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Editing of Chemical Exchange-Relayed NOEs in NMR Experiments for the Observation of Protein–Water Interactions

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An experimental approach for the editing of exchange-relayed NOEs in water-selective NOE experiments is presented. The proposed pulse sequence is based on the application during the NOE mixing time of continuous wave irradiation, which saturates resonances of relaying labile protons in slow chemical exchange with water. The technique can efficiently reduce the contributions of exchange-relayed NOE peaks that often crowd the water-selective NOE spectra and hide direct intermolecular NOEs between water and protein protons. The present approach opens new opportunities for the characterization of hydration by NMR, even in the proximity of polar labile groups.

Key Words: **water; hydration; NOE; chemical exchange relay.**

INTRODUCTION AND PROBLEM DEFINITION

Water is ubiquitously present in biological systems and it has a profound influence on the structure and dynamics of biomolecules (*1, 2*). Specifically, water can affect hydrogen bonds, electrostatic screening, and hydrophobic effects (*3*), and hydration water molecules can also be an integral part of protein (*4*) and DNA (*5*) architecture. The measurement of water-solute NOEs is an important tool for the characterization of biomolecular hydration, providing valuable structural and kinetic information on both interior and surface water molecules (*1, 6–9*).

One of the most common artifacts found in water-selective NOE experiments (*9*) arises from two-step magnetization transfer processes in which magnetization is first transferred from water to labile protein protons via chemical exchange and then from these to other protein protons via dipolar cross-relaxation (*1, 7–13*). The rates of proton exchange with water can be significantly higher than those of dipolar cross-relaxation leading to the frequent appearance of intense exchange-relayed NOE peaks in water-selective NOE spectra. The exchange-relayed intramolecular NOEs can obscure direct intermolecular NOEs with water. Furthermore, ROESY experiments do not allow the distinction between exchange-relayed ROEs and direct ROEs with tightly bound water molecules because these two types of magnetization transfer mechanisms result in crosspeaks with the same sign (*14*). The approach currently taken to rule out exchange-relayed contribution to the observed NOEs is based on the three-dimensional structure of the macromolecule and restricts the assignment of direct NOEs with water only to those hydrogen nuclei which are more than 4.5 Å far from any exchangeable proton (*14*). In this way many direct NOEs between water and protein protons in the proximity of labile groups (i.e., hydroxyls of Thr and Ser residues) will remain ambiguous.

ALTERNATIVE SOLUTION—CONCEPTUAL BASIS

In this communication, we describe an alternative method for the editing of exchange-relayed NOEs. The proposed approach is based on experimental observations and does not require the knowledge of the three-dimensional macromolecular structure. Our method exploits the chemical shift difference between water and labile protons that are in slow exchange with the solvent. Such a chemical shift difference is frequently observed for nitrogen-bound exchangeable protons and for hydroxyl protons inaccessible to water (*15–17*). In addition, the exchange of hydroxyl protons can be further slowed down by the use of low temperatures and pH close to approximately 5–6 (*15, 18*).

The resonances of slowly exchanging labile protons can be easily identified in water-selective NOE and ROE experiments as intense peaks preserving the same sign in both spectra. The relay effect via the identified labile proton can then be reduced by selective irradiation of this exchanging proton during the NOE mixing time (*19–21*). The saturation of the irradiated labile proton is also transferred to water and to the other exchangeable protons, but the resultant intensity reduction of water is negligible given the concentration difference between the solute (usually in the mM range) and water itself (approximately 55 M).

ALTERNATIVE SOLUTION—PULSE SEQUENCE (GRADIENTS AND PHASES)

The ideas outlined above are implemented in the water-selective NOE pulse sequence shown in Fig. 1a. Gradients G_1,

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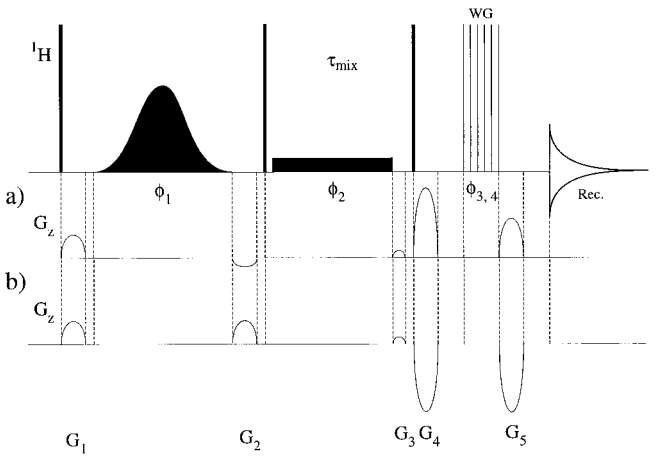

FIG. 1. Pulse sequence for the slow chemical exchange-relay edited water-selective NOE experiment. Thick vertical bars indicate 90° hard pulses. Between the first two 90° pulses water is selectively inverted by a 50-ms gaussian pulse (*22a*). During the mixing period continuous irradiation is applied on a selected resonance corresponding to atoms or group of atoms in slow chemical exchange with water. Water is suppressed before acquisition using the Watergate block implemented with the 3–9–19 pulse train (*30*). (a) Gradients G_1, G_2, G_4, and G_5 are used for coherence transfer pathway selection (*22a*). Gradient G_3 dephases unwanted transverse magnetization at the end of the mixing time. All gradients have a sinus amplitude profile. G_1, G_2, G_4, and G_5 last 2.4 ms, while G_3 0.4 ms. The strengths of G_1–G_5 are 4.27, −2.51, 1.57, 12.55, and 5.77 G/cm, respectively, fulfilling the condition $|G_1| + |G_2| + |G_5| = |G_4|$. Every gradient is followed by a delay for gradient recovery; for G_1–G_3 this is 0.4 ms, for G_4 and G_5 it is 0.8 ms. (b) Same as pulse sequence in Fig. 1a but using only phase cycling to select the desired coherences. The gradient strengths for G_1–G_5 are therefore changed to 4.27, 4.27, 1.57, −5.77, and −5.77 G/cm, respectively. For both sequences the phases are: $\Phi_1 = x, y, -x, -y$; $\Phi_2 = y$; $\Phi_3 = x$; $\Phi_4 = -x$; $\Phi_{Rec} = x, -x$; all other phases are x.

G_2, G_4, and G_5 select the coherence transfer pathway corresponding to water magnetization transferred to the protein protons during the mixing time through dipolar cross-relaxation and chemical exchange (*22a*). During the mixing time the CW pulse is applied with phase y in order to avoid the creation of minor water magnetization components along the x axis, which can result in baseline distortions if not completely dephased by gradient G_3 (Fig. 1a).

ALTERNATIVE SOLUTION—PULSE SEQUENCE (THE CW PULSE STRENGTH)

The CW pulse must be strong enough to ensure that the water magnetization which is continuously transferred to the irradiated labile proton is kept to a constant saturation level. Specifically, effective suppression of exchange-relay artifacts is obtained when the CW pulse inverts the labile proton magnetization at a rate faster than that of chemical exchange. This condition defines a lower limit on the strength of the CW field. In the limit of slow chemical exchange processes in the NOE mixing time scale, a single selective 180° pulse could be used in place of the CW irradiation (*22b*). An upper limit to the strength of the CW irradiation is imposed by the need to avoid significant off-resonance effects on water or other protein protons which are involved in direct NOEs with water molecules. Saturation of multiple overlapped or partially overlapped signals by the CW irradiation is generally not a concern and it can even be advantageous because relay through the several saturated protons may be suppressed in a single experiment.

VALIDATION OF THE PROPOSED METHOD

The proposed pulse sequence has been tested on HEW lysozyme (HEWL) for which the hydration has been previously characterized by NMR (*23, 24*) and for which well-refined X-ray determined structures are available (*25–26*). Figure 2a shows a 1D water-selective NOE experiment of HEWL acquired at pH 4.1 and 36°C using the sequence of Fig. 1a without CW irradiation during the mixing time. This experiment serves as reference and contains both direct NOEs with water and exchange-relayed NOEs, as shown for the Thr methyl region in the blown-up Fig. 3a (see dots and filled squares). The exchange-relayed NOEs can be reduced by irradiating the relaying Thr OH protons which are known to resonate around 5.4–6.2 ppm if in slow exchange with water (*18, 23*). Specifically, one of the exchange peaks of Fig. 2a resonates at 5.93 ppm (see asterisk 1). This resonance contains contributions from the threonine hydroxyl protons T40 OH and T118 OH, as indicated by 2D-e-PHOGSY-NOE-TOCSY and 2D-e-PHOGSY-NOE-NOESY experiments (*24*). A 1D water-selective NOE spectrum was therefore acquired using the pulse sequence of Fig. 1a with an off-resonance irradiation at 5.93 ppm (Fig. 2b). The strength of the continuous pulse used for the irradiation was 213 Hz, allowing almost complete saturation of the irradiated resonance at 5.93 ppm (Fig. 2b). The effect of the CW pulse on the exchange-relayed NOEs of the seven threonine residues present in HEWL can be seen in Fig. 3b, which shows an expanded region of the spectrum of Fig. 2b. The NOE exchange-relayed to T118 $C_\gamma H_3$ at 0.97 ppm is efficiently quenched and so is the NOE resulting from the two-step transfer $H_2O \rightarrow$ T118 OH $\rightarrow$ V120 $C_{\gamma 1}H_3$, which resonates at 1.14 ppm (see arrows in Fig. 3b). These results indicate that the continuous wave pulse at 5.93 ppm during the mixing time edits the NOEs exchange-relayed by T118 OH. In addition, the peak at 1.65 ppm, which contains contributions from the NOE exchange-relayed to T40 $C_\gamma H_3$, is reduced by 36% but is not completely suppressed. Finally, in cases of fast exchange between labile protein protons and water, the proposed technique cannot efficiently suppress exchange-relayed NOEs (i.e., T47 at 1.37 ppm and T51 at 0.34 ppm in Figs. 3a and 3b) and therefore in these circumstances a structure-based approach is still the only valuable alternative.

The observations on T40 and T118 described above are fully consistent with the crystal structure of HEWL. The hydroxyls of both T40 and T118 are involved in hydrogen bonding interactions with the main chain (*26*) explaining the observed

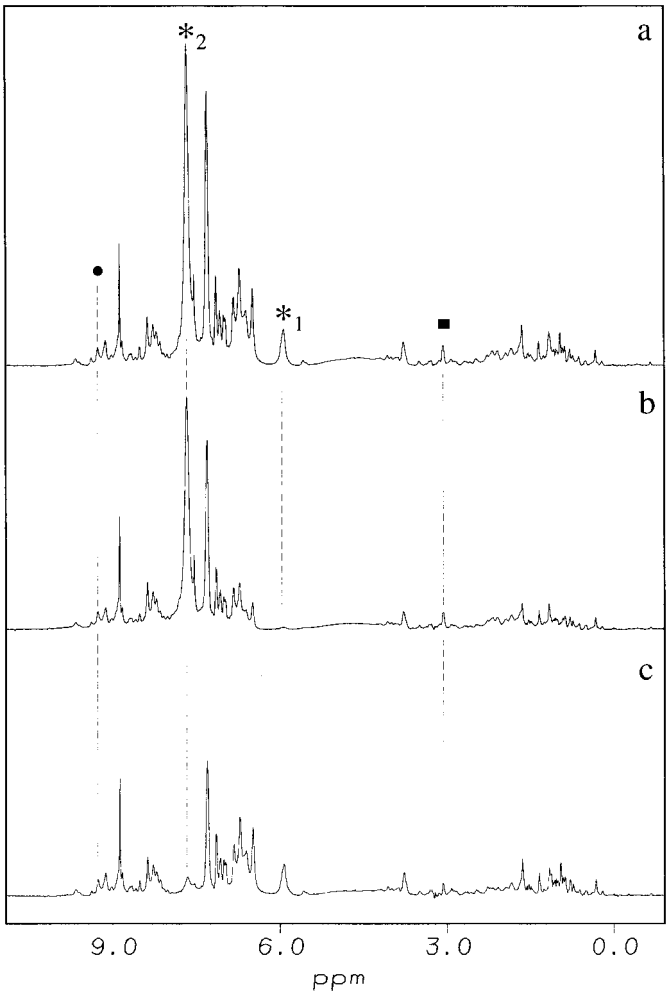

FIG. 2. (a) Reference 1D water-selective NOE experiment of HEWL in the same experimental conditions as in Ref. (*23*) but pH 4.1. The spectrum was acquired on a Bruker AMX500 spectrometer using the sequence of Fig. 1a without the continuous wave pulse during the 50-ms mixing time (*22a*). The number of scans was 17K in order to observe also weak NOEs with water molecules. The relaxation delay after acquisition was 2 s. The residual water peak was removed through convolution filtering. Asterisks (*) indicate representative resonances for which the chemical exchange with water prevails over dipolar cross-relaxation, as indicated by the conserved positive sign in the corresponding ROESY experiment (*23*). The dot (•) indicates a representative direct NOE between water and a protein proton, I55 NH (*23*). The filled square (■) denotes a representative chemical exchange-relayed NOE. A more detailed picture of the NOEs in the aliphatic region can be found in the expansions shown in Fig. 3. (b) 1D water-selective NOE experiment of HEWL acquired and processed as spectrum (a) but with a 213-Hz continuous pulse applied during the mixing time on the chemical exchange peak (*1) at 5.93 ppm. (c) 1D water-selective NOE experiment of HEWL acquired and processed as spectrum (a) but with a 155-Hz continuous pulse applied during the mixing time on the chemical exchange peak (*2) at 7.67 ppm.

slow exchange with water (Fig. 2a, asterisk 1). The crystal structure of HEWL also reveals an important difference between these two Thr residues: T118 $C_\gamma H_3$ lies on the protein surface (*25*) and therefore, as observed (Fig. 3b), the exchange-relayed NOE is the major contribution to the T118 $C_\gamma H_3$ resonance; T40 instead is buried within the core of HEWL (*27*) and faces a large protein cavity containing three water molecules (*25*). Specifically, the T40 $C_\gamma H_3$ pseudoatom in the crystal structure of HEWL (*25*) is 4.2 and 3.7 Å far from the buried water molecules W180 and W131, respectively. The presence of these two water molecules in solution is independently confirmed by the direct intermolecular NOEs between the solvent and the protein protons I55 NH at 9.27 ppm (Figs. 2a and 2b), I55 $C_\gamma H_3$ at 0.91 ppm and I88 $C_\gamma H_3$ at 0.80 ppm (Figs. 3a and 3b), as originally assigned by Otting *et al.* (*23*). Furthermore, these water molecules are within 3 and 4 Å, respectively, from the I55 $C_\beta H$ proton, which resonates at 1.65 ppm as T40 $C_\gamma H_3$ (*28*). The residual signal observed at 1.65 ppm in Fig. 3b contains therefore contributions arising from direct intermolecular NOEs between water and the protein protons I55 $C_\beta H$ and T40 $C_\gamma H_3$. These direct NOE contribu-

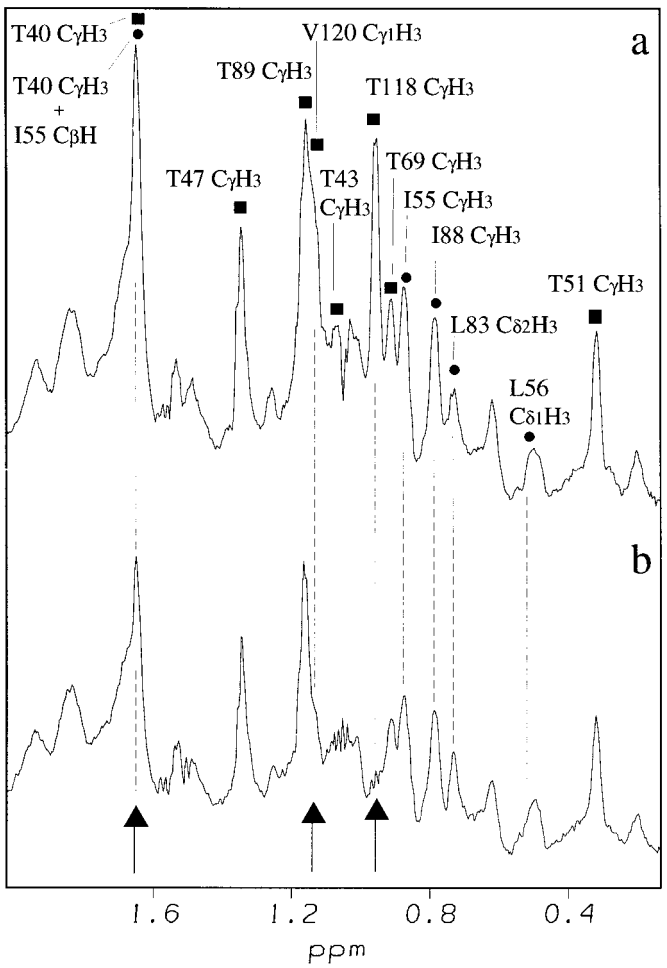

FIG. 3. (a, b) Expanded threonine methyl region of the spectra shown in Figs. 2a and 2b, respectively. Dots (•) indicate contributions to the observed peaks arising from direct intermolecular NOEs with water, while filled squares (■) denote contributions from exchange-relayed NOEs corresponding to the two-step transfer $H_2O \rightarrow$ T OH $\rightarrow$ protein proton (*23*). Arrows indicate the exchange-relayed NOEs which are edited by the 213 Hz continuous pulse at 5.93 ppm. The same nomenclature as in (*23*) is used for the assignments.

tions were obscured by the NOE exchange-relayed through T40 OH in the spectrum acquired without continuous wave irradiation at 5.93 ppm (Fig. 3a).

In addition to slowly exchanging hydroxyl protons, the scheme of Fig. 1a can also be successfully applied to nitrogen-bound labile protons, for which chemical exchange is often slow in the NMR chemical shift time scale. For instance, in HEWL one of the intense chemical exchange peaks (*23*) observed in the 1D water-selective NOE experiment resonates at 7.67 ppm (Fig. 2a, asterisk 2), as expected for nitrogen-bound protons of lysine and/or arginine side chains (*18*). An additional experiment was therefore recorded using the pulse sequence of Fig. 1a with a 155 Hz CW applied at 7.67 ppm. The resulting spectrum is shown in Fig. 2c. The CW quenched the chemical-exchange peak at 7.67 ppm by more than 94% and reduced the chemical exchange-relayed NOE at 3.07 ppm by 37%. This reduction can be explained considering that the peak at 3.07 ppm in Fig. 2a contains contributions from side-chain protons vicinal to the nitrogen bound labile protons resonating at 7.67 ppm, as indicated by 2D e-PHOGSY-NOE-TOCSY experiments. Furthermore, these 2D experiments show that the T89 $C_\alpha H$ proton also contributes to the peak at 3.07 ppm, thus explaining why this resonance is not completely quenched upon irradiation at 7.67 ppm. However, additional contributions to the residual peak at 3.07 ppm cannot be *a priori* ruled out.

APPLICATION TO NOE DIFFERENCE SPECTROSCOPY

The sensitivity of the basic experiment shown in Fig. 1a can be improved if the desired magnetization is selected by phase cycling only, as shown in Fig. 1b. In the sequence of Fig. 1b, gradients do not select coherences but just destroy those which are undesired, thus avoiding the loss of one half of the water magnetization during the mixing time (*24, 29*). For instance, spectra acquired under the same experimental conditions indicated in Fig. 2b but using the sequence in Fig. 1b have a signal-to-noise ratio doubled (*31*) as compared to spectra obtained using the experiment of Fig. 1a. This observation suggests that the water magnetization is not significantly affected by radiation damping during the NOE mixing time of the sequence in Fig. 1b. It should however be noticed that this statement about radiation damping strictly refers only to the experimental conditions employed to acquire the spectra shown (Fig. 2b). At long mixing times or using probeheads with high Q factors, radiation damping may be more dramatic and in that case the sequence from Fig. 1a may be the preferred choice.

EXCHANGE RATE WINDOW OF APPLICABILITY

The method of irradiating the relaying labile proton can be applied only when this is not exchange-broadened beyond detection. Assuming that a signal with a linewidth of $\sim$300 Hz is hardly detectable, an upper limit for k_{ex} of approximately 1000 s^{-1} is deduced. A lower limit for k_{ex} can be computed considering that the exchange relay starts competing with direct dipolar cross-relaxation rates (σ) when $k_{ex} >$ maximum σ between water and macromolecular protons. Assuming a maximum absolute value of approximately 10 s^{-1} for σ (*1, 9, 32*), it can therefore be concluded that the CW method is applicable and useful to edit relay through proton exchange with rates in the 10 to 1000 s^{-1} window. Within this interval fall many labile protons of proteins and DNA in a range of experimental conditions (*10, 15, 33*). Several such protons are difficult to detect even with NOESY spectra which avoid the saturation of water. This is because the rapid transfer of magnetization from protein labile protons to water protons during the NOE mixing time quenches the diagonal peak of the labile proton and the cross-peaks between the labile proton and nonexchangeable protons (*10*). This is also observed for the hydroxyl protons of T40 and T118 of HEWL reported here.

COMPARISON WITH THE 1D-CLEANEX-PM EXPERIMENT

Negative NOEs between fast moving waters and protein protons even in the vicinity of relaying labile groups can also be measured using the 1D-CLEANEX-PM sequence (*34*), which is based on NOE/ROE compensation valid for $\omega_o \tau_c \gg 1$. However, this experiment also eliminates NOEs with long-lived waters and suffers from leakages caused by rapid local side-chain motions (*34*). These problems can be overcome by the proposed CW approach which edits the exchange-relayed NOEs independently of the relayed NOE effective correlation time. On the other hand, the CW approach requires multiple experiments to suppress relay though labile protons resonating at different frequencies and, unlike the CLEANEX-PM experiment, is limited to cases in which the relaying labile proton exchanges with water slowly in the NMR chemical shift time scale.

CONCLUSIONS

Based on the observations described above, we conclude that the editing of the exchange-relayed NOEs via irradiation of the intermediate labile protons (Fig. 1) facilitates the unambiguous interpretation of direct intermolecular NOEs between water and macromolecules without reference to a model. This allows in the future the NMR characterization of hydration in the vicinity of polar groups.

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