# Solvent isotope effect and protein stability

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**Here we present a comparative study of the stability of several proteins in $H_2O$ and $D_2O$ as a function of pH/pH*. We show that the substitution of $D_2O$ for $H_2O$ leads to an increase in the transition temperature and a decrease in the enthalpy of unfolding. The stability of the proteins, however, appears to be largely unchanged as a result of entropic compensation for the decrease in enthalpy. This enthalpy-entropy compensation is attributed to changes in hydration of proteins in $D_2O$ compared to $H_2O$. Analysis of thermodynamic data for the transfer of model compounds from $H_2O$ to $D_2O$ shows that almost all the changes in the enthalpy of unfolding and in the protein-ligand interactions due to water isotopic substitution can be rationalized by changes in hydration of the buried non-polar groups.**

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The interaction of solvent with a polypeptide chain is believed to constitute one of the major driving force in protein folding[1–5] and one of the principal determinants of protein stability[6–9]. In the case of soluble proteins the solvent is water. Direct experimental estimates of the role of water in the stability of proteins can be obtained from solvent perturbation experiments. The mildest perturbant for 'light' water ($H_2O$) is its isotopic 'heavy' form ($D_2O$). $D_2O$ appears to have physical properties not very distinct from light water so that its substitution for $H_2O$ causes the smallest possible solvent perturbation[10–11]. In order to obtain a better understanding of the thermodynamics of the interaction of water with proteins, we have undertaken a study of the solvent isotope effect (that is the effect of $H_2O$ versus $D_2O$) as a function of pH/pH* on the stability of three proteins, bovine ribonuclease A (RNase), horse cytochrome *c* (Cyt-*c*) and hen egg lysozyme (HEL) using high sensitivity microcalorimetry.

## Protein unfolding in $H_2O/D_2O$

Two sets of scanning microcalorimetry experiments were performed: denaturation of deuterated proteins (*d*-proteins) in $D_2O$ and denaturation of undeuterated proteins (*h*-proteins) in $H_2O$. We observe that the pH/pH* dependence of the transition temperature, is not linear, but seems to plateau at pH/pH* values around 5 to 6 (Fig. 1*a*). This is most pronounced for RNase and HEL. This behaviour can be directly attributable to the protonation in this pH/pH* range of titratable groups that influence protein stability (namely Asp and Glu residues involved in salt bridges which titrate with a $pK_a$ of 3.5–4.0, and in the case of Cyt-*c* the haem-ligating histidine, as well). It appears that the changes in transition temperature occur in a parallel fashion for *h*-proteins and *d*-proteins in $H_2O$ and $D_2O$, respectively. At pH/pH* 4 the transition temperatures of the three proteins are only minimally dependent on pH/pH*. This is reassuring because the observed changes in the transition temperatures in $H_2O$ and $D_2O$ under these conditions are least influenced by the possible difference in the determination of the activity of hydrogen or deuterium using a glass electrode. The transition temperature of the *d*-proteins in $D_2O$ is always somewhat higher than that of the *h*-proteins in $H_2O$ (Fig. 1*a*).

In all three cases studied, the enthalpy of unfolding, $\Delta H^{exp}(T_t)$, of *d*-proteins in $D_2O$ is lower than that for *h*-proteins in $H_2O$ (Fig. 1*b*). At 25 °C the difference is of the order of 60 kJ mol$^{-1}$. The slope of $\Delta H^{exp}(T_t)$ as a function of the transition temperature, $T_t$, represents the heat capacity change upon unfolding, $\Delta C_p$, which is an important parameter for calculating the temperature dependence of stability. Using the experimental values of $T_t$, $\Delta C_p$ and $\Delta H^{exp}(T_t)$, we can obtain the Gibbs energy of protein unfolding at 25 °C, $\Delta G$(25 °C), which is a measure of protein stability (Fig. 1*c*). There are distinct differences in the stabilities of the three proteins in $D_2O$ and $H_2O$. In the case of RNase the stabilities in $D_2O$ and $H_2O$ are comparable, while in the case of HEL and Cyt-*c* the stability in $H_2O$ is slightly higher than that in $D_2O$. Changes in the Gibbs energy of unfolding of *d*-proteins in $D_2O$ and *h*-proteins in $H_2O$ are of the order of 6 kJ mol$^{-1}$, a factor of 10 smaller than the changes in the enthalpy of unfolding, obviously due to compensat-

ing changes in entropy. Such enthalpy-entropy compensation phenomena have been observed in many systems, particularly for the transfer[12] of ions and non-electrolytes from $H_2O$ to $D_2O$, and have been hypothesized to be a very specific feature of water[13]. We therefore suggest that the difference in stability is most likely related to the differences in the hydration of buried protein groups in $H_2O$ and $D_2O$. Experimental data on the Gibbs energy of transfer of model compounds from $H_2O$ to $D_2O$, $\Delta^{D_2O}_{H_2O}G$, are limited and absolute values vary considerably due to the small magnitude of the observed changes[14–17]. Nevertheless, these data on model compounds can be used to obtain estimates of the contributions of polar and non-polar groups to $\Delta^{D_2O}_{H_2O}G$. Analysis of the model compound data[14–17] obtained at 25°C shows that both polar and non-polar groups have small negative contributions to the Gibbs energy of transfer from $H_2O$ to $D_2O$, thus predicting a small decrease in protein stability in $D_2O$ relative to $H_2O$. This, however, only provides a qualitative description of the observed changes in stability of proteins due to the solvent isotope effect. Other alternative explanations, such as changes in electrostatic interactions due to small shifts in the $pK_a$'s of protein groups upon solvent isotopic substitution, cannot be excluded.

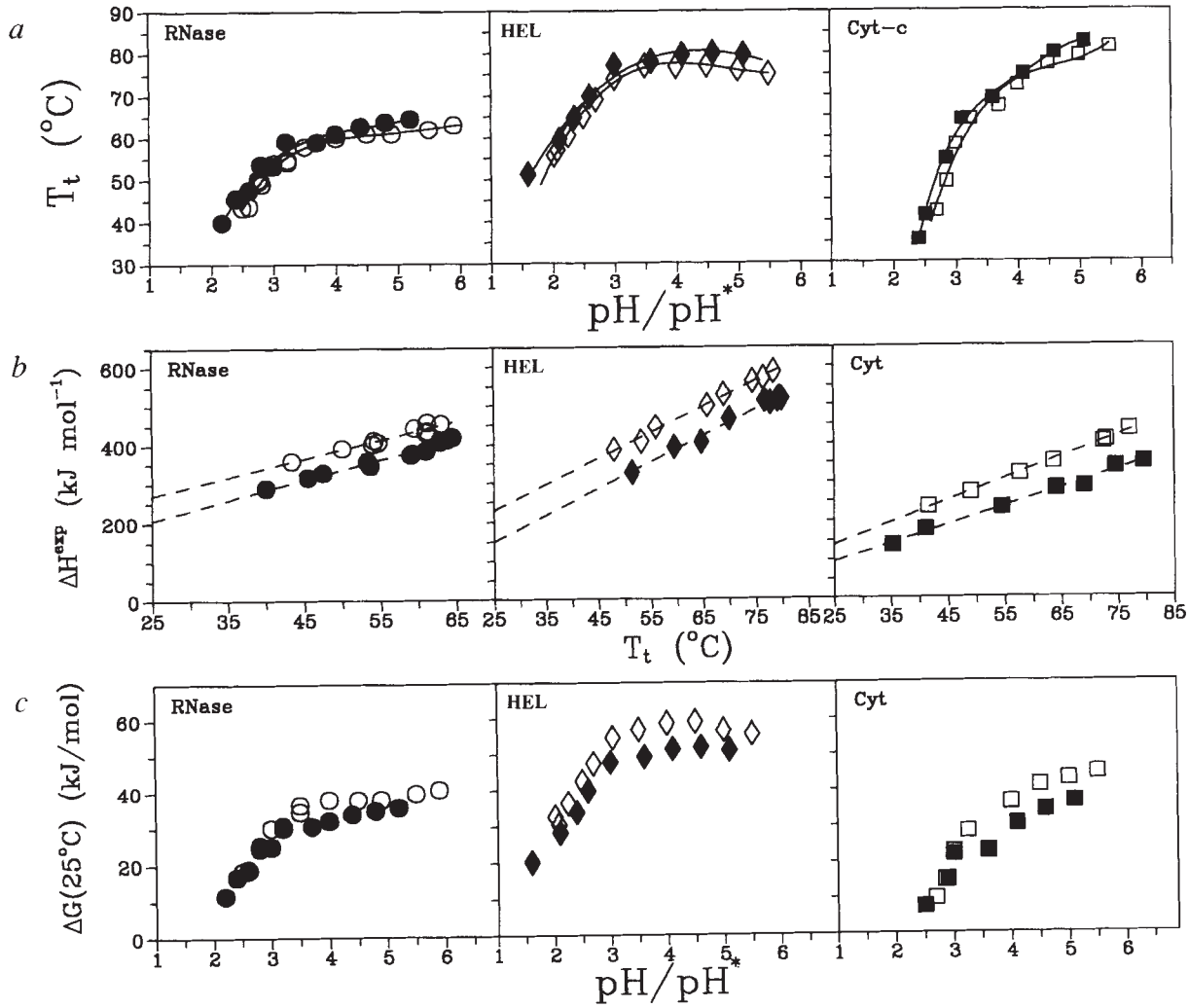


**Fig. 1** Results of calorimetric experiments in $H_2O$ (open symbols) and $D_2O$ (filled symbols) for bovine ribonuclease A (○,●), hen egg lysozyme (□,■) and horse cytochrome *c* (◇,◆). *a*, Dependence of the transition temperature, $T_t$, on the pH/pH*. *b*, Dependence of the enthalpy of unfolding, $\Delta H^{exp}(T_t)$, on the transition temperature, $T_t$. The slopes which represent the heat capacity change upon unfolding ($\Delta C_p$, kJ K$^{-1}$ mol$^{-1}$) are 4.8±0.4 (*d*-RNase), 5.2±0.3 (*h*-RNase), 6.7±0.3 (*d*-HEL), 6.7±0.2 (*h*-HEL), 4.7±0.2 (*d*-Cyt-*c*) and 5.5± 0.3 (*h*-Cyt-*c*). *c*, Dependence of the Gibbs energy of unfolding at $\Delta G$ (25°C), on the pH/pH*. $\Delta G$ (25°C) values were calculated using Gibbs-Helmholtz equation :

$$\Delta G(25°C)=\Delta H^{exp}(T_t)\cdot(1-\frac{298}{T_t})-(T_t-298)\cdot\Delta C_p+\Delta C_p\,298\cdot\ln\cdot[\frac{T_t}{298}]$$

where $T_t$ is transition temperature expressed in degrees Kelvin. It was assumed that $\Delta C_p$ does not depend on temperature and can be taken as the slope of a linear fit of the $\Delta H^{exp}$ ($T_t$) dependence on $T_t$ (Fig. 1*b*). Although in the strictest sense there is a dependence of $\Delta C_p$ on temperature[30], the above assumption would not affect the calculated Gibbs energy significantly, as $\Delta C_p$ is expected to have a very similar temperature dependencies in $D_2O$ and $H_2O$.

The observed differences in the enthalpy of unfolding,

$$\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{exp}=\Delta^{U}_{N}H(D_2O)-\Delta^{U}_{N}H(H_2O)$$

can be subdivided into two components[9]:

$$\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{exp}=\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{hyd}+\Delta^{U}_{N}\Delta^{D}_{H}H^{int}$$

where $\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{hyd}$ reflects differences in the enthalpy of hydration, and $\Delta^{U}_{N}\Delta^{D}_{H}H^{int}$ reflects changes in the enthalpy of internal interactions due to isotopic substitutions of exchangeable groups in the protein. The latter will primarily arise from changes in the strength of hydrogen-bonding interactions. The fact that there is an enthalpy-entropy compensation, specifically attributed to the aqueous solution, implies that hydration is probably responsible for the observed consequences of solvent isotopic substitution. The question, however, is to what extent changes in the hydration of buried protein groups are responsible for the observed behaviour of the enthalpy of unfolding of *d*- and *h*-proteins, and, concomitantly, how large are the changes in the internal interactions.

## Model compounds in $H_2O/D_2O$

A possible way to quantify the observed differences in the enthalpies of unfolding of *d*- and *h*-proteins is suggested from an analysis of the transfer of model compounds from $H_2O$ to $D_2O$. Three different types of compounds (alcohols, amino acids and alkylamides) have been studied extensively[12,17] and their enthalpies of transfer from $H_2O$ to $D_2O$ have been measured at 25 °C. A plot of the enthalpy of transfer of these compounds from $H_2O$ to $D_2O$, $\Delta^{D_2O}_{H_2O}H$, versus the non-polar water-accessible surface area, $ASA_{npl}$ reveals two significant features (Fig. 2). First, the dependence of $\Delta^{D_2O}_{H_2O}H$ on $ASA_{npl}$ within each series of compounds is linear, indicating the additivity of the enthalpy of transfer. The slopes of these dependencies are not very different, with a deviation of only 20% from the average value of -10 J mol$^{-1}$ Å$^{-2}$. This value represents the enthalpic contribution of 1 Å$^2$ of non-polar ASA upon transfer from $H_2O$ to $D_2O$ regardless of the type of compound. Second, the extrapolation to zero non-polar ASA yields values for the three sets of compounds that are close to zero. This indicates that the polar surface, at least to a first approximation, does not contribute significantly to the enthalpy of transfer of these series of compounds from $H_2O$ to $D_2O$ at 25 °C. Correspondingly, since polar groups form hydrogen bonds with water, the enthalpies of these hydrogen bonds in both $H_2O$ and $D_2O$ are very similar, and the small negative $\Delta^{D_2O}_{H_2O}G$ of transfer is due to the small positive changes of entropy of solvent[12]. Thus, analysis of the enthalpy of transfer of model compounds from $H_2O$ to $D_2O$ at 25 °C shows that the contribution of polar groups to the enthalpy of transfer can be neglected and that the major contribution of -10±2 J mol$^{-1}$ Å$^{-2}$ arises from changes in hydration of the non-polar surface. A similar value (-8 J mol$^{-1}$ Å$^{-2}$) has been obtained[18] from the analysis limited to a smaller set of model compounds[19].

## Solvent isotope effect

As a result of protein unfolding, non-polar groups buried in the interior of the protein become exposed to solvent. Knowing the buried non-polar surface area[9], $\Delta^{U}_{N}ASA_{npl}$, of RNase A, HEL and Cyt-*c* and knowing the contribution arising from changes in hydration of non-polar surfaces in $H_2O$ and $D_2O$ obtained from model compounds, we can obtain the enthalpy changes expected for the changes in hydration in $H_2O$ versus in $D_2O$ upon unfolding of these proteins, $\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{hyd}$ (Table 1). It appears that all the differences in $\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{exp}$ can be accounted for by the changes in the hydration of non-polar groups in $D_2O$ and $H_2O$. The enthalpy of protein-ligand interactions can also be rationalized in the same terms (Table 1). For example[20], the difference in the enthalpy of association of concanavalin A (ConA) with α-methyl-D-mannopyranoside (MeMan) in $H_2O$ and $D_2O$ is 2.1 kJ mol$^{-1}$ at 25 °C. According to our estimates (see Methods) 173 Å$^2$ of non-polar surface area is buried upon complex formation, which predicts an enthalpy change of 1.7 kJ mol$^{-1}$. Thus, changes in the hydration of non-polar groups in $D_2O$ compared to $H_2O$ describe, on a quantitative level, the observed changes in enthalpy for protein folding in $D_2O$ and $H_2O$, as well as protein ligand interactions. These changes in enthalpy of hydration of non-polar groups are largely compensated by the changes in entropy, so that the resulting stability remains largely unchanged.

## Implications

The results presented in this paper have three major implications for the study of protein stability: *i*) the contribution of hydration to the stability of proteins is very significant, since even apparently small changes in the nature of solvent water, such as an H to D substitution, leads to

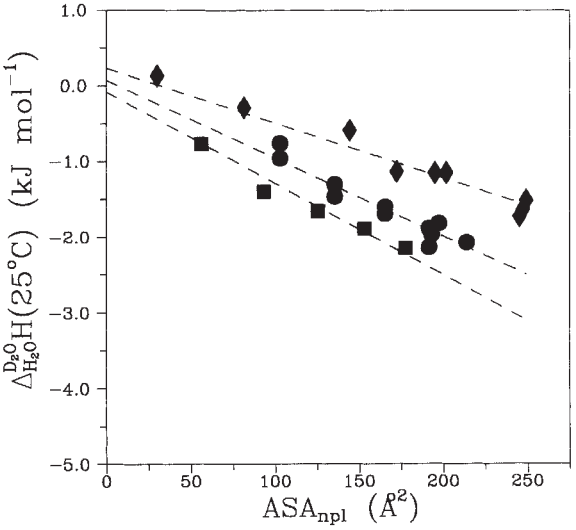


**Fig. 2** Dependence of the enthalpy of transfer from $H_2O$ to $D_2O$, $\Delta^{D_2O}_{H_2O}H$, for amino acids[17] (●), alcohols[17] (■) and alkylamides[12] (♦) at 25 °C on the non-polar water-accessible surface areas, $ASA_{npl}$, of these compounds. The best fit slopes of the lines are -11±1 J mol$^{-1}$ Å$^{-2}$, -12±1 J mol$^{-1}$ Å$^{-2}$, and -8±1 J mol$^{-1}$ Å$^{-2}$, respectively. The average slopeis -10±2 J mol$^{-1}$ Å$^{-2}$.

**Table 1 Solvent isotope effects on thermodynamics of unfolding and binding**

| System | Process | $\Delta ASA_{npl}$ | $\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{exp}$ | $\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{hyd}$ |
|---|---|---|---|---|
| | | Å² | kJ·mol⁻¹ | kJ·mol⁻¹ |
| Cytochrome | unfolding | 4942[1] | -43 ± 11 | -49 ± 9[5] |
| Ribonuclease A | unfolding | 5273[1] | -63 ± 13 | -53 ± 10[5] |
| Lysozyme | unfolding | 6365[1] | -72 ± 15 | -64 ± 13[5] |
| ConA-MeMan | binding | -173[2] | 2.1 ± 0.1[3] | 1.7 ± 0.3[5] |
| FKBP-FK506 | binding | -697[2] | 7.6 ± 2.9[4] | 7.0 ± 1.4[5] |

[1]Taken from ref. 9
[2]Calculated as described in Methods
[3]Data from ref. 20
[4]Data from ref. 31;
[5]Calculated as $\Delta^{U}_{N}\Delta^{D_2O}_{H_2O}H^{hyd}=k\cdot\Delta^{U}_{N}ASA_{npl}$, where k=(-10±2) J·mol⁻¹Å⁻² (see Fig. 2 for details), and assuming that uncertainties arise only from k.

considerable changes in the thermodynamic behaviour of proteins; *ii*) hydration effects, at least to a close approximation, are additive that is, they can be scaled with the water-accessible surface area; *iii*) the study of protein stability requires an analysis of its thermodynamics not only in terms of the Gibbs energy but also in terms of enthalpy and entropy, because the enthalpy-entropy compensation taking place in aqueous solution may obscure important details of the overall energetics involved in protein folding.

## Methods

The purity of the commercially available proteins (Sigma) was checked by SDS-PAGE and found to be >95%. Deuteration of exchangeable protons (amides, hydroxyls, and so on) was carried out under alkaline solvent conditions for a period of several days at 35 °C. Completeness of deuteration was followed by the disappearance of the backbone amide resonances in the NMR spectra. Prior to the calorimetric experiments protein solutions were extensively dialyzed against two changes (2×0.5 l) of the corresponding buffers, based on $D_2O$ or $H_2O$, with the pH controlled using a glass electrode. The apparent pH reading of the glass electrode, pH*, can be related to the activity of deuterium ions, pD, by means of the relationship[21] pD=pH*+0.4. It has been shown, however, that the effect of solvent isotopic substitution on the $pK_a$ of protein groups is just the opposite to the changes of pH* measured by the glass electrode[22]. Thus the same ionization state of proteins in $D_2O$ and $H_2O$ can be approximated at the same readings of the glass electrode. The concentration of proteins in solution was determined spectrophotometrically using extinction coefficients[23] of $E^{1\%,\,1cm}_{278\,nm}$=7.32 for RNase A, $E^{1\%,\,1cm}_{530nm}$=26.9 for HEL and $E^{1\%,\,1cm}_{280nm}$ =9.06 at pH 5.5 for Cyt-c. Details on the procedures used for the calorimetric measurements, sample preparation and data analysis are reported elsewhere[24].

The solvent-accessible surface area was computed by the algorithm of Lee and Richards[25] using the van der Waals radii reported by Chothia[26]. Protein Data Base[27] files 5cna[28] (concanavalin A α-methyl-D-mannopyroniside complex) and 1fkf[29] (FKBP-FK506), were used for the estimation of water-accessible surface area changes upon complexes formation.

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