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Sensitivity-Enhanced Detection of Fast Exchanging Protons by an Exchange-Edited Gradient HEHAHA-HSQC Experiment

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An experiment is presented which allows for the sensitivity-enhanced measurement of proton exchange rates in a HSQC type experiment. Instead of using INEPT type transfer of magnetization from protons to heteronuclei and vice versa, we have used heteronuclear Hartmann-Hahn transfer, which is known to have higher sensitivity in the presence of chemical exchange. Direct NOE's between NH protons and α-protons are suppressed by an exchange-editing step unless the α-resonances are degenerate with the water resonance. A comparison between the exchange-edited HEHAHA-HSQC and a standard exchange-edited HSQC experiment performed on the uniformly ^{15}N-labeled staphylococcal nuclease H124L shows an enhancement of approximately 100% with the former experiment. A set of one-dimensional exchange-edited spectra of urea was used for evaluating the ability to extract exchange rates using the presented experiment.

The study of chemical exchange rates provides valuable insights into the dynamic and conformational properties of biological macromolecules (*1–4*). For example, the exchange rates between labile nitrogen-bonded protons and bulk water reveal information about the formation of hydrogen bonds and the accessibility of solvent molecules which are both indicators of protein secondary structure. The first NMR methods to measure slow and moderately rapid exchange rates were the double resonance (*5*) and EXSY methods (*6*). The latter is often preferred due to its higher resolution compared to the double resonance method. However, in studies of macromolecules, the resolution in homonuclear 2D ^{1}H NMR spectra is still not sufficient in some cases. In those cases, the larger chemical-shift dispersion of heteronuclei may be exploited for exchange measurements by acquiring "exchange-edited" HSQC or HMQC spectra (*7*). The first experiment which allowed for the accurate extraction of exchange rates, without the influence of direct NOE's between α-protons and NH protons from an HSQC type experiment, was the MEXICO experiment (*8*).

In order to extract the exchange rates between NH protons and water, the MEXICO experiment requires the acquisition of a set of 2D spectra with different mixing times on doubly labeled (^{15}N and ^{13}C) material. The water magnetization is alternately aligned along the $+z$ and $-z$ directions and thereby the influence of T_1-relaxation on the recovery of NH resonances is cancelled out. The magnetizations of ^{13}C and ^{15}N-bound protons are brought into the transverse plane where they are subsequently destroyed by a homospoil pulse and, therefore, not detected. Chemical exchange or NOE's from bound water molecules are the only ways to obtain a non-zero magnetization at the NH site and since all the magnetization of ^{13}C and ^{15}N-bound protons is destroyed, there are no direct contributions of the nuclear Overhauser effect between NH protons and any ^{13}C-H protons. However, magnetization that builds up at the NH protons due to chemical exchange or NOE's from bound water molecules may be transferred to surrounding protons by cross-relaxation.

Other 2D water magnetization transfer experiments, which have the ability to measure rapidly exchanging protons, have been reported (*9–14*). One of these simply uses the fact that ^{15}N-labeled material is sufficient if the molecule falls in the range where $\omega\tau_c$ is approximately 1 and the NOE's are 0 (*9*). Some of the others use the so-called water-exchange filter (WEX filter) to selectively observe exchangeable protons (*10, 11*). The WEX filter uses a selective pulse on the solvent signal to selectively excite its magnetization at the beginning of the pulse sequence. However, radiation damping tends to bring the excited magnetization back to equilibrium during the application of the long selective pulse resulting in the incomplete excitation of the solvent signal (*15*). Another way to select for those protons that can transfer magnetization with the solvent, either by chemical exchange or by NOE's, is to selectively invert the transverse magnetization of the water resonance while leaving all other spins unaffected, thus no NOE's between the NH protons and the α-protons can build up (*12*). This selects resonances that are exchanging with the inverted protons and is advantageous because no double labeling is required. A limitation, however, is the loss of information about exchange rates of NH protons whose neighboring α-resonances are degenerate with the water. In this case, the NH

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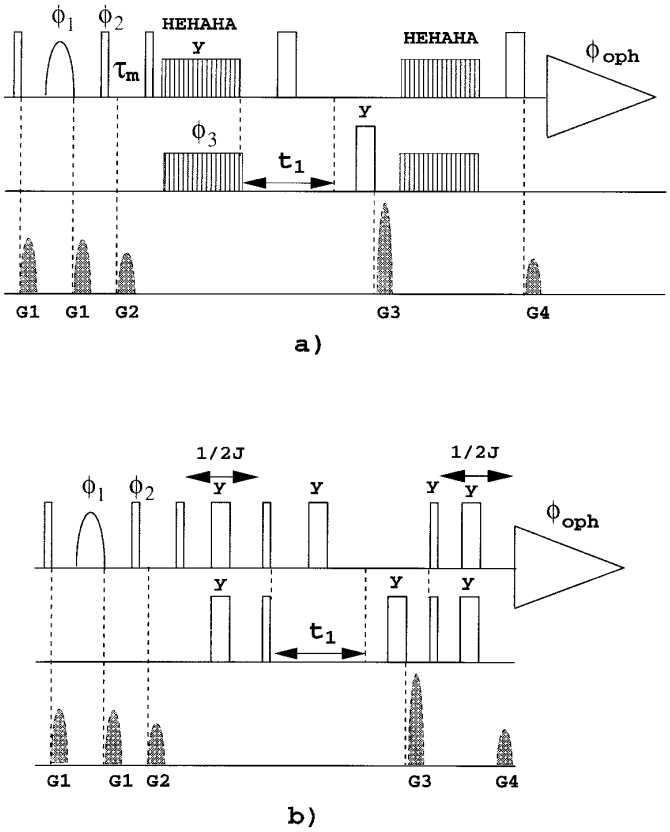

FIG. 1. (a) Pulse sequence of the exchange-edited gradient HEHAHA-HSQC experiment. Thin and thick bars are 90° and 180° pulses, respectively. The pulse of phase ϕ_1 is a 180° selective pulse; in our case it was a Gaussian-shaped pulse of 9 ms. τ_m is the mixing time, i.e., the time when magnetization exchange can take place. HEHAHA stands for heteronuclear Hartmann-Hahn transfer and it was carried out using synchronous DIPSI-2 sequences for a duration of $1/^1J_{NH}$ on both the nitrogen and the proton channel. Trim pulses before or after the isotropic mixing sequences may be used, but we have found that they are not necessary on the hardware used. The 180° pulse in the middle of the evolution delay t_1 serves to defocus any evolution of heteronuclear scalar coupling between the nitrogen and its attached proton. The final 180° nitrogen pulse refocuses the evolution of chemical shifts during the action of the gradient G4. The gradient intensities were of the following ratio: G1:G2:G3:G4 = 10:−7:50:4.98. The phase-cycling scheme used for this experiment is $\phi_1 = x, x, y, y, -x, -x, -y, -y$; $\phi_2 = x, -x$; $\phi_r = x, -x, -x, x$; all other phases are x, unless noted otherwise. Quadrature detection in the t_1-dimension was achieved by inverting the sign of the gradient G4 every other scan and suitable combination of the thus separately detected P- and N-type spectra. (b) Pulse sequence of a standard exchange-edited HSQC, which was used to compare the efficiency of the proposed method. The phases ϕ_1, ϕ_2, and ϕ_r as well as the ratio of gradient field strengths are the same as in Fig. 1a. The delay for the evolution of heteronuclear anti-phase magnetization was experimentally optimized to give maximum signal intensity. It was approximately 2.8 ms.

signals contain both NOE build-up between α-protons and amide protons and exchange rate information. One could filter out the magnetization of all α-protons by the incorporation of a 90° carbon pulse during the selective 180° pulse on the water signal (*13*), however, this would again require the use of double-labeled material, like the MEXICO experiment. Another way to separate magnetization transfer due to chemical exchange from NOE's of α-protons that coincide in chemical shift with the water resonance is the use of a WEX filter in combination with spin-echo filters (*14*). This method utilizes the large difference in relaxation and coupling properties of water and macromolecules to separate their magnetizations. However, like in the original WEX filter, radiation damping during the first selective pulse may cause troubles for the selective excitation of the solvent signal.

The protocol put forth in this manuscript uses an exchange-editing step where the solvent signal experiences a selective 180° pulse, following which it is defocused by gradients (*13*) which eliminates the undesirable effects from radiation damping. For increased resolution, this exchange-editing step is then incorporated into an HSQC type experiment (*16*). In this type of experiment, Krishnan and Rance (*17*) have pointed out that the efficiency of magnetization transfer from protons to a heteronucleus or vice versa, in the presence of chemical exchange is substantially enhanced for the heteronuclear Hartmann-Hahn transfer (HEHAHA) (also known as heteronuclear cross polarization (HCP) or hetero-TOCSY) (*18–21*) as compared to the antiphase magnetization transfer (*22*) which is used in almost all current heteronuclear pulse sequences.

A description of the combination of the aforementioned

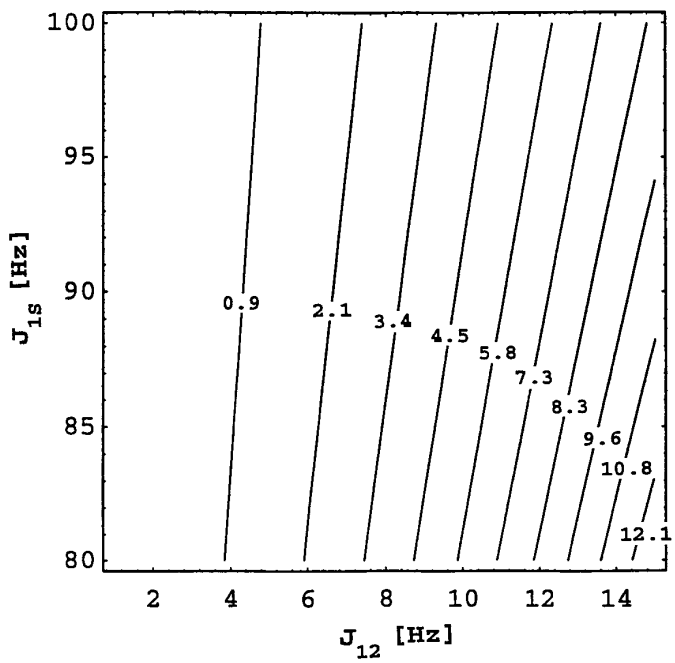

FIG. 2. Plot showing the amount of transverse magnetization present at the site of nucleus I_2 after two simultaneous Hartmann–Hahn mixing periods between nucleus S and I_1 and I_1 and I_2, when the initial state is $\rho(0) = S_x$. J_{1S} is the scalar coupling constant between the heteronucleus and its attached proton and J_{12} is the homonuclear scalar coupling constant. The numbers indicate the percentage of magnetization at site I_2 relative to the amount of magnetization present at site I_1 after a time $t = 1/J_{1S}$ which gives the theoretically maximum transfer between S and I_1.

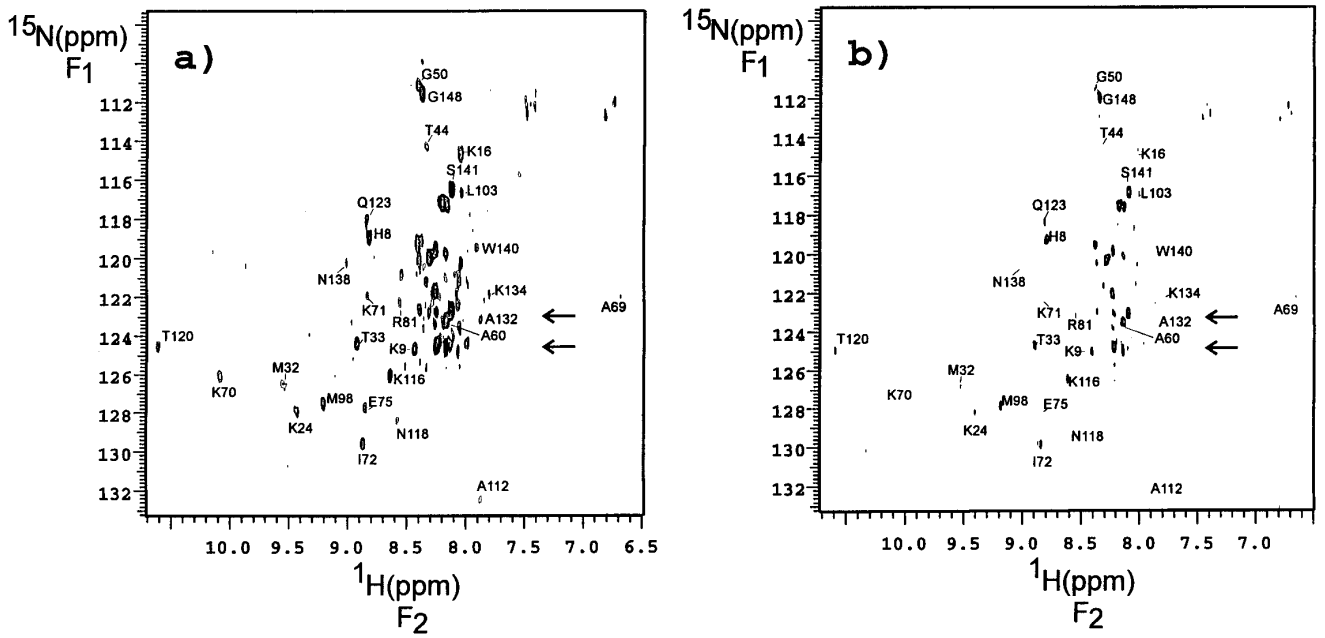

FIG. 3. Exchange-edited HEHAHA-HSQC (a) and standard exchange-edited HSQC (b) spectra of staphylococcal nuclease H124L . The HEHAHA version shows far enhanced sensitivity. Some peaks, like K70, N138, or T44, don't even appear at the shown contour level in the standard HSQC version of this experiment. All acquisition, processing, and display parameters are the same for both spectra. Acquisition parameters included a mixing time of 200 ms and 64 transients of 2K data points for each of the 64 increments. After zero-filling to a matrix of 2048* 1024 points, the data were multiplied by a 60° phase-shifted sine bell window function in both dimensions prior to Fourier transformation. The spectra were recorded in the phase-sensitive mode, by inverting the sign of the last gradient in every other scan. Arrows indicate the frequencies at which the traces, shown in Fig. 4, were taken.

selective exchange-editing step along with the enhanced heteronuclear Hartmann-Hahn transfer substituted into a 2D HSQC type experiment follows for the sensitivity-enhanced measurement of proton exchange rates.

The pulse sequence, which has been designed for this purpose, is depicted in Fig. 1. After excitation of transverse magnetization of all proton spins, a selective 180° Gaussian-shaped pulse of 9 ms inverts the magnetization of the water signal. Two gradients, G1, which are of equal sign, defocus any magnetization, which is not inverted by this selective pulse. Since the selective pulse changes the coherence order by a factor of 2 and it is cycled in steps of 90° (see Fig. 1 caption), the water spins are alternately aligned along the $+y$ and $-y$ axes. The next 90° proton pulse flips this magnetization into the z-direction and chemical exchange takes place in the subsequent mixing time. The next proton pulse brings the magnetization into the transverse plane and the NH signals are transferred to their neighboring ^{15}N by a DIPSI-2 sequence (*23*). The power on the ^{1}H and ^{15}N channels must be matched carefully in order to fulfill the Hartmann–Hahn condition. The evolution delay allows for the evolution of ^{15}N chemical shifts, while the evolution of scalar coupling between ^{15}N and its attached proton is refocused by the 180° proton pulse. The subsequent ^{15}N 180° pulse as well as the final 180° proton pulse defocuses any evolution of chemical shifts during the gradients G3 and G4. After the transfer back to the protons by another DIPSI-2 pulse-train, the last gradient G4 refocuses the magnetization, which has been defocused after the evolution delay by G3. The result, an exchange-edited HSQC type spectrum, is acquired whose sensitivity is superior to a standard exchange-edited HSQC. The improvement achieved depends on the exchange rates of the amide protons. Quadrature detection in the t_1-dimension was achieved by inverting the sign of the gradient G4 every other scan and suitable combination of the thus separately detected P- and N-type spectra (*24*).

A theoretical source of error with this method is that any magnetization which builds up during the last DIPSI-2 sequence may be transferred to a scalar coupling neighbor by homonuclear isotropic mixing since any mixing scheme which can be used for heteronuclear Hartmann–Hahn transfer also leads to homonuclear Hartmann–Hahn transfer if there is a scalar coupled proton present. There are, however, two factors, which prevent substantial loss of magnetization via this pathway. First, the magnetization of NH protons builds up slowly during the isotropic mixing and therefore maximum intensity is present just at the end of the DIPSI-2 sequence, and second, the size of the one-bond N–H scalar coupling is much larger than the commonly observed homonuclear proton coupling constants.

A more quantitative evaluation of the influence of homonuclear Hartmann–Hahn transfer during the HEHAHA transfer can be obtained by a quantum-mechanical treatment. In the following analysis we consider a three-spin system I_1I_2S,

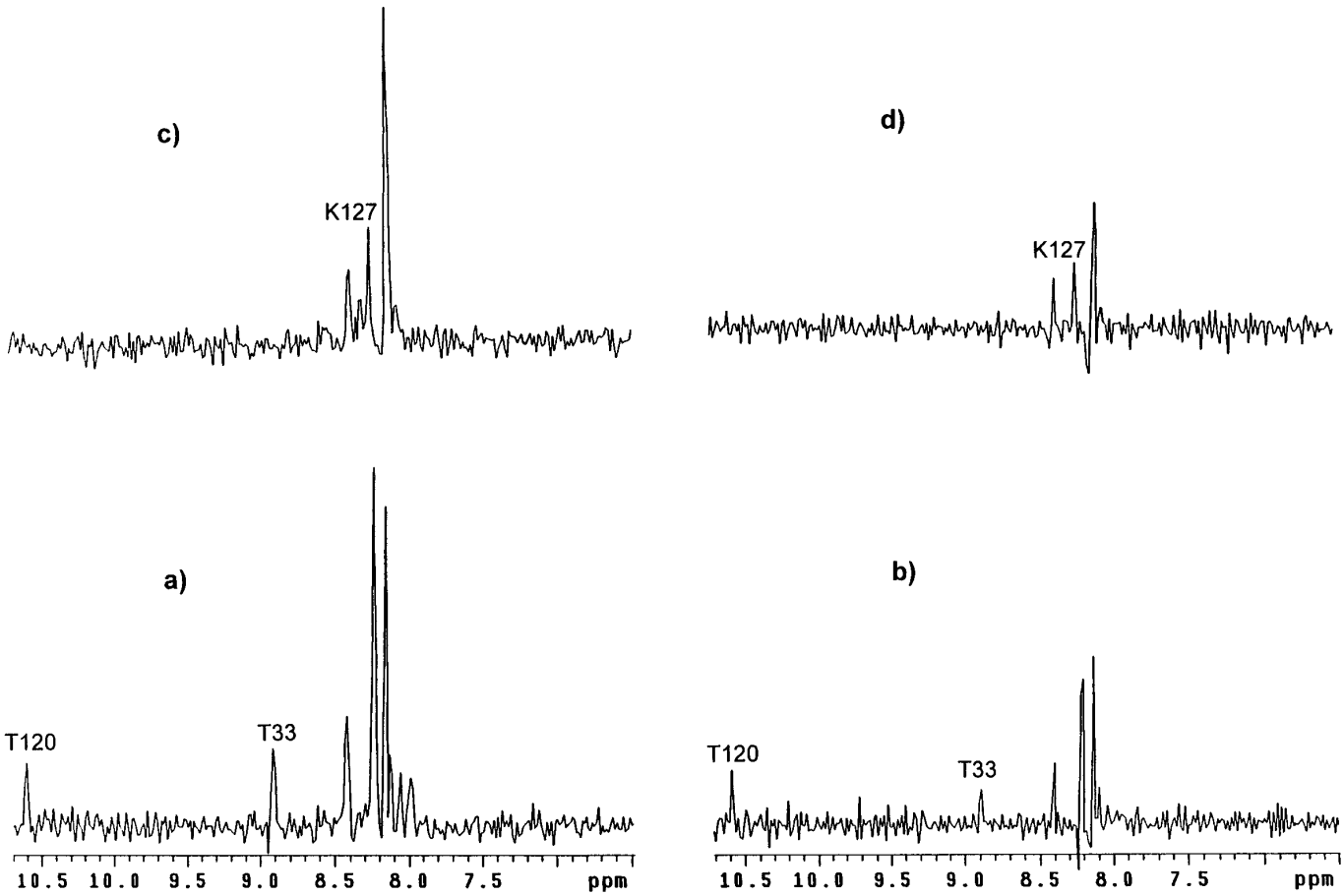

FIG. 4. Traces taken along the F2 axis at the ^{15}N frequency of T33 of the exchange-edited HEHAHA-HSQC spectrum (a) and the standard HSQC version (b) and at the ^{15}N frequency of K127 of the HEHAHA (c) as well as the INEPT type spectrum (d). The same acquisition, processing, and display parameters were used in all cases. In the central part of the traces the gain in sensitivity of the HEHAHA version is about 100%, whereas it is lower at the edges due to the limited bandwidth of the DIPSI-2 sequence.

where I_1 and I_2 represent two protons which are scalar coupled and I_1 is also scalar coupled to the heteronucleus S.

To extract the desired information, one has to solve the Liouville–van Neuman equation,

$$\frac{d\sigma}{dt} = -i[H, \sigma] \qquad [1]$$

whose integrated form

$$\sigma(t) = e^{-iHt/h}\sigma e^{iHt/h} \qquad [2]$$

was solved numerically by the program Mathematica (*25*). In a triply rotating frame, where all spins are on-resonance, the general form of the Hamiltonian H, without including relaxation or chemical exchange, is given by (*26, 27*)

$$H = H_0 + H_{\mathrm{rf}}, \qquad [3]$$

where

$$H_0 = 2\pi J_{12}I_{1x}I_{2x} + 2\pi J_{12}I_{1y}I_{2y} + 2\pi J_{12}I_{1z}I_{2z} + 2\pi J_{1S}I_{1z}S_z \qquad [4]$$

and

$$H_{\mathrm{rf}} = \omega_{I_1}I_{1_x} + \omega_{I_2}I_{2_x} + \omega_S S_x. \qquad [5]$$

J_{1S} is the scalar coupling between the heteronucleus S and its directly attached proton I_1 and J_{12} is the homonuclear scalar coupling constant between I_1 and I_2. As seen in Eq. [4], the full isotropic scalar coupling Hamiltonian needs to be considered for the homonuclear Hartmann–Hahn transfer, whereas the heteronuclear coupling has axial nature at high field conditions (*27*).

The size of the transverse magnetization which is built up at site I_2 as a function of the homonuclear and heteronuclear

scalar coupling constants is shown graphically in Fig. 2 for the case when the Hartmann–Hahn condition ($\gamma_{I1}B_{I1} = \gamma_{I2}B_{I2} = \gamma_S B_S$) is fulfilled and the initial magnetization of nucleus S is along the x-axis. From this plot it is clear that the loss of magnetization by homonuclear Hartmann–Hahn transfer is minimal, e.g., for a $^1J_{NH}$ coupling constant of 90 Hz and $^3J_{HH}$ = 10 Hz, the size of magnetization I_{2X} is just 4.8%. Therefore, the heteronuclear Hartmann–Hahn mixing time is not sufficient to give rise to substantial homonuclear TOCSY transfer. The absence of peaks in regions outside the amide proton region corroborates this interpretation.

As a model system, we used the 149 residue protein staphylococcal nuclease H124L (*28–32*). A demonstration of the performance of this sensitivity-enhanced ^{15}N–^{1}H HEHAHA-HSQC is given in Fig. 3, which shows an exchange-edited HEHAHA-^{15}N–^{1}H HSQC (Fig. 3a) and the standard exchange-edited ^{15}N–^{1}H HSQC version (Fig. 3b). The mixing time, during which chemical exchange or NOE's between water and NH protons can take place, was set to 200 ms. Several peaks, which cannot be seen in the standard exchange-edited HSQC, appear in the HEHAHA version. The faster the protons exchange the higher the gain in signal-to-noise of the HEHAHA-HSQC versus the standard HSQC which identifies the intense peaks in the exchange-edited spectrum as fast exchanging NH protons. Resonances, which were identified with fast exchange from hydrogen/deuterium exchange studies, e.g., K70, K71, M98, and G50 (*33*), are clearly visible in the exchange-edited experiment (Fig. 3a). However, there are some peaks, which have high protection factors, indicating slow exchange with the water that still appear in the spectra in Fig. 3, e.g., the ^{15}N–^{1}H peak of K24. In this case, an NOE between the α-resonance of V23, which lies very close to the water signal and is therefore also inverted by the selective 180° pulse, and the NH of K24 is responsible for the peak in the spectra in Fig. 3. The superior performance of the heteronuclear Hartmann–Hahn transfer over the INEPT type transfer can be better assessed by looking at traces, taken from the 2D spectrum. Traces taken along the F2 axis at the ^{15}N frequencies of 123 and 124.5 ppm are shown in Fig. 4. The gain in signal/noise of the HEHAHA over the conventional INEPT type transfer is about 100% in the central part of the spectrum (around ^{1}H frequencies of, e.g., T33, K9, and K127), whereas peaks that are further away from the transmitter and/or decoupler frequencies show a decreased gain in signal/noise due to the limited bandwidth of the DIPSI-2 sequence which was used to accomplish the heteronuclear Hartmann–Hahn transfer.

To corroborate the use of the exchange-edited HEHAHA-HSQC for the extraction of exchange rates, we have acquired a set of one-dimensional exchange-edited HEHAHA-HSQC spectra of ^{15}N-labeled urea (Fig. 5a) and compared the resultant build-up curve with that obtained using just the exchange-editing module (Fig. 5b). The proton exchange rate is determined by fitting the measured intensities to a function of the form,

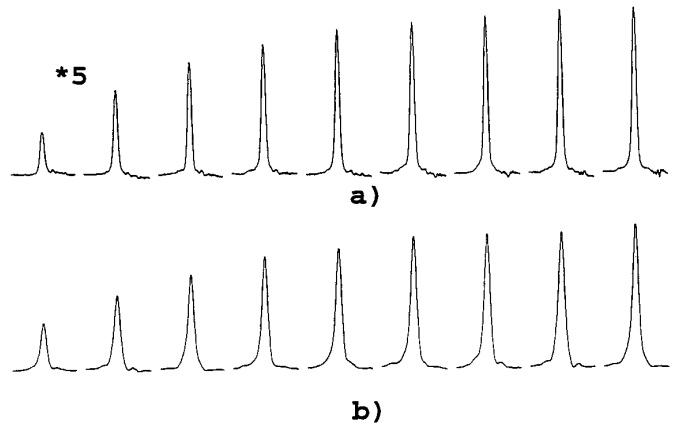

FIG. 5. Build up of magnetization of the NH_2 protons of ^{15}N-labeled urea in H_2O/D_2O (90%/10%) by (a) the exchange-edited HEHAHA-HSQC of Fig. 1a; (b) just a proton-detected exchange-edited set of spectra without heteronuclear filtering by an HSQC type experiment. The spectrum in (a) was plotted with the vertical expansion factors shown. The first spectrum in each set was acquired with a mixing time of 5 ms which was subsequently incremented by 5 ms using 16 scans with 4096 data points for each spectrum at 303 K. Data were multiplied by an exponential window function with a line-broadening of 2 Hz prior to Fourier transformation. The duration of the heteronuclear Hartmann–Hahn mixing sequence was adjusted to 11 ms, which is close to $1/J_{NH}$. The exchange rate obtained by fitting the measured intensities to Eq. [6] is 84 Hz.

$$I(t)/I_{\text{ref}} = 1 - e^{(-kt)}, \qquad [6]$$

where k is the exchange rate between the exchanging proton and water, $I(t)$ is the measured intensity after time t, and I_{ref} is the signal intensity of a reference spectrum, which can be obtained by acquiring a spectrum with a pulse sequence where the selective pulse has been replaced by a hard 180° pulse and the mixing time is set to 0. As expected, inspection of the buildup curves in Fig. 5 shows identical time constants in both cases and gives an exchange rate of 84 Hz.

All experiments were acquired on a 600-MHz Varian Unity INOVA, equipped with a z-axis gradient unit. We used a Gaussian-shaped pulse of 9 ms to selectively invert the solvent resonance. A DIPSI-2 sequence was used to achieve the heteronuclear cross-polarization and the ^{15}N channel was modulated by a GARP (*34*) sequence for the ^{15}N broadband decoupling during the acquisition. The power for ^{15}N broadband decoupling was 0.5 kHz and two matched radiofrequency fields of approximately 3.5 kHz were used for the heteronuclear Hartmann–Hahn mixing, whereby the transmitter was set to the ^{1}H frequency of 8.2 ppm and the decoupler ^{15}N frequency of 122 ppm during the Hartmann–Hahn mixing to ensure proper magnetization transfer in the region of the ^{15}N-^{1}H peaks due to the limited bandwidth of the DIPSI-2 sequence.

A 2m*M* solution of uniformly ^{15}N-labeled staphylococcal nuclease H124L in 1 m*M* DSS, 0.1 m*M* NaN_3, 0.1 m*M* EDTA, and 50 m*M* succinate-d_6, pH 5.1 with 10% D_2O as well as a

solution of 24 mg ^{15}N-labeled urea in 0.5 ml H_2O/D_2O (90%/10%) were used for the experiments. The temperature was 30°C for urea and 45°C for the staphylococcal nuclease H124L.

In conclusion, we have presented a general method which allows for the accurate measurement of chemical exchange rates between water and any exchangeable protons. The sensitivity of this method is far superior to experiments which employ conventional INEPT type magnetization transfer. The method was tested on ^{15}N-labeled staphylococcal nuclease H124L and showed an increase of signal/noise by a factor of approximately 2 over the standard exchange-edited HSQC. Analysis of the possible loss of magnetization by HOHAHA transfer during the final HEHAHA step is shown to be minimal.

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