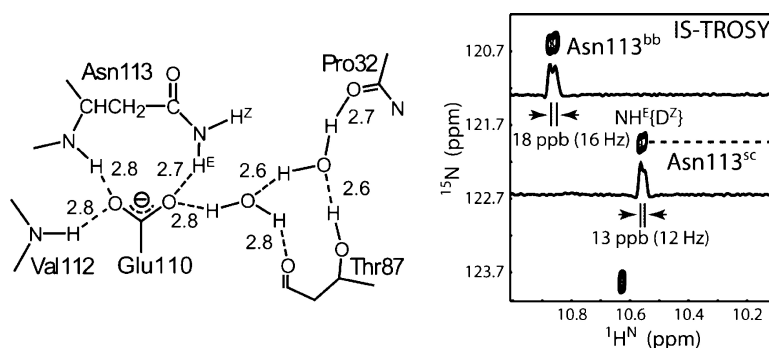


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## NMR Detection of Bifurcated Hydrogen Bonds in Large Proteins

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Hydrogen bonds play critical roles in protein structure, stability, and function. Most hydrogen bonds in proteins are derived from their crystal structures and the use of standard covalent geometry information, because the positions of hydrogen atoms are defined only in a limited number of ultrahigh-resolution crystal structures. On the other hand, NMR structure calculations rely mostly on the distances between hydrogen atoms, and the positions of the heavy atoms involved in hydrogen bonds are defined by standard covalent geometry information. Consequently, NMR structures do not contain independent information about hydrogen bonds.

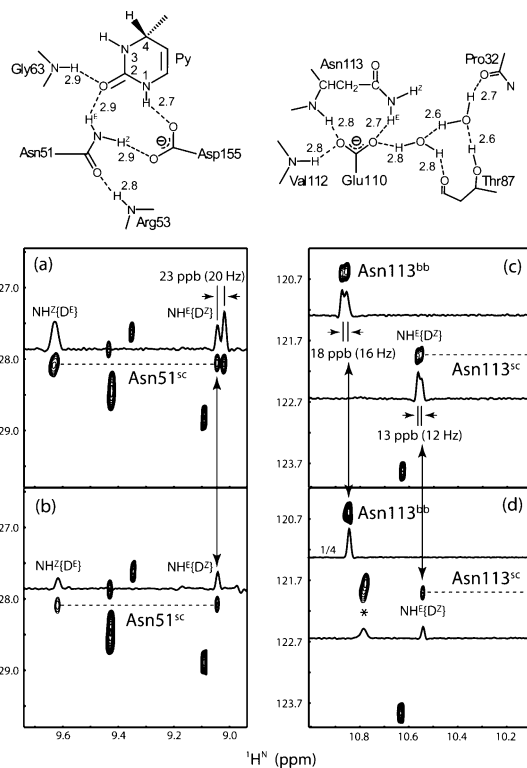
One of the most important advances in NMR spectroscopy in the past 10 years was the direct detection of hydrogen bonds through trans-hydrogen-bond scalar couplings.<sup>1</sup> However, trans-hydrogen-bond scalar couplings are generally small so that they cannot be measured for large proteins. No scalar coupling constants for hydrogen bonds involving water molecules have been measured.

Unlike the electron-mediating scalar couplings, isotope effects are vibrational phenomena in nature and are propagated through either covalent bonds or hydrogen bonds and potentially can be used as an alternative approach for the *direct* detection of hydrogen bonds. A wealth of literatures has documented the usefulness of the isotope effects in NMR analysis of small compounds,<sup>2</sup> small proteins,<sup>3</sup> and nucleic acids.<sup>4</sup> Trans-hydrogen-bond deuterium isotope effects on the chemical shifts of nucleic acids have been reported,<sup>4</sup> but no such effects have been reported on proteins.

In this communication, we report the first observation of trans-hydrogen-bond two-bond H/D isotope effects,  ${}^{2h}\Delta^1\text{H}$ , and trans-hydrogen-bond three-bond isotope effects,  ${}^{3h}\Delta^{15}\text{N}$ , in a large protein using the recently developed isotopomer-selective (IS) TROSY technique<sup>5</sup> and show that such deuterium isotope effects can be used to detect a most common type of bifurcated hydrogen bonds in which a heavy atom, usually oxygen, is involved in two hydrogen bonds.

The protein used for this study was yeast cytosine deaminase (yCD), a 35 kDa homodimeric enzyme that catalyzes the deamination of not only the physiological substrate cytosine but also the prodrug 5-fluorocytosine and is widely used for gene-directed enzyme/prodrug therapy for the treatment of cancer.<sup>6</sup> Two high-resolution crystal structures have been reported for yCD in complex with the transition state analogue 2-pyrimidinone (Py) for the deamination of cytosine, one at 1.14 Å<sup>7a</sup> and the other at 1.6 Å resolution.<sup>7b</sup> We have been studying the catalytic mechanism of yCD and its role in the activation of the prodrug 5-fluorocytosine by biochemical, NMR, and computational methods.<sup>5,8</sup>

Figure 1 shows expanded regions of the  ${}^{15}\text{N}$ - ${}^1\text{H}$  IS-TROSY spectrum of yCD in complex with the transition state analogue 5-fluoro-2-pyrimidinone (5FPy) in either 50%  $\text{H}_2\text{O}/50\%$   $\text{D}_2\text{O}$  (mixture solvent sample) or 95%  $\text{H}_2\text{O}/5\%$   $\text{D}_2\text{O}$  (water sample),



**Figure 1.** Regions of 2D  ${}^{15}\text{N}$ - ${}^1\text{H}$  IS-TROSY spectra of [U- ${}^2\text{H}$ ,  ${}^{13}\text{C}$ ,  ${}^{15}\text{N}$ ]-yCD recorded on a Bruker AVANCE 900 MHz NMR spectrometer equipped with a cryoprobe at 25 °C. Panels (a) and (c) are from the spectrum obtained with a yCD sample in 50%  $\text{H}_2\text{O}/50\%$   $\text{D}_2\text{O}$ , and (b) and (d) are corresponding regions with a sample in 95%  $\text{H}_2\text{O}/5\%$   $\text{D}_2\text{O}$ . The hydrogen-bonding network involving Asn51 and Asn113 are illustrated on top with distances between heavy atoms (Å) indicated for each hydrogen bond. NMR samples were made in 100 mM potassium phosphate buffer, pH 7.0 (pH meter reading), with 100  $\mu\text{M}$   $\text{NaN}_3$  and 20  $\mu\text{M}$  DSS (an internal NMR reference). The samples contained  $\sim 1.5$  mM yCD (protomer concentration) and 20 mM the transition state analogue 5FPy. NMR assignment was achieved with IS-TROSY techniques and reported earlier.<sup>5</sup> The resonance marked with the asterisk in (d) is a folded peak from arginine side chains. The spectrum for panels (a) and (c) was recorded with 16 scans and a 2 s delay time,  $t_{1\text{max}}({}^{15}\text{N}) = 53$  ms and  $t_{2\text{max}}({}^1\text{H}) = 285$  ms, resulting in the experimental time of 3.5 h, and the spectrum for panels (b) and (d) was recorded and processed in the same manner, but with 64 scans, resulting in an experimental time of 13.9 h.

recorded on a 900 MHz NMR spectrometer. The figure contains the side-chain amide resonances of Asn51 and both backbone and side-chain amide resonances of Asn113. Each side-chain amide has two hydrogen atoms, one in the *trans* (*E*) and the other in the *cis* (*Z*) configuration with respect to the carboxamide oxygen. In the mixture solvent, the relative population of each of the four possible isotopomers,  $\text{NH}^E\text{H}^Z$ ,  $\text{NH}^E\text{D}^Z$ ,  $\text{ND}^E\text{H}^Z$ , and  $\text{ND}^E\text{D}^Z$ , is about equal if D/H isotope fractionation factors are close to one. The IS-TROSY experiment exclusively detects the resonances of semideuterated

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isotopomers with high sensitivity.<sup>5</sup> Unexpectedly, with the mixture solvent sample, the resonances of the Asn51 side-chain amide NH<sup>E</sup>D<sup>Z</sup> (panel a) and the backbone amide and side-chain amide NH<sup>E</sup>D<sup>Z</sup> of Asn113 (panel c) show doublets in the <sup>1</sup>H dimension. The splittings are 23, 18, and 13 ppb corresponding to 20, 16, and 12 Hz at the 900 MHz field, respectively. Such splittings can be caused by conformational exchanges, scalar couplings, or isotope effects. Since the doublets became singlets in water (panels b and d), the doublets are not caused by conformational exchange or scalar coupling and are due to the H/D isotope effects of chemically exchangeable hydrogen atoms. Interestingly, the resonances in the spectrum of the water sample have the same <sup>1</sup>H<sup>N</sup> chemical shifts as the corresponding weaker components of the doublets in the spectrum of the mixture solvent sample (see the peaks linked with vertical arrowed lines in Figure 1). Therefore, the weaker component in each doublet of the mixture solvent sample must result from the resonances in the protonated environment and the stronger component from the resonances in the deuterated environment.

In the 1.14 Å crystal structure, as illustrated in Figure 1, the side-chain <sup>1</sup>H<sup>E</sup> of Asn51 (the <sup>15</sup>N<sup>1</sup>H<sup>E</sup>{D<sup>Z</sup>} isotopomer in the IS-TROSY spectrum) is connected to the backbone amide proton of Gly63 through the bifurcated hydrogen bonds, H···O···H, with the O2 oxygen of the transition state analogue. Similarly, the backbone amide proton of Asn113 is connected to the backbone amide proton of Val112 through another pair of bifurcated hydrogen bonds with one of the two side-chain carboxyl oxygen atoms of Glu110, while the other side-chain oxygen of Glu110 connects the side-chain <sup>1</sup>H<sup>Z</sup> of Asn 113 (if the side-chain oxygen and nitrogen positions are swapped as in the 1.6 Å crystal structure) and a bound water molecule through the other bifurcated hydrogen bonds. The two water molecules in this strong hydrogen bond network are observed in both crystal structures. Based on the two high-resolution crystal structures, the only possible explanation for the doublets is that they are due to trans-hydrogen-bond two-bond H/D isotope effects, <sup>2h</sup>Δ<sup>1</sup>H. The conclusion is reinforced by the observation that the weaker component in each doublet corresponds to the isotopomer that both sides of H···O···H bifurcated hydrogen bonds are protons. This could be due to a much stronger dipole–dipole interaction compared to the corresponding semideuterated isotopomer H···O···D and/or a deviation from a unity D/H fractionation factor.

Similar H/D isotope effects have been observed in small compounds with intramolecular bifurcated hydrogen bonds involving two hydroxyl groups.<sup>2a</sup> It is suggested that such isotope effects are due to the weakening of the hydrogen bond with a deuteron and the concomitant strengthening of the hydrogen bond with a proton, resulting in a negative isotope effect.<sup>2a,9</sup> This may also explain the negative isotope effects on the backbone and side-chain amides of Asn113 but not the positive isotope effect on Asn51-<sup>1</sup>H<sup>E</sup>. Furthermore, the isotope effects are asymmetrical. No significant isotope effects on the chemical shifts of the backbone amide resonances of Gly63 and Val112 are observed, and both appear as singlets (see Supporting Information).

Another interesting observation is that, in all three doublets, the stronger component that corresponds to the deuterated isotopomer has a small downfield <sup>15</sup>N shift with respect to the weaker component that corresponds to the protonated isotopomer. The shifts must be from trans-hydrogen-bond three-bond isotope effects on <sup>15</sup>N, <sup>3h</sup>Δ<sup>15</sup>N.

The size and sign of isotope effects on chemical shifts are related to hydrogen bond strength.<sup>2b,10a</sup> Excellent correlations have been reported for some well-defined small intramolecularly hydrogen-

bonded systems<sup>2a</sup> and intermolecularly hydrogen-bonded systems.<sup>2b</sup> The across-hydrogen-bond isotope effects in proteins are expected to be related to hydrogen bond strength as well, but such correlation awaits the careful analysis of more proteins with high-resolution structures and high-level quantum calculations. It is noted that the bifurcated hydrogen bonds (involving Asn113) with the negative but smaller isotope effects have a negatively charged carboxyl oxygen as the acceptor and shorter heavy-atom distances, whereas those (involving Asn51) with the positive but larger isotope effects have a neutral carbonyl oxygen as the acceptor and longer distances. The fact that the isotope effects are asymmetrical indicates that the two hydrogen bonds to the same acceptor atom are different in strength or have different potential energy wells, although the heavy-atom distances of the two hydrogen bonds are the same or very similar according to the crystal structures (Figure 1).<sup>7</sup> Since these bifurcated hydrogen bonds are normal ones (not strong or low-barrier hydrogen bonds) based on their heavy-atom distances (2.7–2.9 Å),<sup>10</sup> the isotope effects may be used to detect most of such bifurcated hydrogen bonds in proteins.

Isotope effects from bound water molecules have never been reported, to the best of our knowledge. Interior bound water molecules are often important for molecular recognition and enzymatic catalysis. The direct detection of bound water molecules through hydrogen-bond mediated isotope effects in the current work is simple and specific, hence a valuable alternative to the NOE-based measurement for the detection of strongly hydrogen-bonded water molecules.<sup>11</sup>

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**Supporting Information Available:** The full <sup>15</sup>N–<sup>1</sup>H IS-TROSY spectrum of yCD. This material is available free of charge via the Internet at <http://pubs.acs.org>

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