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Arginine side chain assignments in uniformly ^{15}N-labeled proteins using the novel 2D HE(NE)HGHH experiment

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Summary

A novel 2D NMR experiment, 2D HE(NE)HGHH, is presented for the assignment of arginine side chain ^{1}H and ^{15}N resonances in uniformly ^{15}N-labeled proteins. Correlations between $^{1}H^{\varepsilon}$, $^{1}H^{\gamma}$ and $^{1}H^{\eta}$ are established on the basis of $^{3}J(^{15}N,^{1}H)$ heteronuclear scalar coupling constants, and sequence-specific assignments are obtained by overlap of these fragments with $^{1}H^{\gamma}$ chemical shifts obtained by assignment procedures starting from the polypeptide backbone. Since guanidino protons exchange quite rapidly with the bulk water, the 2D HE(NE)HGHH pulse scheme has been optimized to avoid saturation and dephasing of the water magnetization during the course of the experiment. As an illustration, arginine side chain assignments are presented for two uniformly ^{15}N-labeled proteins of 7 and 23 kDa molecular weight.

This communication reports a novel 2D NMR experiment, 2D HE(NE)HGHH, that is designed to correlate the $^{1}H^{\varepsilon}$, $^{1}H^{\eta}$, and $^{1}H^{\gamma}$ (and possibly $^{1}H^{\delta}$) resonances of arginine side chains in uniformly ^{15}N-labeled proteins via heteronuclear ^{15}N-^{1}H through-bond connectivities (Fig. 1). With the use of a ^{15}N-^{1}H correlation experiment the $^{15}N^{\varepsilon}$ chemical shift can then also be determined. The new experiment is attractive also for use with proteins that are available in ^{13}C/^{15}N doubly labeled form. Combined with standard NMR assignment techniques, it enables sequence-specific assignments for the peripheral Arg side chain resonances.

The magnetization transfer during the 2D HE(NE)HGHH experiment (Figs. 1 and 2) is similar to that in the 3D HNHB experiment (Archer et al., 1991; Chary et al., 1991). In a first INEPT step, magnetization is transferred from $^{1}H^{\varepsilon}$ to its directly attached $^{15}N^{\varepsilon}$. The subsequent time period of duration τ_2 (Fig. 2) serves to create antiphase magnetization between the $^{15}N^{\varepsilon}$ spin and protons that have long-range couplings with it. During this delay the correlation of $^{15}N^{\varepsilon}$ with its directly attached proton is minimized by setting τ_2 equal to an odd multiple of $1/[2\,{}^{1}J(^{15}N,^{1}H)]$. During the time period t_1 the proton chemical shifts evolve, whereas the $^{15}N^{\varepsilon}$ chemical shifts are refocused by a 180° pulse. Following t_1, antiphase ^{15}N magnetization is refocused with respect to the long-range proton couplings during τ_2, and then transferred back to $^{1}H^{\varepsilon}$ by a reverse INEPT step. The proton signal is detected during t_2.

Successful implementation of the 2D HE(NE)HGHH experiment with uniformly ^{15}N- or ^{15}N/^{13}C-labeled proteins requires some special considerations. First, to ensure that cross peaks corresponding to the correlations $^{15}N^{\varepsilon}$-$^{1}H^{\gamma}$ and $^{15}N^{\varepsilon}$-$^{1}H^{\eta}$ are observed without interference from cross peaks deriving from $^{15}N^{\eta}$ of Arg, the side chains of Asn and Gln, and ^{15}N of the polypeptide backbone, selective 180° $^{15}N^{\varepsilon}$ pulses were inserted in both the forward and reverse INEPT transfers (Fig. 2). Second, since the intrinsic exchange rate of Arg $^{1}H^{N\varepsilon}$ is faster than those of amide protons (Wüthrich, 1986), special care is needed to minimize water saturation during the experiment and to keep the water magnetization along the +z-axis during both the evolution period, t_1, and the detection time, t_2. To this end, the first water-selective 180° proton pulse was inserted just before time point *a* (Fig. 2) to align the water magnetization along the −z-axis. The four subsequent proton pulses are non-selective, so that the water magnetization is along the +y-axis at time point *b* (Fig. 2). To prevent radiation damping, the pulsed field gradients (PFGs) G_1 and G_2 in Fig. 2 are applied while the

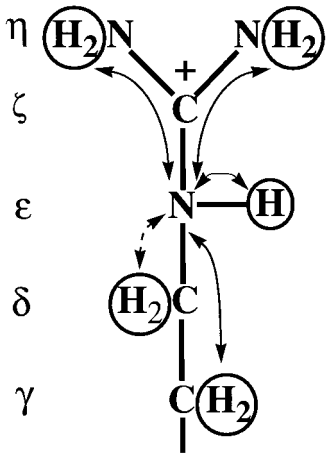

Fig. 1. Magnetization transfer during the 2D HE(NE)HGHH experiment via the $^3J(^{15}N^{\varepsilon},{}^1H^{\gamma})$ and $^3J(^{15}N^{\varepsilon},{}^1H^{\eta})$ couplings (solid arrows), and the $^2J(^{15}N^{\varepsilon},{}^1H^{\delta})$ couplings (broken arrow). The circled nuclei are frequency-labeled during the experiment.

water magnetization is in the transverse plane, and to avoid diffusion losses the strength of these gradients was chosen to be only 2–3 G/cm. The subsequent proton pulse immediately after time point *b* (Fig. 2) restores the water magnetization to the +z-axis. The phase of this pulse is cycled according to x,x,–x,–x, and the water-selective inversion pulse just before *a* is switched on and off in parallel with the phase of this pulse, so that the water-selective inversion pulse is only applied for $\phi_3 = x$. Due to the proton pulse ϕ_5 the water magnetization at time *c* is again transverse. The pulse ϕ_5 is also used to obtain quadrature detection in t_1 by changing its phase according to the States–TPPI method (Marion et al., 1989) (alternative use of States–TPPI with phase ϕ_3 at time *b* would interfere with the alignment of the water resonance along +z during t_1). During the first $\tau_2/2$ period following *c*, radiation damping is prevented by a pair of PFGs and subsequently, with $\tau_2 = 27$ ms during the second $\tau_2/2$ period, the water magnetization is restored along the +z-axis by radiation damping starting with the water magnetization in the transverse plane. Although at 500 MHz the water magnetization could thus not be fully recovered, a sensitivity increase was achieved, and at higher fields radiation damping will be even more efficient. Since both the water magnetization and the signals of interest are thus along +z at point *d*, the PFG G_3 (Fig. 2) can be used to dephase both unwanted magnetization from the protein and residual water magnetization (Bax and Pochapsky, 1992; Wider and Wüthrich, 1993). The last two proton pulses are phase-shifted selective pulses on $^1H^{\varepsilon}$ (see legend to Fig. 2), which leave the water magnetization aligned along the +z-axis for the acquisition time and the relaxation delay.

As an initial test a 2D HE(NE)HGHH spectrum was recorded with a 5 mM sample of a uniformly ^{15}N-labeled mutant form of the 63-residue N-terminal DNA-binding domain of the 434 repressor, a protein with molecular

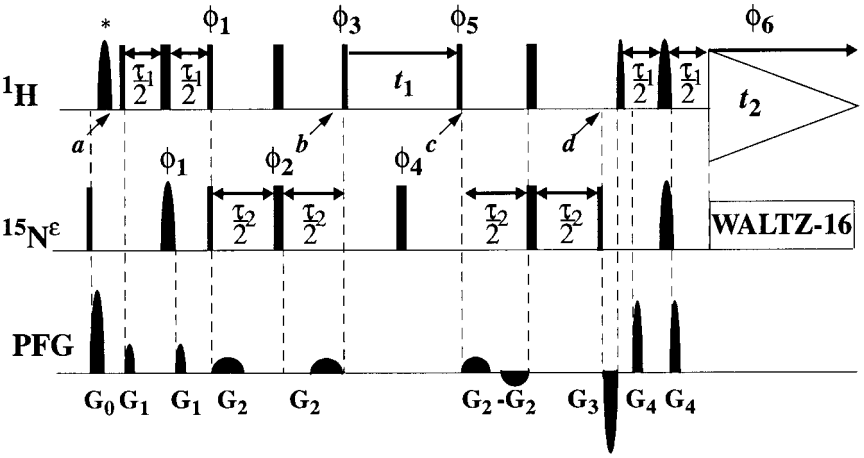

Fig. 2. Experimental scheme of the 2D HE(NE)HGHH experiment. The 90° and 180° pulses are indicated by thin and thick bars, respectively, and phases are indicated above the pulses (where no phase is marked, the pulse is applied along x). The ^1H and ^{15}N carrier positions are set to the solvent line and to 80 ppm, respectively. The first proton pulse (indicated with an asterisk) represents a water-selective I-BURP2 pulse (Geen and Freeman, 1991) of 3.0 ms length and 1.65 kHz peak amplitude. This pulse is applied only when $\phi_3 = x$ (see text); for $\phi_3 = -x$ a delay of the same length was applied. The last two proton pulses are a 90° E-BURP2 pulse of 2.0 ms duration and 2.7 kHz peak amplitude, and a 180° RE-BURP pulse of 2.0 ms duration and 3.1 kHz peak amplitude, which are both applied at 9.2 ppm. The first and last 180°(^{15}N) pulses have a Gaussian shape of 3.0 ms duration and 400 Hz peak amplitude, and have been optimized to avoid excitation of ^{15}N magnetization in the polypeptide backbone, the Asn and Gln side chains, and the Arg $^{15}N^{\eta}$ positions. The lengths of the shaped pulses have been optimized with the Varian program *Pulsetool* for a ^1H frequency of 500 MHz and the aforementioned carrier positions. The duration and amplitudes of the sine-bell-shaped pulsed field gradients (PFGs) are 1 ms and 40 G/cm for G_0, 400 μs and 3 G/cm for G_1, 3 ms and 2 G/cm for G_2, 1 ms and –40 G/cm for G_3, and 200 μs and 30 G/cm for G_4. The first two pairs of PFGs have small amplitudes in order to minimize diffusion losses on the water magnetization. $\tau_1 = 4$ ms, $\tau_2 = 27$ ms, $\phi_1 = y,-y$; $\phi_2 = 8x,8(-x)$; $\phi 3 = x,x,-x,-x$; $\phi_4 = 4x,4y,4(-x),4(-y)$; $\phi_5 = x$; ϕ_6(receiver) = x,2(–x),x,–x,2x,–x. Quadrature detection in t_1 is accomplished by altering the phase ϕ_5 according to the States–TPPI method (Marion et al., 1989). A WALTZ-16 sequence (Shaka et al., 1983) with a field strength of 1.0 kHz is employed to decouple $^{15}N^{\varepsilon}$ during t_2. The time points *a* to *d* are discussed in the text.

weight 7 kDa (Fig. 3B). The correlations $^1H^{\varepsilon}$-$^1H^{\gamma}$ are well separated for each of the seven Arg residues in the protein, five $^1H^{\varepsilon}$-$^1H^{\eta}$ correlations are observed as individual lines and two have been assigned as a group, and there are also four individually assigned $^1H^{\varepsilon}$-$^1H^{\delta}$ cross peaks (Fig. 3B). Sequence-specific assignments of the cross peaks (H. Iwai and K. Wüthrich, to be published) are indicated. Once the $^1H^{\varepsilon}$ and $^1H^{\eta}$ shifts are assigned, the corresponding $^{15}N^{\varepsilon}$ and $^{15}N^{\eta}$ shifts can be deduced from the FHSQC spectrum of Fig. 3A (Mori et al., 1995), provided the peaks are resolved in the ^1H dimension. For some residues two $^1H^{\eta}$ lines and only one $^{15}N^{\eta}$ line are observed, indicating rapid rotational mobility about the N^{ε}-C^{ζ} bond (Yamazaki et al., 1995). In particular, the residue Arg[10] exhibits cross peaks to two $^1H^{\eta}$ resonances with largely different chemical shifts (Fig. 3, A and B). In the three-dimensional protein structure (H. Iwai and K. Wüthrich, to be published), the guanidino group of Arg[10] is involved in a salt bridge with the side chain of Glu[35], which is a likely cause for the different $^1H^{\eta}$ shifts (Yamazaki et al., 1995).

Despite its intrinsic low sensitivity the 2D HE(NE)HGHH experiment works also for larger proteins. 2D HE(NE)HGHH (Fig. 4B) and 2D FHSQC spectra (Fig. 4A)

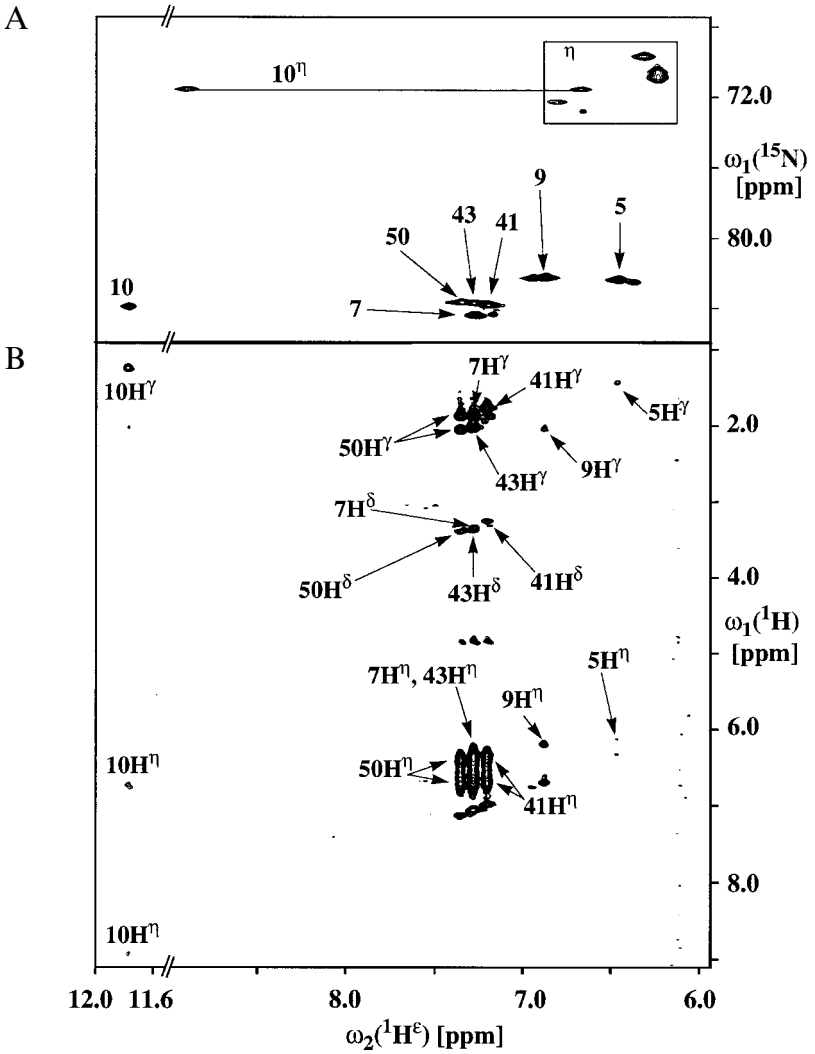

Fig. 3. Two-dimensional NMR spectra recorded with a 5 mM sample of a uniformly ^{15}N-labeled mutant form of the N-terminal 63-residue DNA-binding domain of the 434 repressor, which contains seven Arg residues (solvent 90% H_2O/10% D_2O, pH = 4.8, T = 13 °C). The spectra were recorded on a Bruker DRX 500 spectrometer operating at 500 MHz ^1H frequency. (A) 2D FHSQC experiment (Mori et al., 1995) adapted for Arg side chain detection: the ^{15}N carrier frequency was set at 75 ppm, and the first and last 180° pulses on ^{15}N have a Gaussian shape of 2.0 ms duration to avoid ^{15}N excitation for the polypeptide backbone, Asn and Gln. A data matrix of 128 (t_1) × 1024 (t_2) complex points was collected, yielding $t_{1max}(^1H) = 112$ ms and $t_{2max}(^1H) = 113$ ms. The total measuring time was about 2 h. The Arg N^{ε}–H^{ε} cross peaks are identified by the sequence positions. In the upper part some Arg N^{η}–H^{η} cross peaks are seen and the individual assignment of this correlation is indicated for Arg[10]. (B) 2D HE(NE)HGHH spectrum recorded with the pulse scheme of Fig. 2. Data size 128 (t_1) × 1024 (t_2) complex points, t_{1max} (^1H) = 14 ms, t_{2max} (^1H) = 113 ms. The total measuring time was about 12 h. The spectra were processed using the Bruker program XWINNMR and analysed with the program XEASY (Bartels et al., 1995). Sequence-specific assignments of the individual cross peaks (H. Iwai and K. Wüthrich, to be published) are indicated with the sequence positions and the types of hydrogen atoms that are correlated with $^1H^{\varepsilon}$.

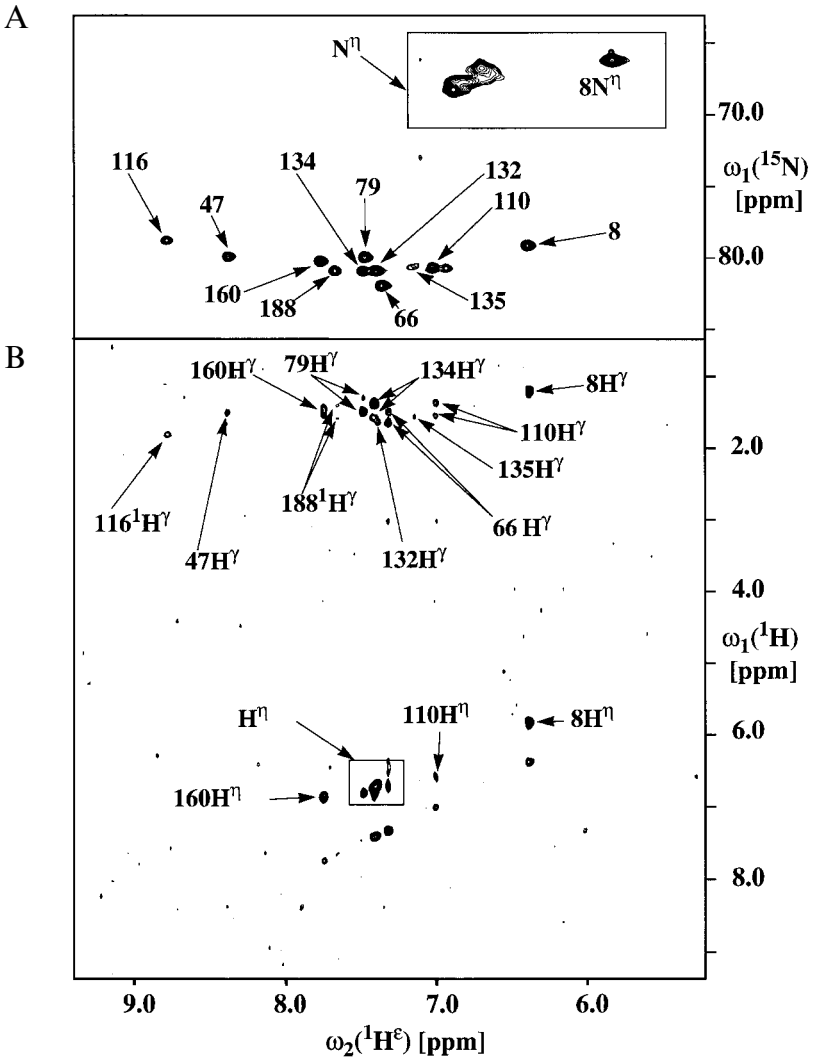

Fig. 4. Two-dimensional NMR spectra obtained with a 1 mM sample of the uniformly ^{15}N-labeled periplasmic chaperone FimC, which contains 11 arginines among its 205 residues (solvent 90% H_2O/10% D_2O, pH = 5.0, T = 38 °C). (A) FHSQC experiment (Mori et al., 1995) adapted for Arg side chain detection as described in the legend to Fig. 3. (B) 2D HE(NE)HGHH spectrum. The spectra were recorded on a Bruker AMX600 spectrometer operating at 600 MHz ^1H frequency. The set-up was as described in Figs. 2 and 3, except that the first water-selective pulse (* in Fig. 2) was omitted and ϕ_3 was –x throughout (Fig. 2). The measuring times were about 2 h for (A) and 36 h for (B). The sequence-specific assignments were obtained from overlap with independently obtained $^1H^{\gamma}$ assignments (M. Pellecchia, P. Güntert, R. Glockshuber and K. Wüthrich, to be published) and are indicated as in Fig. 3.

of the periplasmic chaperone FimC from *E. coli*, a protein of 205 residues with a molecular weight of 23 kDa, were obtained with a 1 mM sample of the uniformly ^{15}N-labeled protein. The correlations between $^1H^{\varepsilon}$ and $^1H^{\gamma}$ of all 11 arginine residues present in FimC are observed as separate lines (Fig. 4B), and individual $^1H^{\varepsilon}$-$^1H^{\eta}$ correlations could be assigned for three residues. The sequence-specific assignments were obtained on the basis of the independently determined $^1H^{\gamma}$ assignments (M. Pellecchia, P. Güntert, R. Glockshuber and K. Wüthrich, to be published). The $^{15}N^{\varepsilon}$ chemical shifts of all 11 arginines and the $^{15}N^{\eta}$ shift of Arg^8 could be deduced from the FHSQC spectrum (Fig. 4A). The appearance of the spectrum in Fig. 4B indicates that the sensitivity of the 2D HE(NE)HGHH experiment is comparable with that of triple resonance experiments that have recently been introduced for arginine side chain detection (e.g., Yamazaki et al., 1995). Comparison with Fig. 3 shows that the N^{ε}-H^{ε} cross peaks in Fig. 4A and the H^{γ}-H^{ε} cross peaks in Fig. 4B are actually better resolved than in the smaller protein, which is due to the larger $^1H^{\varepsilon}$ chemical shift dispersion in FimC than in the 434 repressor DNA-binding domain. On the other hand, for the larger protein less complete data were obtained on $^1H^{\varepsilon}$-$^1H^{\eta}$ correlations. In particular, no $^1H^{\varepsilon}$-$^1H^{\eta}$ peaks could be observed in positions that would be typical for Arg in salt bridges (see Arg^{10} in Fig. 3), although formation of salt bridges with arginines has been implicated by the NMR structure determination of FimC (M. Pellecchia, P. Güntert, R. Glockshuber and K. Wüthrich, in preparation).

The practical interest of the presently introduced 2D HE(NE)HGHH experiment has to be seen in the following context: arginine side chains are frequently involved in catalytic centres of enzymes, they have structural roles in salt bridges and in hydrogen bonds, and they are often functionally important in protein–protein and in particular in protein–DNA recognition. There was thus ample motivation to develop methods for unambiguous assignment of the complete Arg spin systems. A selection of NMR triple resonance experiments to be used with uniformly $^{13}C/^{15}N$-labeled proteins have been proposed for this purpose. Thus, the 3D HNHA-Gly experiment was adapted for detection of arginine side chain $^{1}H^{\varepsilon}$-^{15}N-$^{1}H^{\delta}$ correlations (Wittekind et al., 1993). Vis et al. (1994) proposed the experiments 2D H(N)CZ and 2D H(NCZ)N to detect the correlations $^{1}H^{\varepsilon}$-$^{13}C^{\zeta}$ and $^{1}H^{\varepsilon}$-$^{15}N^{\eta}$, respectively. Yamazaki et al. (1995) presented three 2D NMR triple-resonance pulse schemes, Arg-$H^{\varepsilon}(N^{\varepsilon}C^{\delta})H^{\delta}$, Arg-$H^{\varepsilon}(N^{\varepsilon}C^{\delta})N^{\eta}$ and Arg-$H^{\eta}(N^{\eta}C^{\delta}N^{\varepsilon})H^{\varepsilon}$, which were all optimized to detect the correlations between arginine side chain spins. Finally, Rao et al. (1996) recently presented the two experiments Arg-(H)C(C)-TOCSY-$N^{\varepsilon}H^{\varepsilon}$ and Arg-H(CC)-TOCSY-$N^{\varepsilon}H^{\varepsilon}$, which correlate $^{1}H^{\varepsilon}$ with arginine side chain carbon and proton resonances, respectively, and Farmer and Venters (1996) developed pulse schemes for obtaining Arg assignments in perdeuterated $^{15}N/^{13}C$-labeled proteins. In all the aforementioned experiments the transfer of magnetization is obtained via one-bond heteronuclear scalar coupling constants, and usually a combination of several different experiments is needed in order to connect the guanidino group with the other peripheral Arg side chain resonances. The simultaneous correlation between $^{1}H^{\varepsilon}$, $^{1}H^{\eta}$ and $^{1}H^{\gamma}$ provided by the 2D HE(NE)HGHH experiment makes it, in addition to its unique position for use with proteins labeled only with ^{15}N (see below), an attractive complementation or even an alternative to the use of triple resonance experiments with $^{13}C/^{15}N$-labeled proteins.

For uniformly ^{15}N-labeled proteins the 2D HE(NE)-HGHH experiment offers for the first time a systematic, efficient pathway for assignment of the ^{1}H and ^{15}N spins in guanidino groups of Arg. This is of practical interest for work with smaller proteins, highly attractive for studies of nucleic acid complexes with small ^{15}N-labeled proteins and applicable for work with proteins of all sizes that may be available only in uniformly ^{15}N-labeled form, for example, for studies on the molecular dynamics. Assignments of the Arg guanidino groups can provide a platform for detailed studies of arginine side chain dynamics and hydrogen exchange properties, from which one can expect to elucidate in more detail the roles of Arg residues in protein structure and function, and in particular in specific intermolecular recognition.

The 2D HE(NE)HGHH data can further be useful for distinguishing $^{1}H^{\gamma}$ from $^{1}H^{\beta}$, which often resonate at similar chemical shifts (Wüthrich, 1986). In analogy with the use of the 3D HNHB experiment for studies of χ^{1}, 2D HE(NE)HGHH data might also be combined with NOE constraints for deriving χ^{4} angle restraints (Cai et al., 1995) and possibly stereospecific assignments of arginine $^{1}H^{\gamma}$ and $^{1}H^{\eta}$ resonances.

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