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D/H Amide Isotope Effect in Model α-Helical Peptides

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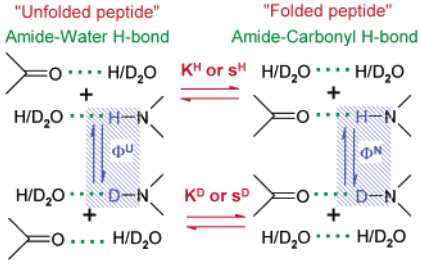
One of the major questions in protein chemistry concerns the role of amide hydrogen bonds (H-bonds) in stabilizing the native state. Recently, D/H amide isotope effects (fractionation factor) [1−3] have been used as a probe to estimate the relative contribution of H-bonds to protein stability.[4−7] The "C_m experiment" uniquely isolates solvent isotope effects,[8,9] and thus can assess the energetic contribution from amide-related H-bonds exclusively (amide−carbonyl and amide−water) by isotope effect measurements.[7] Studies on more than 10 model proteins indicate that the isotope effect is most significant in α-helical proteins, weaker for α/β- and negligible for all β-proteins.[9,10] A linear correlation is found between the isotope effect and the number of helical H-bonds in a protein, the slope being -8.6 ± 0.9 cal mol^{-1} site^{-1}.[10] Several issues remain to be resolved in these studies, including: (i) Is the D/H amide isotope effect denaturant dependent? (ii) Are buried helical H-bonds in proteins different from solvent exposed H-bonds, that is, do they show distinct isotope effects? Here we address these and some related issues by measuring D/H amide isotope effects in model alanine-rich peptides under native conditions.

The C_m experiment was originally developed to measure the D/H amide isotope effect in proteins.[8,9] The experiment determines folding equilibrium constants (K^H and K^D) for both protonated and deuterated proteins (with all amides protonated or deuterated) in the same solvent condition; the resulting stability difference, $\Delta\Delta G^{D-H} = RT \ln(K^D/K^H)$ defines the isotope effect (Scheme 1). The C_m experiment is so termed because experiments carried out near the midpoint of the GdmHCl-induced unfolding transition (C_m) where $\Delta G^{fold} \approx 0$ have intrinsically highest sensitivity. The experiment reports the isotope effect for the equilibrium: amide−carbonyl ↔ amide−solvent (water).[7,9] In principle, GdmHCl at ca. 1 M concentration can influence the isotope effect directly by binding to the amide group or indirectly by changing the properties of bulk water, or both. It is important, then, to compare isotope effects for different proteins in the same reference solvent conditions. However, in practice isotope effects are measured at different concentrations of GdmHCl due to differences in the C_m values from one protein to another. To compare measured isotope effects for different proteins, it is essential to calibrate the GdmHCl concentration dependence of the D/H amide isotope effect.

We show here that model peptides can ideally serve this purpose. Isolated alanine peptides are marginally stable[11] and can have ∼50% helix content at low temperature in aqueous solution. Their free energies can be evaluated by fitting the experimental CD values to helix-to-coil transition models.[12,13] Since D/H amide isotope effects can be measured in the absence of GdmHCl, the GdmHCl dependence of the D/H amide isotope effects can be determined by comparing the measured values with those obtained for proteins

Scheme 1. C_m Experiment Illustrated in a Thermodynamic Cycle[a]



[a] The isotope effect, $\Delta\Delta G_{fold}^{D-H}$, is related to the ratio of s^D to s^H. The vertical arms are fractionation factors of all amide protons in a model peptide either in the unfolded state when backbone amides are H-bonded to water, or in the folded state when backbone amides are H-bonded to amides themselves.

at different concentrations of GdmHCl. Instead of K^H and K^D values for proteins,[7,9,10] experiments on isolated helices yield values of s^H and s^D, where s^H is the Zimm−Bragg site-averaged helical propagation parameter when all amides are in the protonated form, and s^D, the corresponding parameter when all amides are deuterated. As illustrated in Scheme 1, $\Delta\Delta G^{D-H} = RT \ln(s^D/s^H)$ defines the isotope effect.

The model alanine-rich peptides used in this study and their sequences and detailed characterization by CD and NMR have been reported previously.[14,15] According to Scheme 1, we need to measure values of s^H and s^D for fully protonated and deuterated peptides under identical solvent conditions. Equivalently, we can monitor the change in the helical content (CD signal) of peptides as the peptide amides exchange from deuterium to protium. Experimentally, fully deuterated peptides are diluted 50-fold into H_2O buffer at pH 4.5 and 4 °C, where the peptide equilibrates to the new solvent condition in less than a second, the stability of the deuterated peptide can be determined from the initial CD value before significant backbone amide exchange occurs (Figure 1). As hydrogen exchange (HX) proceeds, the helix content of the peptide varies and the CD signal changes. Once HX is complete, the helix content of the fully exchanged (protonated) peptide is measured. The difference in helicity (fitted s values) before and after HX defines $\Delta\Delta G_{fold}^{D-H}$. The results and derived isotope effects for these model alanine peptides are summarized in Table 1.

The isotope effects (Table 1) determined here for these model peptides are significantly larger than the mean value of -8.6 ± 0.9 cal mol^{-1} site^{-1} reported for helical H-bonds in proteins.[10] There may thus be a substantial GdmHCl effect in isotope effects. Krantz et al. [9] observed different isotope effect values for dimeric (10.8 cal mol^{-1} site^{-1}) and monomeric (6.8 cal mol^{-1} site^{-1}) versions of the coiled coil protein GCN4. The respective C_m of the GdmHCl-induced unfolding transitions are 2.7 and 6.1 M for dimeric (at the concentration studied) and monomeric GCN4 proteins, respectively. If we attribute the entire observed difference between the two GCN4

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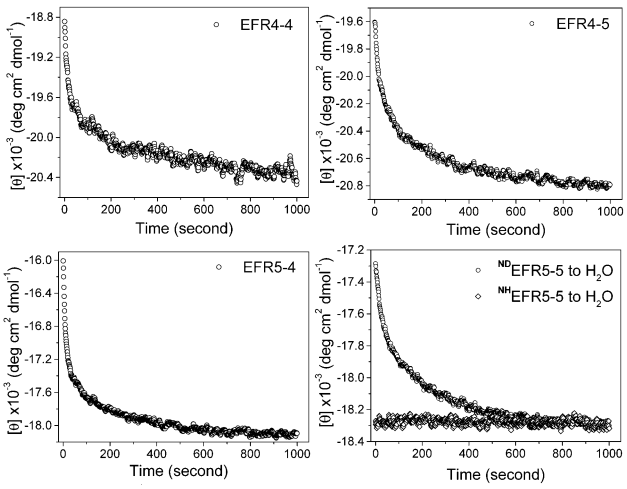

Figure 1. Change in stability upon HX for model peptides. The initial and final CD values determine s^D and s^H (for D- and H-peptides). For all four peptides, helical contents are monitored by CD at 222 nm and results show that deuterated molecules gain stability when they exchange in H_2O. For EFR5-5, a control experiment, in which ^{NH}EFR5-5 is diluted into H_2O, shows the CD signal remains a constant value after dilution. For the residue E in each peptide, a change of the side chain protonation state upon the switch of the solvent from D_2O to H_2O (pH = 4.5) could happen which should cause a shift of the CD signal. We assume here that the change of the protonation state is complete before the first data point.

Table 1. Observed Helicity and Isotope Effects for Model Peptides

peptide[a]	$-[\theta]^H_{222} \times 10^{-3}$ (deg cm^2 dmol^{-1})[b]	$-[\theta]^D_{222} \times 10^{-3}$ (deg cm^2 dmol^{-1})[b]	^Hf_H[c]	^Df_H[c]	$\Delta\Delta G^{D-H}$ (cal mol^{-1})[d]
EFR4-5	20.8	19.6	0.612	0.576	17.3
EFR4-4	20.4	18.8	0.600	0.554	17.5
EFR5-5	18.3	17.3	0.538	0.508	11.2
EFR5-4	18.1	16.0	0.532	0.471	18.6

[a] EFR4-5: Ac-OOAAAAA**E**AAA**F**AAAA**R**AAAAOOY-NH_2; EFR4-4: Ac-OOAAAAA**E**AAA**F**AAA**R**AAAAOOY-NH_2; EFR5-5: Ac-OOAAAA**E**AAAA**F**AAAA**R**AAAAOOY-NH_2; EFR5-4: Ac-OOAAAA**E**AAAA**F**AAA**R**AAAAA OOY-NH_2). [b] In 30 mM sodium acetate at pH 4.5 and 4 °C. The final concentrations are ~50 μM, and the molar CD values for protonated (H-) peptides are normalized to the values reported [14,15]. $[\theta]^H_{222}$ is the molar ellipticity for H-peptides, and $[\theta]^D_{222}$ that for deuterated (D-) petides. Initial data point is used to calculate $[\theta]^D_{222}$ for each peptide. The reported values are the averages of 2−3 measurements. [c] The relationship between fraction helicity and molar ellipticity is $f_H = [\theta]_{222}/[\theta]_{222}°$, where $[\theta]_{222}° = -34\,000$ is the estimated molar ellipticity at 222 nm for an α-helix of 24-mer.[19] ^Hf_H[b] is the fraction helicity of H-peptides, and ^Df_H[b] that of D-peptides. [d] The site-averaged isotope effects were calculated using the equation $\Delta\Delta G^{D-H} = RT \ln(s^D/s^H)$. Both s^D and s^H were fitted using the Zimm−Bragg helix-to-coil transition model,[12,19,20] with the nucleation parameter (0.004) and values of the helix propensities for Ala, Glu, Phe, Arg, Tyr, and Orn as described.[15] On the basis of the precision of repetitive measurents for each peptide, the error of the final reported isotope effect ($\Delta\Delta G^{D-H}$ values) should be less than ±5 cal mol^{-1}.

proteins to the GdmHCl solvent effect, we can extrapolate an isotope effect value of 14 cal mol^{-1} site^{-1}, which lies in the range we derived for model peptides.

For three (EFR4-4, EFR4-5, EFR5-4) of the peptides studied, the observed isotope effects are still larger than the value extrapolated above. This may reflect an intrinsic structural difference between the helical H-bonds in proteins and those in isolated helices. Conceivably helical H-bonds in isolated helices are more optimal geometrically, with less strain arising from packing in proteins with different complexity of topology. Even in a protein with a topology as simple as GCN4, Fourier transform infrared (FTIR) studies[16,17] demonstrate that there is a distortion of the local helical structure compared to that in standard α-helical polypeptides such as polyalanine. Similarly NMR studies[18] detect a periodicity in both HX rates and amide proton chemical shifts in GCN4. These observations suggest that the backbone ϕ,ψ angles or H-bond lengths may differ from those in an undistorted helix and even from site-to-site within GCN4. For a protein with still greater complexity in topology, further distortions due to packing may be inevitable. Their helical H-bonds may have less optimal geometry and smaller observed isotope effects. Even in the two versions of GCN4, dimeric GCN4 may have less strain than the cross-linked version since the Cys-Gly-Gly tether might introduce additional strain at least to the local tethered region. If so, the derived GdmHCl effect may be slightly overestimated, and the extrapolated isotope effect value of 14 cal mol^{-1} site^{-1} would be the upper limit for GCN4 after correcting for the GdmHCl solvent effect.

Two factors—the GdmHCl solvent effect and steric (strain) effect—can thus explain why the isotope effects are larger in isolated helices than in dimeric GCN4 and still lower in cross-linked GCN4. However, the difference between the isolated helix and GCN4 may reflect the difference in amino acid composition between model alanine peptides and GCN4.

By applying the scale factor (74 ± 27) [7] which relates the strength of an H-bond to its isotope effect, we can further derive a value of −1.2 ± 0.4 kcal/mol for the energetic contribution from amide-related H-bonds in isolated helices. This value is slightly larger than that for GCN4 (−1.0 ± 0.4 kcal/mol) after correcting for the GdmHCl solvent effect. Thus, fully or partially buried H-bonds in helical proteins may not be energetically more favorable than H-bonds that are solvent-exposed in isolated helices when the two are compared properly. The difference is consistent with the picture discussed above in which undistorted H-bonds in an isolated helix experience less strain.

In summary, we report here that D/H amide isotope effects are GdmHCl concentration-dependent. Our results suggest that the buried helical H-bonds in proteins are not necessarily energetically more favorable than solvent-exposed H-bonds in isolated monomeric helices.

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