A Simple Method to Distinguish Intermonomer Nuclear Overhauser Effects in Homodimeric Proteins with C_2 Symmetry

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Characterization of the structure of homodimeric proteins by NMR has proven to be particularly challenging due to the difficulty in distinguishing intermonomer from intramonomer contacts. The problem is magnified when the monomers are arranged in parallel, as in coiled coil dimers. There have been few successful attempts to characterize homodimers by NMR. Structures of homomultimers, which have been solved using NMR,[1−7] have employed notable experimental and computational strategies. Here, we present an efficient and straightforward approach to identify protein−protein or, more generally, protein−ligand contacts using NMR. The method is particularly useful for the characterization of dimers with C_2 symmetry. It relies on mixing completely deuterated[8] ^{15}N-labeled protein with protonated unlabeled protein. In a 3D ^{15}N-dispersed NOESY-HSQC spectrum of this mixed dimer, all cross peaks between NH and aliphatic protons must be intermonomer contacts. We have used this method to characterize the dimerization interface of an N-terminal fragment of the dimeric yeast transcriptional activator PUT3.[9]

Numerous approaches have been used to identify intermonomer contacts in dimeric proteins. One class of experiments uses asymmetric isotope labeling, in combination with isotope editing techniques,[10−19] to resolve intermonomer nuclear Overhauser effects (NOEs) from intramonomer NOEs of homodimeric proteins.[3−5] These experiments involve mixing ^{13}C-labeled protein with unlabeled protein to produce a heterodimer. ^{13}C half-filtered experiments, which contain a ^{13}C selection period followed by a ^{13}C purge filter, are performed on the heterodimer to identify NOE interactions from the side chains of the ^{13}C -labeled monomer to the side chains of the unlabeled monomer.

Another method for characterizing intermonomer contacts uses two-dimensional NOESY spectra on proteins which are selectively deuterated by residue type.[1,20] Samples with different residue types deuterated are mixed, and 2D NOESY spectra are acquired on the resultant heterodimer. Intermonomer NOESY cross peaks involving the deuterated residues decrease in intensity upon mixing. Furthermore, computational methods whereby the protein symmetry is maintained during the structure calculations have been used to characterize intermonomer contacts in homodimers.[7]

We have developed a simple approach to unambiguously obtain distance constraints across the dimer interface. The first step in this approach is to prepare completely deuterated ^{15}N-labeled protein dimer. The dimer is then dissolved in H_2O to allow amide exchange. A 1D spectrum is acquired in which only amide resonances are observed (Figure 1). Furthermore, only amide to amide cross peaks are observed in NOESY spectra as the region where amide to aliphatic cross peaks reside is entirely empty (Figure 2A). The deuterated ^{15}N-labeled protein is then mixed with unlabeled protonated protein to produce a mixture of homodimers and heterodimers. After mixing, all cross peaks in the region connecting the NH with aliphatic resonances (upper left-hand quadrant of the NOESY) must be intermonomer NOEs. These cross peaks are best resolved using a 3D ^{15}N-dispersed NOESY-HSQC (Figure 2B). Complete deuteration is essential for this approach, but can readily be achieved (see below).

The general protocol for producing and purifying PUT3 has been published previously.[9] The cells were adapted to deuterium in the following way. PUT3 plasmid was transformed into *Escherichia coli* strain BL21 (DE3) pLysS, and the cells were grown at 37 °C in M9 media with 33% D_2O/ 67% H_2O containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) overnight. They were then added in a 1:100 ratio by volume to M9 media containing 50% D_2O/ 50% H_2O and grown at 37 °C overnight. A 1:100 ratio by volume of the cells adapted to 50% D_2O was added to M9 media containing 99.99% D_2O and given deuterated sodium acetate (at 6 g/L) as the sole carbon source[8] and 99% ^{15}N-labeled ammonium chloride (at 1g/L) as the sole nitrogen source. After 83 h, the cells grew to 0.5 absorbance at 600 nm. $ZnSO_4$ was added to the culture to a final concentration of 20 μM, and the cells were induced, harvested, and purified.[9] With this procedure we were able to achieve 98% deuteration (Figure 1).

To ensure that the NH-to-CH cross peaks of the mixed heterodimer were not due to residual (<2%) aliphatic protons, we recorded a 3D NOESY-HSQC prior to mixing the labeled and unlabeled protein. WATERGATE water suppression[21] and an optimization of the experiment as described in ref 22 were used (mixing time 200 ms). After mixing, this experiment was repeated on the same spectrometer with identical conditions. A Shigemi tube was used to bring the total concentration of PUT3 dimer to 2 mM, which consists of ∼1 mM heterodimer.

Coiled coil dimerization interfaces with C_2 symmetry are among the most common known DNA binding motifs. These dimers contain helical monomers arranged in parallel resulting in intermonomer contacts between symmetry-related protons which reside along the diagonal of NOESY spectra. Coiled

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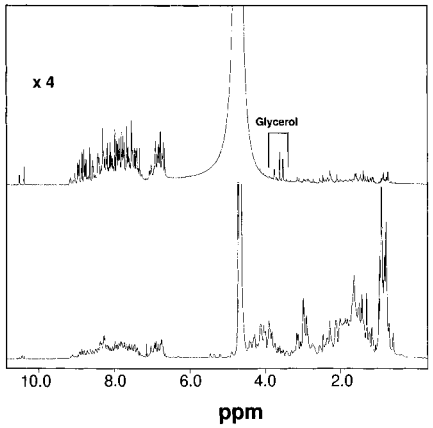

Figure 1. 1D spectra acquired at 750 Mz of deuterated ^{15}N-labeled (top) and unlabeled (bottom) PUT3. Very weak solvent presaturation was applied to avoid saturation transfer of the amide protons. The spectrum of the deuterated ^{15}N-labeled protein is magnified four times relative to the spectrum of the unlabeled protein. Peaks from residual glycerol are marked in the top spectrum. Comparison of the height of the methyl peaks indicates that the deuteration is greater than 98%.

coil dimers maximize the number of ambiguous NOEs as many cross peaks may contain both intermonomer and intramonomer contributions. Consequently, the structure of these proteins is particularly challenging to study using NMR.

The approach described here is more sensitive and has better resolution than techniques relying on ^{13}C -labeling and isotope-filtering experiments. The latter experiments select for cross peaks between ^{13}C -attached protons and ^{12}C-bound protons. In proteins with C_2 symmetry, many of the strongest intermonomer NOEs are between side chain protons which are symmetry-related partners. Consequently, these cross peaks disappear in the diagonal (Figure 3A). The method described here is superior for dimers with C_2 symmetry as the cross peaks between the ^{15}N-bound amide protons and the aliphatic protons are remote from the diagonal and are unambiguously due to intermonomer contacts (Figure 3B). Furthermore, the pulse sequence of the 3D ^{15}N-dispersed NOESY-HSQC is significantly shorter than the ^{13}C half-filter experiments which contain two editing/selection modules on both sides of the NOESY. Finally, since spin diffusion is greatly reduced in deuterated proteins, a long NOESY mixing time can be used to measure distances greater than 5 Å.[23]

In the 3D NOESY-HSQC spectrum taken before mixing the deuterated ^{15}N-labeled protein with unlabeled protein, we observed slight protonation of the methyl group of two of the valines, the γ-protons of arginine and lysine, and the δ-proton of lysine. The protonation was very slight, however, as it was barely above the level of the noise. Similarly, deuteration of another protein with deuterated sodium acetate as the sole carbon source using BL21(DE3) resulted in slight protonation of the methyl protons of alanine, leucine, and isoleucine.[24]

Although a complete analysis of the NOESY-HSQC spectrum of the heterodimer is still in progress, we can conclude that we observe over 50 intermonomer cross peaks, most of which were from methyl protons. However, the sensitivity of this experiment was high enough to distinguish a few NOE cross peaks to β-protons.

We attempted to use the ^{13}C half-filtered NOESY experiments to identify intermonomer contacts. We recorded three different variations of ^{13}C half-filtered NOESY spectra.[17,18,25] We found the resolution and sensitivity of ^{13}C half-filtered experiments

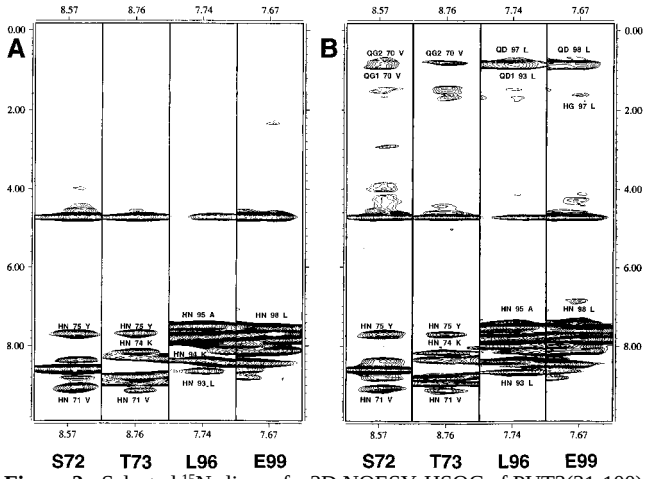

Figure 2. Selected ^{15}N slices of a 3D NOESY-HSQC of PUT3(31-100) (A) deuterated ^{15}N-labeled homodimer and (B) mixed heterodimer. The deuterated ^{15}N-labeled homodimer shows cross peaks from the amide protons to other amide protons and H_2O. The mixed heterodimer, however, also contains cross peaks to aliphatic protons of the nondeuterated monomer. Amide proton assignments are provided at the bottom of each 2D slice and the cross peak assignments are indicated within the spectrum.

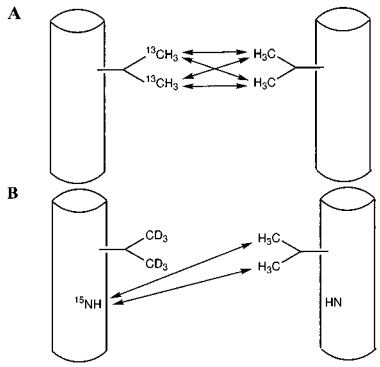

Figure 3. Schematic diagram illustrating NOEs of interest in (A) the ^{13}C half-filtered experiments and (B) the NOESY-HSQC experiment. In A, the peaks to be analyzed in the ^{13}C half-filtered experiments would be primarily along the NOESY diagonal. To unambiguously identify an intermonomer NOE, it has to be ascertained that the peak observed is not due to an imperfect half-filter. In B, NOEs over larger distances can be observed because of deuteration of the ^{15}N-labeled monomer. Furthermore, all NOEs to non-amide protons must be intermonomer.

insufficient to distinguish the intermonomer NOEs. However, our method does not allow one to obtain unambiguous intermonomer NOEs for alipathic to aliphatic and amide to amide protons. ^{13}C half-filtered experiments could provide this complementary information. Arrowsmith *et al.*[1] used selective deuteration by residue type to identify NOEs which exist only as intermonomer contacts in the *E. coli* trp repressor. However, atoms which have intermonomer NOE interactions in coiled coil proteins also have intramonomer NOE interactions. Hence, the resultant decrease in NOE intensity would be more difficult to observe. The nature of the coiled coil dimer required experiments with higher sensitivity and resolution than these experiments. Our approach is highly suited for the characterization of protein−protein contacts in general. We were able to solve the structure of PUT3 using only the side chain-to-backbone intermonomer NOEs provided by this experiment. The high sensitivity and resolution of the 3D ^{15}N-dispersed NOESY-HSQC experiment coupled with the simplicity and straightforward nature of this technique make it ideal for studying protein−protein interactions.

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