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Detection of very weak side chain–main chain hydrogen bonding interactions in medium-size $^{13}C/^{15}N$-labeled proteins by sensitivity-enhanced NMR spectroscopy

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Abstract

We describe the direct observation of very weak side chain–main chain hydrogen bonding interactions in medium-size $^{13}C/^{15}N$-labeled proteins with sensitivity-enhanced NMR spectroscopy. Specifically, the remote correlation between the hydrogen acceptor side chain carboxylate carbon $^{13}CO_2^{\delta}$ of glutamate 54 and the hydrogen donor backbone amide ^{15}N of methionine 49 in a 12 kDa protein, human FKBP12, is detected via the *trans*-hydrogen bond $^{3h}J_{NCO2\delta}$ coupling by employing a novel sensitivity-enhanced HNCO-type experiment, CPD-HNCO. The $^{3h}J_{NCO2\delta}$ coupling constant appears to be even smaller than the average value of backbone $^{3h}J_{NC'}$ couplings, consistent with more extensive local dynamics in protein side chains.

The recent NMR direct detection of scalar couplings between hydrogen donor and acceptor moiety spins in both nucleic acids (Dingley and Grzesiek, 1998; Pervushin et al., 1998; Dingley et al., 1999; Majumdar et al., 1999a, b; Liu et al., 2000a) and proteins (Cordier and Grzesiek, 1999; Cordier et al., 1999; Cornilescu et al., 1999a, b; Wang et al., 1999; Liu et al., 2000b) has opened a new approach for monitoring hydrogen bonds and provided critical parameters for the characterization of structure and dynamics of biological macromolecules in solution.

The scalar couplings in proteins linking the backbone amide moiety and the carbonyl carbon nucleus, $^{2h}J_{HC'}$ and $^{3h}J_{NC'}$, of two residues involved in an N-H$\cdots$O=C hydrogen bond are generally smaller than 1.0 Hz (Cordier and Grzesiek, 1999; Cornilescu et al., 1999a), a value consistent with recent quantum chemical calculations (Dingley et al., 1999; Scheurer and Brüschweiler, 1999). In proteins, backbone–backbone hydrogen bonds are exclusively formed with the amide nitrogen as the hydrogen donor and the carbonyl oxygen as the hydrogen acceptor and most conveniently detected via *trans*-hydrogen bond $^{3h}J_{NC'}$ couplings by performing HNCO-type experiments (Kay et al., 1990; Grzesiek and Bax, 1992). On the other hand, a wider variety of donor–acceptor combinations is possible for hydrogen bonds involving side chains. Along with backbone–backbone hydrogen bonds, such side chain interactions play a key role in stabilizing structures of polypeptides and protein–nucleic acid complexes. Most importantly, side chains involved in hydrogen bonding are often crucial in the regulation of enzymatic reactions and molecular recognition at interfaces (Jeffrey and Saenger, 1991). Despite the importance of understanding these interactions, NOEs between hydrogen-bonded side chains are typically difficult to observe since the relevant protons are often separated by large distances (> 5 Å). However, even in cases where protons on the donor and acceptor side chains are potentially close enough to observe an NOE, poor chemical shift dispersion often hinders definite assignment of signals. Direct NMR detection of hydrogen bonds involving amino acid side chains through *trans*-hydrogen bond *J*-couplings

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should provide a promising alternative for monitoring these otherwise inaccessible interactions. Backbone amide to side chain carboxyl hydrogen bonds have been observed very recently (Cornilescu et al., 1999b) for a small immunoglobulin binding domain (56 a.a. residues) of streptococcal protein G. Hydrogen bonding interactions involving side chains are expected to be generally weaker than the corresponding backbone–backbone interactions because side chains often display extensive local dynamics (Fischer et al., 1998; Liu et al., 2000c), although the *trans*-hydrogen bond $^{2h}J_{NN}$ scalar coupling constant between imidazole ^{15}NH nitrogens of the His24–His119 pair in apomyoglobin is surprisingly as large as 10 Hz (Hennig and Geierstanger, 1999). Therefore, availability of sufficient sensitivity in NMR experiments poses the ultimate limitation to the successful detection of these very weak correlations, in particular in relatively larger proteins.

In this communication, we report the direct observation of an N-H$\cdots$O=C$^{\delta}$ hydrogen bonding interaction between the Met 49 backbone amide and Glu 54 side chain carboxylate moieties in the 12 kDa human FK506-binding protein, FKBP12 (107 a.a. residues), via the three-bond $^{3h}J_{NCO2\delta}$ coupling between backbone amide ^{15}N and glutamate carboxylate carbon $^{13}CO_2^{\delta}$ by novel sensitivity-enhanced NMR spectroscopy.

To detect this extremely small coupling, we have developed a new HNCO-type experiment, CPD-HNCO (Liu et al., 2000b), which employs band-selective composite-pulse decoupling (CPD) applied to the backbone $^{13}C^{\alpha}$ during the entire amide ^{15}N-$^{13}CO_2^{\delta}$ defocusing/refocusing periods. This key feature of the pulse sequence serves to quench the lifetime broadening (Abragam, 1961; Bax et al., 1990; London, 1990; Peng et al., 1991; Harbison, 1993) stemming from the relaxation contribution to backbone ^{15}N from the *J*-coupling mediated $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ dipole–dipole (DD) interaction (Liu, 1999; Liu et al., 2000b, d), thereby significantly enhancing sensitivity relative to the conventional CT-HNCO experiment (Grzesiek and Bax, 1992). The pulse sequence of the CPD-HNCO experiment is shown in Figure 1 with a brief description of the magnetization transfer pathway outlined in the figure caption.

Figure 2 shows (b) the 2D CPD-H(N)CO spectrum, together with (a) the [^{15}N,^{1}H]-HSQC and (c, right panel) a region of the 2D H(C)CO$_2$ spectra of human FKBP12. The unique peak shown in the dashed region of (b) and highlighted in the left panel

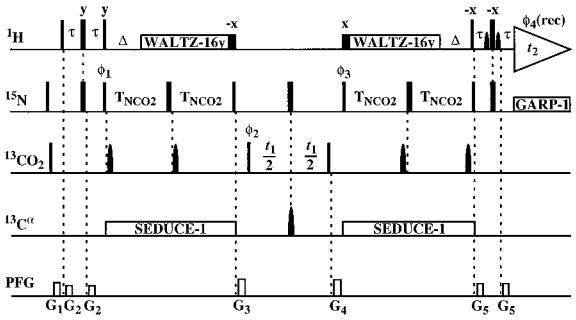

Figure 1. The pulse sequence of the 2D CPD-H(N)CO experiment used for the direct detection of protein side chain–main chain hydrogen bonding interactions through *trans*-hydrogen bond $^{3h}J_{NCO2\delta}$ coupling between glutamate carboxylate $^{13}CO_2^{\delta}$ carbon and backbone amide ^{15}N spins. Narrow and wide rectangular black bars indicate non-selective 90° and 180° pulses, respectively. Unless indicated otherwise, the pulses are applied with phase x. The ^{1}H, ^{15}N, and ^{13}C carrier frequencies are set at 4.77 ppm (water), 119.0 ppm and 181.0 ppm (center of glutamate/aspartate carboxylate $^{13}CO_2$), respectively. The non-selective ^{1}H pulses are applied using a 47.2 kHz field strength. The selective ^{1}H 90° pulses, indicated with black shaped bars, used for water flip-back (Grzesiek and Bax, 1993) are weak rectangular pulses of duration 1.7 ms. Proton decoupling is achieved using the WALTZ-16 (Shaka et al., 1983) sequence succeeded or preceded by water flip-back pulses at 3.6 kHz field strength. The ^{15}N pulses are applied with a field strength of 6.1 kHz and ^{15}N decoupling using the GARP-1 (Shaka et al., 1985) sequence with 1.0 kHz is applied during acquisition. The non-selective $^{13}CO_2$ carbon pulses are applied at 18.5 kHz field strength and the corresponding selective 180° pulses are applied with the Gaussian profile truncated at 5% amplitude having 101 μs pulse length. Backbone $^{13}C^{\alpha}$ band-selective decoupling is achieved by using the WALTZ-16 sequence with phase modulated pulses having the SEDUCE-1 (McCoy and Mueller, 1992) profile (250 μs 90° pulse length) applied at 56.0 ppm. The delays employed are: $\tau = 2.65$ ms, $\Delta = 5.5$ ms, and $T_{NCO2} = 66.5$ ms. The phase cycling scheme used is: ϕ_1 = x, $-$x; ϕ_2 = 2(x), 2($-$x); ϕ_3 = 4(x), 4($-$x); ϕ_4(rec) = x, $-$x, $-$x, x, $-$x, x, x, $-$x. Quadrature detection in the ω_1(^{13}C) dimension is achieved using States-TPPI (Marion et al., 1989) phase cycling of ϕ_2. The durations and strengths of the z-axis pulse field gradients (PFG) employed are: $G_1 = 1.5$ ms, 20 G/cm; $G_2 = 0.5$ ms, 7 G/cm; $G_3 = 0.8$ ms, 10 G/cm; $G_4 = 0.8$ ms, 13 G/cm; $G_5 = 0.3$ ms, 17 G/cm. Magnetization begins on the backbone amide proton and is transferred to the attached nitrogen via the INEPT (Morris and Freeman, 1979) step. The ^{15}N magnetization is then partially transferred to the hydrogen-bond-linked glutamate carboxylate carbon, $^{13}CO_2^{\delta}$, via the *trans*-hydrogen bond $^{3h}J_{NCO2\delta}$ coupling through the 'long-range' ^{15}N-$^{13}CO_2^{\delta}$ defocusing period. Throughout the transfer, band-selective decoupling is applied to the backbone $^{13}C^{\alpha}$. The period, $2 \times T_{NCO2\delta}$, is set to $2/^{1}J_{NC'} = 133.0$ ms for optimal refocusing of the backbone sequential $^{1}J_{NC'} = 15$ Hz coupling. Subsequently, the $^{13}CO_2^{\delta}$ magnetization is frequency-labeled during the t_1 evolution period, and then transferred back to the remote backbone amide proton for detection via the same pathway.

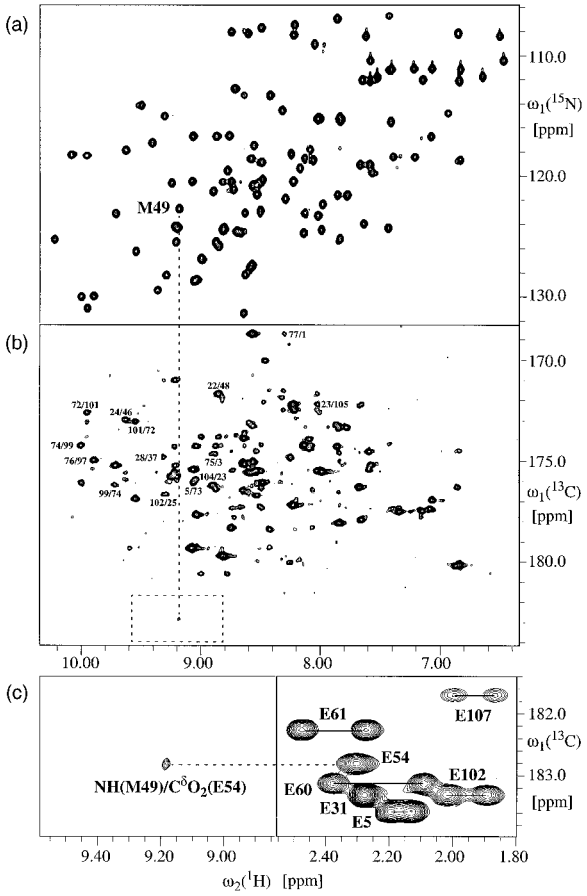

Figure 2. (b) The 2D CPD-H(N)CO spectrum, together with (a) the [^{15}N,^{1}H]-HSQC and (c, right panel) a region of the 2D H(C)CO_2 (Pellecchia et al., 1997) spectra of human FKBP12. The unique peak shown in the dashed region of (b) and highlighted in (c, left panel), correlating Met 49 backbone amide (in a) and Glu 54 carboxylate $^{13}CO_2^{\delta}$ resonances (in c, right panel), clearly indicates the existence of the backbone–side chain N-H$\cdots$O=C^{d} hydrogen bonding interaction. Amide resonances in (a) were assigned in previous publications (Rosen et al., 1991; Xu et al., 1993). Resonances of glutamate $^{13}CO_2^{\delta}$ (c, right panel) were assigned to individual residues based on the reported human FKBP12 proton chemical shifts (Rosen et al., 1991) and the two $^{1}H^{\gamma}$ resonances that correlate with individual $^{13}CO_2^{\delta}$ are linked to each other with solid horizontal lines. Fourteen 'strong' backbone–backbone hydrogen bond correlation peaks are indicated in (b) with the residue number of an amide followed by the residue number of hydrogen-bond-linked carbonyl. All spectra were recorded with acquisition times of $t_{2\,\max}(^{1}H)$ = 128 ms and $t_{1\,\max}(^{15}N)$ = 51 ms for (a), $t_{1\,\max}(^{13}C)$ = 55 ms for (b), and $t_{1\,\max}(^{13}C)$ = 62 ms for (c, right panel), respectively. Four scans per t_1 increment for (a), 400 scans for (b), and 32 scans for (c, right panel) were used, which resulted in experimental times of 37 min, 2.2 days, and 3.2 h for respective spectra. The NMR samples contained either 3.1 mM $^{13}C/^{15}N$-labeled protein dissolved in 250 μL of 93% H_2O /7% D_2O with a Shigemi microcell or 1.5 mM protein in 500 μL of D_2O with 25 mM sodium acetate-d_3 at pH 5.0. NMR spectra were collected at 25 °C on a Varian Inova 600 MHz (^{1}H) instrument equipped with a z-axis pulsed field gradient probehead.

of (c), correlating the Met 49 backbone amide and Glu 54 carboxylate $^{13}CO_2^{\delta}$ resonances (c, right panel), clearly establishes the existence of the backbone–side chain N-H$\cdots$O=C$^{\delta}$ hydrogen bond. The extremely weak intensity of this resonance prevents a direct quantification of the three-bond $^{3h}J_{NCO2\delta}$ coupling within a reasonable measuring time. This peak is not observable in the 2D CT-H(N)CO spectrum with the same sample under identical experimental conditions, demonstrating the advantage of using the CPD sensitivity-enhanced NMR experiment. The diatomic distance between Met 49 amide nitrogen and one of the Glu 54 carboxylate oxygens is 2.5 Å, as measured in the crystal structure of free bovine FKBP12. Similar distances are found in the crystal structures of human and bovine FKBP12 in complex with FK506 and rapamycin, respectively, suggesting that this interaction is an integral feature of the protein architecture and not an artifact of particular crystallization conditions. The Glu 54 side chain has not been defined in the reported solution structure of unligated FKBP12 (PDB accession code: 1FKR). The 2.5 Å N-H$\cdots$O=C$^{\delta}$ distance is even shorter than the average diatomic distance (2.9 Å) between the carbonyl oxygen and the amide nitrogen of the 14 NMR detected backbone–backbone hydrogen bonds (Figure 2b) in the high resolution crystal structures of unligated FKBP12 (PDB accession code: 1FKK) and its complexes with FK506 and rapamycin (PDB accession codes: 1FKF, 1FKB, 1FKJ, 1FKL). Following comparison of the peak intensity of this side chain–backbone correlation signal with those backbone–backbone hydrogen bonding correlation resonances (Figure 2b) and given that the reported $^{3h}J_{NC'}$ coupling constants range from 0.25 to 0.92 Hz (Cordier and Grzesiek, 1999; Cordier et al., 1999; Cornilescu et al., 1999a), this *trans*-hydrogen bond *J*-coupling must be smaller than 0.2 Hz.

Two other backbone amide–Glu/Asp carboxylate hydrogen bonds are formed in the Thr 14–Asp 11 and Gly 51–Glu 60 residue pairs in the free bovine FKBP12 crystal structure (PDB accession code: 1FKK). These two hydrogen bonds were not detectable, despite our best efforts using NMR. In the free bovine FKBP12 crystal structure, the diatomic distances between the amide nitrogen of Gly 51 and the carboxylate oxygens of Glu 60 are both larger than 3.5 Å (3.53 Å and 3.83 Å, respectively), indicative of a weak hydrogen bond. On the other hand, the amide moiety of Thr 14 is involved in bifurcated hydrogen bonding interactions with both the side chain alkyl hydroxyl group of the same residue and the carboxylate

group of Asp 11. The diatomic distances of N(T14)-$O^{\gamma 1}$(T14) and N(T14)-CO_2^{δ}(D11) are 2.6 Å and 2.8 Å, respectively. The major contribution to the $^{3h}J_{NC'}$ coupling stems from Fermi contact (FC) that is dominated by two orbitals, each of which involves all hydrogen bonding atoms of amide and hydrogen acceptor (Dingley et al., 1999; Scheurer and Brüschweiler, 1999). These FC contributions originate from atomic orbitals bearing the σ bond feature across the hydrogen bond (Levine, 1991). Bifurcation of hydrogen bonding interactions might disperse the polarization of the electron cloud and reduce the electronic orbital overlap between the single hydrogen donor with the *two* hydrogen acceptors, reducing the *J*-coupling constant across the hydrogen bond.

In conclusion, we have identified a *trans*-hydrogen bond three-bond *J*-coupling between the backbone amide and the glutamate carboxylate moieties of distinct residues in a 12 kDa protein. The success in detecting the very weak main chain–side chain hydrogen bonding interactions through sensitivity-enhanced NMR experiments should provide a more efficient approach for monitoring the tertiary structure and dynamics, enzymatic activity and biomolecular recognition events of medium-sized proteins in solution without the need for perdeuteration.

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