Intermolecular $^{31}P-^{15}N$ and $^{31}P-^{1}H$ Scalar Couplings Across Hydrogen Bonds Formed between a Protein and a Nucleotide

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Hydrogen bonds (H-bonds) are of central importance for maintenance of three-dimensional conformations of proteins and nucleic acids and play key roles in recognition of ligand molecules and in modulation of enzymatic reactions.[1−3] Many such ligands contain phosphate groups; examples are nucleotides, phospholipids, coenzyme A (CoA), NAD, DNA, and RNA.[4] Intermolecular H-bonds formed between the ligand phosphates and protein amide groups are crucial for their interactions.[5] However, identifications of these H-bonds by means of NMR have been difficult. Here we report the detection of intermolecular three-bond $^{31}P-^{15}N$ and two-bond $^{31}P-^{1}H$ J couplings across $N-H\cdots O^{-}-P$ H-bonds.

Recently, several groups observed scalar couplings across H-bonds in nucleic acid base pairs and proteins, providing tools for direct identification of individual H-bonds in these macromolecules.[6−11]

Prompted by these recent reports, we investigated the scalar coupling between the H-bond donating ^{15}N and accepting ^{31}P nuclei ($^{3h}J_{NP}$) of human Ras p21 Q61L-substituted protein (C-terminal truncated form: residues 1−171) complexed with GDP [Ras(Q61L)·GDP]. We obtained $\{^{31}P\}$ spin−echo difference ^{15}N constant-time TROSY spectra of a $^{15}N,^{2}H$-labeled protein (see Supporting Information). The pulse scheme used was analogous to $\{^{15}N\}$ spin−echo difference ^{13}C constant-time HSQC[13] and the total constant time (T_A) was set to 60 ms. Only the backbone ^{15}N resonance of Ala18 exhibited a change of signal intensity relative to the reference spectrum. The absolute value of the measured $^{3h}J_{NP}$ was 4.62 ± 0.01 Hz (Table 1).

Having detected the presence of $^{3h}J_{NP}$, we next used a new pulse scheme, $^{3h}J_{NP}$ HNPO (Figure 1), to observe correlations between $^{1}H_N$, ^{15}N, and ^{31}P of the H-bond donor and acceptor groups. This experiment is conceptually similar to the 3D $^{3h}J_{NC'}$ HNCO and TROSY-HNCO experiments,[10,15] and the $^{15}N-^{31}P$ dephasing delay, T, was set to $1/(2^{3h}J_{NP})$ (108 ms). Figure 2 illustrates results of the two-dimensional (2D) HN(PO) and H(N)PO experiments. By virtue of the large $^{3h}J_{NP}$ coupling and the use of TROSY,[14,15] the sensitivity of the HNPO experiment was markedly high. This set of 2D experiments clearly established the correlation (^{1}H, ^{15}N, ^{31}P) = (9.38, 126.4, −14.0 ppm), which corresponds to the H-bond between the protein backbone amide group of Ala18 and the α-phosphate of GDP. No other correlation was observed in these experiments.

To further investigate the nature of this Ala18 α-phosphate H-bond, we analyzed the size of $^{2h}J_{HP}$ by using a quantitative J_{HP} [$^{15}N,^{1}H$] HSQC experiment with de- and rephasing delays T_B of 60 ms (see Supporting Information), which is conceptually related to the $\{^{31}P\}$ spin−echo differences $^{1}H-^{13}C$ HSQC experiment.[16] The result showed that $^{2h}J_{HP}$ has an absolute value of 3.36 ± 0.09 Hz. (Table 1). The presence of this J_{HP} coupling was also shown by a $\{^{31}P$-selected$\}$-($^{15}N,^{1}H$) HSQC experiment (data not shown). The resultant spectrum looks similar to the 2D HN(PO) (Figure 2A). Further, a (^{31}P, ^{1}H) HSQC experiment with de- and rephasing delays of 60 ms for both INEPT and reverse INEPT periods established a $^{2h}J_{HP}$ correlation between the amide proton of Ala18 and the α-phosphate of GDP (Figure 2C).

We also measured $^{3h}J_{NP}$ and $^{2h}J_{HP}$ in wild-type Ras·GDP (truncated form: residues 1−171). Crystal structures,[17,18] NMR solution structures,[19,20] H-exchange rates of backbone amide protons with solvent[20,21] and ^{1}H chemical shifts[19,21,22] suggest that backbone amide groups of five residues of Ras, Gly13, Gly15, Lys16, Ser17, and Ala18, form H-bonds with either the α- or β-phosphate group of GDP in wild-type Ras·GDP. Nevertheless, our experiments showed that only the H-bond between Ala18 and the α-phosphate transmits observable scalar coupling in wild-type Ras·GDP, as in Ras(Q61L)·GDP. The measured absolute values of $^{3h}J_{NP}$ and $^{2h}J_{HP}$ were 4.44 ± 0.06 and 3.9 ± 0.8 Hz, respectively, which is similar to that observed for Ras(Q61L)·GDP. These observations indicate that the Ala18 α-phosphate H-bond in Ras(Q61L)·GDP is similar to that in wild-type Ras·

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Table 1. H-Bond Lengths and Angles of the Crystal Structure of Human Wild c-Ha-Ras Complexed with GDP[a] and the $^{3h}J_{NP}$ and $^{2h}J_{NP}$ Couplings Observed for Ras(Q61L)·GDP[b]

donors	acceptors	d(N−O) (Å)	αPOH (deg)	αOHN (deg)	$^{3h}J_{NP}$ (Hz)	$^{2h}J_{HP}$ (Hz)
Gly13	O2β	2.76	107.5	167.5	<0.27	<0.52
Gly15	O1β	2.74	107.6	163.0	<0.35	<0.92
Lys16	O1β	2.77	115.9	162.5	<0.35	<0.98
Ser17	O3β	3.05	120.1	156.6	<0.29	<0.66
Ala18	O2α	2.85	173.4	143.5	4.62 ± 0.01	3.36 ± 0.09

[a] PDB code 1Q21.[17,18] [b] For determination of the H-bond distances and angles, hydrogens were added to the crystal structure with the program CNS version 0.5,[26] using idealized covalent geometry,[12] Uncertainties and upper-bounds of the coupling constants are defined at 1 σ (see Supporting Information).

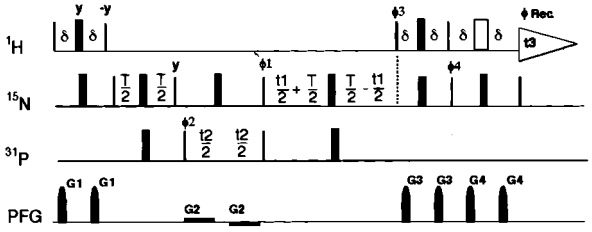

Figure 1. Pulse scheme of the $^{3h}J_{NP}$ HNPO experiment, used to detect intermolecular trans-H-bond J connectivities between proteins and ligands. Narrow and wide black bars indicate nonselective 90° and 180° pulses, respectively. An open bar indicates the selective 3−9−19 ^{1}H 180° pulse for WATERGATE gradient-tailored water suppression.[24] All pulse phases are x, unless indicated otherwise. The ^{1}H, ^{15}N, and ^{31}P carriers are positioned at 4.62 (water), 119.94, and −9.3 ppm, respectively. Delay durations: $\delta = 2.7$ ms; $T = 108$ ms ($= {}^1/_2\ {}^{3h}J_{NP}$). Phase cycling: $\phi_1 = \{y, -y, x, -x\}$; $\phi_2 = \{4x, 4(-x)\}$; $\phi_3 = \{-y\}$; $\phi_4 = \{-y\}$; $\phi_{rec} = \{y, -y, -x, x, -y, y, x, -x\}$. The phase of the second ^{1}H 90° pulse is y or $-y$, depending on the brand of spectrometer. To obtain Rance−Kay style quadrature data in the ^{15}N(t_1) dimension, a second FID for each t_1 value is recorded with $\phi_1 = \{y, -y, -x, x\}$; $\phi_3 = \{y\}$ and $\phi_4 = \{y\}$, and the data are processed as described by Kay et al.[25] Quadrature detection in the ^{31}P(t_2) dimension is achieved by incrementing ϕ_2 in States-TPPI manner.

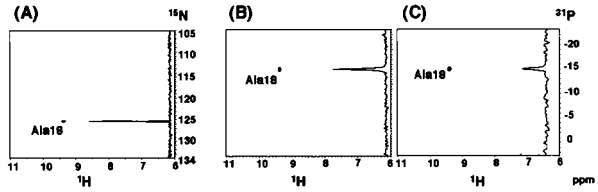

Figure 2. Plots of 2D $^{3h}J_{NP}$ HNPO and (^{31}P,^{1}H) HSQC spectra of the uniformly ^{15}N/^{2}H-enriched human c-Ha-Ras·Q61L (residues 1−171) complexed with GDP [Ras(Q61L)·GDP], taken at 303 K. (A, B) Regions of 2D HN(PO) (A) and H(N)PO (B) spectra are shown, in which [ω_1 (^{15}N), ω_3 (^{1}H$_N$)], and [ω_2 (^{31}P), ω_3 (^{1}H$_N$)] frequencies were recorded, respectively. (C) (^{31}P,^{1}H) HSQC spectrum, showing the $^{2h}J_{HP}$ connectivity between Ala18 H$_N$ and the α phosphorus of GDP. For each spectrum, only one cross-peak was observed. Cross sections taken at the ^{1}H chemical shift of the cross-peaks are also shown. All of the spectra were recorded with a 2.0 mM sample of Ras(Q61L)·GDP containing 20 mM sodium phosphate buffer (pH 6.5), 40 mM NaCl, 5 mM $MgCl_2$, 0.01% NaN_3 and 10% D_2O on a Bruker DRX 800 MHz spectrometer equipped with a triple-axis pulsed field gradient ^{1}H/^{15}N/^{13}C/^{31}P probehead optimized for ^{1}H detection.

GDP, which is consistent with the fact that the backbone ^{1}H^N and ^{15}N chemical shifts of Ras(Q61L)·GDP (Ito, unpublished results) are almost identical to those of Ras·GDP,[19,21,22] except for several residues at and around position 61, thus indicating their structural similarity. In particular, those of the H-bond-forming residues, Gly13, Gly15, Lys16, Ser17, and Ala18, of the two proteins are very similar, suggesting that the H-bonding in the two complexes is equivalent.

It is intriguing that only the H-bond involving Ala18, but not the other four residues, yields observable $^{3h}J_{NP}$ and $^{2h}J_{HP}$ connectivities in both the wild-type Ras·GDP and Ras(Q61L)·GDP. Consistent with this, only the mainchain amide of Ala18 yields doublet signals in the (^{15}N,^{1}H) TROSY spectrum[14] of Ras(Q61L)·GDP (see Supporting Information). The doublet pattern indicates that the signs of $^{3h}J_{NP}$ and $^{2h}J_{HP}$ are the same. Structural fluctuations can lead to a reduction of the trans-H-bond J coupling, for the reason that the H-bond exists only part of the time, as was observed for fraying stem ends of the DNA triplex.[8] However, studies of backbone dynamics of wild-type Ras·GDP using ^{15}N relaxation parameters show no evidence of large structural fluctuations in these H-bond-forming residues.[19,20] Thus, it is unlikely that structural fluctuations are a major cause of the missing couplings from Gly13, Gly15, Lys16, and Ser17.

Table 1 summarizes geometric parameters of the five possible intermolecular H-bonds that have phosphates as acceptor groups: the N−O distances and the α(POH) and α(OHN) angles. Electron orbital overlap decreases with the N−O distance in the limit of weak H-bonding;[8,12,23] thus, a shorter N−O distance is expected to correlate with a larger trans-H-bond coupling, as has been observed for N−H···O═C′ H-bonds in proteins.[12] However, the Ala18 α-phosphate H-bond distance is not the smallest among the five bonds. Thus, the unique presence of $^{3h}J_{NP}$ and $^{2h}J_{HP}$ couplings across this H-bond cannot be simply explained by the H-bond length alone, although the quality of the correlation is limited by the accuracy of the atomic coordinates in the 2.2 Å crystal structure.[17] The α(OHN) angles of all the H-bonds are confined to a relatively narrow range, so that their variability is not a factor. On the other hand, only the α(POH) angle of the Ala18 α-phosphate H-bond is close to 180°, whereas all of the other H-bonds have rather acute α(POH) angles. Therefore, it is possible that the linear arrangement of the ^{31}P nucleus relative to the hydrogen and H-bond accepting oxygen nuclei is a prerequisite for the presence of observable trans-H-bond couplings.

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Supporting Information Available: Pulse schemes of the {^{31}P} spin−echo difference ^{15}N constant-time TROSY and quantitative $^{2h}J_{HP}$ (^{15}N,^{1}H) HSQC experiments, and (^{15}N,^{1}H) TROSY and ^{31}P spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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