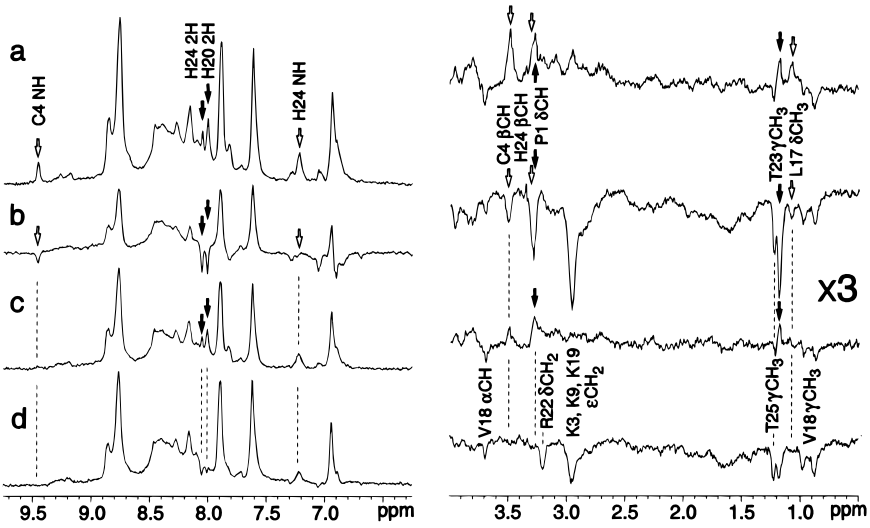

Figure 2. NH and CH regions of an 8 mM zinc finger peptide (CP1), at pH 7.0 and 10 °C, recorded on a 500 MHz Varian UnityPlus spectrometer by water-selective 1D versions of (a) NOESY, (b) ROESY, (c) a spin-echo-filtered WEX II (NOESY), and (d) CLEANEX-PM. Selective excitation, flip-back, and suppression schemes for water were the same among the sequences (a−d) with the parameters in the legend of Figure 1. Water was on resonance during these experiments. In each case, 64 scans were acquired, the mixing time was 150 ms, predelay 2 s, and LB = 3 Hz. The spin-locking field in ROESY and CLEANEX-PM was 4.95 kHz. The length of the spin−echo filter was 40 ms. During the mixing period in a and c, a strong gradient of 20 G/cm was first applied for 2 ms to destroy transverse magnetization, after which the strength was reduced to 0.05 G/cm throughout the period to prevent radiation damping. Open and solid arrows indicate intramolecular[1g] NOEs/ROEs and exchange-relayed NOEs/ROEs, respectively. The vertical scale expanded in the CH region is 3 times that in the NH region. Val 18 has two $C_\gamma H_3$ groups, namely at 0.98 and 0.88 ppm.

sequence. In this sequence, the length of the CLEANEX-PM exactly defines the mixing time because magnetization exchanged from water to protein during the excitation scheme is dephased prior to the mixing.[1g,4]

Figure 2 compares spectra of NH and CH regions of a zinc finger consensus peptide (CP1, MW: 3.0 kDa)[12] obtained at pH 7.0 and 10 °C with 150 ms mixing time by water-selective 1D versions of (a) NOESY, (b) ROESY, (c) a spin-echo-filtered WEX II (NOESY), and (d) CLEANEX-PM. Low-temperature (10 °C) was used to enhance the observation of cross-relaxation peaks in the slow motion limit. In the NH regions of Figure 2a−c, some well-assigned intramolecular NOEs/ROEs and exchange-relayed NOEs/ROEs are indicated by open and solid arrows in the spectra, respectively. Figure 2c shows the removal of intramolecular NOE from Cys 4 $C_\alpha H$ to its NH (9.46 ppm) by a 40 ms spin−echo filter,[1g] in which $C_\alpha H$ protons excited by the selective pulse are relaxed and/or evolved into an antiphase state during the filtering. Figure 2d shows that CLEANEX-PM can further eliminate exchange-relayed peaks from His 24 ring NH to 2H (8.06 ppm) and from His 20 ring NH to 2H (8.01 ppm). At 7.21 ppm, CP1 has a ROE from His 24 $C_\alpha H$ to its NH. This negative peak is canceled in ROESY by the positive chemical exchange peak, while the pure exchange peak is visible in the spin-echo-filtered WEX II and CLEANEX-PM spectra. When studying the pure exchange peaks in these four spectral types (e.g., signal at 7.61 ppm), it is clear that the spectral intensities are quite different, which can be attributed to different relaxation mechanisms during the mixing period.[2,7b] In the spin-echo-filtered WEX II, the intensities are reduced mainly due to water relaxation during the filtering.[1g] To extract chemical exchange rates, initial slope analysis can be applied to CLEANEX-PM in the same manner as in the spin-echo-filtered WEX II spectroscopy.[1g] It should be noted that NOEs from slowly-moving bound water, which is integrated inside the protein structure, are suppressed in CLEANEX-PM, while NOEs from hydration water,[1a] which tumbles fast and has short residence time in contacting protein surfaces, should appear as negative peaks.

In the CH region, negative intermolecular NOE peaks are intermixed with positive intramolecular and/or exchange-relayed NOE peaks (Figure 2a,c) or correspondingly negative ROE peaks (Figure 2b), complicating the data interpretation. In CLEANEX-PM, the NOE contributions from Cys 4 $C_\alpha H$ to its $C_\beta H$ (3.50 ppm) and to Leu 17 $C_\delta H_3$ (1.08 ppm), from His 24 $C_\alpha H$ to its $C_\beta H$ (3.30 ppm), and possibly exchange-relayed contributions from other sources to Pro 1 $C_\delta H$ (3.29 ppm) are eliminated. NOESY and ROESY show that Thr 23 has a larger exchange-relayed NOE/ROE effect than Thr 25 on $C_\gamma H_3$, where $C_\gamma H_3$ of Thr 25 is a negative peak in NOESY. The exchange-relayed effect is suppressed to a large extent in CLEANEX-PM, resulting in roughly the same intensities for $C_\gamma H_3$ of Thr 23 and Thr 25. These intensities are comparable to the $C_\gamma H_3$ of Val 18, which does not have any nearby fast exchanging hydroxyl or amine protons to complicate the intermolecular NOE peaks. We therefore conclude that these negative peaks in CLEANEX-PM are due to intermolecular NOEs, although we cannot completely rule out the possible existence of small exchange-relayed contributions for protons in side chains with rapid internal motion. Null contribution from relaxed peaks during mixing was also confirmed in difference spectroscopy by turning off the water selective 180° pulse.

In conclusion, application of CLEANEX-PM in conjunction with a gradient during the mixing period can effectively suppress intramolecular and exchange-relayed NOEs/ROEs, radiation damping, and TOCSY effects, resulting in better detection of water−macromolecule chemical exchange and intermolecular NOE interactions.

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