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Effect of Deuteration on the Accuracy of HN–HN Distance Constraints

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The effect of deuteration on the measurement of HN–HN distances in moderately sized (15 kDa) proteins is discussed. Data are presented for a 15 kDa protein which is 95% deuterated on the H_α position, and partially (70%) deuterated at other aliphatic sites. Deuteration of the protein increases the signal intensity of HN–HN cross peaks in NOESY spectra such that dipolar couplings between protons 4–5 Å apart are readily detected. Experimental data and computer simulations show that either perdeuteration or partial deuteration of the protein increases the accuracy of amide–amide distance constraints. Thus, partial deuteration can be used to obtain more accurate long-range distance constraints for structure determination by NMR.

INTRODUCTION

The incorporation of deuterium into proteins at selected sites has been traditionally used to simplify the proton NMR spectra by removal of resonance lines (*1–3*). Recently, partial deuteration has been utilized to decrease the spin–spin relaxation rates of ^{1}H spins. The overall goal of this approach is to improve resolution by a reduction in proton linewidth, but at the same time maintain a sufficiently high proton density to detect scalar and dipolar coupling between protons. The degree of change in the spin–spin relaxation rates can be controlled by randomly varying the fraction of protons replaced with deuterons (see Ref. *4* for review).

Partial deuteration also decreases the spin–spin relaxation rate of heteronuclear spins because most of the relaxation of these spins is due to the attached proton. This leads to an increase in signal intensity of triple resonance experiments, thereby permitting the application of these techniques to larger proteins for resonance assignment (*5*). Most recently, complete deuteration of proteins has been employed to minimize the spin–spin relaxation rate of heteronuclear spins. Perdeuteration of proteins has made possible the application of a wide variety of triple resonance experiments to larger proteins (30–40 kDa) to obtain assignments of N, HN, C_α, CO, and C_β resonances (*6–9*).

Perdeuteration also has a substantial effect on the transfer of magnetization by dipolar coupling (NOE) between amides. An increase in the intensity of HN–HN cross peaks in NOESY spectra occurs because of line-narrowing of the resonances as well as the restriction of magnetization transfer to amide protons (*10–13*). This increased intensity allows the detection of NOEs between protons separated by 5–6 Å. It has been suggested that HN–HN NOEs can be used to determine the global fold of large proteins (*12–14*). Additionally, it has been shown that the selective protonation of Ile, Leu, and Val residues in otherwise perdeuterated proteins can lead to NMR structures of large proteins which are of moderately high resolution (*15*).

Since perdeuteration has had a large impact on the application of NMR to large proteins, it is surprising that this labeling scheme has not been used to enhance the structure determination of smaller (10–15 kDa) proteins. The ability to measure longer distance constraints (i.e., 5–6 Å) would be advantageous. Furthermore, because the magnetization transfer pathways are restricted to amide protons, the error in converting an NOE cross-peak intensity to a distance should be reduced in deuterated proteins, thus providing more accurate distance constraints.

The reluctance in obtaining data from deuterated midsized proteins may be related in the cost of obtaining perdeuterated samples. Although growth in D_2O with deuterated acetate is relatively inexpensive and results in complete deuteration of aliphatic protons (*16*), many proteins are not highly expressed in this medium. Alternatively, proteins can be produced by growth of bacterial cultures on labeled glucose in minimal medium or in a fully deuterated rich medium (*10*), both of which are expensive. An inexpensive way of achieving partial deuteration is to simply grow bacteria in D_2O on normal carbon sources. This method results in nearly complete replacement of the H_α protons and replacement of approximately 70% of the remaining aliphatic protons (see later discussion). We show that the NOESY spectra from such partially deuterated protein has all of the attributes of spectra obtained from perdeuterated protein. Increased signal intensity is observed in these spectra, and NOE cross peaks between distant amide protons (4–5 Å) are readily observed. We also show with a combination of computer simulation and experimental data that

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the accuracy of distance constraints is improved with either perdeuteration or partial deuteration.

RESULTS AND DISCUSSION

A 130 residue protein, Rho130, was used for these studies (*17, 18*). This protein consists of an amino-terminal three helix bundle (residues 1–48) and a carboxy-terminal β-sheet domain (*17, 18*). The acquisition of the three-dimensional ^{15}N-separated NOESY spectra has been described elsewhere (*17, 18*). The levels of deuteration at aliphatic sites were estimated by analysis of 1D-^{1}H and 2D-^{13}C/^{1}H-HSQC spectra of the protein. This sample was produced using normal ^{13}C-labeled glucose as the carbon source in minimal medium, and partially deuterated by growing the cells in 95% D_2O. A comparison of the amide proton to alpha proton ratios in 1D-^{1}H spectra showed that the H_α's were approximately 95% deuterated. The estimation of the level of deuteration at other sites was obtained from the relative intensity of CHD_2, CH_2D, and CH_3 peaks in a 2D-^{13}C/^{1}H-HSQC spectrum. From these data, it was estimated that approximately 70% of the aliphatic protons were deuterated. The level of deuteration varied with the amino acid type and the site of deuteration. For example, Ser H_β were deuterated to a level of 30% while Thr H_β were deuterated to 90%. Methyl groups were deuterated to a level of ~40%.

NOE peak intensities were simulated using the program FIRM (*21*) with an overall rotational correlation time of 6×10^{-9} s. A three-step methyl group rotation with a correlation time of 5×10^{-11} s was used to model methyl rotations. The leakage rate was set to zero for these calculations. The coordinates of the protons in Rho130 were obtained by adding protons to the 1.5 Å crystal structure of Rho130 (*19*) using InsightII (Molecular Simulations Incorporated, San Diego, CA). In the case of fully deuterated protein the only protons considered in the simulations were the exchangeable protons (amide and hydroxyl). All protons were used in the simulation of NOEs in fully protonated protein. For the case of partially deuterated protein, it was necessary to consider an ensemble of molecules because of incomplete deuteration of the aliphatic carbons. For each member of this ensemble all exchangeable protons were present, and the level of deuteration at other sites was chosen randomly to give an average level of deuteration for the H_α proton of 95% and an average level for the other nonexchangeable protons of 70% over the ensemble. A total of 100 proteins made up the ensemble, and the NOE intensities for each interproton pair were averaged over the ensemble. In all cases, interproton distances were obtained from the NOE intensity using the isolated spin approximation. This assumes the peak intensity is proportional to $1/r^6$. The scaling factor for conversion of intensities to distances was obtained from NOE intensities for a set of protons separated by 2.7 Å.

There are two anticipated effects of deuteration on HN–HN NOEs: an increase in overall peak intensity and a more reliable estimation of interproton distances from these intensities. The first effect, increased intensity, has been observed experimentally by others and is illustrated in Fig. 1. Note that the spectra for protonated and partially deuterated Rho130 samples were taken using the same total aquisition times. While the T_1 relaxation times of the amide protons in the deuterated proteins are longer than those for protonated proteins, there was still a significant increase in NOE cross-peak intensity for the partially deuterated protein. Thus, in the partially deuterated sample, the loss in sensitivity due to a relatively short (compared to T_1) recycle delay is negligible as compared to the overall increased intensity of the partially deuterated NOE cross peaks.

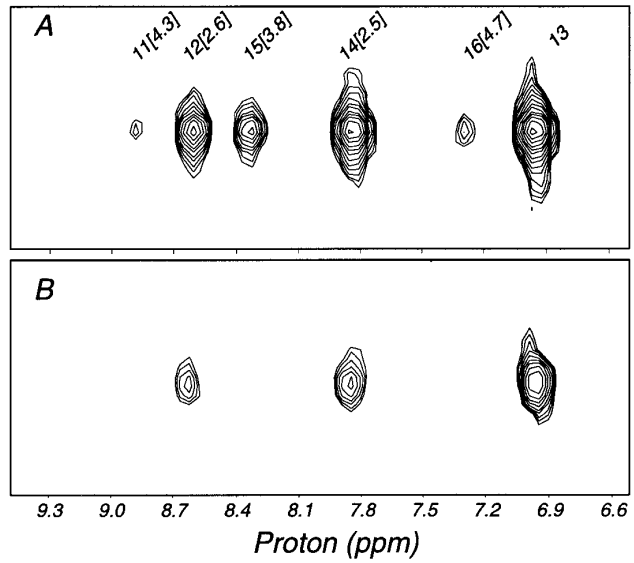

FIG. 1. Deuteration increases the intensity of HN–HN NOEs. A portion of an HN–HN HSQC-NOESY obtained on partially deuterated Rho130 (A) and fully protonated Rho130 (B) are shown. The spectra show NOEs from Glu13 to the amide protons of the indicated residues (*11, 12, 14, 15, 16*). The distance from the HN proton of Glu13 to the residues is contained within the square brackets. Glu13 is the third residue in an α-helix. The spectra for panel A were obtained from a 1.0 mM sample on a 600 MHz Bruker DRX spectrometer using a mixing time of 180 ms. The spectra in panel B were obtained from a 1.5 mM sample on a 500 MHz Varian Unity Plus spectrometer using a mixing time of 120 ms.

NOESY spectra of the partially deuterated protein (Fig. 1A) shows peaks between amides which are separated by as much as 4.7 Å while the same region of the protonated sample only shows peaks for amides which are separated by 2.6 Å (Fig. 1B). This observed increase in intensity is also seen in the numerical simulations (see Table 1). The increase in cross-peak intensity for the deuterated protein is largest for shorter interproton distances at longer mixing times. In a fully protonated sample the magnetization from HN protons is transferred to aliphatic protons in close proximity to the amide instead of being transferred to other amide protons. This effect is especially large for long mixing times, leading to an overall decrease in the intensity of the cross peak for interproton distances in the range of 2–4 Å. For both the fully deuterated and the partially deuterated sample, the intensities of HN–HN cross

TABLE 1
NOE Peak Intensity[a]

Mixing time (ms)	100			150			200			250		
HN–HN distance (Å)	D	PD	P	D	PD	P	D	PD	P	D	PD	P
2–3	872	778	527	1163	985	558	1390	1120	537	1560	1204	494
3–4	182	173	141	272	251	177	358	318	195	437	374	201
4–5	50	48	50	77	73	71	105	96	86	133	118	96
5–6	20	19	21	30	29	31	41	41	42	53	53	50
2–6	249	224	163	333	286	182	404	336	188	464	373	186

[a] NOE peak intensities are shown in arbitrary units. D, fully deuterated; PD, partially deuterated; P, protonated.

peaks are two- to threefold higher than the corresponding peaks from a fully protonated sample for distances in this range.

As the interproton distance increases, the intensity difference of cross peaks for the deuterated and protonated samples becomes smaller. In fact, for NOEs from protons separated by 5–6 Å, the cross-peak intensities in all three samples are predicted to be similar. This relative increase in the intensity of cross peaks in the protonated sample is due to the relay, or spin diffusion, of magnetization from one HN proton to another via an intervening, non-amide, proton. The preceding analysis does not consider the effect of deuteration on the linewidths of the cross peaks, which results in a factor of 2–3 increase in the signal-to-noise for deuterated samples versus protonated samples. Therefore, cross peaks from either fully deuterated or partially deuterated proteins will be three- to sixfold more intense than corresponding peaks in protonated samples.

Deuteration also increases the accuracy of interproton distances calculated from NOE crosspeak intensities. Figure 2 shows scatter plots of the actual interproton distance versus the measured interproton distance for fully deuterated, partially deuterated, and fully protonated samples. These plots show that partial or full deuteration results in a smaller discrepancy between the actual versus measured distance. This effect is more pronounced at larger distances and with longer mixing time. Table 2 shows the effect of deuteration levels and mixing time on the agreement between the interproton distances from the model structure and the distances calculated from NOE peak intensities. As expected, in the case of short interproton

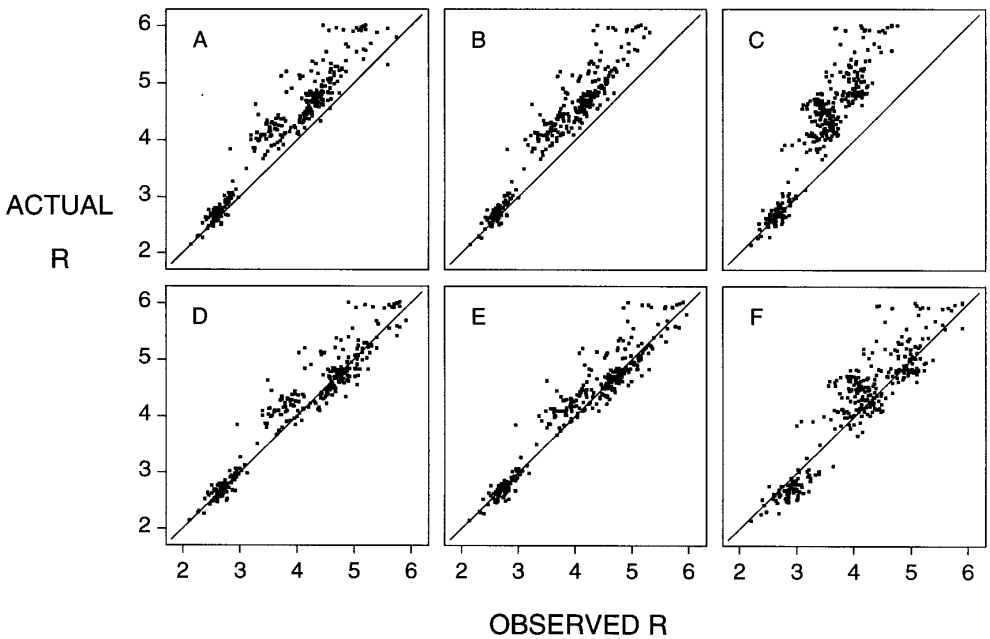

FIG. 2. Effect of deuteration on distance measurements. The actual HN–HN distance is plotted versus the HN–HN distance obtained from the simulated NOE intensities. Points are shown for all interamide distances of 6 Å or less. The mixing time used in these simulations was 200 ms. (A) Results obtained for fully deuterated protein. (B) Results of calculations for partially deuterated protein. (C) Results obtained for fully protonated protein. (D–F) Effect of applying a correction factor of the form, $R = \gamma(R - 2.2) + 2.2$ to the data presented in panels A–C. γ is 1.2, 1.25, and 1.5 for the deuterated, partially deuterated, and protonated sample, respectively.

TABLE 2
Real Space *R* Factors[a]

Mixing time (ms)	100			150			200			250		
HN–HN distance (Å)	D	PD	P	D	PD	P	D	PD	P	D	PD	P
2–3	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.04	0.04	0.04
3–4	0.06	0.07	0.09	0.09	0.10	0.12	0.11	0.12	0.15	0.13	0.14	0.16
4–5	0.05	0.06	0.12	0.07	0.09	0.16	0.09	0.11	0.19	0.11	0.13	0.22
5–6	0.07	0.09	0.16	0.11	0.12	0.21	0.13	0.15	0.25	0.15	0.18	0.29
2–6	0.05	0.06	0.11	0.07	0.09	0.15	0.09	0.11	0.18	0.11	0.13	0.21

[a] The real space *R* factor

$$R = \sum_{i=1}^{N} |d - d_0| / \sum_{i=1}^{N} d_0$$

was calculated for the indicated shells of interproton distances. d_0 is the distance based on the crystal structure of Rho130. d was calculated for each NOE mixing time and is the distance calculated from the simulated NOE intensity using the standard $1/r^6$ relationship. In these calculations the protein was fully deuterated (D), partially deuterated (PD), or fully protonated (P).

distances (2–3 Å), all three levels of deuteration show an *R* factor of 3–4% at all mixing times. In all cases the *R* factor increases as the interproton distance becomes larger or the mixing time increases. However, it is clear that the *R* factors for either the fully or partially deuterated samples are significantly lower than those obtained for the fully protonated sample. For example, consider the *R* factors for distances of 4–5 Å at a mixing time of 100 ms. Note that protonated protein distances have an *R* factor of 0.12, whereas partially deuterated protein shows an *R* factor of 0.06. Most importantly, note that the *R* factors are similar for both deuterated samples, indicating that complete removal of the H_α proton as well as 70% of the remaining aliphatic protons are sufficient to attenuate spin diffusion for all practical purposes.

In practice, the choice of a mixing time should include a consideration of the desired error in the distances which are obtained from the peak intensities. Further, these errors should be the basis of error limits on the distance constraints. For example, if a 10% error is desired for interproton distances in the range of 4–5 Å, a 100 ms mixing time should be used for a fully protonated sample. However, a 200 ms mixing time would give the same error in the case of either partially or fully deuterated samples (see Table 2). Under these conditions, the peak intensities for the deuterated samples would be at least twofold greater than those found in the protonated sample (see Table 1).

Additional improvement in the accuracy of distance measurements can be obtained by the empirical adjustment of measured distances to account for the effects of spin diffusion (*20*). The application of this correction to the simulated data can reduce further the disparity between the true distance and the measured distance. The success of the application of the corrective factor on the distances is different for deuterated and protonated proteins (see Fig. 2, panels D–F). In the case of the deuterated samples, the distribution of the data around a line of unit slope becomes more symmetrical after application of a correction factor. However, for the case of the protonated data, it is not possible to use a linear scaling factor to accomplish this over the entire range of distances. Therefore, data from either perdeuterated or partially deuterated samples is more amenable to correction by a linear scaling factor than data from protonated samples.

The predicted increase in accuracy for NOE distance measurements after scaling is also realized experimentally. In the NMR structures of Rho130 (*18*), the NOE *R* factor (*21*) before relaxation matrix refinement is 20% for the amide–amide constraints obtained from the partially deuterated sample and 45% for constraints obtained from the protonated sample. These values are higher than those presented in Table 2 because they also contain a contribution from the experimental signal-to-noise.

In summary, the use of partially deuterated samples is an economical way to enhance the information content of NOESY spectra. The increased signal intensity in NOESY spectra permits the measurement of dipolar coupling between distant amide protons. More importantly, the accuracy of distance measurements is significantly improved. With linear scaling of the data to account for residual spin diffusion effects it is possible to lower the *R* factor for long range distances (4–6 Å) to less than 10%, thus providing accurate long-range structural constraints.

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