Equilibrium Protium/Deuterium Fractionation of Backbone Amides in U-13C/15N Labeled Human Ubiquitin by Triple Resonance NMR

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The equilibrium D/H isotope fractionation factor, Φ, of a given amide in a protein corresponds to the population ratio of deuterated over protonated states, when equilibrated in a 50% D2O/50% H2O solvent mixture. Fractionation is one of the simplest manifestations of the Heisenberg uncertainty principle, Δp x = C, where p x represents the momentum of a particle in the x dimension and x is its position, and its theoretical basis is well understood.1-3 As a result of its larger mass, the lowest vibrational energy level of 2H is lower than that of 1H. The same applies to the solvent. However, if the difference between the lowest vibrational energy levels of a proton and a deuterium at a protein site is larger than the energy difference in the solvent, the protein site will become enriched in deuterium. Several theoretical studies of isotope fractionation have also been reported in recent years.4-6 Although it is generally believed that fractionation factors for regular, weak hydrogen bonds in peptide systems are larger than one, calculations on peptide clusters4-5 indicate the possibility for Φ values considerably smaller than one.

Recent experimental studies have reported backbone amide fractionation numbers in proteins ranging from smaller than 0.7 to larger than 1.4.7-9 These values were based on measurement of the intensities of 15N-1H correlations in a range of H2O/D2O solvent mixtures. Interpretation of these results, however, is complicated by magnetization exchange between the solvent and the protein, a process which itself is also a function of the deuteration level. For example, solvent presaturation and/or the use of an interscan delay shorter than the (long) solvent deuteration level. For example, solvent presaturation and/or the use of an interscan delay shorter than the (long) solvent deuteration level. For example, solvent presaturation and/or

Here we report a different method for measuring backbone amide D/H fractionation, based on the HACACO experiment10 which is commonly used for protein backbone resonance assignments: If the 1H resonance of residue i (1H1i) is correlated with 13Ci, for a sample equilibrated in 50% D2O/50% H2O, the 1H1i-13Ci correlation will show a splitting in the 13Ci dimension as a result of the two-bond isotope shift, corresponding to protonated and deuterated states of Ni+1. This isotope shift is 0.084 ± 0.005 ppm and is readily resolved in the 13Ci dimension of the 2D correlation spectrum. Assuming that the chance to find a 1H at a given exchangeable site is independent of whether adjacent sites are protonated or deuterated, the relative intensity of the two “doublet” components is solely a function of the isotopic fractionation at Ni+1.

Figure 1 shows a small region of the 1H-13C spectrum of human ubiquitin, recorded at 600 MHz in 50/50 H2O/D2O, correlating intraresidue 1H9 and 13Ci resonances. The splitting in the 13Ci dimension corresponds to the two-bond isotope shift, and the ratio of the intensities for a 1H9-13Ci correlation is used to derive the fractionation value of residue i + 1. Acquisition times used: 1H, 57 ms; 13C, 110 ms. Digital resolution: 1H, 2.2 Hz; 13C: 0.9 Hz. Dry powder protein (VLI Research, Southeastern, PA) was first dissolved in 50/50 H2O/D2O; the pH was raised to 9 by adding 50/50 NaOD/NaOH, and equilibrated for 4 h at 45 °C; the pH was then lowered to 4.7 by adding 50/50 HCl/NaCl, using a pH meter pre-equilibrated for 1 week in 50/50 H2O/D2O, 3 M KCl. Subsequently the sample was lyophilized and redissolved in 50/50 H2O/D2O transferred to an NMR tube that had been prewashed with 50/50 H2O/D2O, and subsequently dried. The pH of the sample was not remeasured, but the same procedure applied to an unlabeled protein sample yielded no significant pH change after the final lyophilization. The ubiquitin sample was sealed with parafilm, heated to 45 °C overnight, and stored in a sealed desiccator containing drierite. No change in fractionation was observed over a 5-month period.

Table 1 shows a small region of the 1H-13C correlation spectrum recorded for a sample containing 3 mg of U-13C/15N human ubiquitin, dissolved in 250 µL of 50/50 D2O/H2O, pH 4.7, at 27 °C. The spectrum was recorded in 8.5 h on a Bruker AMX-600 spectrometer, using the regular 3D HACACO pulse scheme11 (without D2O presaturation) in which the 13C evolution period was kept at zero. A relatively long 13C dimension as a result of the two-bond isotope shift, corresponding to protonated and deuterated states of Ni+1. This isotope shift is 0.084 ± 0.005 ppm and is readily resolved in the 13Ci dimension of the 2D correlation spectrum. Assuming that the chance to find a 1H at a given exchangeable site is independent of whether adjacent sites are protonated or deuterated, the relative intensity of the two “doublet” components is solely a function of the isotopic fractionation at Ni+1.

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<table>
<thead>
<tr>
<th>Residue</th>
<th>Hδ Value (ppm)</th>
<th>Reproducibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>K6</td>
<td>4.01</td>
<td>±0.01</td>
</tr>
<tr>
<td>E18</td>
<td>4.02</td>
<td>±0.01</td>
</tr>
<tr>
<td>T22</td>
<td>4.03</td>
<td>±0.01</td>
</tr>
<tr>
<td>T66</td>
<td>4.04</td>
<td>±0.01</td>
</tr>
</tbody>
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as a result of HDO $T_1$ noise. These residues are therefore not considered below.

Before quantitatively interpreting the intensity ratio, small corrections need to be made for the difference in $T_2$ relaxation of $H_2^O, C_\alpha$, and $C_i$ during the pulse scheme. First, transverse relaxation of $^1H$ during the $I_{CH}$ dephasing intervals of the HACACO experiment (3 ms each) is different in the case where $N_{i+1}$ is protonated versus deuterated. The $H_\alpha$-H$\bar{\alpha}$ distance is obtained from ubiquitin’s X-ray coordinates and the orientation of the H$\alpha$-H$\bar{\alpha}$ relative to the unique axis of the axially symmetric diffusion tensor is also known. Therefore, these losses can be predicted accurately, taking into account that due to increased viscosity the rotational diffusion in 50/50 D$_2$O/H$_2$O is 10% slower compared to a 5/95 D$_2$O/H$_2$O mixture. The increase in magnetization loss during the $^1H$-dephasing periods for the case where H$\alpha_{i+1}$ is a proton versus a deuteron ranges from 4.2% (R42) to 0.1% (Q31). Differential relaxation during the HACACO pulse scheme caused by $^{13}C_\alpha$-H$\bar{\alpha}^{13}O$ dipolar interactions results in a small ($\leq 0.4\%$) decrease of the correlation for which H$\alpha_{i+1}$ is a proton relative to the deuteron case. Finally, when comparing duplicate experiments, we find that the peak height ratio is more reproducible than the integrated cross peak ratio. When calculating the ratio of the integrated intensities from the peak heights, corrections need to be made for the difference in $^1H$ and $^{13}C$ line widths of the two “doublet” components. Assuming slow exchange of $^1H$ relative to the two-bond isotope shift, the difference in transverse $^{13}C$ relaxation for $^{13}C_i$-H$\bar{\alpha}^{13}O$ versus $^{13}C_i$-H$\bar{\alpha}^1O$ is quite uniform ($\sim 0.4$ s$^{-1}$). Its effect on the peak height is calculated by reprocessing the data with the additional 0.4 s$^{-1}$ exponential line broadening in the $^{13}C$ dimension. Similarly, the effect of differential $^1H_{\alpha}$-H$\bar{\alpha}^1O$ relaxation on the peak intensity is obtained by reprocessing the data with the corresponding additional line broadening in the $F_2$ dimension. This latter correction ranges from an additional 7% attenuation for residues with short $^1H_{\alpha}$-H$\bar{\alpha}^1O$ distances (2.1 Å) to 0.2% for long distances (3.6 Å). Individual $\Phi$ values and correction factors used are available as Supporting Information.

All fractionation factors measured in our study fall in the relatively narrow range of 1.01–1.21. As expected on the basis of previous calculations, we find no clear correlation between hydrogen bond length and fractionation but, on average, the $\alpha$-helical residues (E24–E34) yield slightly lower fractionation numbers (1.07 ± 0.03) than those involved in $\beta$-sheet type hydrogen bonds (1.14 ± 0.04), in agreement with a previous study by Bowers and Klevit. The data also do not support a statistically significant correlation between fractionation and the N-H$\cdots$O angle, or a combination of this angle and hydroxyl bond length (not shown). However, the data indicate a larger $\Phi$ value when the H$\cdots$O=C angle falls in the 180 ± 40° range (Figure 2). This finding coincides with a recent theoretical study

Figure 2. Fractionation values ($\Phi$) for backbone amides in ubiquitin hydrogen bonded to a backbone carbonyl, as a function of the H$\cdots$O=C angle. Only non-bifurcated hydrogen bonds with a H$\cdots$O distance smaller than 2.8 Å and a N-H$\cdots$O angle larger than 120° are included in the figure, which showed a sharp increase in the hydrogen bond potential energy surface for H$\cdots$O=C angles outside this range.

With the exception of D39 (Φ = 1.01), amides hydrogen bonded to water in the X-ray structure (L8, T12, T14, E16, D39, L43, G47, and K63) all have $\Phi$ values in a relatively narrow range (1.07–1.16). Solvent exposed amide hydrogens for which no hydrogen-bonded water molecule is reported in the crystal structure (T14, T22, Q49, D52, and T66) have $\Phi$ values in the same range (1.07–1.17), close to the $\Phi = 1.1$ value, reported for a random coil. The random coil amides of R74 and G75 have $\Phi$ values of 1.11 and 1.09, confirming that the H$_2$O/D$_2$O ratio of the solvent indeed is very close to one.

In summary, we have presented a very simple scheme for quantitative measurement of $^1H$/$^2H$ fractionation. For obtaining highly accurate data, small corrections to the measured resonance intensity ratios are needed which require knowledge of the local structure and dynamics of the protein. Similar corrections are needed when deriving fractionation values from $^1H$-$^15N$ correlation intensities of HSQC spectra, recorded as a function of solvent composition. Our data indicate that all fractionation values in human ubiquitin are larger than one, consistent with the notion that backbone-backbone hydrogen bonds in proteins are weak.

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Supporting Information Available: Tables containing the observed intensity ratios, relaxation correction factors, and $^1H$/$^2H$ fractionation values and a listing of the hydrogen bond lengths, hydrogen bond angles, and acceptor atoms, taken from the crystal structure (4 pages). See any current masthead page for ordering and Internet access instructions.

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References