Four-Dimensional ^{15}N-Separated NOESY of Slowly Tumbling Perdeuterated ^{15}N-Enriched Proteins. Application to HIV-1 Nef

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Dipolar interactions are the main source of ^{1}H line broadening in protein NMR. It has long been recognized that this dipolar broadening can be reduced by deuteration.[1−7] However, deuteration reduces the number of ^{1}H spins available for ^{1}H detection, and the sensitivity gain associated with a decrease in ^{1}H line width is therefore offset by the depletion of protons. However, when an NMR study of a perdeuterated protein in H_2O solution is conducted, the exchangeable backbone amides are protonated and their resonances are narrowed about 2-fold by the decrease in homonuclear dipolar interactions. In NOESY experiments, deuteration therefore results in a much slower decay of the diagonal amide intensity as a function of mixing time and makes it possible to increase sensitivity by the use of long mixing times.[6] Spin diffusion effects will occur under these conditions, but as the average interproton distance for these exchangeable nuclei is considerably larger than the average interproton distance in a nondeuterated protein, these effects remain manageable and interpretable. A previous report emphasized the utility of such an approach for delineating α-helical domains in proteins.[6] Here we demonstrate that a four-dimensional (4D) version of the NOESY experiment can be applied successfully to obtain a large number of H^N−H^N NOE interactions in a N-terminal deletion mutant of the HIV-1 protein nef.

Aggregation problems of HIV-1 nef were reported in a previous NMR study.[9] For the N-terminal deletion mutant, nef-(39−206), used in the present study, we find aggregation to be reduced at low ionic strength and high pH values. ^{1}H and ^{15}N relaxation indicates a rotational correlation time, τ_c, of 16 ns at pH 8, 35 °C, using a 600 μM sample concentration. A decrease in sample concentration further reduces τ_c but leads to sensitivity problems when NOESY spectra are recorded. In the absence of deuteration, *J*-correlation-based sequential backbone assignment of such a relatively dilute and slowly tumbling protein presents a major challenge. In contrast, triple resonance experiments on a deuterated protein with $\tau_c = 16$ ns are readily feasible and yield high-quality spectra.[7,10] Here we demonstrate that structurally useful information can be obtained from the NOE interactions between H^N protons in perdeuterated nef.

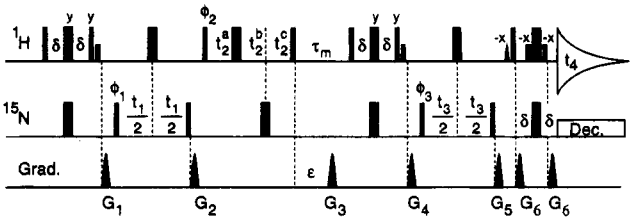

Figure 1. Pulse sequence of the ^{15}N-separated HSQC−NOESY−HSQC experiment. Narrow and wide pulses correspond to flip angles of 90° and 180°, respectively. Low-power ^{1}H pulses are applied to the H_2O resonance ($\gamma B_2 = 200$ Hz). The sine-bell-shaped $90°_{-x}$ pulse has a duration of 2.4 ms. Delays: $\delta = 2.25$ ms; $\epsilon = 170$ ms; $\tau_m = 200$ ms. For the first t_2 increment, $t_2^a = t_2^c = 2.25$ ms and $t_2^b = 0$ and increments are −90 μs (t_2^a), 326.6 μs (t_2^b), and 416.6 μs (t_2^c). The spectrum is recorded as a 19* (t_1) × 24* (t_2) × 19* (t_3) × 512* (t_4) matrix, with spectral widths of 912 Hz (F_1 and F_3), 1200 Hz (F_2), and 9259 Hz (F_4), and the rf carrier on H_2O. Unless indicated, all pulses are applied along the *x* axis. Phase cycling: $\phi_1 = x, -x$; $\phi_2 = 45°$; acquisition = $x, -x$. Quadrature detection in the t_1, t_2, and t_3 dimensions is achieved using the States-TPPI protocol, changing ϕ_1 (t_1), ϕ_2 (t_2), and ϕ_3 (t_3) in the ususal manner. Gradients are sine-bell shaped with an amplitude of 20 G/cm at their center and durations of $G_{1,2,3,4,5,6} = 2.15$, 1.35, 5.0, 3.0, 1.0, and 0.3 ms.

The pulse sequence of the 4D ^{15}N/^{15}N-separated NOESY experiment (Figure 1) is very similar to that of 4D ^{13}C/^{13}C-separated NOESY,[11] except for a mixed-constant-time[12] t_2 evolution period and the use of selective H_2O pulses, which ensure that the water magnetization is aligned along the *z* axis during the application of pulsed field gradients.[13] Radiation damping during the first fraction, ϵ, of the NOE mixing period, τ_m, also returns H_2O magnetization to the *z* axis. Experimentally, we find that, at the start of ^{1}H data acquisition, ~75% of the equilibrium H_2O magnetization has been returned to the +*z* axis. As the nef study is carried out at an unusually high pH, amide exchange with solvent is relatively rapid and replenishes the amide proton magnetization during the delay between transients.[13−16] This is particularly important for a perdeuterated protein where the amide ^{1}H T_1 value is 2−3-fold longer relative to the protonated protein. Although we had expected that a gradient-enhanced coherence selection version for the last HSQC step would yield improved sensitivity,[14−16] we were unable to achieve a sufficiently high flip-back of H_2O magnetization to the *z* axis with our 8-mm probe setup, and so a conventional water-flip-back HSQC[13] was used instead.

To facilitate interpretation of the 4D NOESY spectrum, it is important to record it at the highest possible resolution. Using a two-step phase cycle combined with pulsed field gradients for artifact suppression, acquisition times in the t_1, t_2, t_3, and t_4 dimensions were 21, 20, 21, and 55 ms. These acquisition times are relatively long compared to those in most other 4D NMR studies and result in high spectral resolution, and therefore all NOE cross peaks could be assigned uniquely to the corresponding pairs of interacting amide protons.

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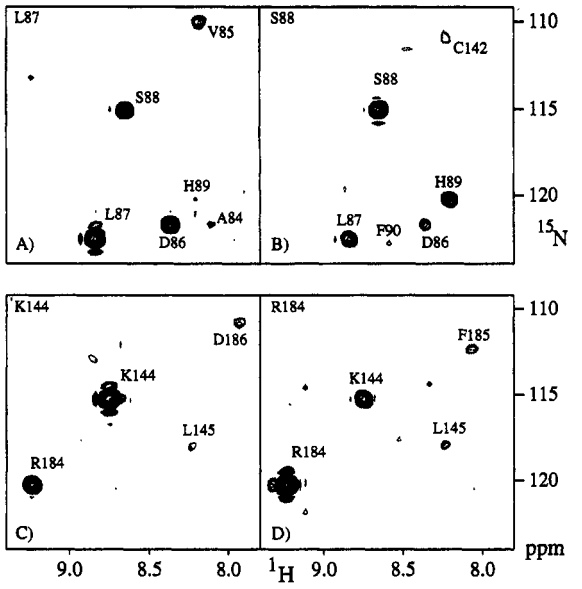

Figure 2. (F_1, F_2) cross sections through the 600-MHz 4D NOESY spectrum of HIV-1 nef(39−206) (0.6 mM, pH 8.0, 35 °C, 200 ms NOE mixing), taken at the (F_3, F_4) frequencies of the amides of (A) Leu^{87}, (B) Ser^{88}, (C) Lys^{144}, and (D) Arg^{184}. The protein was deuterated at a level of >95% for the C$^{\alpha}$ sites, and at *ca.* 85−90% for most side chains, by using *Escherichia coli* expression in a medium containing 98% D_2O, $^{15}NH_4Cl$, and M9 minimal medium. It was processed with the program NMRPipe,[17] using linear prediction in the F_3 dimension and zero filling to 64 × 64 × 64 × 1024. Dashed contours correspond to resonances which have been aliased in the F_1 dimension. The spectrum was recorded using a Nalorac 8-mm triple-resonance PFG probe and a Bruker AMX-600 spectrometer, equipped with a home-built gradient power supply (R. Tschudin, unpublished results).

Figure 2 shows (F_1, F_2) cross sections taken through the 4D spectrum at the (F_3, F_4) frequencies of the amides of Leu^{87}, Ser^{88}, Lys^{144}, and Arg^{184}. Residues Leu^{87} and Ser^{88} are located in one of the long and well-defined α-helices and correspondingly exhibit strong $d_{NN}(i,i\pm1)$ NOE cross peaks to the preceding and following amides. Weaker $d_{NN}(i,i\pm2)$ and $d_{NN}(i,i\pm3)$ connectivities, which contain a significant contribution from spin diffusion, can also be observed. However, it is important to note that, even for the long NOE mixing time used (200 ms), direct interactions dominate the spectrum, as evidenced by the weakness of the cross peak between Leu^{87} and His^{89} (Figure 2A) and by strong NOE interactions between Leu^{87} and Ser^{88}, and also between Ser^{88} and His^{89}. A structurally very informative NOE between Ser^{88} and Cys^{142} amides in Figure 2B does not contain a significant spin diffusion contribution as these amides do not exhibit strong NOEs to a common third amide proton.

Panels C and D of Figure 2 show H^N-H^N NOE connectivities observed for the amides of Lys^{144} and Arg^{184}, which are part of a short triple-stranded antiparallel β-sheet. Both cross sections show a strong NOE between Lys^{144}-H^N and Arg^{184}-H^N. Both amides also show a weak NOE to the sequential amide, but the NOE to their preceding amide is missing. Distances between sequential H^N protons in an antiparallel β-sheet are typically *ca.* 4.5 Å, and the 4D spectrum indicates that the cutoff range for observing NOE cross peaks in the present experiment is at this distance.

The H^N-H^N NOEs observed for residues 76−140 are summarized in Figure 3. The short-range NOE pattern confirms

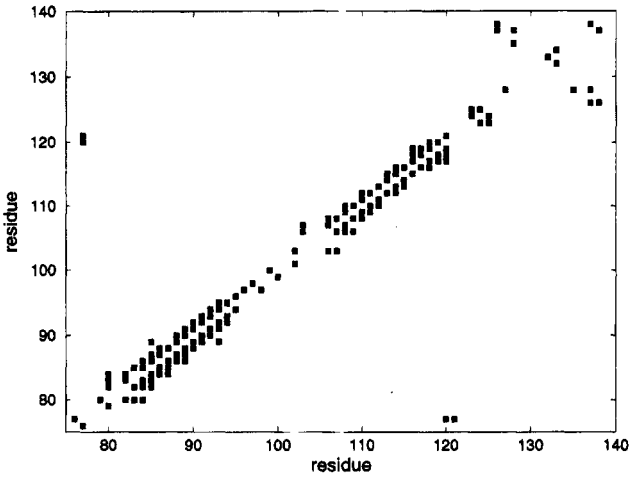

Figure 3. Matrix representation of the H^N-H^N NOEs observed for residues 76−140.

the presence of two α-helices, spanning from Tyr^{81} to Glu^{93} and from Arg^{105} to His^{116}, followed by a short antiparallel β-sheet. Interestingly, a pair of long-range NOEs is observed between the N-terminus of the first helix and what appears to be a 3−10 helical extension at the C-terminus of the second α-helix, suggesting an antiparallel arrangement.

Perdeuteration of a protein significantly narrows the ^{13}C and amide proton resonances and extends the triple resonance *J*-correlation methodology to proteins with correlation times too long for standard multidimensional NMR.[7,8,10] The 4D H^N-H^N NOE experiment, demonstrated in the present work, complements these methods for making sequential assignments as the vast majority of amides with hydrogen exchange rates of less than *ca.* 20 s^{-1} show $d_{NN}(i,i+1)$ NOEs. The good spectral dispersion of the $^1H^N-^{15}N$ shift correlation map and the high resolution at which the 4D NOESY spectrum can be recorded make spectral interpretation particularly simple. However, most importantly, relatively long interproton distances can be measured with little spin diffusion. Therefore, not only is it possible to determine the secondary stucture, but one can also obtain the global fold of the protein.[18] The side-chain $N^{\epsilon1}-H^{\epsilon1}$ resonances of the five Trp residues in nef are also found to be useful in this respect.

Calculations indicate that 4D H^N-H^N NOESY on perdeuterated proteins will yield high-quality spectra up to correlation times of ~25 ns. It therefore may become possible to use this approach for obtaining structural information on slowly tumbling proteins such as, for example, detergent-solubilized domains of integral membrane proteins.

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Supporting Information Available: One figure, comparing the ^{15}N-filtered 2D H^N-H^N NOE spectra obtained for deuterated and protonated ^{15}N-labeled nef (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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