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

Andy C. LiWang† and Ad Bax*

Laboratory of Chemical Physics
National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health
Bethesda, Maryland 20892-0520

Received August 30, 1996

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% D_2O/50% H_2O solvent mixture. Fractionation is one of the simplest manifestations of the Heisenberg uncertainty principle, $\Delta p_x\, \Delta x = C$, 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 ^{2}H is lower than that of ^{1}H. The same applies to the solvent. However, if the difference between the lowest vibrational energy levels of a proton and a deuteron 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 clusters[4,5] 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 ^{15}N−^{1}H correlations in a range of H_2O/D_2O 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 T_1 attenuates resonances of protons in rapid exchange with solvent and, through the NOE, the intensities of other protons in their vicinity. The attenuation factor is roughly proportional to the H_2O/D_2O ratio and therefore decreases the apparent Φ value. Other factors affecting these measurements include the change in solvent viscosity as a function of the H_2O/D_2O ratio (increasing the apparent Φ) and intramolecular dipole−dipole contributions between exchangeable protons (decrease of Φ). As these effects are present during the dephasing and rephasing delays of the 2D NMR experiment, they affect the integrated resonance intensities.

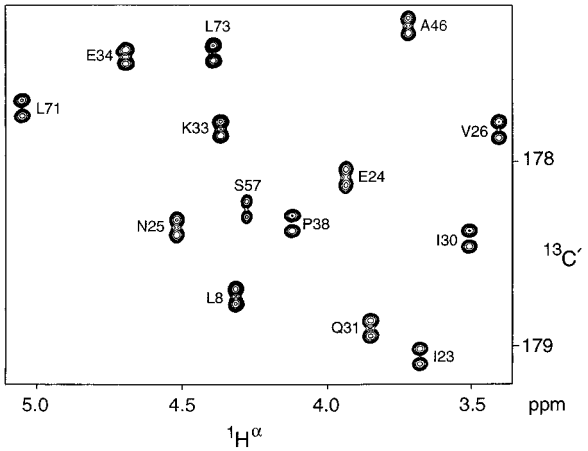

Figure 1. Small region of the HA(CA)CO spectrum of human ubiquitin, recorded at 600 MHz in 50/50 H_2O/D_2O, correlating intraresidue ^{1}H$^\alpha$ and ^{13}C′ resonances. The splitting in the ^{13}C′ dimension corresponds to the two-bond isotope shift, and the ratio of the intensities for a ^{1}H$^\alpha_i$−^{13}C′$_i$ correlation is used to derive the fractionation value of residue $i + 1$. Acquisition times used: ^{1}H$^\alpha$, 57 ms; ^{13}C′, 110 ms. Digital resolution: ^{1}H$^\alpha$, 2.2 Hz; ^{13}C′: 0.9 Hz. Dry powder protein (VLI Research, Southeastern, PA) was first dissolved in 50/50 H_2O/D_2O; 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/DCl, using a pH meter pre-equilibrated for 1 week in 50/50 H_2O/D_2O, 3 M KCl. Subsequently the sample was lyophilized and redissolved in 50/50 H_2O/D_2O, transferred to an NMR tube that had been prewashed with 50/50 H_2O/D_2O, 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.

Here we report a different method for measuring backbone amide D/H fractionation, based on the HACACO experiment[10] which is commonly used for protein backbone resonance assignments: If the ^{1}H$^\alpha$ resonance of residue i (^{1}H$^\alpha_i$) is correlated with ^{13}C′$_i$ for a sample equilibrated in 50% D_2O/50% H_2O, the ^{1}H$^\alpha_i$−^{13}C′$_i$ correlation will show a splitting in the ^{13}C′$_i$ dimension as a result of the two-bond isotope shift, corresponding to protonated and deuterated states of N_{i+1}. This isotope shift is 0.084 ± 0.005 ppm and is readily resolved in the ^{13}C′ dimension of the 2D correlation spectrum. Assuming that the chance to find a ^{1}H 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 N_{i+1}.

Figure 1 shows a small region of the ^{1}H$^\alpha_i$−^{13}C′$_i$ correlation spectrum recorded for a sample containing 3 mg of U−^{13}C/^{15}N human ubiquitin, dissolved in 250 μL of 50/50 D_2O/H_2O, 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 scheme[11] (without HDO presaturation) in which the ^{13}C$^\alpha$ evolution period was kept at zero. A relatively long ^{13}C′ evolution period of 110 ms was used, yielding a 2D spectrum with high ^{13}C′ resolution. The experiment was repeated four times, twice with an interscan delay of 1.2 s and twice with 2.2 s. The reproducibility in the doublet intensity ratios indicates a random error of ±0.01 in the averaged value and shows no effect of the interscan delay on the intensity ratio. For four residues (K6, E18, T22, and T66) the H$^\alpha$ of the preceding residue resonates very close to the HDO resonance and reproducibility of the intensity ratio was up to 10-fold worse

† Present address: Department of Chemistry, 1393 Brown Building, 331 WTHR, Purdue University, West Lafayette, IN 47907-1393.

(1) Kreevoy, M. M. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds; Elsevier: Amsterdam, 1976; pp 1−31.
(2) Cleland, W. W. *Methods Enzymol.* **1980**, *64*, 105−125.
(3) Schowen, K.; Schowen, R. L. *Methods Enzymol.* **1982**, *87*, 551−606.
(4) Edison, A. E.; Weinhold, F.; Markley, J. L. *J. Am. Chem. Soc.* **1995**, *117*, 9619−9624.
(5) Edison, A. E.; Markley, J. L.; Weinhold, F. *J. Phys. Chem.* **1995**, *99*, 8013−8016.
(6) Scheiner, S.; Cuma, M. *J. Am. Chem. Soc.* **1996**, *118*, 1511−1521.
(7) Loh, S. N.; Markley, J. L. In *Techniques in Protein Chemistry*; Angeletti, R., Ed.; Academic Press: San Diego, 1993; Vol. 4, pp 517−524.
(8) Loh, S. N.; Markley, J. L. *Biochemistry* **1994**, *33*, 1029−1036.
(9) Bowers, P. M.; Klevit, R. E. *Nature, Struct. Biol.* **1996**, *3*, 522−531.
(10) Ikura, M., Kay, L. E.; Bax, A. *Biochemistry* **1990**, *29*, 4659−4667.
(11) Grzesiek, S.; Bax, A. *J. Magn. Reson. Ser. B* **1993**, *102*, 103−106.

S0002-7863(96)03055-7 CCC: $12.00

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^{α}_i, C^{α}_i, and C'_i during the pulse scheme. First, transverse relaxation of $^1H^{\alpha}$ during the $^1J_{CH}$ de/rephasing intervals of the HCACO experiment (3 ms each) is different in the case where N_{i+1} is protonated versus deuterated. The $H^{\alpha}_i-H^{N}_{i+1}$ distance is obtained from ubiquitin's X-ray coordinates[12] and the orientation of the $H^{\alpha}_i-H^{N}_{i+1}$ relative to the unique axis of the axially symmetric diffusion tensor is also known.[13] Therefore, these losses can be predicted accurately, taking into account that due to increased viscosity the rotational diffusion in 50/50 D_2O/H_2O is 10% slower compared to a 5/95 D_2O/H_2O mixture.[14] The increase in magnetization loss during the $^1H^{\alpha}$−de/rephasing periods for the case where H^{N}_{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 $^{13}C^{\alpha}_i-^1H^{N}_{i+1}/^2H^{N}_{i+1}$ dipolar interactions results in a small ($\leq$0.4%) decrease of the correlation for which H^{N}_{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 $^1H^{\alpha}$ and $^{13}C'$ line widths of the two "doublet" components. Assuming slow exchange of H^N relative to the two-bond isotope shift, the difference in transverse $^{13}C'$ relaxation for $^{13}C'-\{^2H^{N}_{i+1}\}$ versus $^{13}C'-\{^1H^{N}_{i+1}\}$ 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 $^1H^{\alpha}_i-\{^1H^{N}_{i+1}/^2H^{N}_{i+1}\}$ 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 $^1H^{\alpha}_i-^1H^{N}_{i+1}$ distances (2.1 Å) to 0.2% for long distances (3.6 Å). Individual Φ 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,[5] we find no clear correlation between hydrogen bond length and fractionation but, on average, the α-helical residues (E24−E34) yield slightly lower fractionation numbers (1.07 ± 0.03) than those involved in β-sheet type hydrogen bonds (1.14 ± 0.04), in agreement with a previous study by Bowers and Klevit.[9] The data also do not support a statistically significant correlation between fractionation and the N−H···O angle, or a combination of this angle and hydrogen bond length (not shown). However, the data indicate a larger Φ value when the H···O═C angle falls in the 180 ± 40° range (Figure 2). This finding coincides with a recent theoretical study

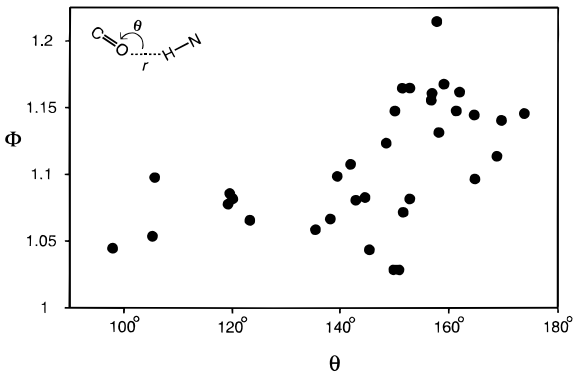

Figure 2. Fractionation values (Φ) for backbone amides in ubiquitin hydrogen bonded to a backbone carbonyl, as a function of the H···O═C angle. Only non-bifurcated hydrogen bonds with a H···O distance smaller than 2.8 Å and a N−H···O angle larger than 120° are included in the figure.

which showed a sharp increase in the hydrogen bond potential energy surface for H···O═C angles outside this range.[15]

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 Φ 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 Φ values in the same range (1.07−1.17), close to the Φ = 1.1 value, reported for a random coil.[16] The random coil amides[13] of R74 and G75 have Φ values of 1.11 and 1.09, confirming that the H_2O/D_2O ratio of the solvent indeed is very close to one.

In summary, we have presented a very simple scheme for quantitative measurement of $^2H/^1H$ 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 $^1H-^{15}N$ 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.

Acknowledgment. We thank Nico Tjandra for useful discussions and unpublished data and Frank Delaglio and Dan Garrett for assistance and use of their software. This work was supported by the AIDS Targeted Anti-Viral Program of the Office of the Director of the National Institutes of Health. A.C.L. is supported by an American Cancer Society postdoctoral fellowship (PF-4030).

Supporting Information Available: Tables containing the observed intensity ratios, relaxation correction factors, and $^2H/^1H$ 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.

JA9630553

(12) Vijay-Kumar, S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1987**, *194*, 531−544.
(13) Tjandra, N.; Feller, S. E.; Pastor, R. W.; Bax, A. *J. Am. Chem. Soc.* **1995**, *117*, 12562−12566.
(14) *The Merck Index*, 9th ed.; Merck & Co.: Rahway, NJ, 1976; p 385. Tjandra, N. Unpublished results.
(15) Adalsteinsson, H.; Maulitz, A. H.; Bruice, T. C. *J. Am. Chem. Soc.* **1996**, *118*, 7689−7693.
(16) Englander, S. W.; Poulsen, A. *Biopolymers* **1969**, *7*, 379−393.