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^{15}N-edited Three-Dimensional NOESY-HMQC with Water Flipback: Enhancement of Weak Labile ^{1}H Resonances of Protein Side Chains Contacting DNA

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Two pulse sequences are described that employ a modified water flipback technique to enhance the signal intensity of weak side chain resonances at the protein–DNA interface of the vnd/NK-2 homeodomain/DNA complex in an ^{15}N-edited three-dimensional NOESY-HMQC spectrum. The pulse sequences presented employ water flipback pulses at the beginning of the NOESY mixing time, optimizing the direct NOE transfer of magnetization from the water to the protein by maximizing the *z*-component of the water magnetization. In one of the pulse sequences, radiation damping during the the indirect ^{1}H and ^{15}N evolution times is suppressed. A modified version of the WATERGATE water suppression technique is employed during the HMQC portion of the experiment. The signal enhancement is demonstrated for the resonances of the side chain amide of Asn51, an invariant homeodomain residue whose contact with the DNA is critical for binding. An ancillary advantage of the experiment is the ability to observe NOE transfer of magnetization from water. The information present in the water resonance plane of the three-dimensional spectrum is illustrated in a comparison with the corresponding HMQC spectrum of the protein/DNA complex. © 1998 Academic Press

Key Words: **water flipback; WATERGATE; homeodomain; protein/DNA complex; NOESY-HMQC; radiation damping.**

INTRODUCTION

Recent improvements in NMR experimental techniques, such as the use of pulsed field gradients and greater magnet strength, have allowed increasingly larger macromolecular systems to be studied. Many of these systems include complexes, between protein and DNA, for instance, the study of which naturally focuses on the protein side chain interactions at the complex interface. NMR experiments originally designed for protein backbone resonances are not necessarily suited or adequate for studying the typically weaker and broader resonances of these protein side chains. Conformational exchange and chemical exchange with water often make the observation of side chain resonances difficult. The water flipback technique (*1*), originally designed to deal with these exchange problems for backbone amide resonances and recently discussed in a review of water suppression techniques (*2*), is similarly effective for enhancing the signal of weak side chain resonances. In this paper we demonstrate the importance of water flipback and describe two pulse sequences that result in improvement of signal intensity in the ^{15}N-edited three-dimensional NOESY-HMQC experiment with flipback and WATERGATE (*3*) water suppression.

In earlier NMR studies of the 20 kDa complexes of the vnd/NK2 homeodomain and of the Antennapedia homeodomain with DNA, many of the resonances of side chains contacting the DNA were extremely weak or not observed, such as the critical contact made by the invariant homeodomain residue Asn51 (*4*, *5*). The earlier experiments used standard ^{15}N-edited three-dimensional NOESY pulse sequences that included presaturation of the water resonance for water suppression. The inclusion of flipback in a standard three-dimensional NOESY experiment was motivated by the need to observe these important protein–DNA contacts. Observations of crosspeaks between the side chain resonances of Asn51 and the DNA are indispensable for the proper characterization of the interactions between the homeodomain recognition helix and the DNA.

RESULTS

Three ^{15}N-edited three-dimensional NOESY-HMQC experiments were performed on the 1.3-m*M* ^{15}N-labeled vnd/NK-2 homeodomain protein bound to DNA. These experiments were NOESY-HMQC with water presaturation, NOESY-HMQC with WATERGATE water suppression, and NOESY-HMQC with WATERGATE and water flipback (pulse sequences shown in Fig. 1). WATERGATE was included in the NOESY-HMQC with presaturation, which allowed very mild ($\gamma B_2 = 13$ Hz) presaturation while still giving acceptable water suppression. Results comparing these experiments are shown in Fig. 2 for the Asn51 side chain NH_2 resonances. NOESY-HMQC with WATERGATE and flipback shows the strongest cross-

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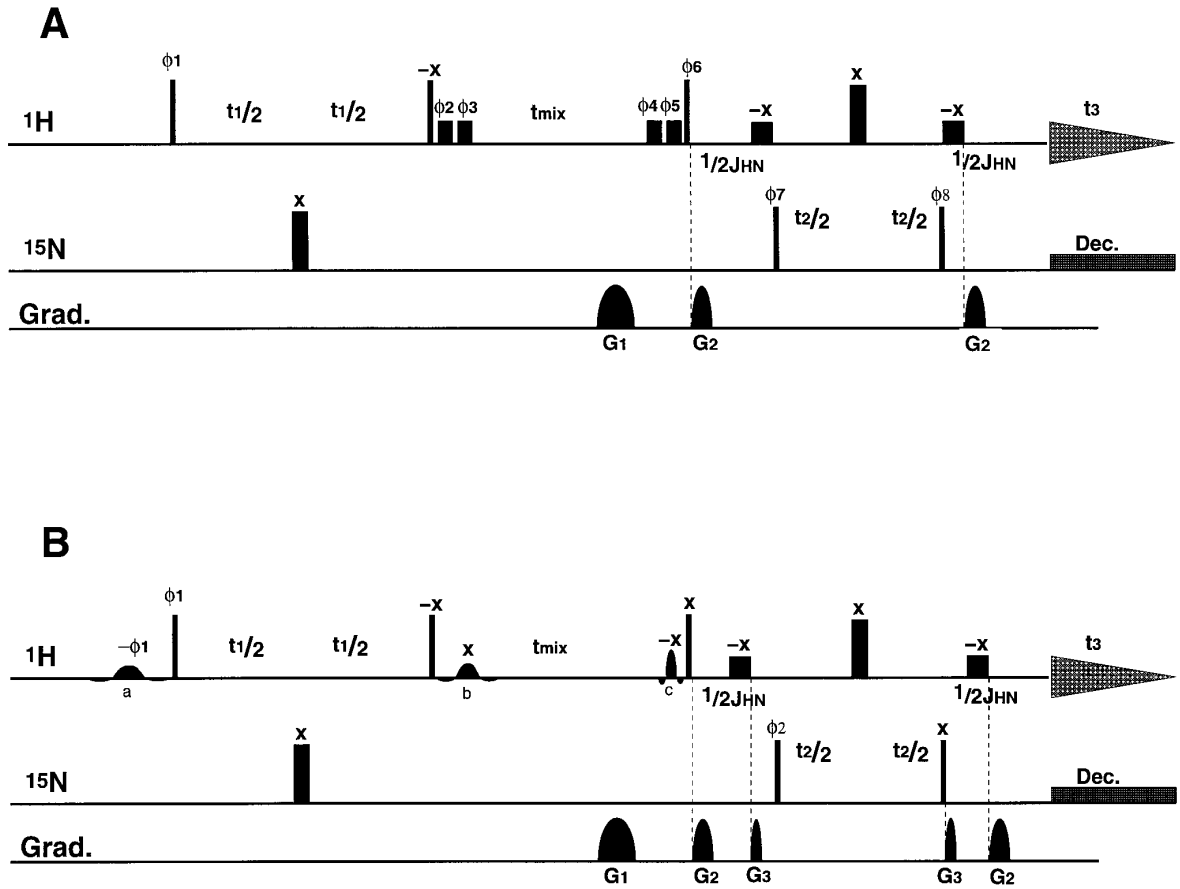

FIG. 1. (A) Pulse sequence of the ^{15}N-edited NOESY-HMQC experiment with flipback during the NOESY mixing time, and WATERGATE during HMQC, with no 45° phase-shifted quadrature detection in t_1 and no radiation damping suppression. High-power ^{1}H and ^{15}N 90° and 180° flip-angle pulses correspond to the narrow and wide tall rectangles, respectively. Low-power ^{1}H pulses applied at the H_2O resonance correspond to the shorter rectangles. The low-power ^{1}H pulses during the mixing time have flip angles of 45° and γB_2 = 25 Hz, and the low-power ^{1}H pulses during the HMQC have flip angles of 90° and γB_2 = 250 Hz. The H_2O resonance was used as the RF carrier frequency. The States protocol was used for quadrature detection in the t_1 dimension by changing ϕ_1. The States protocol was used for the t_2 dimension by changing ϕ_7. The phase cycle differs for real versus imaginary t_1 data points. For the real t_1 data points; ϕ_1 = x; ϕ_2 = −y; ϕ_3 = y; ϕ_4 = ϕ_5 = −x; ϕ_6 = x; ϕ_7 = x, −x; ϕ_8 = x, x, −x, −x; acq = x, −x, −x, x. For the imaginary t_1 data points; ϕ_1 = y, −y; ϕ_2 = −y, y; ϕ_3 = −y, y; ϕ_4 = ϕ_5 = −x, x; ϕ_6 = x, −x; ϕ_7 = x, −x; ϕ_8 = x, x, −x, −x; acq = x, −x, −x, x. Gradients are sine-bell shaped with an amplitude of 25 G/cm at their center and durations of $G_{1,2}$ = 2.5 and 1.5 ms, followed by recovery delays equal to the duration of the gradient. (B) Pulse sequence of the ^{15}N-edited NOESY-HMQC experiment with water flipback, 45° phase-shifted quadrature detection in t_1, radiation damping suppression during t_1 and t_2, and modified WATERGATE during HMQC. High-power ^{1}H and ^{15}N 90° and 180° flip-angle pulses correspond to the narrow and wide tall rectangles, respectively. Low-power ^{1}H 90° pulses correspond to the shorter rectangles. Pulses indicated by a, b, and c correspond to shaped (sinc) ^{1}H 90° pulses. All ^{1}H pulses are applied at the H_2O frequency. Shaped pulse a before the t_1 evolution period keeps the water magnetization along + z during the indirect ^{1}H evolution, preventing radiation damping during t_1. Shaped pulse b puts the water magnetization along + z during the mixing time. Shaped pulse c ensures the water magnetization is along + z during the acquisition and recovery time. The modified WATERGATE is achieved with the two low-power 90° and high-power 180° ^{1}H pulses and the two gradients G2 during the HMQC portion of the sequence. The two gradients G3 dephase the water magnetization before and refocus it after the ^{15}N evolution, suppressing radiation damping during t_2. Shaped pulses a and b are at the same power, with a = ∼10 ms and b = ∼5 ms (b is shorter since it brings water up from being transverse to + z), and shaped pulse c is higher power, with c = ∼2 ms. The low-power ^{1}H pulses for WATERGATE are 1 ms in duration. The phase ϕ_1 = −45°, and the States-TPPI protocol was used for quadrature detection in the t_1 dimension by changing ϕ_1 and the receiver phase. Phase ϕ_2 = x, −x for the HMQC, and the TPPI protocol was used for quadrature detection in the t_2 dimension by incrementing ϕ_2. Gradients are sine-bell shaped with an amplitude of 25 G/cm at their center, with durations $G_{1,2,3}$ = 5.0, 1.5, and 0.2 ms, followed by recovery delays equal to the duration of the gradient.

peaks, especially from the DNA, with the intensity at least 50% greater than the results using WATERGATE without flipback. Three of the crosspeaks from the DNA are from the non-labile H8 protons of two adenine bases contacted by Asn51, while the fourth, the one not present in the WATERGATE without flipback spectrum, is from the NH_2 of one of the adenines. With presaturation the crosspeaks from the DNA sink to the noise level. The absence of the crosspeaks from the non-labile protons in the presaturation spectrum presumably is due to spin diffusion with the water magnetization during the recovery time reducing the magnetization of the non-labile spins. Surprisingly, the intensity of the crosspeak from Ile47 CH_3^{γ} in the

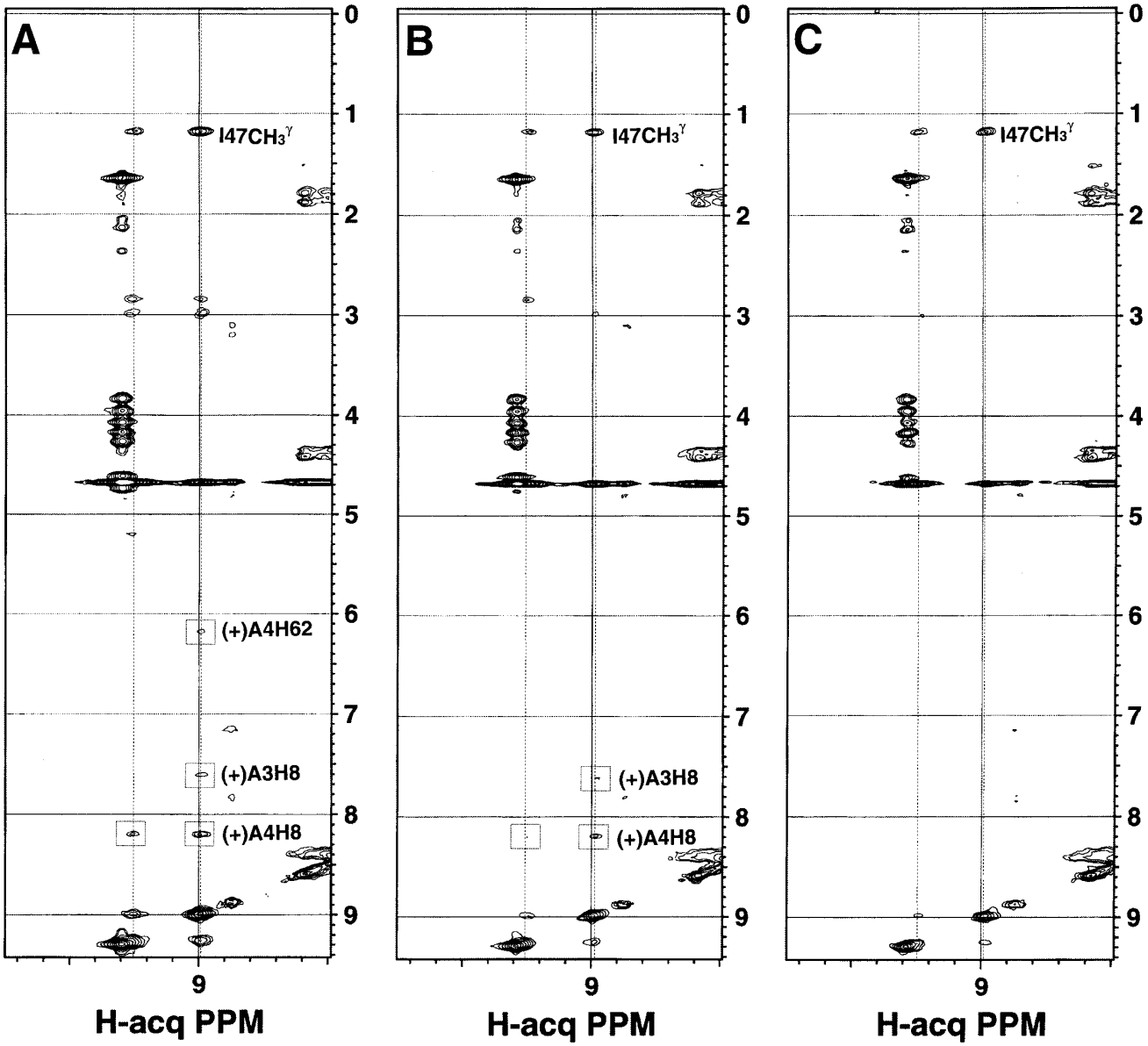

FIG. 2. NOESY signals of the Asn51 side chain NH_2 resonances of the vnd/NK-2 homeodomain-DNA complex. The resonance frequencies are indicated by the vertical lines. The boxes indicate crosspeaks from DNA resonances. (A) ^{15}N-edited NOESY-HMQC with water flipback and WATERGATE using the pulse sequence in Fig. 1A. (B) Same as (A), but with the flipback pulses removed from the mixing time. (C) Same as (B), but with mild presaturation of the H_2O resonance (γB_2 = 13 Hz) during the mixing time and the recovery delay (1.4 s for the three spectra). The three spectra were recorded as 128* (t_1) × 13* (t_2) × 512* (t_3) matrices with spectral widths of 5700 Hz (F1), 1520.5 Hz (F2), and 8333.3 Hz (F3). The mixing time was 140 ms. The number of scans per data point was 36. The spectrum in (B) was recorded at a receiver gain a factor of four less than the spectra in (A) and (C) due to poorer water suppression. The spectra were each processed the same way, with linear prediction used to double the data size in the t_1 and t_2 dimensions, and sine-bell squared window functions for all three dimensions. In order to compare the spectra, each is displayed with a threshold equal to five times the rms noise level of the spectrum. The stronger resonance seen to the left, at 9.29 ppm, belongs to the backbone amide of Ala28.

presaturation experiment spectrum is comparable to that obtained with the flipback experiment, despite the drastic reduction in the intensities of the other crosspeaks. This is likely due to some amount of shielding of this methyl group from the solvent and to the slightly lower rms noise level in the presaturation experiment. Several other resonances of side chains contacting the DNA not observed in earlier studies, Gln50 and Arg53, for instance, are clearly evident in the new flipback experiment. Overall improvement in the intensity of backbone amide resonances is also seen, consistent with what was first observed in the original two-dimensional water flipback experiments of Grzesiek and Bax (*1*). Incorporation of the additional signals observed in the spectrum obtained with flipback has significantly improved the quality of the vnd/NK-2 homeodo-

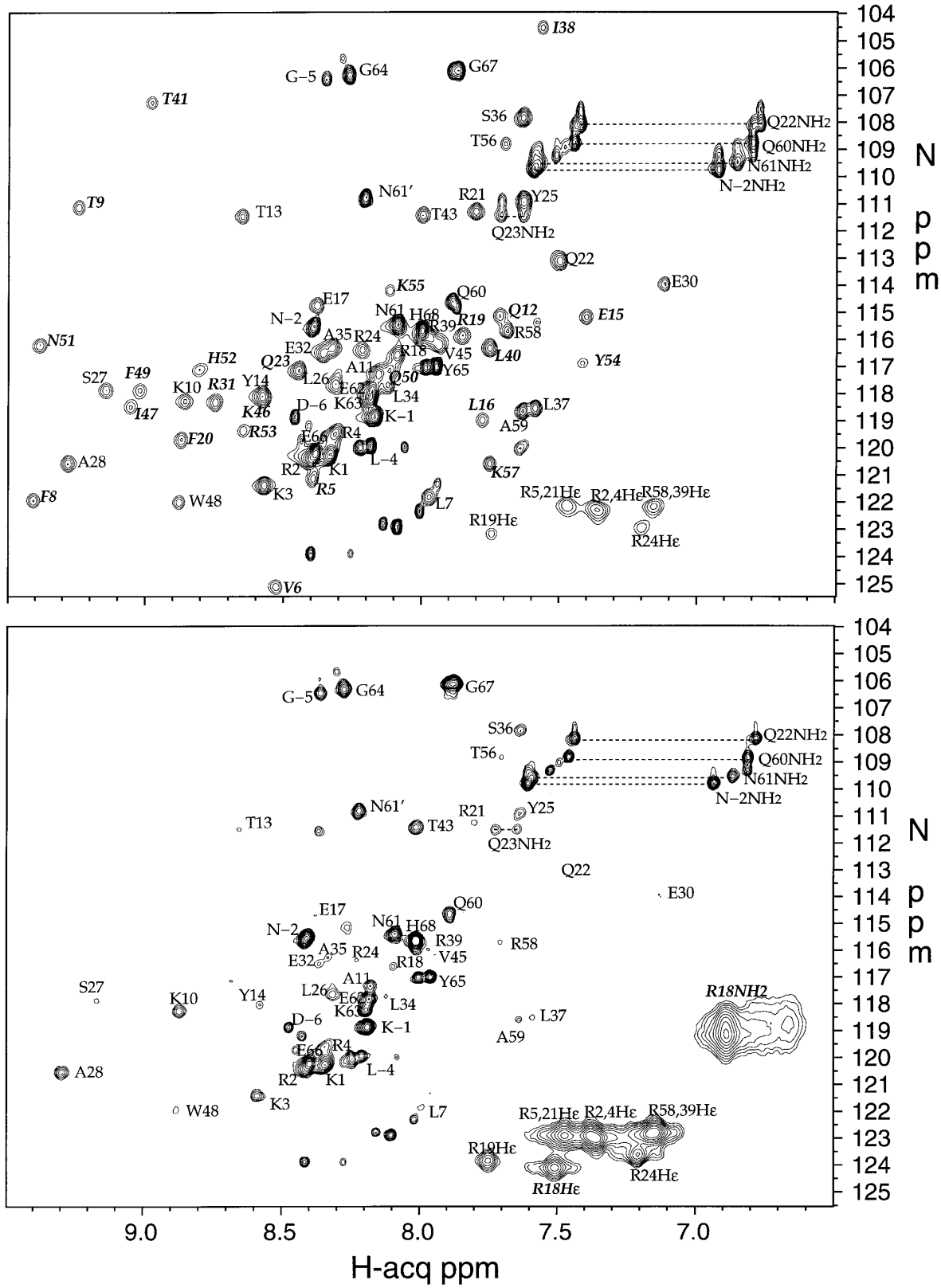

FIG. 3. The H_2O resonance plane (4.644 ppm in the F1 dimension) from the ^{15}N-edited NOESY-HMQC of the vnd/NK2 DNA complex (below) with the normal HMQC spectrum of the complex (above) for comparison. The ^{15}N-edited NOESY-HMQC was acquired with the pulse sequence shown in Fig. 1B as a 128* (t_1) × 128 real (t_2) × 512* (t_3). The spectral widths are the same as the spectra in Fig. 2, and 2 scans were taken per data point. A relatively short mixing

main-DNA structure currently being determined in our lab, especially in the region where the recognition helix contacts the DNA.

Spectra were recorded for the purposes of examining the water plane using the NOESY pulse sequence with radiation damping suppression (Fig. 1B) and the pulse sequence without radiation damping suppression (Fig. 1A). The spectrum recorded with radiation damping suppression (Fig. 3) is shown together with the normal HMQC for comparison. Resonances of buried amides have little or no intensity in the H_2O resonance plane, whereas those protons with fast ^{1}H exchange, exposed arginine side chain amine protons, for instance, have the strongest signals, often stronger than their diagonal peak intensities. Overall, the crosspeaks in the water resonance plane are significantly more intense than observed in typical ^{15}N-edited 3D NOESY-HMQC experiments. The use of the radiation damping suppression pulse sequence resulted in a much larger fraction of the water magnetization being returned to $+z$ at acquisition. Using the pulse sequence with no suppression of radiation damping (Fig. 1A) with t_2 set to 40 ms, only 20% of the water magnetization is returned to $+z$, whereas with the pulse sequence in Fig. 1B approximately 60% is returned. Radiation damping during the mixing time can return any transverse component of the water magnetization to $+z$, and suppression of radiation damping during t_1 had a negligible effect on the amount of returned water magnetization at acquisition. Indeed, past experiments have relied on radiation damping during the mixing time to bring a significant fraction of the water magnetization back to $+z$ by the end of the mixing time (*6, 7*). For protein ^{1}H resonances that interact strongly with water, their equilibration will be slowed when less water magnetization is returned to $+z$ at acquisition. Since this reduction in returned water is a function of t_2, this effect should be manifested as an increase in linewidth in the F_2 dimension; however, it is interesting to note that no significant reduction ($<$5%) in ^{15}N linewidth was observed using radiation damping suppression. The use of radiation damping suppression will likely become more important for higher field experiments (greater than 600 MHz) since radiation damping increases as a function of field strength. The inclusion of water flipback significantly enhances the quality of the three-dimensional ^{15}N-edited NOESY-HMQC spectra.

EXPERIMENTAL

The NOESY pulse sequence with radiation damping suppression, shown in Fig. 1B, utilizes three low-power shaped ^{1}H 90° pulses for water flipback. The first flipback comes directly before the first high-power ^{1}H 90° pulse at the beginning of the t_1 evolution period. This flipback pulse is opposite in phase to the first high-power ^{1}H pulse, so that the water magnetization is returned to $+z$, where it remains during t_1. Keeping the water magnetization along $+z$ during t_1 eliminates losses of water magnetization that would occur as a consequence of radiation damping. The second flipback comes directly after the second high-power ^{1}H 90° pulse, and the water magnetization remains at equilibrium along $+z$ for the entire mixing time, thus optimizing the direct NOE transfer of magnetization from the water to the protein. These first two low-power shaped pulses ($\sim$10 ms and $\sim$5ms duration) are highly selective in order to minimize the impact of the flipback pulses on resonances neighboring the water resonance. Both are applied at the same power, but the second pulse is shorter since the water magnetization goes from being transverse to $+z$, so the feedback field from the water magnetization responsible for radiation damping actually enhances, rather than hinders, the low-power RF field. The third flipback pulse counteracts the third high-power 90° ^{1}H pulse, returning the water magnetization to $+z$ at the beginning of the HMQC portion of the experiment. The two low-power and one high-power ^{1}H pulses during the HMQC have zero net effect on the water magnetization, so that the magnetization ends up along $+z$ at the end of the sequence.

A modified version of WATERGATE (*3*) water suppression was employed simultaneously with the HMQC portion of the pulse sequence. The low-power 90° pulses for WATERGATE are executed before and after the ^{15}N evolution time, with the high-power 180° ^{1}H refocusing/decoupling pulse also serving as the WATERGATE high-power 180° pulse. In addition, two short gradients are applied between the low-power ^{1}H 90° pulses and the ^{15}N 90° pulses. The first of these gradients dephases the transverse magnetization, including the water magnetization, so that no radiation damping occurs during t_2, and the second refocuses the magnetization. The standard WATERGATE sequence executes the low-power 90° pulses immediately before and after the high-power 180° pulse. However, executing the low-power 90° pulses with the gradients and the ^{15}N evolution delay separating them from the high-power 180° pulse results in only a moderate reduction in water suppression for the ^{15}N evolution times ($<$60 ms) used in the three-dimensional experiments presented here. The HMQC add/subtract phase cycling of the ^{15}N 90° pulses eliminates nearly all of the remaining signal arising from residual transverse water magnetization.

Phase sensitive methods for the indirect ^{1}H dimension involve incrementing the phase of one of the ^{1}H pulses. This

time of 32 ms was used to reduce the effect of spin diffusion. The recovery delay was 1.4 s. The processing was done as noted for the spectra in Fig. 2, but with linear prediction also in the t_3 dimension. The HMQC, with 128 real (t_1) $\times$ 1024* (t_2) points and sweepwidths 1763.7 Hz (F1) and 9615.4 Hz (F2) and linear prediction in the t_1 dimension, was recorded using only the HMQC portion of the the ^{15}N-edited NOESY-HMQC in Fig. 1B, including the water flipback immediately preceding the HMQC. Resonances appearing in one spectrum and not the other are indicated by italics.

incrementation also affects the phase of the water magnetization, and this must be taken into account in the implementation of water flipback. Care must be taken to produce water crosspeaks that are phaseable along with the rest of the spectrum. Since the carrier frequency is set at the water resonance, NOE signals from the water should only be present for the real indirect ^{1}H time points using typical phase sensitive indirect detection methods. Shifting the phase of the first pulse in Fig. 1B by -45 degrees, however, causes NOE signals from water to be present in equal amounts during the real and imaginary indirect time points (*6, 8*), producing crosspeaks from water that are phaseable. Since the water magnetization is brought to $+z$ by the soft pulse at the beginning of the mixing time for all scans, rather than 45° from $+z$, as would be the case without any flipback pulses, the NOE signal from water is enhanced by the square root of two relative to the other NOE signals in the spectrum (with a slight adjustment for the short time water magnetization which is not along $+z$ during the flipback pulses). Since we require that the water magnetization remain along $+z$ during the mixing time, the States phase sensitive indirect detection for t_1 must be implemented using both the phase of the first high-power ^{1}H 90° pulse and the receiver phase, that is, the first pulse has $-45°$ phase for the real t_1 points and 45° for the imaginary t_1 points.

The NOESY pulse sequence shown in Fig. 1A employs an alternate technique for obtaining phaseable crosspeaks from water. In this pulse sequence no $-45°$ phase shift is used for phase sensitive detection in t_1. Instead, the first pulse has 0° phase for the real t_1 points and has 90° phase for the imaginary t_1 points. Since the carrier frequency is set at the water resonance, NOE signals from the water should only be present for the real indirect ^{1}H time points for this pulse sequence. The water NOE signals can be canceled for the imaginary time points by additional phase cycling (see Fig. 1A legend), thus producing crosspeaks from water with the correct phase. Flipback is implemented in the pulse sequence by two pairs of 45° flip-angle low-power ^{1}H pulses during the NOESY mixing time. For the real indirect ^{1}H dimension the water magnetization is already brought back to $+z$ by the first two high-power ^{1}H 90° pulses with phases x and $-x$. The following two 45° low-power pulses have opposite phase, yielding a net zero degree pulse. However, for the imaginary indirect ^{1}H dimension the two high-power ^{1}H pulses have phases y and $-x$ so that the magnetization is aligned along the x-axis at the beginning of the mixing time. In this latter case the two 45° low-power pulses, now with the same phase, bring the water magnetization back to $+z$. The second pair of 45° pulses ensures that the water magnetization is along $+z$ after the third high-power ^{1}H pulse. Pairs of 45° low-power pulses were used for the flipback in order to balance the effect of the pulse sequences for both the real and imaginary time points in the indirect ^{1}H dimension. These 45° low-power pulses (5-ms duration) are highly selective in order to minimize the impact of the flipback pulses on resonances neighboring the water resonance. Using shaped pulses for these selective pulses further reduces distortion of neighboring resonances, and while the spectra shown in Fig. 1 were recorded with rectangular low-power pulses, we subsequently have adopted the use of shaped pulses for all flipback pulses (as in the pulse sequence of Fig. 1B). Because of the additional phase cycling required to cancel the water NOE signal during the imaginary time points, the minimum phase cycle for pulse sequence 1A (Fig. 1A) is four scans, compared to pulse sequence 1B (Fig. 1B) where only a two scan phase cycle is required. Pulse sequence 1A could also be modified to suppress radiation damping in a manner similar to that employed in pulse sequence 1B. That is, a selective 90° pulse could be placed before the first high-power 90° pulse and another selective 90° pulse after the second high-power 90° pulse, and an additional pair of gradients could be added to the HMQC/WATERGATE portion of the sequence.

All spectra reported here are from the complex of the vnd/NK-2 homeodomain bound to its cognate DNA at a 1.3-m*M* concentration at 308 K, 80 m*M* NaCl, and pH 6.0. The protein consists of a 77 amino acid peptide containing the 60 residue homeodomain plus 17 flanking residues, and the DNA is a duplex of 16 base pairs. Further sample preparation details have been described previously (*5, 9*). The spectra were recorded on a Bruker AMX 600-MHz spectrometer. All spectra were processed with nmrPipe, and the spectra were plotted using nmrDraw (*10*).

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