Water Exchange Filter (WEX Filter) for Nuclear Magnetic Resonance Studies of Macromolecules

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Abstract: A pulsed field gradient NMR method is described in which only exchangeable proton signals are selectively observed. In this so-called water exchange filter (WEX filter), complete saturation of water is attained rapidly (within 0.5-1.0 ms) by frequency selective gradient phase encoding of water protons. The WEX filter has several advantageous features. First, if exchangeable proton peaks are well resolved, exchange rates can be calculated from the dependency of the peak intensities on the exchange mixing time in a 1D approach instead of in a conventional 2D experiment. Second, to resolve overlapping exchangeable peaks, the WEX filter can be combined with any phase-sensitive 1D and 2D experiment. Third, complete saturation is achieved within a millisecond, avoiding sensitivity losses. Finally, contrary to conventional methods like inversion transfer and 2D EXSY, the WEX filter does not suffer from radiation damping. In this paper we demonstrate the use of the WEX filter for a sample of 2 mM ubiquitin in 90/10 H_2O/D_2O and calculate the exchange rate for a side-chain proton in the unfolded consensus zinc finger peptide CP1.

The study of exchangeable protons by NMR can provide important insight into conformational and dynamic properties of macromolecules. When exchange is slow on the NMR time scale, deuterium substitution studies can be successfully applied.[1-5] For faster exchange, 1D and 2D proton magnetization transfer methods are necessary. In 1D approaches, such as saturation transfer and inversion transfer experiments, the water resonance is perturbed and the resulting effect on the peak intensities of labile protons is observed.[6-11] However, in order to selectively observe exchangeable protons, these 1D experiments require difference spectroscopy, which may impair the accuracy of the measurements. This problem becomes significant especially when exchangeable and nonexchangeable peaks overlap. Furthermore, saturation transfer experiments are hampered by incomplete saturation, while inversion transfer measurements may suffer from improper definition of the exchange period.[12] Two-dimensional exchange spectroscopy (EXSY) is free from these problems.[12-14] and has been used to measure amide proton exchange.[10,15] However, 2D EXSY is time-consuming and can still not resolve exchange rates for exchanging protons which have similar chemical shifts. In order to resolve overlapping exchangeable peaks, 3D methods such as NOESY-HMQC have been used,[16] and it is only recently that attempts have been made to separate overlapping cross peaks in 2D experiments.[17-19] The availability of high-quality shielded gradients for high-resolution NMR has provided new approaches to study proton exchange.[19-21] Here we report a gradient sequence in which only exchangeable proton signals are selectively observed. In this so-called water exchange filter (WEX filter), complete saturation of water is attained rapidly (within 0.5-1.0 ms) by frequency-selective gradient phase encoding of water protons. Coherence selection without contamination of axial peak contributions is achieved analogously to a gradient-selected stimulated echo experiment.[22-24] Many features of 2D-EXSY are accomplished in a 1D experiment. The WEX filter can be combined with any phase-sensitive 1D or *n*D experiment, and in this first paper, we show its effect using detection by a WATERGATE sequence.[25]

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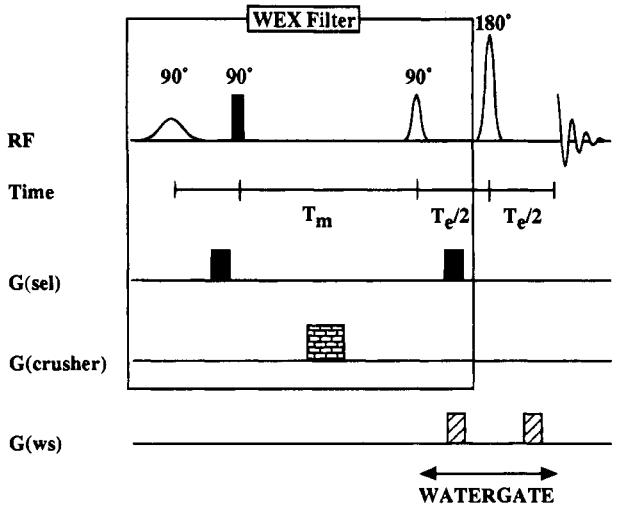

Figure 1. Water exchange filter (solid box) combined with WATERGATE detection. The first 90° pulse is selective to water. The second 90° pulse is nonselective and defines the start of exchange. The third 90° pulse can be either selective on the NH protons or hard (we used soft pulses) and is followed by the detection sequence, in this case a WATERGATE with a selective NH 180° pulse. *G*(sel), *G*(crush), and *G*(ws) are gradients for coherence selection, crushing, and water suppression, respectively, and should preferably be in different directions.

Figure 1 shows the WEX-filter sequence (solid box) combined with WATERGATE detection. The first soft 90° pulse excites water protons selectively. After a short gradient phase encoding pulse (0.5 ms), a hard 90° pulse is applied. This converts the dephased magnetization into a vertical plane. Half of this magnetization and protein protons excited by the second 90° pulse are spoiled by a crusher gradient during the mixing time (T_m). The relevant mixing process starts at this second 90° pulse. The third 90° pulse transforms longitudinal magnetization into observables. Magnetization newly excited by the third pulse is dephased by the second gradient coherence selection pulse, which rephases only signals originating from the selective water 90° pulse.[22,23] Thus, both exchanged and nonexchanged water protons are refocused, while macromolecular protons that do not result from exchange are dephased. This third pulse can be either hard or selective on the region of interest. In our example, we use a selective 90° pulse on the low-field spectral region, and residual water signal is suppressed by a WATERGATE sequence.[25] Note that, since water is on resonance, exchanged magnetization is in-phase after the third rf pulse and pure absorption amide signals are obtained after the WATERGATE spin echo. Also, the quality of coherence selection is improved when different gradient directions are used for *G*(sel), *G*(crush), and *G*(ws).

Figure 2 shows an example of application of the WEX-filtered WATERGATE sequence to a 2 mM solution of ubiquitin in 90/10% H_2O/D_2O. Compared to a simple 1D WATERGATE spectrum, the WEX filter removes all nonexchangeable (e.g., aromatic) protons. As expected, the peak intensities of the exchangeable protons are characteristic functions of T_m. For example, intensities of peaks indicated by solid arrows develop as fast as T_m = 12.8 ms and begin to decay around 122.8 ms. On the other hand, those indicated by open arrows can be seen only after 82.8 ms of mixing time and keep evolving over 302.8 ms. These results clearly show the potential of the WEX filter to identify exchangeable protons and to monitor the extent of the exchange. It appears as if some broad negative resonances

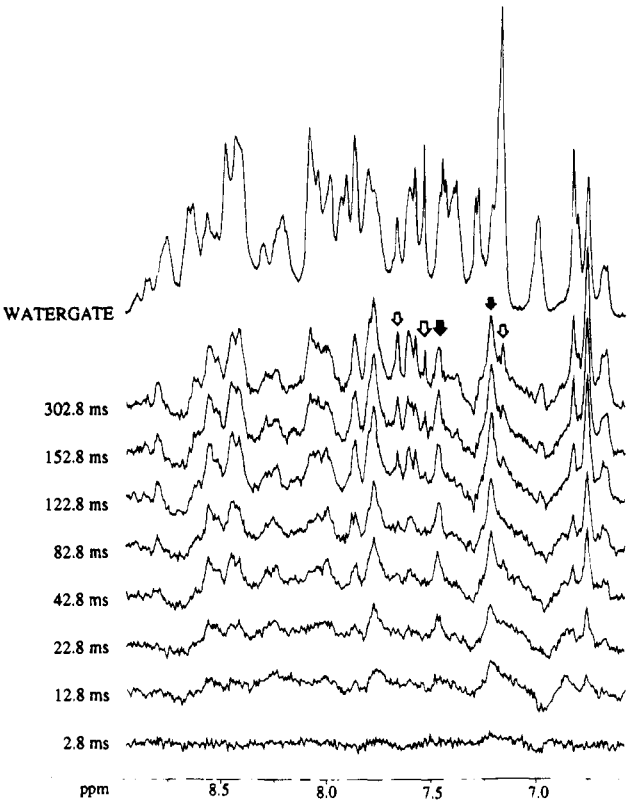

Figure 2. T_m dependence of WEX-filtered WATERGATE spectra (64 scans) for 2 mM ubiquitin in 90/10% H_2O/D_2O. The top spectrum was acquired without the WEX filter. Experiments were performed on a Bruker DMX-500 spectrometer, equipped with triple-axis gradients (0.50 T/m in three directions). Predelay was 1.5 s. A digital filter was applied for the 5.9−9.9 ppm region. The selective pulses in the WEX filter were Gaussians on water (4.9 ms) and on the exchangeable proton region (1.1 ms; third 90° and the 180° pulse). *G*(sel) was 0.5 ms (0.1 and 0.05 T/m for *x* and *z*), *G*(crush) was 2 ms (0.4 T/m for *x* and *y*), and *G*(ws) was 0.5 ms (0.2 T/m for *x*, *y*, and *z*). Gradient recovery time was 250 μs.

are growing at 7.3 and 7.0 ppm, but these are actually small and not growing when a correct base line is drawn between the spectral edges, and we attribute them to small phasing artifacts.

The evaluation of actual rate constants is slightly more complex than in an EXSY experiment, since some diffusion weighting may enter the equations.[20,26] Including the effect of diffusion attenuation in the two-site exchange model of Jeener et al.,[14] one can derive the following T_m dependence for the signal intensities of cross peaks and diagonal peaks in an EXSY experiment:

$$a_{AB} = a_{BA} = \frac{-X_A(R_D - k)}{R_{1A} - R_{1B} + cD_A - cD_B + 2R_D + k} [e^{-(R_{1B}+cD_B)T_m} - e^{-(R_{1A}+cD_A+2R_D+k)T_m}] \quad (1)$$

and

$$a_{AA} = X_A \exp^{-(R_{1A}+cD_A+2R_D+k)T_m} \quad (2)$$

in which A and B denote exchangeable amide/amine protons and water protons. X is the equilibrium mole fraction (X_B = 1), R_1 is the longitudinal relaxation rate, and R_D is the intermolecular dipolar relaxation rate (water−NH NOE); $k_{BA} = X_A k$, and k_{BA} and k are the reverse (water−amide) and normalized rate constants. D is the diffusion constant, $c = \gamma^2 G^2 \delta^2$, and G and δ are the gradient strength and length. γ is

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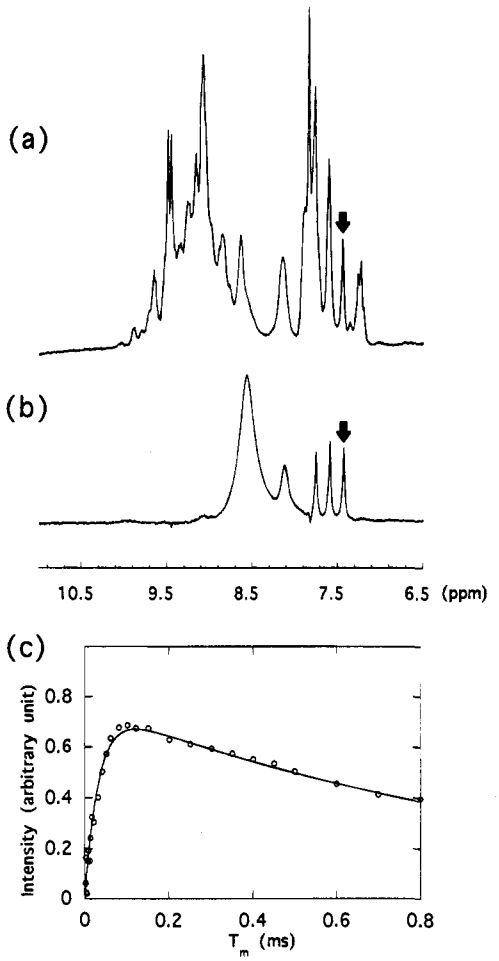

Figure 3. (a) NH-filtered and (b) WEX-filtered spectra (256 scans) of the unfolded zinc finger peptide CP1 (1.5 mM, pH = 2.9) in 90/10 H_2O/D_2O. T_m was 2 ms for spectrum a and 50 ms for spectrum b; the T_m dependence of the intensity of the peak indicated in spectra a and b is given in spectrum c. The solid line is fitted (eq 1) using one effective relaxation rate for all signal contributions besides k. Experiments were performed on a GE Omega 400 MHz spectrometer, equipped with triple-axis gradients (1.30 T/m in three directions). The repetition time was 1.5 s. The selective pulses in the WEX filter were a one-lobe sinc on water (8.0 ms) and cosine-modulated four-lobe sincs (3.5 ms, five cosine cycles) on the exchangeable proton region. G(sel) was 1.0 ms (0.02 T/m for x and y), G(crush) was 1 ms (0.05 T/m for z), and G(ws) was 1.5 ms (0.08, 0.06, and 0.03 T/m for x, y, and z). Gradient recovery time was 300 μs

the gyromagnetic ratio (26.75×10^7 rad/Ts), and it is assumed that the mixing time and diffusion time are equal. These equations reduce to the usual EXSY ones by setting the cD terms and R_D to 0. In our approach, two 1D experiments replace the 2D EXSY, namely, a WEX-filter experiment, in which signal intensities obey eq 1, and an experiment (NH-filter) in which the first 90° pulse of Figure 1 is an amide proton selective pulse,[24] for which the signal intensity obeys eq 2. In the WEX filter, intermolecular (water−NH) NOEs contribute to the signal, while intramolecular NH−NH or NH−CH(aromatic) NOEs (neglected in the equations) may contribute after exchange. Intramolecular NOEs with nonaromatic CH protons are possible if these carbon-bound protons (e.g., Hα resonances and some aliphatics) are excited by the selective water pulse. In the NH-filter experiment, intramolecular NOEs are only important before exchange and mostly influence slowly exchanging protons. As is customary in other methods, we neglect these small contributions in the first approximation. In order to evaluate the rate constant k, both the EXSY and WEX-filter approaches require the acquisition of signal intensities as a function of T_m and an additional experiment to determine the effective water relaxation rates $R_{1B} = 1/T_{1B}$ and $R_{1B} + cD_B$, respectively. In this case the time gain for WEX-filter approach is substantial. As a first illustration, Figure 3 shows spectra with amide-region selective excitation (Figure 3a) and water-selective excitation (Figure 3b) for a 1.5 mM solution of the unfolded consensus zinc finger peptide CP1[27] at pH 2.9. At this pH, the backbone amide proton signals are not detected due to slow exchange, and only five side chain peaks can be observed. Figure 3c shows the T_m dependency of the only side chain peak that is also resolved in the NH-filtered spectrum (indicated by arrow). Neglecting diffusion (or, basically, including it in the relaxation), the solid line is the fitted curve to eq 1 using an experimentally determined $1/T_{1B} + cD_B$ of 0.860 s^{-1} and $1/T_{1A} + cD_A$ of 30.2 s^{-1}, and k was estimated to be 22.3 s^{-1}, which is in the expected order of magnitude for a side chain exchangeable proton.

Equations 1 and 2 show that the WEX filter extends the NOESY-type experiment to include another molecular dynamics process, namely, molecular diffusion. The contribution of the diffusion parameter cD can be regulated by choosing appropriate values for gardient strengths and lengths, as long as good coherence selection remains assured. To obtain an impression of the influence of diffusion, the magnitude of the parameter cD can be compared to the relaxation and exchange rates. Typical relaxation rates are $1/T_{1A} = 10$ s^{-1} and $1/T_{1B} = 1$ s^{-1} for NH and water, respectively, while exchange rates are often on the order of 50−0.5 s^{-1}. Using $D_{1A} = 0.5 \times 10^{-9}$ m^2/s and $D_{1B} = 3.0 \times 10^{-9}$ m^2/s (free water at 37 °C) as typical diffusion constants and $G = 0.1$ T/m and $\delta = 1$ ms, one can calculate rates $cD_A = 0.36$ s^{-1} and $cD_B = 2.15$ s^{-1}. Although this is on the order of magnitude of the parameters that are to be measured, it is important to realize that the inclusion of diffusion does not increase the complexity of the measurements, since its effect is automatically included in the effective relaxation rate. In reality, flexibility is increased, because exchange can now also be studied independently of relaxation by the GEXSY approach.[20] The above calculation also indicates that diffusion influences the free water term much more than the NH term,[26] and the term cD_A can probably often be safely neglected in many experiments. One interesting aspect of this diffusion editing possibility is that it potentially allows studying the bound water,[19,28] which may actually also have a different relaxation rate.

In conclusion, the WEX filter has several advantageous features. First, to resolve overlapping exchangeable peaks, phase-sensitive 2D experiments such as DQF-COSY, HMQC, for HSQC can substitute for WATERGATE. Secondly, complete saturation is achieved within 1 ms, avoiding sensitivity losses. Thirdly, contrary to conventional inversion transfer and 2D EXSY, the WEX filter does not suffer from radiation damping because water remains dephased throughout the sequence. Finally, variable diffusion weighting can be introduced to achieve weighting of free and bound water[19,28] and to study lifetimes.[20] Potential disadvantages are signal loss due to stimulated echo coherence selection (factor 2) or saturation transfer of dephased water (due to incoherent or coherent gradient dephasing) in rapid scan experiments.

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