Improved NMR Structures of Protein/Ligand Complexes Using Residual Dipolar Couplings

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Three-dimensional structures of protein/ligand complexes have played an important role in discovering new pharmaceutical agents.[1,2] When used for drug discovery, structures must be accurately defined to guide the synthesis of ligands with improved shape and charge complementarity to the binding pocket. Although NMR structures of protein/ligand complexes could be used to aid in ligand design, structures that are obtained from NOE-derived short-range distance restraints are not always well-defined by the NMR data. Recently, improvements in the accuracy and precision of NMR structures of proteins have been obtained using additional restraints derived from residual dipolar couplings.[3] Dipolar couplings yield the orientations of bond vectors relative to the molecular alignment tensor and thus provide long-range structural information.[4−8] One-bond ^{15}N−H residual dipolar couplings have also been used to orient two proteins relative to each other in the structure determination of a protein/protein complex.[9] Here we describe the use of residual dipolar couplings in the structure determination of a small, unlabeled ligand complexed to an isotopically labeled protein. Using this approach, more precise and more accurate structures of protein/ligand complexes can be determined by NMR, which is important for structure-based ligand design.

The strategy involves supplementing the intra- and intermolecular NOEs which define the atoms in close proximity with residual dipolar couplings to yield the relative orientation of the protein and the ligand in the complex. Ideally, heteronuclear residual dipolar couplings would be measured using isotopically labeled protein and ligand. However, unlike proteins which can be ^{15}N- and ^{13}C-labeled using standard methods, small organic molecules that serve as drug leads cannot generally be isotopically labeled. Therefore, the ^{13}C−^{1}H residual dipolar couplings for the ligand must be measured at natural abundance. To suppress the signals of the carbon-attached protons of the protein, perdeuteration is necessary. The protein must also be uniformly ^{15}N-labeled for measuring the ^{15}N−^{1}H residual dipolar couplings to allow the ligand to be oriented with respect to the protein.

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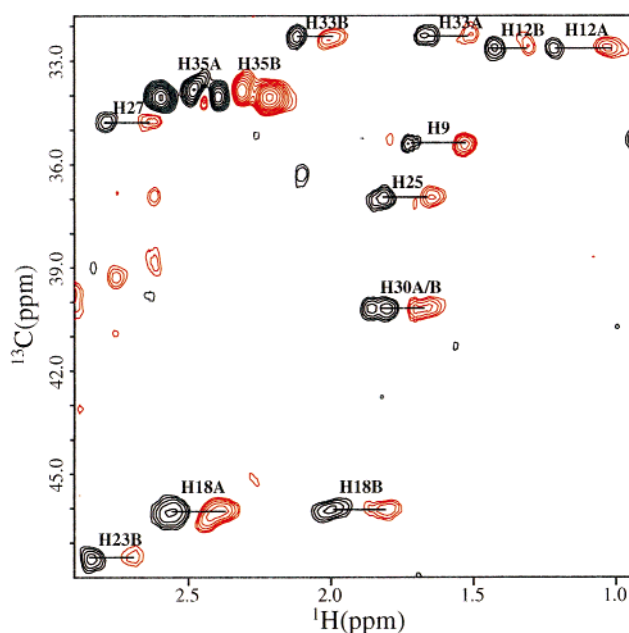(12) Clore, G. M.; Starich, M. R.; Gronenborn, A. M. *J. Am. Chem. Soc.* **1998**, *120*, 10571−10572.

Figure 1. Portion of a ^{13}C/^{1}H correlation spectrum of unlabeled FK506 complexed to [^{15}N,^{2}H]FKBP (1:1) at a concentration of 1 mM in potassium phosphate buffer (50 mM, pH 6.5). Pf1 phage were prepared as previously described[11] and were added at a concentration of 17 mg/mL to orient the FKBP/FK506 complex. The spectrum was recorded at 800 MHz on a Bruker DRX-800 spectrometer without ^{13}C-decoupling during the proton acquisition period using an unrefocused INEPT sequence. The total acquisition time was 36 h. Positive and negative contours are shown in black and red, respectively. Coupling constants were measured from the differences in the position of the peaks obtained from spectra recorded using the isotropic and aligned samples.

This strategy for improving NMR-derived structures of protein/ligand complexes was tested using the FKBP/FK506 complex.[10] Structures of the complex determined only from NOEs were compared to those generated with additional restraints derived from residual dipolar couplings. To increase the size of the residual dipolar couplings, the FKBP/FK506 complex was partially aligned using a colloidial suspension of phage.[11,12] ^{15}N−^{1}H residual dipolar couplings were measured from an ^{15}N/^{1}H correlation spectrum that was not decoupled in the $\omega 1$ dimension. The ^{13}C−^{1}H dipolar couplings of the ligand were measured from a ^{13}C/^{1}H correlation spectrum using an unrefocused INEPT sequence without ^{13}C decoupling during the proton acquisition period ($\omega 2$). Although the line width in the proton dimension is broadened by unresolved ^{1}H−^{1}H dipolar couplings, the advantage of using this experiment compared to an $\omega 1$-coupled ^{13}C/^{1}H correlation spectrum is that the ^{13}C−^{1}H couplings can be measured for each proton of the methylene groups. In addition, deleting the refocusing period of the pulse sequence reduces the loss in sensitivity due to T_2 relaxation and unoptimal coherence transfers involving non-uniform ^{13}C−^{1}H couplings.

Figure 1 depicts a portion of the $\omega 2$-coupled ^{13}C/^{1}H correlation spectrum used to measure the ^{13}C−^{1}H residual dipolar couplings for unlabeled FK506 when complexed to [^{15}N,^{2}H]FKBP aligned with phage. The spectrum was acquired in 36 h on a 800 MHz NMR spectrometer. A total of 22 ^{13}C−^{1}H dipolar couplings were measured from the differences in the positions of the peaks obtained from spectra recorded in the presence (aligned) and absence (isotropic) of phage. The values observed range from −39 to +40 Hz.

NMR structures of the FKBP/FK506 complex were generated from 1466 intramolecular NOEs for FKBP, 21 intramolecular
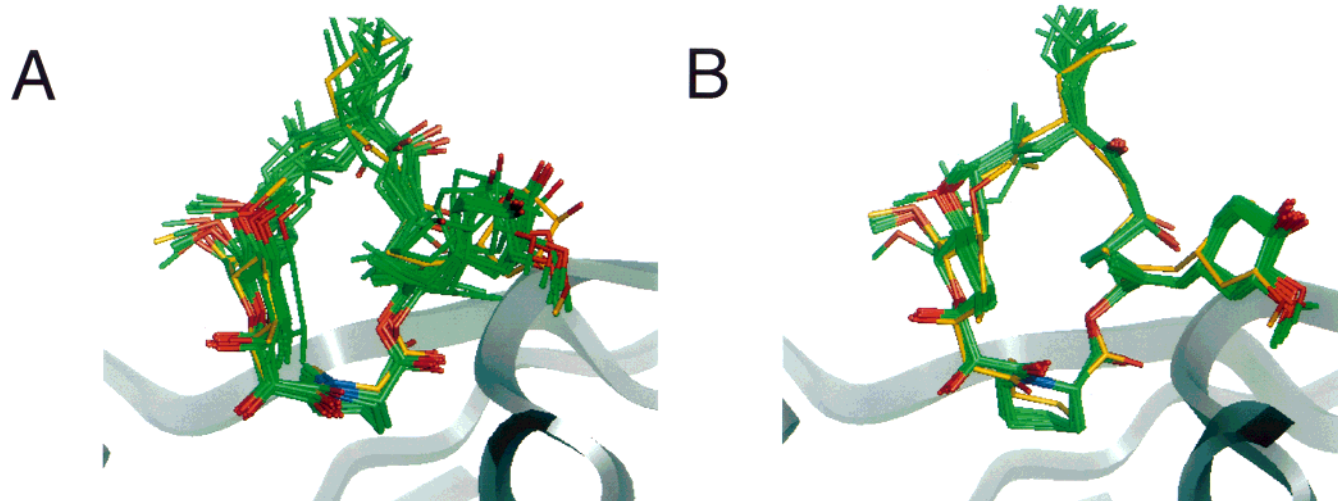
Figure 2. Superposition of FK506 from 15 NMR structures (green carbon atoms) and the X-ray crystal structure (orange carbon atoms) of the FKBP/FK506 complex.[10] The NMR structures were calculated (a) with the NOE data and (b) with the NOEs and residual dipolar couplings. Structures were calculated with a modified version of the X-Plor program.[14] A total of 1511 NOE-derived distance restraints, 100 hydrogen bond restraints, and 82 backbone dihedral restraints[15] were included in the structure calculations. For the structures calculated with residual dipolar coupling restraints, 17 individual C$-$H and 5 methyl residual dipolar couplings were obtained for the ligand and scaled to 57 protein N$-$H residual dipolar couplings.[3] Coefficients for the axial and rhombic terms were 15.0 and 0.81, respectively, and were obtained as described elsewhere.[3] The refinement protocol consisted of high-temperature torsion angle dynamics[16] from a linear form of the protein and the ligand at an arbitrary position. No residual dipolar restraints were included in this initial round. Several low-energy structures resulting from the initial simulations were used as starting structures for the next stage of refinement. For the structures calculated with residual dipolar coupling restraints, this refinement stage began with 5 ps of dynamics at 3000 K during which the chemical terms along with distance, torsion, and residual dipolar restraints were employed. Force constants for the square-well NOE, harmonic torsional, and harmonic residual dipolar coupling potential energy terms were 2 kcal mol^{-1} Å^{-2}, 200 kcal mol^{-1} rad^{-2}, and 0.1 kcal mol^{-1} Hz^{-2}, respectively. Following the high-temperature dynamics, the conformation was cooled to a final temperature of 100 K over the course of 12 ps. During the annealing stage, the force constant for the residual dipolar coupling restraints was increased to 0.5 kcal mol^{-1} Hz^{-2} and the NOE force constant was increased to 30 kcal mol^{-1} Å^{-2}. Resulting low-energy conformations were then subjected to 2500 additional steps of energy minimization with a residual dipolar coupling force constant of 1.0 kcal mol^{-1} Hz^{-2} and distance and torsional force constants of 30 kcal mol^{-1} Å^{-2} and 200 kcal mol^{-1} rad^{-2}, respectively. The same protocol was followed for the control set except for the exclusion of restraints derived from the residual dipolar couplings. X-plor energies for the control set indicated good covalent and noncovalent geometries ($E_{bond} = 1.4 \pm 0.3$, $E_{angle} = 29.9 \pm 1.4$, $E_{impr} = 3.5 \pm 0.2$, $E_{LJ} = -614 \pm 24$) and good agreement to the experimental dihedral and distance restraints ($E_{cdih} = 0.00 \pm 0.00$, $E_{noe} = 3.5 \pm 0.9$). Although slightly higher, the energies for the conformations refined using dipolar couplings were also good ($E_{bonds} = 6.3 \pm 0.6$, $E_{angle} = 58.3 \pm 4.4$, $E_{impr} = 5.0 \pm 0.5$, $E_{LJ} = -671 \pm 16$, $E_{cdih} = 0.04 \pm 0.03$, $E_{noe} = 13.6 \pm 2.8$, and $E_{sani} = 14.1 \pm 2.4$). No significant violations (>0.4 Å for NOEs, >3 Hz for residual dipolar coupling restraints) were observed in any of the final structures. The NOE data and residual dipolar couplings are consistent with a single conformation.

NOEs for FK506, and 24 intermolecular NOEs. Figure 2A depicts the superposition of FK506 from 15 NMR structures of the FKBP/FK506 complex generated without residual dipolar couplings. The atomic root-mean-square deviation about the mean coordinate position is 0.67 ± 0.2 Å for the heavy atoms of FK506. As shown in Figure 2B, a marked improvement in the quality of the NMR structures is obtained when the residual dipolar couplings for the protein and the ligand are included in the structure calculations. The root-mean-square deviation for the heavy atoms of the ligand drops to 0.29 ± 0.1 Å. No significant improvement in the precision of the ligand was observed when only the residual

dipolar couplings of the protein were used in the structure calculations. In addition to increasing the precision of the NMR structures of the complex, the accuracy is also improved as judged by a comparison with the X-ray crystal structure of the FKBP/FK506 complex.[10] The root-mean-square deviation between FK506 in the X-ray structure compared to the ensemble of NMR structures generated with and without residual dipolar couplings was 0.74 ± 0.1 Å and 1.12 ± 0.3, respectively (Figure 2).

In summary, more precise and more accurate structures of protein/ligand complexes can be determined by NMR when residual dipolar couplings are used to orient the protein and ligand in the complex. These high-quality NMR structures will be extremely useful for structure-based ligand design and drug discovery. ^{13}C$-^1$H residual dipolar couplings for unlabeled ligands can be measured using today's technology and will become even more practical in the future due to the large gains in sensitivity observed with cryogenic NMR probe technology.[13]

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