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A study of protein–water exchange through the off-resonance ROESY experiment: Application to the DNA-binding domain of AlcR

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Summary

In this communication a new NMR experiment for the safe observation and quantification of water–protein exchange phenomena is presented. It combines a water-selective pulse, offering chemical shift-based separation, and the off-resonance ROESY dynamic filter, which permits the elimination of the unwanted intramolecular dipolar cross relaxation of protein protons. Moreover, pulsed field gradients are used for the suppression of radiation damping and the solvent signal. The straightforward incorporation of this sequence in heteronuclear experiments is demonstrated for the case of the DNA-binding domain of the alcohol regulator protein.

Water, being the natural environment of living matter, plays a predominant role in the construction of the puzzle of nature as well as in the structuration of individual species. Therefore, the study of the interactions between water and biomolecules can elucidate important features with regard to their conformational or dynamic behaviour (Otting et al., 1991; Gerothanassis, 1994).

One aspect of these studies is the investigation of the exchange processes between water and labile protons in proteins, which can reveal the solvent-exposed or solvent-protected regions as well as solute intramolecular hydrogen bonding interactions and, together with dynamic information, can provide evidence of conformational interconversions in given, less structurally defined regions. The latter process can be principally detected by two NMR methods: (i) deuterium substitution in the case of slow exchange; and (ii) proton magnetisation transfer experiments in the case of fast exchange. Here, water is perturbed by means of saturation inversion NOESY/ROESY schemes and this perturbation is subsequently transferred to the exchangeable protons of the protein, after which it is observed and quantified.

Since in the latter type of experiments the source of magnetisation is the water singlet, selective excitation techniques can be used to reduce the dimensionality of the spectra and thus the experimental time. However, radiation damping of the concentrated (110 M) water spins competes with the long 90° soft (or DANTE-like) excitation pulse, affecting the sensitivity of the experiment. Elegant solutions based on the use of Q-switched probes (Otting and Liepinsh, 1995a) or pulsed field gradients (Dalvit and Hommel, 1995; Böckmann and Guittet, 1996) can be used in commercial spectrometers. Alternatively, one can take profit of radiation damping for selective excitation of water through difference spectroscopy (Otting and Liepinsh, 1995b; Böckmann et al., 1996) at the expense of an ill-defined mixing period.

In addition to the problems arising from radiation damping during selective pulses, excitation of the nearby H^{α} protons can provoke artefacts due to dipolar coupling (Fig. 1d). These signals can be suppressed either by isotope filtering of the H^{α} protons (Grzesiek and Bax, 1993), a method demanding ^{13}C-labelled proteins, or by relaxing the H^{α} protons (Mori et al., 1996) in the case of nonenriched compounds. To achieve this goal, the addition of a T_2 filter is required prior to the mixing time of the WEX II sequence. Therefore, it results in a decrease in sensitivity due to the T_2 relaxation of water protons, in

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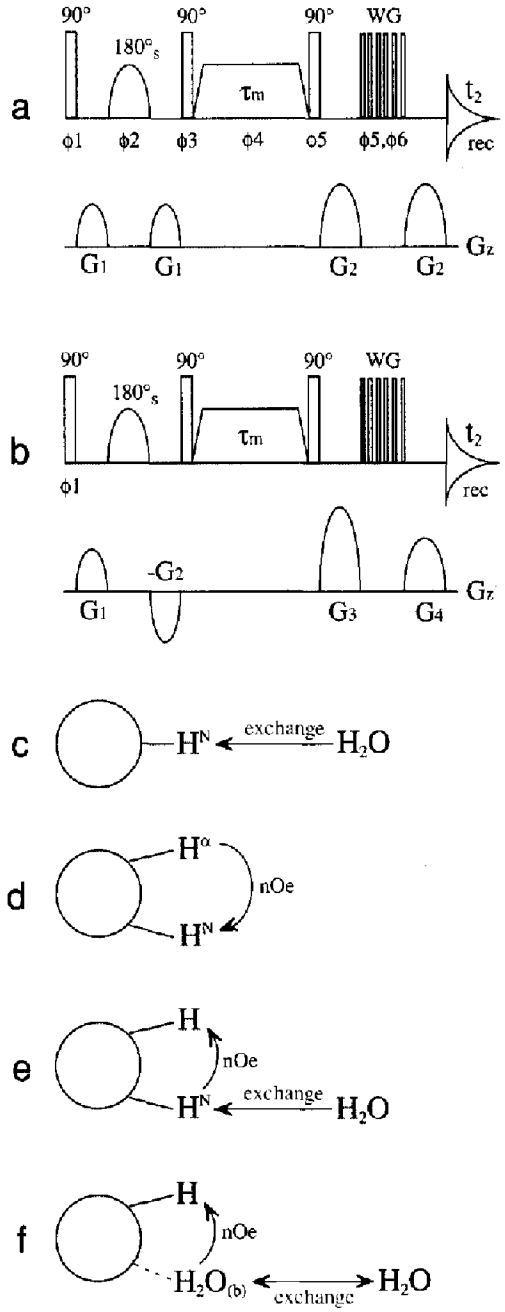

Fig. 1. Selective 1D off-resonance ROESY pulse sequences for the study of protein–water exchange: with phase cycling (a); and with gradients for coherence transfer pathway selection (b). Panels c–f show the different pathways leading to cross peaks in classical NOESY and ROESY spectra. In the off-resonance ROESY experiment with $\theta = 35.5°$, the pathways shown in green can be detected while those shown in red are suppressed or attenuated. The phase cycling is: (a) $\phi1 = x,-x$; $\phi2 = x$; $\phi3 = 8(x)$, $8(-x)$; $\phi4 = 16(x),16(-x)$; $\phi5 = x,x,-x,-x,y,y,-y,-y$; $\phi6 = -x,-x,x,x,-y,-y,y,y$; Rec = x,–x,–x,x,y,–y,–y,y,–x,x,x,–x,–y,y,y,–y; (b) $\phi1 = x,-x$; Rec = x,–x.

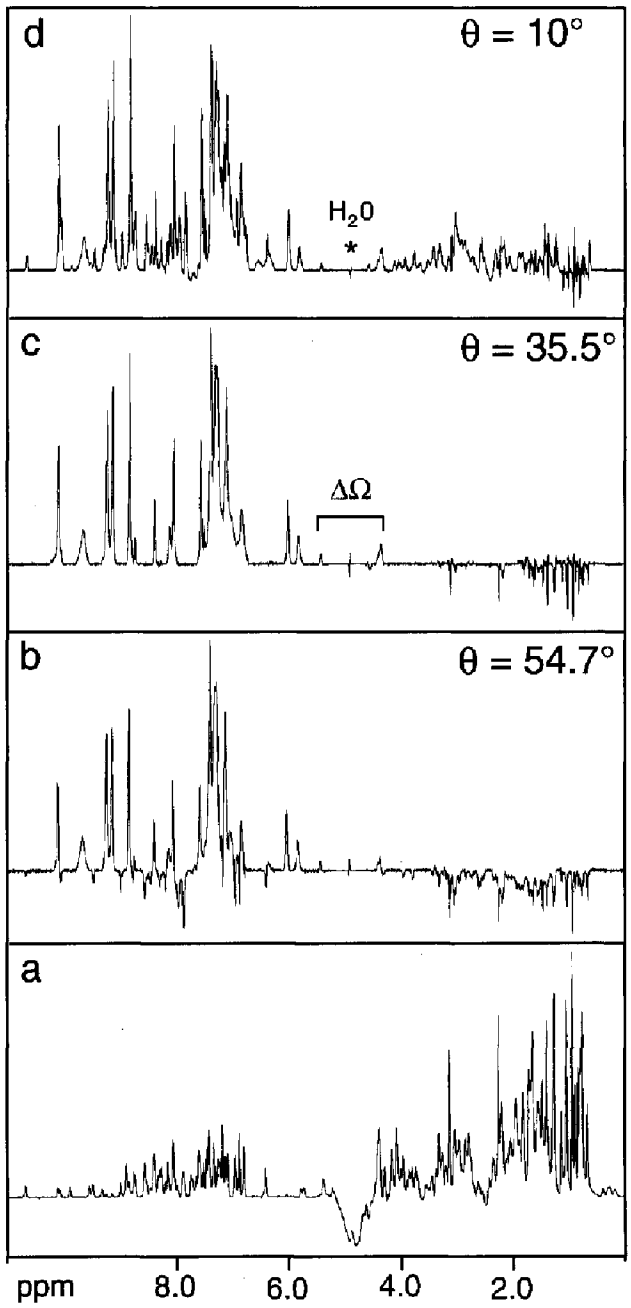

Fig. 2. (a) 1D spectrum of BPTI. (b–d) BPTI–water exchange studies using the sequence of Fig. 1a at different angles θ under the following conditions: solute concentration 4 mM, temperature 25 °C, pH = 6.0. A 100 ms, 7.8 kHz trapezoidal spin-lock pulse was applied during the mixing period; 16 dummy scans and 64 scans were acquired for each experiment (interscan delay 5.9 s, total acquisition time 8 min). The gradients G_1 and G_2 have a semi-sinus amplitude profile, a duration of 2 ms and strengths of –8.16 G/cm and 11.04 G/cm, respectively.

addition to that arising from radiation damping during the water-selective E-BURP excitation pulse.

Chemical exchange and homonuclear dipolar cross relaxation are traditionally investigated through the classical NOESY (Jeener et al., 1979) and ROESY (Bothner-By et al., 1984) experiments. Quite recently, a new method – the off-resonance ROESY – has been proposed (Desvaux et al., 1995), which does not suffer from the inherent limitations of the experiments mentioned above and offers the possibility to explore simultaneously molecular structures and dynamics (Berthault et al., 1996).

The off-resonance ROESY technique can also be very

useful for the study of protein–water exchange. Assuming a rigid macromolecule experiencing isotropic rotational motion, e.g. $\omega\tau_c > 5$, a particular angle $\theta = 35.5°$ between the effective spin-lock axis and the static magnetic field can be used so that dipolar cross relaxation between protons of the macromolecule vanishes and thus exchange phenomena can be selectively observed. This angle θ constitutes a dynamic filter and no elongation of the sequence is necessary (Fig. 1a).

A somewhat related concept was proposed earlier by Fejzo et al. (1990) and applied by Liepinsh et al. (1992) for the study of chemical exchange phenomena in macromolecules, by interleaving the NOESY and ROESY mixing periods. However, the proposed sequence suffers from the well-known offset effect and Hartmann–Hahn transfer associated with the on-resonance spin-lock schemes, but absent in the case of off-resonance irradiation used in this study.

Contrary to the T_2 filter, our new method is able to suppress not only $H^\alpha \rightarrow H^N$ NOE transfers but also indirect pathways of the chemical exchange–NOE type (Fig. 1e). Internal motions may complicate the situation, since perfect cancellation of dipolar couplings cannot be achieved for one value of θ; in any case, strong attenuation of the unwanted dipolar interactions is again obtained. In the case of long-lived water $H_2O_{(b)}$ molecules buried in the interior of the protein, the water–protein vector may experience a significantly shorter τ_c than that of the protein, thus resulting in the incomplete elimination of the corresponding cross peak, which is then only attenuated (Fig. 1f).

To evaluate the potentiality of this approach, bovine pancreatic trypsin inhibitor (BPTI) was chosen as a test compound. Figure 2 presents a series of 1D selective off-resonance ROESY experiments at different θ angles. The first part of the pulse sequence consists in selective excitation of the water via a gradient version of the spin-pinging scheme (Dalvit and Hommel, 1995). The use of defocusing–refocusing gradients suppresses the radiation damping effect during the selective 180° pulse and reduces the phase cycling of the spin-pinging module. In order to demonstrate the efficiency of the proposed scheme in the suppression of the unwanted $H^\alpha \rightarrow H^N$ dipolar transfer, we have used a reduced selectivity ($\Delta\Omega = 0.94$ ppm) universal 180° RE-BURP shaped DANTE sandwich, which avoids the problems associated with the nonperfect amplifier linearity (Roumestand et al., 1995). In this way we are sure that sufficient H^α protons are excited by the

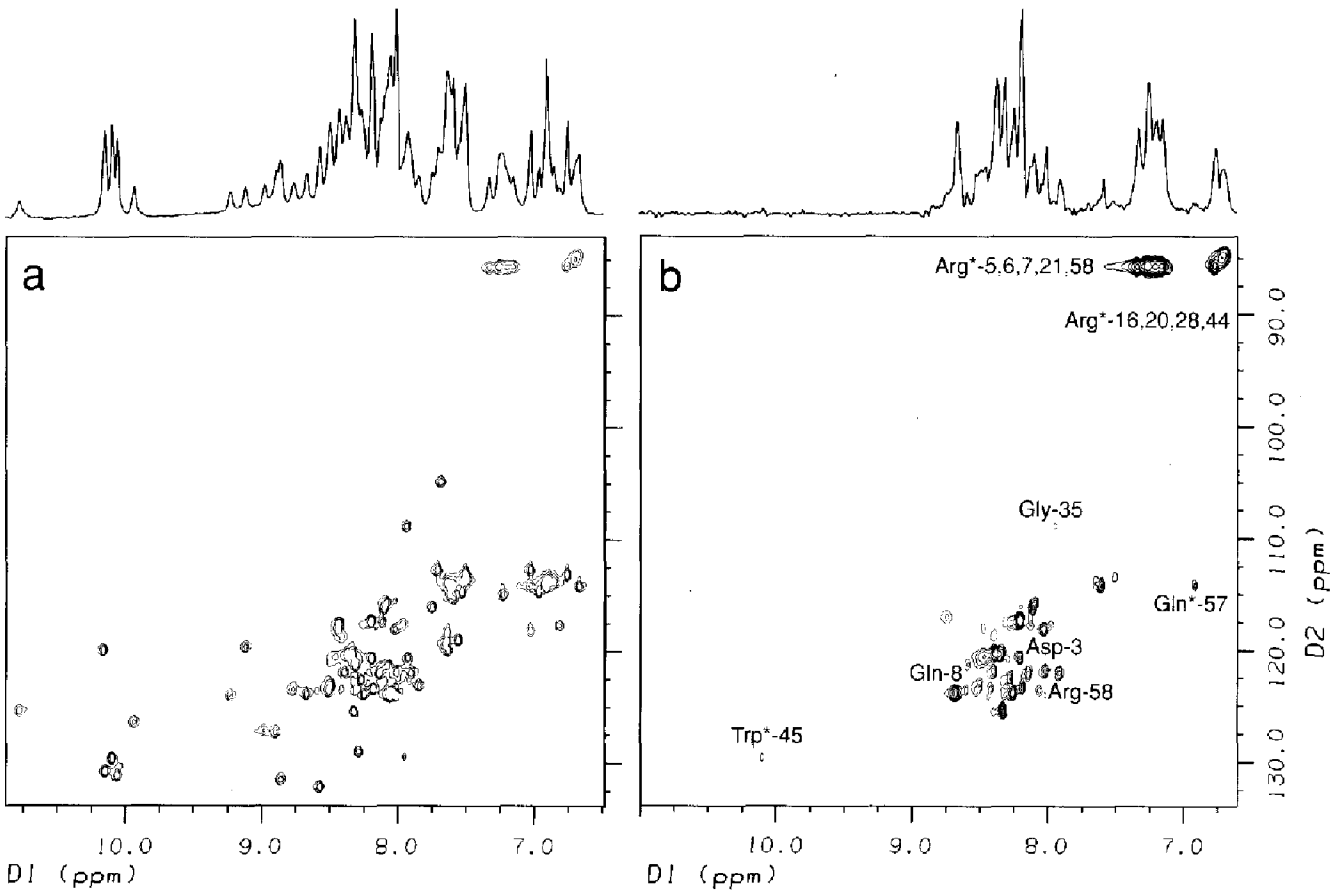

Fig. 3. ^1H-^{15}N 2D HMQC (a) and pseudo-3D off-resonance ROESY-HMQC (b) spectra of AlcR under the following conditions: concentration 1.5 mM, temperature 20 °C, interscan delay 2 s. The experimental times were 6 h (48 scans per increment) and 13 h (96 scans per increment), respectively. Signals assigned so far are shown in red (asterisks denote side-chain peaks). 1D projections are given in the upper part.

preparation module, thus making the suppression of the H^{α}-H^{N} cross peaks more demanding. For routine application of the sequence, a soft Gaussian 180° pulse, offering a better band width-duration product (Hajduk et al., 1993), could be used.

The second part of the sequence comprises the mixing module of the trapezoidal off-resonance spin-lock pulse, bracketed by two hard 90° pulses. The offset of the spin-lock carrier frequency (ΔF) relative to the spectrum center can be calculated according to the equation:

$$\Delta F = \frac{\gamma B_1}{\tan\theta} \qquad (1)$$

where γB_1 is the amplitude of the rf field.

The sequence ends with the WATERGATE module (Piotto et al., 1992) for solvent suppression. Care should be taken to avoid an integer ratio between the two pairs of z gradients (G_1 and G_2), which would result in the creation of echoes. Optionally, a homospoil gradient and a flip-back pulse can be added just after the spin-lock pulse to defocus transverse magnetisation and to return the water magnetisation to the z-axis, leading to an increase of the repetition rates with less signal loss.

The spectra presented here were acquired at 14.1 T on a Bruker AMX-600 spectrometer equipped with a bath cooling unit and a 5 mm HCN triple resonance probe containing a self-shielded z gradient coil (maximum delivered gradient strength 48 G/cm). The trapezoidal spin-lock pulses (comprising 4 ms adiabatic rotations) were created in ASCII format, converted to binary format through the SHAPE program and used as input for the selective excitation unit. The two-offset version of the off-resonance ROESY experiment was used, providing almost negligible angular dispersion (Desvaux and Goldman, 1996). Spectral processing was carried out with the FELIX 95.0 software package (Biosym/MSI, San Diego, CA, U.S.A.). The residual solvent peak was further suppressed by the application of a convolution processing filter.

At an angle $\theta = 54.7°$ (Fig. 2b), the amide region contains negative signals due to the ROE effect between nearby H^{α}-H^{N} protons, together with the exchange peaks which have a positive phase. However, the separation of these two phenomena, feasible in the case of well-resolved signals, becomes impossible in the case of overlap, due to their mutual annulation.

As expected, at an angle $\theta = 10°$ these unwanted peaks are inverted (see the experiment shown in Fig. 2d), since the NOE effect dominates the ROE. In this case, distinction between exchange and dipolar cross peaks becomes quite problematic because their respective signals have the same phase.

Contrary, inspection of the amide region of the spectrum presented in Fig. 2c reveals the absence of cross peaks due to dipole–dipole coupling, since at an angle $\theta = 35.5°$ the mutual cancellation of NOE and ROE effects is achieved. Therefore, chemical exchange can be safely observed and quantified. It should also be noted that the spectrum of Fig. 2c was identical to the row at the water frequency (parallel to the F2 axis) of a 2D off-resonance ROESY spectrum at $\theta = 35.5°$. Examination of this map confirmed the absence of cross peaks involving protein H^{α} protons (with the exception of some weak H^{α}-H^{β} anti-phase signals due to zero-quantum coherences), thus demonstrating the efficiency of the method.

The signals observed in the aliphatic region of the spectrum in Fig. 2c also need to be discussed. In addition to the 2D experiment, we repeated the 1D selective experiment at $\theta = 35.5°$ with an elongation of the spin-pinging module to act as an additional T_2 filter (duration approximately 50 ms). The resulting spectrum was essentially identical to that presented in Fig. 2c, implying that an $H^{\alpha} \rightarrow H^{\beta}$, H^{γ} etc. dipolar transfer pathway should be excluded. Therefore, these signals may be interpreted either as an indirect two-step pathway (exchange–ROE) water $\rightarrow H^{N} \rightarrow$ side-chain protons, or as a ROE effect between water/protein OH and nearby protein side-chain protons. Due to their significant mobility, vectors comprising some side-chain or water protons should experience a sufficiently shorter τ_c than the average one of the protein backbone, thus escaping the dynamic filter of the off-resonance ROESY experiment.

This basic experiment can be further improved by using pulsed field gradients for coherence selection (Fig. 1b). Phase cycling can be reduced and possible subtraction artefacts can be suppressed at the expense of recording only half of the signal. Moreover, this scheme offers the possibility to perform diffusion experiments by modulating the strength of the gradient pairs. In this way, the translational diffusion coefficients obtained for each exchangeable proton can be correlated with the lifetime of the proton at the protein site (Moonen et al., 1992). This experiment was performed using 2 ms refocusing–defocusing gradients, as follows: $G_1 = +8.16$ G/cm; $G_2 = -4.80$ G/cm; $G_3 = +11.04$ G/cm; and $G_4 = +24.00$ G/cm, so that the following condition is fulfilled:

$$|G_1| + |G_2| + |G_4| = |G_3| \qquad (2)$$

Moreover, two homospoil gradients were added before and after the off-resonance spin-lock pulse (1 ms × −2.88 G/cm and −6.24 G/cm, respectively) and the resulting spectrum was identical to that of Fig. 2c.

The availability of ^{15}N-enriched proteins offers the possibility to test this sequence as part of a heteronuclear experiment. This is demonstrated for the DNA-binding domain of the alcohol regulator protein (AlcR), a protein which is under study in our laboratory. AlcR is a transcription factor from *Aspergillus nidulans*, required for the activation of the genes encoding the ethanol metabolising

enzymes. The fragment studied here consists of 65 amino acids and contains a zinc cluster motif of the type Cys-X2-Cys-X6-Cys-X16-Cys-X2-Cys-X6-Cys (Kulmburg et al., 1991).

Deuterium exchange experiments revealed that only three of the AlcR labile protons remain 2 h after dissolving the lyophilised protein in D_2O at 20 °C. Therefore, it is of great interest to investigate water–AlcR exchange in a faster regime.

The uniformly ^{15}N-labelled DNA-binding domain of AlcR was cloned, overproduced, purified and concentrated in a buffer of pH = 6.0. Figure 3a presents the ^1H-^{15}N spectrum of AlcR recorded with the HMQC sequence. Substitution of the first 90° proton pulse by the selective 1D off-resonance ROESY sequence ($\theta = 35.5°$) results in the 2D spectrum displayed in Fig. 3b; here the cross peaks represent only those NH protons that are in fast exchange with the solvent.

In a similar manner, we can incorporate the proposed sequence in any homo- or heteronuclear experiment that can serve for the attribution of the protein–water exchange peaks. In particular, its combination with the Fast-HSQC (Mori et al., 1995) can minimise the water saturation and facilitate the quantification of the exchange signals through a buildup procedure, by modulation of the ROESY mixing time. The analysis of the exchange rates in free AlcR as well as in the complex formed with DNA will be presented in a future communication, together with the structural and dynamic information (N. Birlirakis, R. Cerdan and E. Guittet, work in progress).

In conclusion, we have proposed and demonstrated the efficiency of the off-resonance ROESY technique for the in-depth investigation of protein–water exchange as well as its possible incorporation in heteronuclear pulse sequences. In the absence of significant internal motions, the proposed sequence handles the problems associated with radiation damping and suppresses the unwanted NOE and exchange–NOE transfers implicating protein protons.

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