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New pulsed field gradient NMR experiments for the detection of bound water in proteins

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Summary

New H_2O-selective homonuclear and heteronuclear 2D NMR experiments have been designed for the observation of protein hydration (PHOGSY, *P*rotein *H*ydration *O*bserved by *G*radient *S*pectroscop*Y*). These experiments utilize selective excitation of the H_2O resonance and pulsed field gradients for coherence selection and efficient H_2O suppression. The method allows for a rapid and sensitive detection of H_2O molecules in labelled and unlabelled proteins. In addition, it opens a way to measure the residence time of water bound to proteins. Its application to uniformly ^{15}N-labelled FKBP-12 (FK-506 binding protein) is demonstrated.

The importance of water molecules for structure and function of proteins has been widely recognized and demonstrated (Levitt and Park, 1993; Surridge, 1994). They have been shown to influence substrate specificity, molecular recognition and protein folding (Ottwinowski et al., 1988; Quiocho et al., 1989; Sundaralingam and Sekharudu, 1989). Until recently, X-ray diffraction has been the only technique for the observation of protein hydration. With the advent of multidimensional NMR methods and isotope labelling techniques, it is now possible to detect water molecules which are either tightly bound in the interior of proteins or in fast exchange with the protein surface (Otting and Wüthrich, 1989; Clore et al., 1990; Forman-Kay et al., 1991; Otting et al., 1991a,b; Clore and Gronenborn, 1992; Gerothannasis et al., 1992; Gemmecker et al., 1993; Grzesiek and Bax, 1993; Qian et al., 1993; Xu et al., 1993; Qi et al., 1994). The standard NMR techniques employed to observe H_2O bound to proteins in solution are the heteronuclear $^{15}N/^{13}C$-edited 3D ROESY and NOESY (Fesik and Zuiderweg, 1988; Marion et al., 1989; Clore et al., 1990,1991; Ikura et al., 1990; Zuiderweg et al., 1990) and the homonuclear 3D NOESY-TOCSY (Oschkinat et al., 1988; Vuister et al., 1988) experiments. These experiments have the disadvantage of being time-consuming and having an intrinsic low digital resolution. New experiments have been proposed to reduce the dimensionality of these experiments to two (Grzesiek and Bax, 1993; Kriwacki et al., 1993; Qi et al., 1994), in which alternate sets of scans are recorded with the selectively excited H_2O aligned along the z- and –z-axis, respectively. The difference of these spectra results in a 2D spectrum containing only the desired cross peaks arising from H_2O.

We report here on a new and different method for the observation of hydration water on proteins, which takes advantage of selective excitation of the H_2O resonance and of pulsed field gradients (PFG) for coherence selection (Maudsley et al., 1978; Bax et al., 1980; Counsell et al., 1985; Sotak et al., 1988; Hurd, 1990; Vuister et al., 1991; Mori et al., 1994; Stonehouse et al., 1994; Dalvit and Bovermann, 1995) and efficient H_2O suppression. Its principle is based on the recently developed 1D H_2O-selective NOE and ROE experiments (Dalvit, 1995). This method allows for a rapid and sensitive detection of water molecules in labelled and unlabelled proteins.

Figure 1 shows the pulse sequences for the 2D H_2O-selective heteronuclear ^{13}C- or ^{15}N-edited ROESY (Fig. 1A), NOESY (Fig. 1B) and the homonuclear 2D NOESY-TOCSY (Fig. 1C) experiments. The H_2O-selective 180° pulse located between the first two PFGs inverts the H_2O coherence. The function of the first two PFGs, which have equal strength and opposite sign, is to refocus

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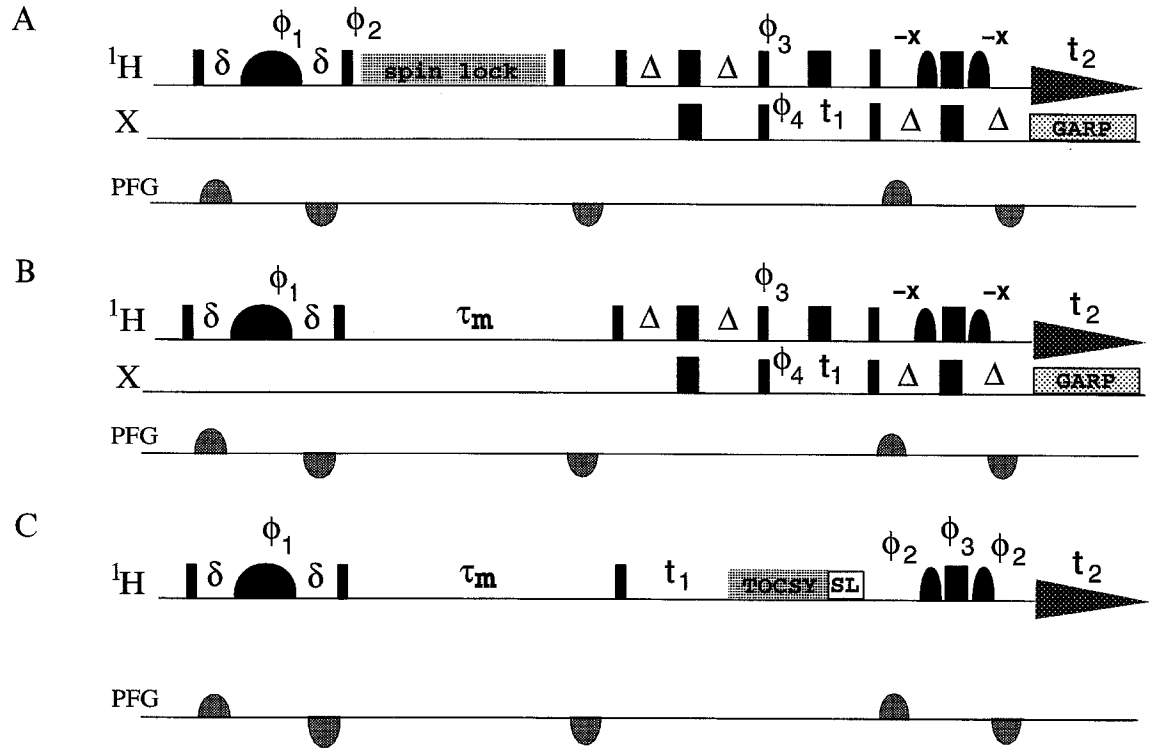

Fig. 1. Pulse sequences for the PFG 2D H_2O-selective heteronuclear X (= ^{15}N,^{13}C)-edited (A) ROESY and (B) NOESY; and for (C) homonuclear NOESY-TOCSY experiments. The H_2O-selective 180° Gaussian pulse located between the first two PFGs has a typical length of 50 ms in order to achieve sufficient selectivity. The narrow and broad bars represent 90° and 180° pulses, respectively. The two H_2O-selective 90° Gaussian pulses located between the last two PFGs have lengths of 1.4 ms. The value δ is equal to the length of the PFG plus 100 μs of gradient recovery, and Δ is equal to 1/(4J) where J is the one-bond heteronuclear coupling constant. The spin-lock in (A) is achieved with a continuous pulse of reduced power and phase y (Bothner-By et al., 1984; Bax and Davis, 1985). The TOCSY step in (C) comprises a DIPSI-2 (Shaka et al., 1988) or a WALTZ-16 (Shaka et al., 1983) composite pulse sequence. X-decoupling during acquisition (A,B) is performed with a GARP (Shaka et al., 1985) composite pulse sequence. Phases in (A,B) are: $\phi_1 = 2(x),2(-y),2(-x),2(y)$; $\phi_2 = (y)$; $\phi_3 = (y)$; $\phi_4 = (x,-x)$; and $\phi_{rec} = (x,-x,-x,x)$. EXORCYCLE (Bodenhausen et al., 1977) phase cycling was used for the composite pulse in (C). The phases in (C) are: $\phi_1 = 4(x),4(-y),4(-x),4(y)$; $\phi_2 = (-x,y,x,-y)$; $\phi_3 = (x,-y,-x,y)$; and $\phi_{rec} = 2(x,-x),2(-x,x)$. The phase of the spin-lock pulse is y. All other pulses have phase x, unless indicated otherwise. The length of the five sine-shaped PFGs was 1.15 ms. The third PFG destroys magnetization not aligned along the z-axis. The other four PFGs are used for coherence selection.

all the magnetization that is not affected by the selective pulse. The magnetization of H_2O is defocused immediately after the first 90° hard pulse and it remains defocused throughout the entire pulse sequence. This avoids problems arising from radiation damping. During the spin-lock period depicted in Fig. 1A and the mixing times in Figs. 1B and C, the magnetization of H_2O is partially transferred via chemical exchange or via dipolar cross-relaxation to some protons of the protein. The magnetization of these spins is then transferred by an INEPT sequence (Figs. 1A,B) (Morris and Freeman, 1979) to the directly coupled heterospin and labelled in t_1 with the heterospin frequency. The reverse INEPT sequence, following the evolution period, deserves some explanation. The ^{1}H 180° pulse in the original sequence is replaced by a sandwich pulse sequence comprising two H_2O-selective $(90°)_{-x}$ pulses and a $(180°)_x$ hard pulse (Piotto et al., 1992). The function of the last two PFGs, which are of alternate sign and symmetrically displaced from the composite pulse, is to refocus all the magnetization originating from H_2O that has been transferred during the spin-lock or the mixing times to other spins via chemical exchange or relaxation processes and inverted by the ^{1}H composite pulse sequence (Dalvit, 1995). Consequently, the H_2O magnetization which (through the composite sequence) experiences a 0° pulse will not be observable. The sum of the absolute strengths of the last two gradients has to be equal to the sum of the first two PFGs to achieve refocusing of the desired coherence. Furthermore, the last two PFGs must differ in strength in order to avoid rephasing of the unwanted magnetization which was refocused by the second PFG in the pulse sequence. This method selects in each scan only the desired coherence (Stonehouse et al., 1994; Dalvit and Bovermann, 1995), thus avoiding all problems inherent to subtraction techniques. A similar scheme can be designed for unlabelled proteins, as shown in Fig. 1C. For these experiments we propose the acronym PHOGSY (*P*rotein *H*ydration *O*bserved by *G*radient *S*pectroscop*Y*).

The protein FKBP-12 was used to test the new experi-

ments. FKBP-12 is the FK-506-binding protein; it consists of 107 amino acids (MW = 11 700). All experiments were recorded at 295 K on a Bruker DMX-500 spectrometer equipped with B_0 gradient hardware. A Bruker 10 A amplifier unit was used for the gradient strength. The performance of the new experiments is demonstrated in Fig. 2, showing the H_2O-selective ^{15}N-edited ROESY (Figs. 2A,B) and NOESY (Fig. 2C) spectra, acquired on a 2 mM uniformly ^{15}N-labelled sample of FKBP-12 with the pulse schemes of Figs. 1A and B, respectively. In the ROESY spectra, positive cross peaks correspond to NH which are in chemical exchange with H_2O, while negative cross peaks arise from cross-relaxation between NHs and H_2O or $C^{\alpha}H$ protons resonating close to the H_2O chemical shift. In addition, negative ROEs are seen for NHs which are spatially close to fast exchanging protons. In the NOESY spectra all cross peaks are positive. In total, five residues can be identified that are proximal to tightly bound H_2O molecules. The criteria for identification of a bound water molecule close to an observed NH were that (i) neither a fast exchangeable proton nor (ii) any $C^{\alpha}H$ proton resonating at the H_2O chemical shift is close (<4 Å), according to the crystal structure of FKBP complexed with ascomycin (Kallen, J., personal communication). Three molecules of bound H_2O are detected. One molecule is close to the NHs of Gly^{86}, His^{94} and Ala^{95}, another one is close to the NH of Tyr^{82} and the third is in proximity to the NH of Glu^{54}. The same three H_2O molecules have been detected previously using 3D ^{15}N-edited ROESY experiments (Xu et al., 1993). The H_2O-selective 2D ^{15}N-edited ROESY spectrum of Figs. 2A and B required only 10 h measuring time, which compares favourably to the 3.3 days needed to record the 3D ^{15}N-edited ROESY spectrum (Xu et al., 1993).

The PFG strength should be kept small to reduce problems of signal attenuation arising from H_2O spatial diffusion which occurs between the first and last PFG. However, by monitoring the signal intensity as a function of the strength of the PFGs, it would be possible to extract the values of the exchange lifetimes of the amide protons and of the bulk water–bound water in a similar way as described previously (Van Zijl and Moonen, 1990; Moonen et al., 1992; Kriwacki et al., 1993). Furthermore, this method allows differentiation between ROE/NOEs stemming from H_2O and from a $C^{\alpha}H$ resonance degenerate with the H_2O signal. This is experimentally demonstrated in Fig. 3. Cross sections taken at the ^{15}N frequencies of three selected residues from two 2D H_2O-selective ^{15}N-edited NOESY experiments recorded with different PFG strength are shown. The exchange peak of Gly^{89} and

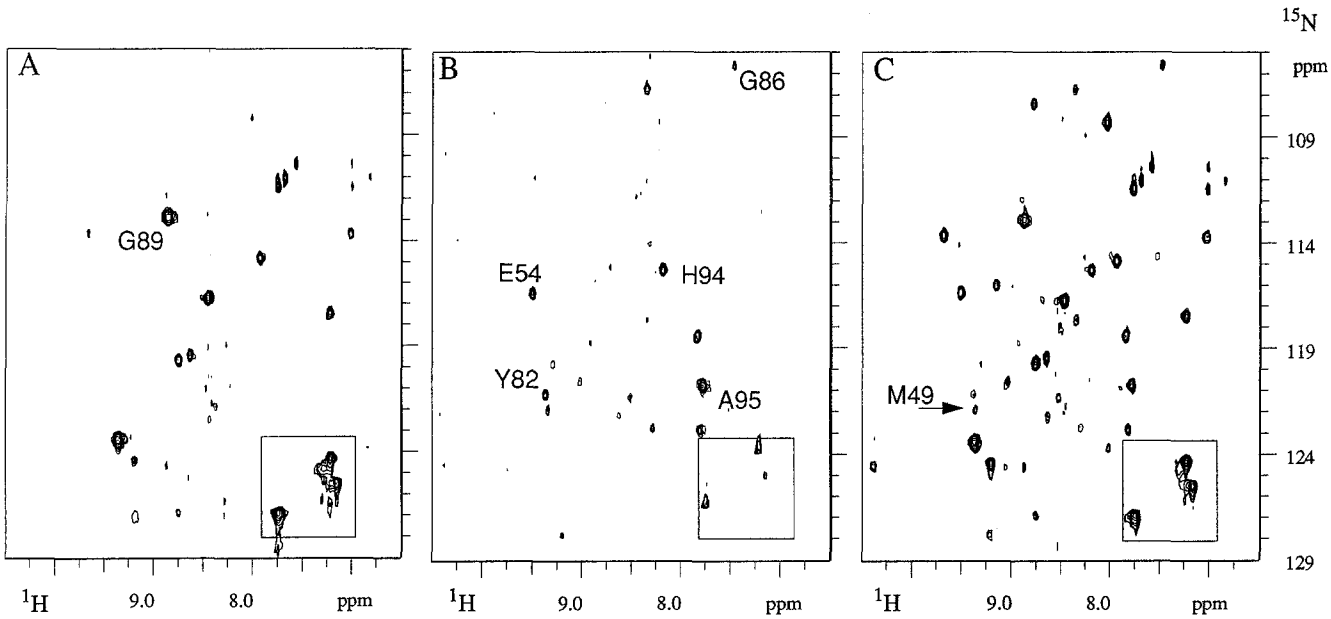

Fig 2. Two-dimensional ^{15}N-edited (A,B) ROESY and (C) NOESY spectra, recorded with the pulse sequences of Figs. 1A and B, respectively. Positive cross peaks (exchange) and negative cross peaks (ROE) are shown in (A) and (B), respectively. Spectra have been acquired at 295 K on a 2 mM sample of complexed FKBP in H_2O (5% D_2O), pH 6.5. Resonance assignments of the complex are based on published data (Rosen et al., 1991) and were confirmed using standard 2D and 3D NMR methods. Residues identified as being in close contact with bound H_2O are indicated. In addition, the cross peak of Gly^{89} due to chemical exchange and of Met^{49} due to intramolecular ROE with the $C^{\alpha}H$ Phe^{48} are labelled. The exchange cross peaks inside the small boxes are folded signals of the side-chain NHs of lysines and arginines. (A,B) 128 scans and (C) 64 scans were recorded for each of the 128 t_1 increments. The repetition time was 2.17 s, amounting to a total measuring time of 10 h for (A,B) and 5 h for (C). The spectral widths in ω_1 and ω_2 were 1368 and 6009 Hz, respectively. The data were multiplied with a cosine window function (Demarco and Wüthrich, 1976) in both dimensions prior to Fourier transformation. The lengths of the H_2O-selective 180° and 90° Gaussian pulses were 50 and 1.4 ms, respectively. The spin-lock (A,B) and mixing periods (C) were 40 and 100 ms long, respectively. The five sine-shaped PFGs of length 1.15 ms had strengths of –6.4, 6.4, –2.3, 5.3 and –7.5 G/cm, respectively.

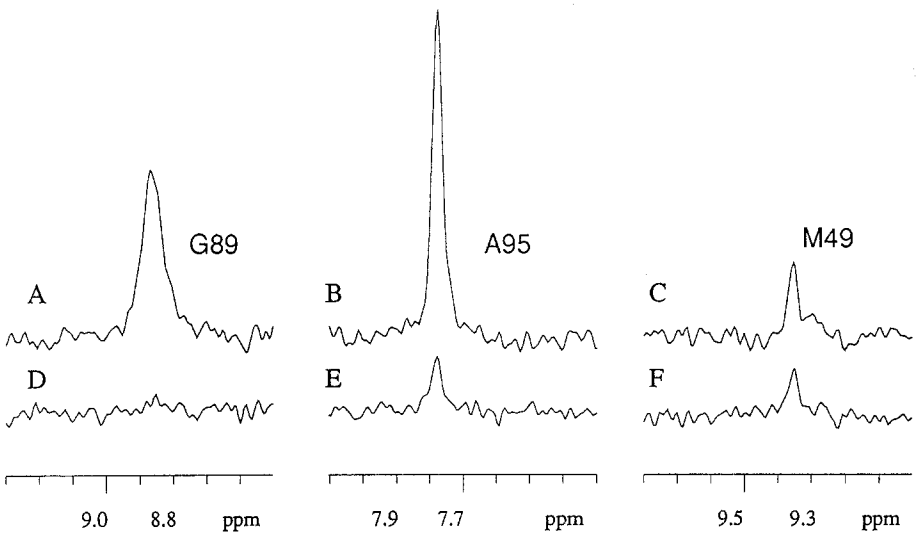

Fig. 3. Cross sections for three NHs, taken from two 2D H_2O-selective ^{15}N-edited NOESY spectra recorded with different PFG strengths. The cross peaks of Gly^{89} (A,D) due to exchange with bulk H_2O, of Ala^{95} (B,E) due to NOE with bound H_2O and of Met^{49} (C,F) due to NOE with $C^{\alpha}H$ Phe^{48}, which is almost degenerate with the H_2O signal, are shown. The spectra were recorded with the pulse sequence of Fig. 1B and 128 scans were acquired for each of the 128 t_1 increments. The strengths of the five 1.152 ms sine-shaped PFGs were –6.4, 6.4, –2.3, 5.3 and –7.5 G/cm in (A–C) and –28.6, 28.6, –2.3, 25.4 and –31.8 G/cm in (D–F). Other parameters are the same as in Fig. 2.

the NOE peak of Ala^{95}, arising from bound H_2O clearly visible in the experiment with weak PFG (Figs. 3A,B), are completely absent (Fig. 3D) or strongly attenuated (Fig. 3E) in the spectra recorded with stronger PFGs due to the diffusion of bulk H_2O. In contrast, the intramolecular NOE to Met^{49} (Fig. 3C) which originates from $C^{\alpha}H$ Phe^{48} resonating at the H_2O frequency is only slightly attenuated in Fig. 3F because of the slow protein diffusion rate.

The new PHOGSY experiments reported here provide a rapid and sensitive means for the observation of the hydration of proteins in solution. In addition, they should allow measurements of the residence time of H_2O bound to proteins.

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