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Enthalpic stabilization of an SH3 domain by D_2O

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Abstract: The stability of a protein is vital for its biological function, and proper folding is partially driven by intermolecular interactions between protein and water. In many studies, H_2O is replaced by D_2O because H_2O interferes with the protein signal. Even this small perturbation, however, affects protein stability. Studies in isotopic waters also might provide insight into the role of solvation and hydrogen bonding in protein folding. Here, we report a complete thermodynamic analysis of the reversible, two-state, thermal unfolding of the metastable, 7-kDa N-terminal src-homology 3 domain of the *Drosophila* signal transduction protein drk in H_2O and D_2O using one-dimensional ^{19}F NMR spectroscopy. The stabilizing effect of D_2O compared with H_2O is enthalpic and has a small to insignificant effect on the temperature of maximum stability, the entropy, and the heat capacity of unfolding. We also provide a concise summary of the literature about the effects of D_2O on protein stability and integrate our results into this body of data.

Keywords: deuterium oxide; NMR spectroscopy; protein folding; protein stability; SH3 domain; solvent isotope effect; thermodynamics

Introduction

Water, arguably the most important molecule for life on Earth,[1,2] is essential for the stability, folding, and structure of proteins that drive biology. The hydrogen bonds between protein and water help shape the free energy landscape of folding, guiding a protein towards its stable, folded state. In many experimental techniques, however, the signal from H_2O interferes with that from the protein, and D_2O is used as the solvent. Nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy, small angle X-ray scattering, and small angle neutron scattering are techniques that often incorporate this substitution. Additionally, examining proteins in D_2O, which is only a small perturbation of the system, can provide insight into the role of hydration and hydrogen-bonding in protein folding.[3–8] Nonetheless, this solvent substitution can affect proteins,[9] and it is important to be cognizant of these effects when conducting experiments in D_2O. The aim of the present study is three-fold: provide a complete thermodynamic analysis of globular protein folding in H_2O and D_2O, concisely summarize similar literature studies, and compare our results to those in the literature.

We chose the metastable, 7-kDa N-terminal src homology 3 domain of the *Drosophila* signal transduction protein drk (SH3) to probe solvent isotope effects on protein stability. Even under non-denaturing conditions, a large population of SH3 is unfolded.[10] SH3 has one tryptophan, which we labeled with a fluorine atom.[11] This residue experiences different solvent exposure in the folded and unfolded states, resulting in two ^{19}F resonances in slow exchange on the NMR timescale,[10,12] one for the folded state and one for the unfolded ensemble (Fig. 1). The presence of only two

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resonances is consistent with two-state folding. The areas under the resonances can be integrated to obtain the relative populations of each state and thus a modified standard-state free energy of unfolding:

$$\Delta G_U^{\circ\prime} = -RTln\frac{population\ unfolded}{population\ folded} \quad (1)$$

where R is the gas constant and T is the absolute temperature. The temperature dependence of $\Delta G_U^{\circ\prime}$ is used to construct a protein stability curve (Fig. 2)[13] which provides a complete thermodynamic picture of folding in H_2O and D_2O via the integrated Gibbs–Helmholtz equation:

$$\Delta G_{\mathrm{U}}^{\circ\prime} = \Delta H_{\mathrm{U}}^{\circ\prime} - T\Delta S_{\mathrm{U}}^{\circ\prime} + \Delta C_p^{\circ\prime}\left[T - T_{\mathrm{ref}} - T\ln\left(\frac{T}{T_{\mathrm{ref}}}\right)\right] \quad (2)$$

where T_{ref} is a reference temperature, $\Delta H_{\mathrm{U}}^{\circ\prime}$ and $\Delta S_{\mathrm{U}}^{\circ\prime}$are the modified standard state enthalpy and entropy of unfolding, respectively, and $\Delta C_p^{\circ\prime}$ is the modified standard state heat capacity of unfolding which is assumed to be temperature independent within the range studied.

Results and Discussion

Heat-induced unfolding of SH3 in H_2O and D_2O

The metastability of this SH3 domain allows stability curves to be constructed and analyzed[13–15] at reasonable temperatures (5°C–45°C). The stability curve in D_2O is simply shifted above the curve in H_2O (Fig. 2). This shift indicates that SH3 stability is greater in D_2O at all temperatures and that the effects of D_2O are mainly enthalpic.[9,14] $\Delta C_p^{\circ\prime}$, as well as $\Delta G_{\mathrm{U}}^{\circ\prime}$, $\Delta S_{\mathrm{U}}^{\circ\prime}$, and $\Delta H_{\mathrm{U}}^{\circ\prime}$ at any temperature can be calculated by fitting the data to Equation 2.

The thermodynamic parameters (Table I) paint a picture of the solvent isotope effect. At 318 K,

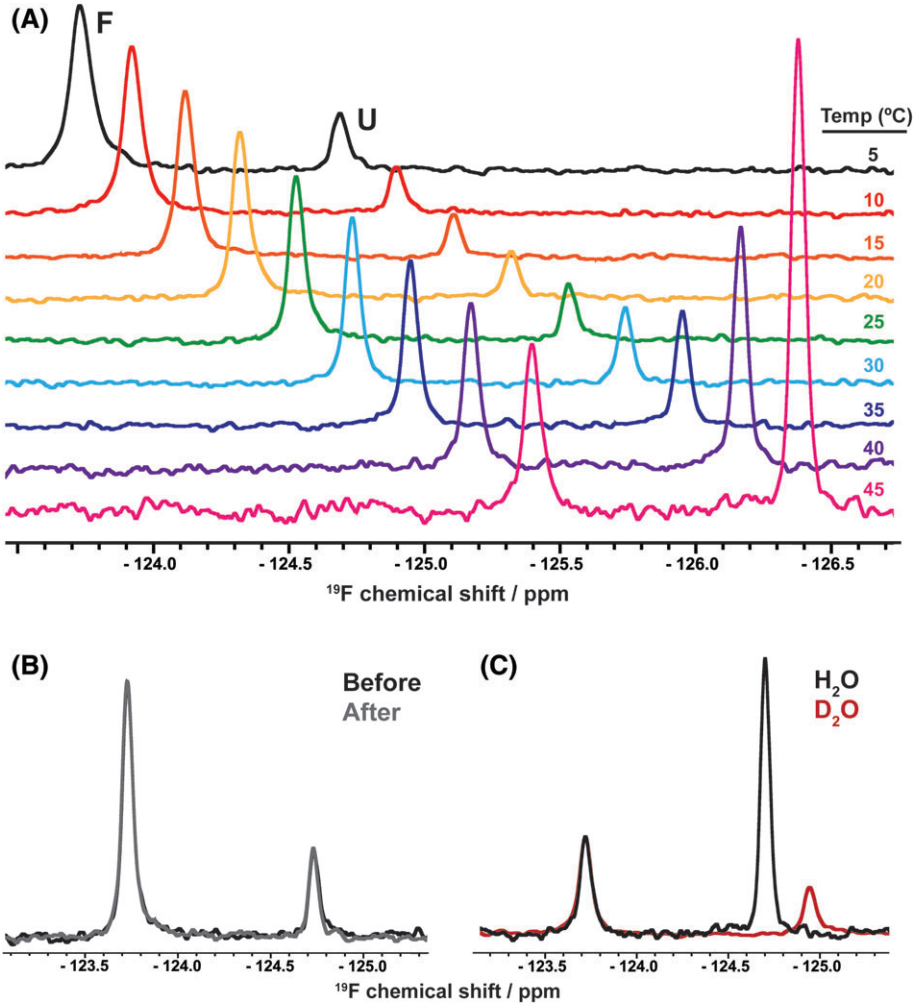

Figure 1. Two-state, temperature-dependent, reversible SH3 folding in H_2O and D_2O monitored with ^{19}F NMR. (A) One-dimensional spectra in H_2O at nine temperatures normalized to the intensity of the folded state peak at 5°C. Subsequent spectra are offset by –0.2 ppm to aid visualization. The downfield resonance is the folded state (F), and the upfield resonance is the unfolded ensemble (U). (B) Spectra at 25°C before (black) and after (gray) an experiment where the temperature ranged from 5°C to 45°C over approximately 180 min indicating the reversibility of denaturation. (C) Spectra in H_2O (black) and D_2O (red) at 45°C. The D_2O spectrum is normalized to the chemical shift and intensity of the folded state resonance in H_2O.

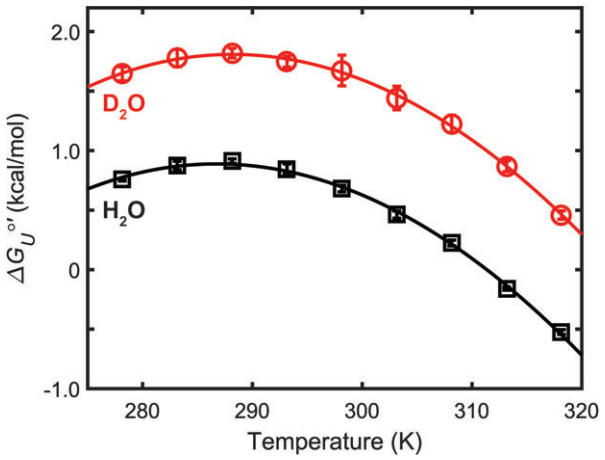

Figure 2. Temperature dependence of SH3 stability in H_2O and D_2O. Error bars represent standard errors of the mean from three independent trials.

approximately half way between T_m in D_2O and H_2O, the heavy water stabilizes the protein by nearly 1 kcal/mol [Fig. 1(C)]. This increase is also visible by comparing the T_m (the melting temperature, where $\Delta G_U^{\circ\prime} = 0$). D_2O increases this value by approximately 12 K, indicating an increased thermal stability. The higher stability in D_2O is often rationalized in terms of the increased difficulty of cavity creation in D_2O compared with H_2O.[9,16,17]

Breaking $\Delta G_U^{\circ\prime}$ into its enthalpic and entropic components shows only an effect on the enthalpy of unfolding. In both H_2O and D_2O, the enthalpy and entropy of unfolding are large and positive making it difficult to determine which one dominates at 318 K. Examining the enthalpy at the temperature of maximum stability, T_s, is more useful due to the small uncertainty in the value at this temperature. At T_s the entropy is zero,[13] and therefore differences in enthalpy are identified. Additionally, T_s is nearly the same in H_2O and D_2O. The $\Delta H_{U,T_S}^{\circ\prime}$ in H_2O is 0.89 ±0.03 kcal/mol, whereas in D_2O, it is twice as large (1.8 ±0.1 kcal/mol). The curvature is the same in both solvents. This is quantitatively demonstrated by a minimal change in $\Delta C_p^{\circ\prime}$. In summary, at all temperatures, the increased stability of SH3 in D_2O is dominated by $\Delta H_U^{\circ\prime}$.

Origins of the D_2O effect

The molecular origins of this enthalpic stabilization are difficult to pinpoint, especially since they cannot be directly measured. D_2O stabilization of proteins is often attributed to an increase in hydrogen bond strength in heavy water,[18–22] which is also consistent with the observation that D_2O reduces protein flexibility.[23,24] Although a change in solvent hydrogen bond strength would be reflected in a change in enthalpy because there are numerous solvent–solvent, solvent–protein, and protein–protein hydrogen bonds formed during protein folding, there are multiple contributions to the enthalpy of unfolding.[25] In addition to the enthalpy from hydrogen bond formation and breakage, solvation enthalpy also plays a significant role in protein folding.[9] Protein unfolding involves solvation of groups that are buried in the folded state. The enthalpy of solvation for a particular protein is, therefore, based on its sequence, structure, and changes in the solvent accessible surface area upon unfolding. Solvation enthalpies are typically based on studies of small molecules, which are then used in combination to calculate a net enthalpy of protein solvation.[9,25] These solvation enthalpies are large in magnitude and opposite in sign for apolar versus polar groups, often resulting in small estimated net enthalpy changes of both signs for an entire protein. It is likely that the observed increase in the enthalpy of SH3 unfolding in D_2O arises from a combination of solvation and hydrogen-bond effects.

The heat capacity of unfolding, $\Delta C_p^{\circ\prime}$, which is related to solvation changes upon unfolding, is often difficult to quantitatively interpret due to the relatively large uncertainty in its value (~10%, Table I).[9] Additionally, we assume that $\Delta C_p^{\circ\prime}$ does not change with temperature, which is not necessarily true,[13,25,26] but a good assumption over a small range, like the one used here.[13,27] For SH3, $\Delta\Delta C_p^{\circ\prime}$ ($\Delta C_{p,D_2O}^{\circ\prime} - \Delta C_{p,H_2O}^{\circ\prime}$) is 0.0 ±0.1 kcal/mol/K, meaning that any change is too small to interpret, which suggests that $\Delta C_p^{\circ\prime}$ is constant from 5 °C to 45 °C. This conclusion is consistent with other observations.[9,22,28] As suggested in the previous paragraph, the change in the heat capacity of transfer of hydrophilic versus hydrophobic protein groups from light- to heavy-water are often large and

Table I. *Equilibrium thermodynamic parameters for SH3 in H_2O and D_2O*

Condition	$\Delta G_{U,318.15K}^{\circ\prime}$ (kcal/mol)[a]	$\Delta H_{U,T_m}^{\circ\prime}$ (kcal/mol)[b]	$\Delta H_{U,T_s}^{\circ\prime}$ (kcal/mol)[b]	$\Delta H_{U,318.15K}^{\circ\prime}$ (kcal/mol)[c]	$T\Delta S_{U,318.15K}^{\circ\prime}$ (kcal/mol)[c]	$\Delta C_P^{\circ\prime}$ (kcal/mol/K)[b]	T_m (K)[b]	T_s (K)[b]
H_2O	−0.52 ±0.02	22 ±2	0.89 ±0.03	28 ±1	28 ±1	0.86 ±0.09	311 ±1	287 ±1
D_2O	0.46 ±0.04	33 ±2	1.8 ±0.1	29 ±1	28 ±1	0.89 ±0.07	323 ±1	288 ±1

[a]Uncertainties determined from standard error of the mean from triplicate experimental analysis.
[b]Uncertainties determined from 95% confidence intervals of fit to the integrated Gibbs–Helmholtz equation.
[c]The temperature half way between T_m in H_2O and D_2O. Values from Kirchhoff's equations and uncertainties by error propagation from the uncertainties in $\Delta C_p^{\circ\prime}$, T_{ref} (T_m or T_s), and $\Delta H_{U,ref}^{\circ\prime}$ ($\Delta H_{U,T_m}^{\circ\prime}$ or $\Delta H_{U,T_s}^{\circ\prime}$)

opposite in sign,[9] resulting in a minimal and uncertain change in $\Delta C_p^{\circ\prime}$.

Literature studies find D_2O is primarily stabilizing

The effects of D_2O on protein stability have been of interest for decades (Table II). The purpose of Table II is to highlight peer-reviewed publications in which the stability of a protein is directly compared in H_2O and D_2O. The majority of studies reveal that D_2O stabilizes proteins. The parameter most used to assess the influence of heavy water is its effect on the melting temperature, T_m. Although the degree to which D_2O increases the T_m of a particular protein varies, our data is in accord with the literature[19,21,22,28,30–32,36,37] in that the T_m of a protein increases upon changing H_2O to D_2O. In all studies, but one,[32] that report $\Delta G_U^{\circ\prime}$ along with T_m, an increased melting temperature is accompanied by an increase in the free energy of unfolding.[22,28,35] In the case of phycocyanin,[30] an increase in the activation free energy of denaturation is observed.

Additional equilibrium thermodynamic parameters describing protein stability became more commonplace with the advent of highly accurate calorimeters. Differential scanning calorimetry is particularly useful because the melting temperature, enthalpy, and heat capacity of unfolding can be measured.[40,41] All but one study[32] reports results similar to ours: D_2O increases the enthalpy of unfolding.[22,30,36,40] One report[28] shows minimal changes to $\Delta H_U^{\circ\prime}$ and increases in both T_m and $\Delta G_U^{\circ\prime}$. Attempts have been made to describe the molecular basis of this change in enthalpy, often attributed to increased hydrogen-bond strength. As described here, however, there are likely multiple contributions making it difficult to ascribe its effects to hydrogen bonding or solvation alone. Some investigators also report the entropy of unfolding[28,30]; but like enthalpy, it contains multiple contributions.[25] In summary, our results correspond to almost all published observations: D_2O increases the stability ($\Delta G_U^{\circ\prime}$),[22,28,30,34,35,37] the melting temperature (T_m),[19,21,22,28,30–32,36,37] and the enthalpy of protein unfolding ($\Delta H_U^{\circ\prime}$).[22,30,36,37]

D_2O affects many biological processes

Although we focus on protein stability, the effects of D_2O on many biological processes have been investigated with the potential for widespread impact on the fundamental roles of water in biology and therapeutics. D_2O affects protein–carbohydrate, protein–peptide, and protein–nucleic acid interactions,[42] with effects on the enthalpy of binding. In addition to binding, D_2O enhances protein oligomerization and aggregation,[36,43–52] by what has been suggested to be the promotion of hydrophobic interactions. Given the influence of heavy water on biomolecular reactions,

Table II. *Effects of D_2O on Protein Stability*

Protein	Method	Effect of D_2O	Parameter(s) examined
Ovalbumin[29]	Urea, polarimetry	Stabilizing	$t_{1/2}$
Ribonuclease[19,21]	Heat, polarimetry	Stabilizing	T_m
Phycocyanin[30]	Heat, absorbance spectroscopy, fluorescence quenching	Stabilizing	T_m, $\Delta H^{\circ\prime\ddagger}$, $\Delta S^{\circ\prime\ddagger}$, $\Delta G^{\circ\prime\ddagger}$
Staphylococcal nuclease[31]	Heat, GdnSCN, GdnHCl, NMR, CD, fluorescence spectroscopy	Stabilizing	T_m
Bovine ribonuclease A[32]	DSC	Small	$\Delta H^{\circ\prime}$, $\Delta G^{\circ\prime}$, T_m
Hen egg lysozyme[32]	DSC	Destabilizing	$\Delta H^{\circ\prime}$, $\Delta G^{\circ\prime}$, T_m
Cytochrome c[32]	DSC	Destabilizing	$\Delta H^{\circ\prime}$, $\Delta G^{\circ\prime}$, T_m
Malate dehydrogenase[33]	Enzyme assay	Stabilizing	Residual enzyme activity
Staphylococcal nuclease[34]	Urea, CD, FTIR	Stabilizing	m-Value, $\Delta G^{\circ\prime}$
Domain 1 of rat CD2[35]	GdnHCl, stopped-flow fluorescence spectroscopy	Stabilizing	$\Delta G^{\circ\prime}$, $\Delta G^{\circ\prime\ddagger}$, $k_{I\text{-}F}$, $k_{F\text{-}I}$, m-values
NTL9[28]	Heat, urea, GdnHCl, far-UV CD	Stabilizing	T_m, $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$, $\Delta S^{\circ\prime}$, m-values, $\Delta C_p^{\circ\prime}$
β-lactoglobulin[36]	DSC, DLS	Stabilizing	T_m, $\Delta H^{\circ\prime}$
Ribonuclease A[37]	Heat, urea, CD, fluorescence spectroscopy, HDX NMR	Stabilizing	T_m, ΔH°, $\Delta C_p^{\circ\prime}$
Ribonuclease T1[37]	Heat, urea, CD, fluorescence spectroscopy, HDX NMR	Stabilizing	T_m, ΔH°, $\Delta C_p^{\circ\prime}$
Polyproline type II helix[38]	CD	Stabilizing	Polyproline II content
Hen egg lysozyme[22]	DSC	Stabilizing	T_m, $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$, $\Delta S^{\circ\prime}$, $\Delta C_p^{\circ\prime}$
Bovine serum albumin[22]	DSC	Stabilizing	T_m, $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$, $\Delta S^{\circ\prime}$, $\Delta C_p^{\circ\prime}$
Bovine serum albumin[39]	Heat, far-UV CD	Stabilizing	Molar ellipticity

Abbreviations: $t_{1/2}$, half-time of denaturation reaction; GdnSCN, guanidine thiocyanate; GdnHCl, guanidine hydrochloride; NMR, nuclear magnetic resonance spectroscopy; CD, circular dichroism spectropolarimetry; $k_{I\text{-}F}$, rate of intermediate to folded state reaction; $k_{F\text{-}I}$, rate of folded state to intermediate reaction; NTL9, N-terminal domain of the ribosomal protein L9; UV, ultraviolet; $\Delta C_p^{\circ\prime}$, change in heat capacity; DSC, differential scanning calorimetry; DLS, dynamic light scattering; HDX, hydrogen–deuterium exchange.

D_2O is also expected to affect whole organisms. Research in this field began as soon as the deuterium isotope was discovered[53] and isolated[54] in the 1930s. High concentrations of D_2O have deleterious effects on organismal growth and survival from microorganisms like *Escherichia coli*[55,56] and yeast[57,58] to algaes,[59,60] plants,[58,61,62] and animals such as mice[58,60,63] and dogs[55]. However, the natural abundance of deuterium in nature is approximately 156 ppm.[64,65] More recent studies show that low concentrations may be necessary and even beneficial,[66,67] with interesting recent hypotheses on the use of heavy isotopes for increasing human longevity.[68] Finally, there is some interest in the use of D_2O as an excipient[52,69] because of the observation that D_2O can stabilize vaccines.[70]

Understanding the effects of isotopic waters is key to understanding biology, including protein folding. We focused on the equilibrium thermodynamics of D_2O on protein stability, because stably folded proteins are often a pre-requisite to proper biological function. We anticipate that our results and those of others compiled here will be of use for understanding the effects of the molecule that unites all of life on Earth.

Materials and Methods

Protein expression and purification

5-Fluorotryptophan-labeled SH3 was expressed and purified as described.[71,72]

NMR

NMR samples were prepared as described.[14,15,71] Briefly, 1 mg of fluorine-labeled, SH3 was resuspended in NMR buffer (50 mM HEPES, bis–tris propane, sodium acetate/acetic acid, pH 7.2) made using H_2O or 99.9% D_2O. pH readings are direct measurements and uncorrected for the D_2O isotope effect.[73] For samples prepared in H_2O, a coaxial-insert containing D_2O was used to lock the spectrometer. 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS, Cambridge Isotope Laboratories, Cambridge, UK) was used as a chemical shift reference. One-dimensional ^{19}F spectra were acquired at 5°C increments between 5°C and 45°C on a Bruker Avance III HD spectrometer operating at a ^{19}F Larmor frequency of 470 MHz equipped with a Bruker QCI cryoprobe.

Data processing and analysis

Data were processed as described using Topspin 3.2.[14,15,71] The parameters shown in Table I were calculated using Kirchhoff's equations and the integrated Gibbs-Helmholtz equation as described[14] using MATLAB R2016a.

Note added in proof. After our manuscript was accepted, we learned about work that more completely explains the stabilizing effect of D2O. [Pica A, Graziano G (2018) Effect of heavy water on the conformational stability of globular proteins. Biopolymers, 2017].

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