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# Protein hydration studied with homonuclear 3D $^1$H NMR experiments

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## SUMMARY

Homonuclear 3D $^1$H NOESY-TOCSY and 3D $^1$H ROESY-TOCSY experiments were used to resolve and assign nuclear Overhauser effect (NOE) cross peaks between the water signal and individual polypeptide proton resonances in $H_2O$ solutions of the basic pancreatic trypsin inhibitor. Combined with a novel, robust water-suppression technique, positive and negative intermolecular NOEs were detected at 4°C. The observation of positive NOEs between water protons and protein protons enables more precise estimates of the very short residence times of the water molecules in the hydration sites on the protein surface.

With the use of two-dimensional (2D) homonuclear $^1$H NOESY and $^1$H ROESY and the corresponding three-dimensional (3D) $^{15}$N-correlated experiments with the uniformly $^{15}$N-labeled protein, a small number of hydration water molecules bound to the protein basic pancreatic trypsin inhibitor (BPTI) were recently identified (Otting and Wüthrich, 1989, 1990; Wüthrich and Otting, 1991). Special interest lies in the fact that the observed four water molecules are located in the interior of the protein and that none, or at most very few, of the over 60 surface hydration sites reported in crystal structures of the same protein (Deisenhofer and Steigemann, 1975; Wlodawer et al., 1984, 1987) were observed with these NMR experiments. Similar results were reported for interleukin 1β (Clore et al., 1990). The present paper describes experimental schemes for homonuclear $^1$H 3D NOESY-TOCSY (Vuister et al., 1988) and 3D ROESY-TOCSY for the study of protein hydration, which include a novel, robust water-suppression scheme. These experiments enabled the identification of numerous previously unassigned NOEs between water protons and

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protons of BPTI, including NOEs with water molecules bound with very short residence times to hydration sites on the protein surface.

The practical importance of the experiments described here is related to the facts that both positive and negative NOEs with water protons may be present, and that the corresponding NOESY cross peaks may be overlapped in the 2D spectra. Because of rapid exchange on the chemical shift time-scale, all NOEs with the water, including the aforementioned interior water molecules, are observed at the chemical shift of the bulk water (Otting et al., 1991). Therefore, they are all observed on a single frequency axis in the 2D spectra (Otting and Wüthrich, 1989), which may contain additional signals manifesting chemical exchange or NOEs with rapidly exchanging amino, hydroxyl and carboxyl protons of the polypeptide chain. Heteronuclear 3D $^{15}$N-correlated [$^1$H,$^1$H]-NOESY and 3D $^{15}$N-correlated [$^1$H,$^1$H]-ROESY experiments with uniformly $^{15}$N-labeled proteins (Clore et al., 1990; Otting and Wüthrich, 1990; Wüthrich and Otting, 1991) resulted in improved resolution of the cross peaks with $^{15}$N-bound proton resonances. A similar strategy for studies of NOEs between carbon-bound polypeptide protons and water protons will probably be more limited, because of the expense involved in the preparation of concentrated samples of uniformly $^{13}$C-labeled protein for the 3D $^{13}$C-correlated experiments. The presently proposed use of homonuclear 3D experiments with unlabeled proteins is a more economic alternative for studies with small and medium-size molecules.

In experiments used for the observation of intermolecular NOEs between water protons and polypeptide protons, no solvent suppression by preirradiation of the water signal can be employed (Otting and Wüthrich, 1989). Instead, the water magnetization must be eliminated *after* the mixing period during which the NOE transfer of magnetization from the water protons to the protein protons has taken place. For observation of hydration water molecules the NOEs must be recorded with short mixing times and at low temperature (see below). Since, under these conditions, radiation damping does not bring the water magnetization completely back to the z-axis at the end of the mixing period, application of most of the commonly used water-suppression schemes (Guéron et al., 1991) would give unsatisfactory results. An acceptable degree of water suppression in experiments with short mixing times and at temperatures around 0°C can, however, be achieved using the spatial radiofrequency inhomogeneity of a spin-lock pulse of about 2 ms duration, which effectively defocuses any magnetization not aligned along the spin-lock axis (Bax and Davis, 1985; Otting and Wüthrich, 1988). Figure 1 shows the experimental schemes of homonuclear $^1$H 2D NOESY, 2D ROESY, 3D NOESY-TOCSY and 3D ROESY-TOCSY with solvent suppression through spin-lock pulses. The carrier frequency is set at the water resonance. In the NOESY experiments, the water suppression is achieved with the use of two short spin-lock pulses, $SL_{\varphi 4}$ and $SL_{\varphi 5}$, which are phase-shifted by 90° relative to each other (Fig. 1, A and C), similar to the water-suppression scheme used by Messerle et al. (1989) with 3D $^{15}$N-correlated [$^1$H,$^1$H]-NOESY. The first spin-lock pulse, $SL_{\varphi 4}$, is applied immediately following the third NOESY($\frac{\pi}{2}$) pulse and selects in-phase magnetization along the y-axis, which then evolves during the delay $\tau$ under the chemical shift Hamiltonian. The following spin-lock pulse, $SL_{\varphi 5}$, selects the in-phase magnetization that has precessed to the x-axis during the delay $\tau$. With the carrier frequency at the water resonance, the water signal is strongly suppressed by the spatial radiofrequency inhomogeneity of the second spin-lock pulse. The spectral excitation profile in this experiment is uniform in all dimensions except along the $\omega_2$ frequency axis, where it depends on the offset $\Omega$ from the carrier frequency with $\sin\Omega\tau$. The first spin-lock pulse is important in NOESY experi-

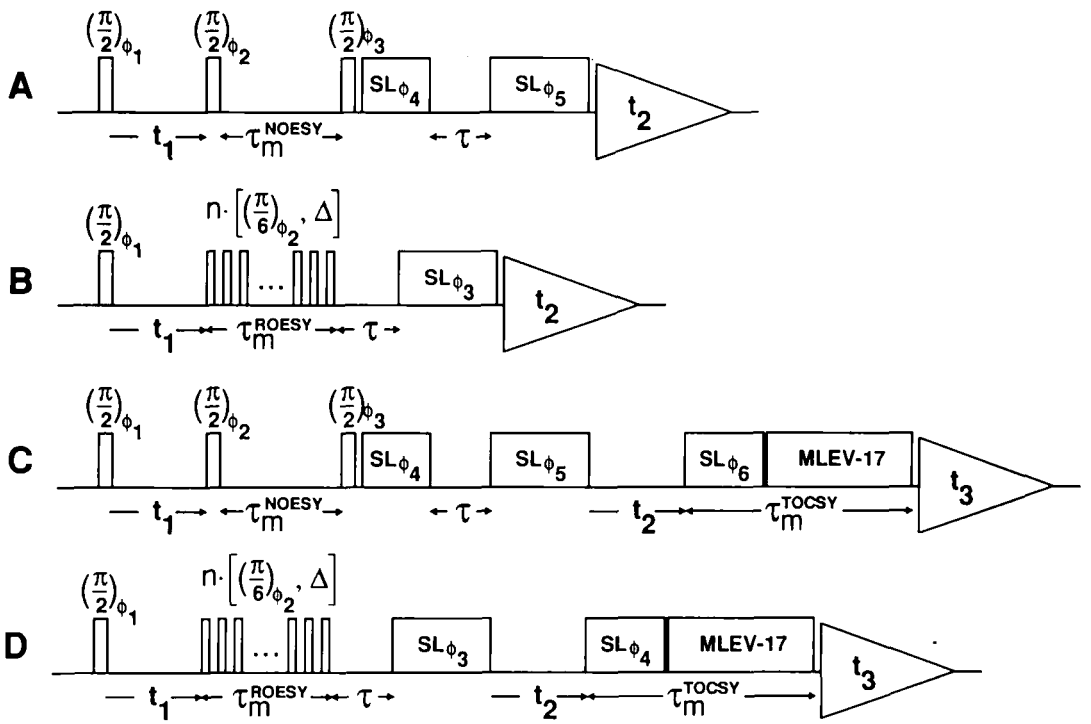


Fig. 1. Experimental schemes for investigations of protein hydration in $H_2O$ solution. Phase cycles: (A) 2D NOESY: $\varphi_1 = 2[8(x),8(-x)]$; $\varphi_2 = [16(x,-x)$; $\varphi_3 = 8[(x,x,-x,-x)$; $\varphi_4 = 4[4(y),4(-y)]$; $\varphi_5 = 16(x),16(-x)$; receiver $= 2[2(x,-x,-x,x),2(-x,x,x,-x)]$. (B) 2D ROESY: $\varphi_1 = 2[2(x,y),4(-x,-y),2(x,y)]$; $\varphi_2 = 2[2(x,y,-x,-y),2(-x,-y,x,y)]$; $\varphi_3 = 4(y,-x),8(-y,x),4(y,-x)$; receiver $= 2[2(x,y), 4(-x,-y), 2(x,y)]$. (C) 3D NOESY-TOCSY: $\varphi_1 = 4(x),4(-x)$; $\varphi_2 = 4(x,-x)$; $\varphi_3 = 8(x)$; $\varphi_4 = 8(y)$; $\varphi_5 = 2(x,x,-x,-x)$; $\varphi_6 = 8(x)$; receiver $= 2(x,-x),2(-x,x)$; this phase cycle may be extended to 16 steps by additional phase alternation of $\varphi_6$ and of the phase of the MLEV-17 (Bax and Davis, 1985) mixing sequence. (D) 3D ROESY-TOCSY: $\varphi_1 = 4(x),4(-x)$; $\varphi_2 = 4(x,-x)$; $\varphi_3 = 8(y)$; $\varphi_4 = 2[2(x),(2(-x)]$; receiver $= 4(x),4(-x)$; the phase cycles of the schemes (A), (C) and (D) may be extended two-fold by a two-step CYCLOPS (Hoult, 1975; Otting, 1990). For quadrature detection in $\omega_1$, time-proportional phase incrementation (TPPI) (Marion and Wüthrich, 1983) is applied to the phase $\varphi_1$ in all four schemes (A)–(D). In schemes (C) and (D), TPPI is further applied independently to $\varphi_1$–$\varphi_5$, and $\varphi_1$–$\varphi_3$, respectively, for quadrature detection in $\omega_2$. All pulses are applied with the same power amplitude. In the ROESY mixing schemes (B) and (D), a series of n $(\frac{\pi}{6})$ pulses separated by delays, $\Delta$, is used (Kessler et al., 1987). Typical spin-lock durations are: in (A) and (C), $SL_{\varphi 4} = 0.5$ ms, $SL_{\varphi 5} = 2$ ms, $SL_{\varphi 6} = 1$ ms; in (B) and (D), $SL_{\varphi 3} = 2$ ms, $SL_{\varphi 4} = 1$ ms. The spectral excitation profile in $\omega_2$ depends on the delay $\tau$ with $\sin\Omega\tau$, where $\Omega$ is the angular frequency relative to the carrier.

ments with short mixing times, where it randomizes the water magnetization that is not aligned along the z-axis at the end of the mixing period. In the ROESY schemes (Fig. 1, B and D) the mixing sequence of n $(\frac{\pi}{6})$ pulses separated by delays, $\Delta$, (Kessler et al., 1987) provides a comparable spin-lock effect to that of the spin-lock $SL_{\varphi 4}$ of the NOESY schemes. Sufficient suppression of the water signal is therefore achieved with a single additional spin-lock pulse, $SL_{\varphi 3}$, which is phase-shifted by 90° relative to the phase of the mixing sequence.

Figure 2 shows the spectral region ($\omega_2 = 0.5-4.7$ ppm, $\omega_3 = 0-4.7$ ppm) of 2D cross-sections taken through a $^1H$ 3D NOESY-TOCSY spectrum and a $^1H$ 3D ROESY-TOCSY spectrum of

BPTI, respectively, at the $\omega_1$ frequency of the water resonance, $\delta(H_2O) = 5.00$ ppm. The spectral excitation in the $\omega_2$ dimension corresponds to a sin[0.66($\delta$-5.0)] dependence, where $\delta$ is the chemical shift in ppm. The peaks on the diagonal come from the transfer of magnetization from the water line to the protein resonances during the NOESY or ROESY mixing, respectively. The fact that $\omega_2$ equals $\omega_3$ shows that no magnetization transfer occurred during the TOCSY mixing period. In Fig. 2 the diagonals thus correspond to the one-dimensional cross-sections along $\omega_2$ through the water $\omega_1$ resonance position in the corresponding 2D NOESY and 2D ROESY spectra (Otting and Wüthrich, 1989). However, because the digital resolution in the $\omega_1$ dimension of the 3D experiments is only about 0.1 ppm per point, the water signal in the 3D NMR spectra is not as well resolved from the nearby $C^{\alpha}H$ resonances of the protein as in the 2D spectra. In particular, the $\alpha$-proton resonance of $Cys^{38}$ overlaps with the water signal, and the $\alpha$-proton resonances of $Tyr^{10}$, $Gln^{31}$, $Tyr^{35}$, $Asn^{43}$ and $Asn^{44}$ are within 0.15 ppm of the water signal. To distinguish NOEs with the water from NOEs with one of these $\alpha$-protons, a second experiment could be recorded at a different temperature, where the water-resonance position would be sufficiently different to exclude overlap with the same $\alpha$-protons. In the present case, high-resolution 2D NOESY experiments showed that in the spectral region of Fig. 2, strong NOEs with the $\alpha$-proton resonances near the water line are exclusively intra-residual connectivities with the $\beta$-protons.

It is an important advantage of the homonuclear 3D NMR experiments over the corresponding 2D spectra that the assignment of the NOE peaks on the diagonal is not based solely on comparison with the chemical shift of a single protein proton resonance (Otting and Wüthrich, 1989), but is further supported by the off-diagonal peaks. These arise because the TOCSY mixing (Fig. 1, C and D) transfers magnetization precessing during $t_2$ to scalar coupled protons, which are then detected during $t_3$. The assignment of the diagonal NOE cross peak can then be based on comparison with some or all chemical shifts in the spin system to which the involved polypeptide proton belongs. In crowded regions of the diagonal, two or several overlapped NOE peaks may thus be individually assigned without ambiguity. In the experiments of Fig. 2, $\tau_m^{TOCSY}$ was 27 ms, which provides a particularly efficient relay of magnetization between geminal protons (Braunschweiler and Ernst, 1983; Rance, 1989). As a result, the NOE peaks on the diagonal may be weaker than the TOCSY relay peaks derived from them, which have the same $\omega_2$ frequency but different $\omega_3$ chemical shift. In the 3D NOESY-TOCSY spectrum, the positive and negative signs of the diagonal peaks are carried over to the cross peaks, which thus reflect the sign of the water-protein NOEs (remember that positive NOESY cross peaks correspond to negative NOEs, and vice versa). A first observation to be made from the 3D NOESY-TOCSY spectrum of Fig. 2A is that, throughout, the positive peaks are much stronger than the negative peaks. The positive peaks correspond to the signals that were previously identified in 2D NMR spectra and assigned to NOEs with four water molecules located in the interior of the BPTI structure (denoted as W111, W112, W113 and W122 in the BPTI crystal structures; Deisenhofer and Steigemann, 1975; Wlodawer et al., 1984, 1987) and to exchange cross peaks or NOE cross peaks with rapidly exchanging protons of the polypeptide chain (Otting and Wüthrich, 1989). The weak negative NOESY cross peaks escaped detection in the earlier studies, and their identification and assignment is the principal new result achieved with the improved experiments of Fig. 1. These NOEs are between water protons and solvent-accessible protons on the protein surface. The negative sign of the cross peaks shows that the vector connecting the interacting protons of the protein and the water undergoes spatial rearrangements in the fast motional regime, i.e., $\tau_c << 1$ ns. It is imperative that short

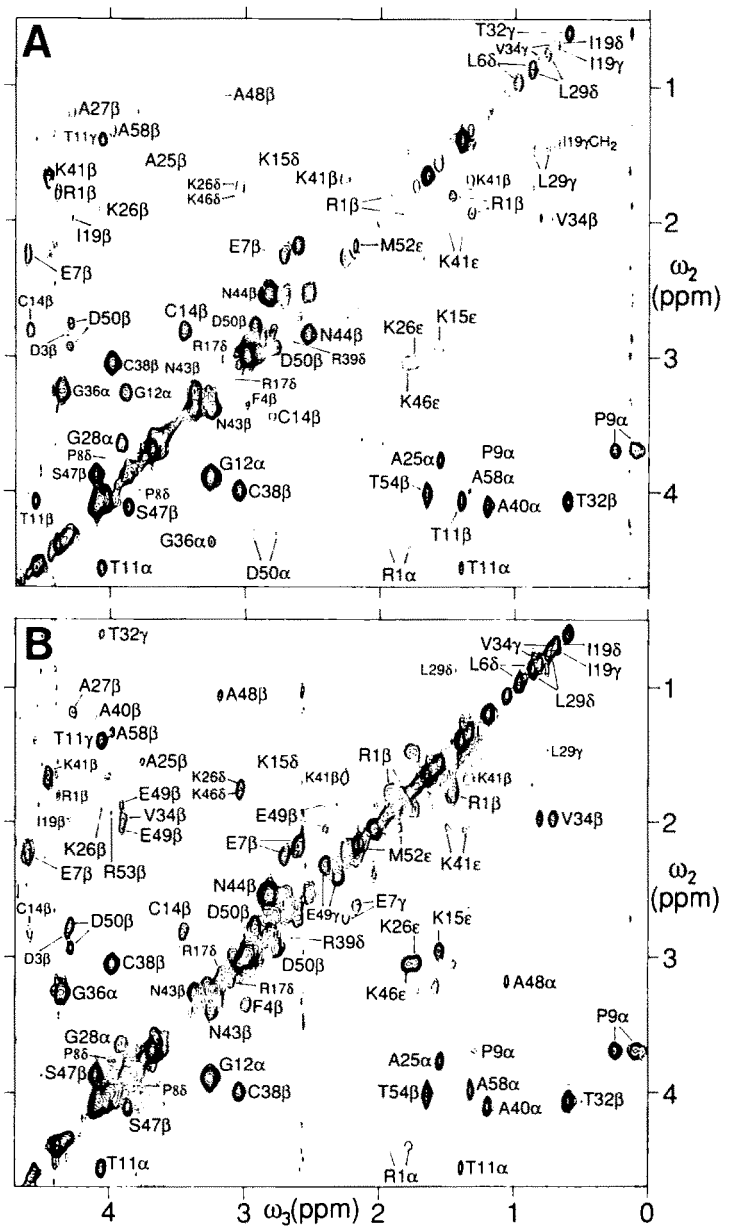


Fig. 2. 2D cross-sections taken at the $\omega_1$ frequency of the water signal through a homonuclear $^1$H 3D NOESY-TOCSY spectrum (A) and a $^1$H 3D ROESY-TOCSY spectrum (B) recorded with a 20 mM solution of BPTI in 90%$H_2O$/10%$D_2O$,pH = 3.5 at 4°C on a Bruker AM500 spectrometer. The experimental schemes of Fig. 1, C and D were used. ($\tau_m^{NOESY}$ = 50 ms, $\tau_m^{ROESY}$ = 25 ms, $\tau_m^{TOCSY}$ = 27 ms; $SL_{\varphi 4}$(NOESY) = 0.5 ms, $SL_{\varphi 5}$ = 3ms, $SL_{\varphi 6}$ = 1 ms, $SL_{\varphi 3}$ (ROESY) = 3 ms, $SL_{\varphi 4}$(ROESY) = 1 ms, $t_{1max}$ = 9.6 ms, $t_{2max}$ = 21.5 ms, $t_{3max}$ = 86 ms, time domain data size 100 × 256 × 1024 points, 16 scans per FID, total experimental time about one week). Before Fourier transformation, the data were multiplied with cosine windows, and after Fourier transformation they were baseline-corrected in all three dimensions using polynomials. In (A), negative contour levels are plotted with dashed lines. In (B), only negative levels were plotted. The peaks are identified with the assignment of the proton interacting with the water (or with an α proton near the water resonance; see text).

mixing times $\tau_m^{NOESY}$ (Fig. 1) are used for studies of these surface hydration sites. Otherwise the weak negative NOESY cross peaks tend to be cancelled by spin diffusion, which can relay positive magnetization from the cross peaks with the more stably bound water to other resonances of the protein.

The presence of the weak negative cross peaks in the homonuclear $^1H$ 3D NOESY-TOCSY spectrum was confirmed by the presence of corresponding peaks in a 3D ROESY-TOCSY spectrum (Fig. 2B). All the cross peaks observed in Fig. 2B are negative, since the ROE has the same sign for all correlation times (Bothner-By et al., 1984). To account for the more rapid cross-relaxation during the ROESY mixing period (Farmer et al., 1988), the mixing time, $\tau_m^{ROESY}$, was chosen only half as long as $\tau_m^{NOESY}$ in the 3D NOESY-TOCSY experiment (Fig. 1). Despite the short mixing time used, the occurrence of some intraresidual spin diffusion is indicated by the fact that the strong 3D NOESY cross peaks of Thr[11] $C^{\alpha}H$, Gly[12] $\alpha CH_2$, Gly[36] $\alpha CH_2$ and Asn[44] $\beta CH_2$ are accompanied by the symmetry-related cross peak on the other side of the diagonal (Fig. 2A), whereas these are absent or very weak in the ROESY spectrum (Fig. 2B).

A potential pitfall of the water-suppression scheme used in Fig. 1 lies in the possibility that coherent and incoherent magnetization transfer during the spin-lock pulses could dominate the results obtained. To obtain an estimate for the contributions from the spin-lock pulses, a 2D reference spectrum was recorded with $\tau_m^{NOESY} = 0$, using the experimental scheme of Fig. 1A. In this spectrum the spin-lock pulses produced weak ROESY cross peaks and strong lysyl $\varepsilon CH_2$-$NH_3^+$ TOCSY cross peaks. With regard to the 3D experiments presented in Fig. 2, this control experiment indicates that the spin-lock pulses used for water suppression will, in general, contribute only a small amount of ROESY mixing, which can in practice only result in observable 'spin diffusion' peaks when magnetization is transferred from strong water-protein cross peaks present after $\tau_m^{NOESY}$, or $\tau_m^{ROESY}$. In the experiments with BPTI, the $\varepsilon NH_3^+$ resonances of Lys[41] and Lys[46] give by far the strongest cross peaks with the water, which is due to chemical exchange (Otting and Wüthrich, 1989). During the precession delay, $\tau$, the $\varepsilon NH_3^+$ magnetization precesses in the opposite sense to that of the $\varepsilon CH_2$ spins. The TOCSY effect of the spin-lock pulse following $\tau$ (Fig. 1) therefore transfers magnetization of the opposite sign to $\varepsilon CH_2$. Overall, we conclude that the information contained in the 3D spectra of Fig. 2 is not falsified by the use of the spin-lock pulses for water suppression, except that the 3D cross peaks $H_2O(\omega_1) - \varepsilon CH_2(\omega_2) - \delta CH_2(\omega_3)$ of Lys[41] and Lys[46] may contain intensity from the $H_2O(t_1) - \varepsilon NH_3^+(\tau) - \varepsilon CH_2(t_2)$ pathway in addition to that due to the direct NOE between water and the $\varepsilon CH_2$ group. The importance of this undesired pathway could be assessed more quantitatively by recording additional experiments with different NOE mixing times. Alternatively the water-suppression element $\tau - SL$ could be used only immediately before $t_3$ (Fig. 1, C and D), where the TOCSY effect of the spin lock pulse would be dominated by the preceding TOCSY mixing scheme.

In conclusion, the homonuclear 3D NMR experiments of Fig. 1, C and D, greatly help to assign water-protein cross peaks in $H_2O$ solution. In cases of overlap between positive and negative NOEs with the water, the third dimension is indispensible for the observation of the weaker peaks. The novel water-suppression scheme of Fig. 1 can be used with the short NOESY mixing times required for the observation of weak NOEs in the presence of strong NOEs of opposite sign. Although this water-suppression scheme results in non-uniform spectral excitation along $\omega_2$, and care has to be taken to account for possible magnetization transfer during the spin-lock pulses, its reliability and ease of use make it an attractive element for general use in complex experimental schemes.

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