Solvent isotope effect on thermodynamics of hydration

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Abstract

Partial molar heat capacities of five linear alcohols (methanol, ethanol, n-propanol, n-butanol, n-pentanol) and five N-substituted amides (n-propionamide, N-methylformamide, N-methylacetamide, N-methylpropionamide, N-ethyl-acetamide) in aqueous D_2O solution have been measured at 25°C. The heat capacities of transfer of these compounds from H_2O to D_2O were calculated using previously reported (Makhatadze et al., Biophys. Chem. 64 (1997) 93) values of partial heat capacities of alcohols and amides in aqueous H_2O solutions. It is shown that the sign and magnitude of the heat capacity change upon transfer from H_2O to D_2O depends on the relative amount of polar and non-polar solvent accessible surface areas of solute. Analysis shows that transfer of non-polar surface from H_2O to D_2O is accompanied by a positive heat capacity change. In contrast, transfer of polar surface from H_2O to D_2O occurs with negative heat capacity change. Estimates show that the solvent isotope effect on the heat capacity changes upon protein unfolding can be predicted using the changes of the polar and non-polar surface area changes upon protein unfolding and the transfer data of model compounds. Analysis of the thermodynamic functions of transfer of non-polar compounds from H_2O to D_2O shows puzzling behavior which contradicts current definitions of the hydrophobic effect.   © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Quantitative understanding of the role of hydration for protein folding and stability is the key in efforts to solve the protein folding problem. Several experimental and theoretical approaches are currently used to address this problem [1–9]. Experimental approaches include measurements of the effects of changes of covalent structure of proteins by site-directed mutagenesis or chemical modifications on protein stability [6–9], analysis of semi-empirical correlations between structure and stability [6,7], and solvent perturbation experiments [10–12]. In the latter experiments, perturbations of the solvent were traditionally achieved by adding alcohols or other organic solvents such

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as dimethylsulfoxide (DMSO) [13]. However, it seems that these co-solvents provide very large perturbations which lead to certain difficulties in extrapolating the observed property changes back to the properties in aqueous solution. On the other hand, the isotopic form of water, heavy water (D_2O), provides a natural perturbant with physical properties close to but still distinct from those of light water (H_2O) (Table 1). Keeping this in mind we measured the solvent isotope effect on protein stability using three small globular proteins (lysozyme, ribonuclease A, and cytochrome c) as models [14]. We showed that D_2O affects the thermodynamics of protein unfolding in a complex way. The thermostability (temperature of midpoint of transition) of proteins in the presence of D_2O increases by several degrees. The enthalpy of unfolding in D_2O decreases by approx. 20% as compared to the enthalpy of unfolding in H_2O. Decrease in enthalpy of unfolding is largely compensated by a decrease in the entropy. Nevertheless, the resultant Gibbs energy of unfolding, which defines stability of a protein, is higher in H_2O than in D_2O. Detailed analysis of the enthalpy of transfer of model compounds (alcohols, amide and amino acids)

from H_2O to D_2O at 25°C [15,16] showed that [14]: (1) the enthalpy of transfer of a polar surface is close to zero; and (2) the enthalpy of transfer of a non-polar surface is negative and on the order of 10 J/(mol Å^2). In this study, in order to assess the temperature dependence of enthalpies of transfer, we have measured the partial molar heat capacities of five linear alcohols (methanol, ethanol, n-propanol, n-butanol, n-pentanol) and five N-substituted amides (n-propionamide, N-methylformamide, N-methylacetamide, N-methylpropionamide, N-ethylacetamide) in aqueous D_2O solution. From these data and previously reported values for the partial molar heat capacities of the same compounds in aqueous H_2O solution [17], we have calculated the heat capacity change upon H_2O to D_2O transfer. The heat capacity changes upon transfer from H_2O to D_2O have been analyzed assuming additivity of contributions of polar and non-polar parts of these molecules. Our results show that the heat capacity change upon transfer of non-polar groups from H_2O to D_2O is positive in difference to the negative heat capacity of transfer for polar groups.

2. Materials and methods

Methanol, ethanol, n-propanol, n-butanol, n-pentanol, n-propionamide, N-methylformamide, N-methylacetamide, N-methylpropionamide, and N-ethylacetamide were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and were used without further purification. Solutions of these compounds in D_2O were prepared by mixing the weighed components, the compound of interest and 99.9 atom %D deuterium oxide (Isotec Inc., cat. No. 82-70046). Five solutions for each compound were prepared at concentrations ranging from 0.3 to 1.5 wt.%. All solutions were incubated for at least 24 h prior to measurements.

The apparent molar heat capacity, $C_{p,s}^D$, of the solute at temperature T was determined by measuring the heat capacity difference, ΔC_p^{app}, between the solvent (D_2O) and the solution at this temperature using the differential scanning microcalorimeter developed in The Johns Hopkins University (operating principles are described elsewhere [18]), using the equation:

Table 1
Comparison of some physical properties of light water, H_2O, heavy water, D_2O, and methanol, CH_3OH[a]

Property	H_2O	D_2O	CH_3OH
Molecular weight	18.02	20.03	32.04
Melting point (°C)	0	3.81	−97.7
Boiling point (°C)	100.00	101.42	64.7
Critical temperature (°C)	374.1	371.1	512.6
Critical volume (cm^3/mol)	55.3	55.0	117.8
Density at 25°C (g/cm^3)	0.99705	1.1045	0.7872
ΔH fusion (kJ/mol)	6.01	6.34	0.90
ΔH vaporization (kJ/mol)	44.02	45.40	37.40
C_p (25°C, J/(deg mole))	75.27	84.35	81.04
P_{vap} (25°C, kPa)	23.75	20.51	17.0
Compressibility (bar^{-1})	4.45	4.59	10.10
Length of hydrogen bond (Å)	2.765	2.766	–
Dipole moment (25°C)	1.84	1.84	1.7
Dielectric constant (25°C)	78.304	77.937	32.63
Polarizability (25°C)	1.45	1.26	3.32

[a]Compiled from data reported by Arnett and McKelvey [16], Nemethy and Scheraga [25] and the *Handbook of Chemistry and Physics* [28].

$$C_{p,s}^D = \frac{C_p^D \cdot \bar{\nu}_s}{\bar{\nu}_D} - \frac{\Delta C_p^{\text{app}}}{m_s} + \left[\frac{C_p^D \cdot \bar{\nu}_H \cdot \rho_D}{m_s} - C_p^H \frac{m_H}{m_s} \right]$$

$$(1)$$

where C_p^D, $\bar{\nu}_D$, C_p^H, and $\bar{\nu}_H$ are the molar heat capacity and molar volume of D_2O and H_2O, respectively, $\bar{\nu}_s$ is the partial molar volume of the solute, m_s is the mass of the solute in the calorimetric cell, M_W^s is the molar mass. Eq. (1) includes the heat capacity and partial molar volume of H_2O because solute prior to being mixed with D_2O contained exchangeable protons. These protons upon exchange will form H_2O molecules. See Appendix for the complete derivation of the Eq. (1). All experiments were performed at a heating rate of 1°C/min. In the concentration range studied, no concentration dependence for the apparent molar heat capacity was found. Thus the measured values of $C_{p,s}^D$ were considered as the partial molar heat capacity values, i.e. C_{p,s_0}^D. Reported values of C_{p,s_0}^D are thus averaged values obtained at five different concentrations.

The apparent molar volume of the studied compounds in D_2O, $\bar{\nu}_s^D$, was determined from the density of solution, ρ_1, and the density of the solvent (D_2O), ρ_D, using equation:

$$\bar{\nu}_s^D = \frac{\rho_D - \rho_1 + c}{\rho_D \cdot c} - \left[\bar{\nu}_H \cdot \frac{n}{2} \frac{M_W^H}{M_W^s} - \frac{n}{2} \frac{M_W^H}{M_W^s} \bar{\nu}_D \right]$$

$$(2)$$

where c is the molality of the solution. Eq. (2) includes an additional term in square parenthesis which arises from the fact that solute molecules prior to mixing contained exchangeable protons (see Appendix for complete derivation of Eq. (2)). The density was measured by vibrational densitometer DMA-60/602 (Anton Paar, Austria) at a fixed temperature using water and air as a standard for calibration. The partial molar volume at infinite dilution, $\bar{\nu}_{s,0}^D$, was obtained by linear extrapolation of the apparent molar volumes, $\bar{\nu}_s^D$, to zero concentration. The details of the partial molar volume determinations are considered elsewhere [17]. Partial molar volumes of methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-

propionamide, *N*-methylformamide, *N*-methylacetamide, *N*-methylpropionamide and *N*-ethylacetamide are presented in Table 2. Measurements were limited to 25°C due to the hygroscopic nature of D_2O.

3. Results and discussion

Temperature dependencies of the partial molar heat capacities of methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-propionamide, *N*-methylformamide, *N*-methylacetamide, *N*-methylpropionamide and *N*-ethylacetamide in D_2O for the temperature range from 5 to 100°C are presented in Fig. 1. These temperature dependencies were obtained by Eq. (1) using experimentally measured partial molar volume, $\bar{\nu}_{s,0}^D$, of these compounds in D_2O (Table 2) at 25°C and assuming that the temperature dependence of $\bar{\nu}_{s,0}^D$ in D_2O is the same as in H_2O [17]. This assumption was made due to the technical difficulties of measuring partial molar volumes in D_2O at high temperatures because of the high hygroscopic nature of this solvent. Correspondingly, we consider that the values of partial molar heat capacity are reliable only at 25°C and limit further discussion to this temperature. Comparison of the partial molar heat capacities of methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-propionamide, *N*-methylformamide, *N*-methylacetamide, *N*-methylpropionamide and *N*-ethylacetamide in D_2O and in H_2O at 25°C are presented in Table 2. The heat capacity change upon transfer of a compound from H_2O to D_2O defined as:

$$\Delta_{\text{H}_2\text{O}}^{\text{D}_2\text{O}} C_p(25°C) = C_{p,s_0}^{\text{D}_2\text{O}}(25°C) - C_{p,s_0}^{\text{H}_2\text{O}}(25°C) \quad (3)$$

The heat capacity of transfer, $\Delta_{\text{H}_2\text{O}}^{\text{D}_2\text{O}} C_p(25°C)$, for five alcohols and five amides are shown in the fourth column of Table 2. The $\Delta_{\text{H}_2\text{O}}^{\text{D}_2\text{O}} C_p(25°C)$ values are negative for four of these compounds, methanol, ethanol, *n*-propionamide, and *N*-methylformamide, but positive for the other six. It appears that the sign and magnitude of $\Delta_{\text{H}_2\text{O}}^{\text{D}_2\text{O}} C_p(25°C)$ values correlates with the amount of surface area of these compounds. Fig. 2 shows that, with the increase of the relative amount

Table 2
Thermodynamic parameters of methanol, ethanol, n-propanol, n-butanol, n-pentanol, n-propionamide, N-methylformamide, N-methylacetamide, N-methylpropionamide, N-ethylacetamide at 25°C

Compound	$\bar{\nu}_{s,0}^D$ $(\bar{\nu}_{s,0}^H)^a$ (cm^3/mol)	C_{p,s_0}^D $(C_{p,s_0}^H)^a$ (J/K/mol)	$\Delta_{H_2O}^{D_2O}C_p^b$ (J/K/mol)	ASA$_{pol}^a$ (Å^2)	ASA$_{npl}^a$ (Å^2)
Methanol	37.49 (38.11)	144 (156)	−12	47	107
Ethanol	54.71 (55.09)	260 (268)	−8	47	139
n-Propanol	70.82 (70.73)	384 (361)	23	47	167
n-Butanol	87.31 (86.53)	484 (447)	37	47	202
n-Pentanol	102.78 (102.46)	594 (544)	50	47	231
n-Propionamide	62.72 (71.10)	233 (257)	−24	96	124
N-Methylformamide	54.45 (56.46)	132 (163)	−31	77	107
N-Methylacetamide	74.93 (73.88)	268 (253)	15	62	161
N-Methylpropionamide	88.34 (89.65)	391 (344)	47	51	211
N-Ethylacetamide	90.78 (90.20)	390 (347)	43	55	198

[a] Makhatadze et al. [17]
[b] Eq. (3); estimated error in the $\bar{\nu}_{s,0}^D$ values is on the order of 0.5−1%. Estimated error in the $C_{p,0}^D$ values is on the order of 3%. Partial molar volumes in D$_2$O, $\bar{\nu}_{s,0}^D$, and in H$_2$O, $\bar{\nu}_{s,0}^H$; partial molar heat capacities in D$_2$O, C_{p,s_0}^D, and in H$_2$O, C_{p,s_0}^H, heat capacity changes upon transfer from H$_2$O to D$_2$O, $\Delta_{H_2O}^{D_2O}C_p$ and water accessible polar, ASA$_{pol}$, and non-polar, ASA$_{npl}$, surface areas.

of the non-polar surface area, negative $\Delta_{H_2O}^{D_2O}C_p(25°C)$ values observed in the case of some compounds become more positive (Fig. 2). This indicates that the polar surface area makes a negative contribution to the heat capacity of transfer from H$_2$O to D$_2$O. The non-polar surface behaves in just the opposite way: it makes a positive contribution to the heat capacity change upon transfer from H$_2$O to D$_2$O. To assess quantitatively these effects we have analyzed

$\Delta_{H_2O}^{D_2O}C_p(25°C)$ according to the following equation:

$$\Delta_{H_2O}^{D_2O}C_p(25°C) = \Delta_{H_2O}^{D_2O}\hat{C}_p^{pol} \cdot \text{ASA}_{pol}$$
$$+ \Delta_{H_2O}^{D_2O}\hat{C}_p^{npl} \cdot \text{ASA}_{npl} \qquad (4)$$

where $\Delta_{H_2O}^{D_2O}\hat{C}_p^{pol}$ and $\Delta_{H_2O}^{D_2O}\hat{C}_p^{npl}$ are the heat capacity change upon transfer from H$_2$O to D$_2$O of 1 Å^2 of polar and non-polar surface area, respec-
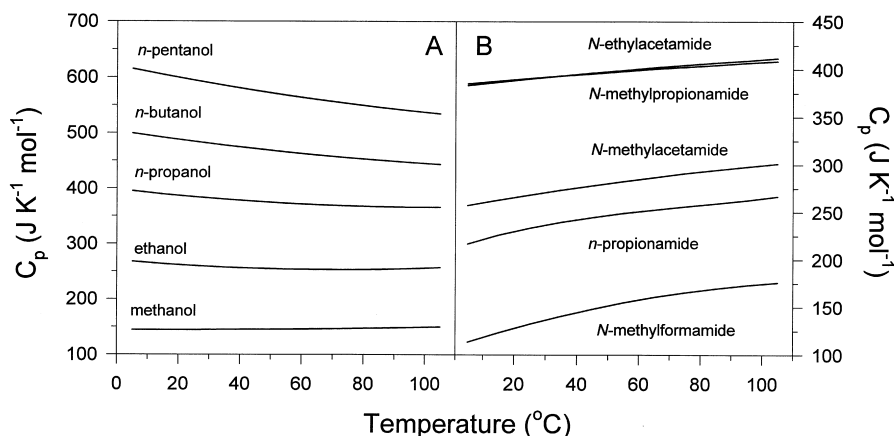

Fig. 1. Temperature dependence of the apparent partial molar heat capacity of the studied compounds in aqueous D$_2$O solution. A: alcohols; B: N-substituted amides. See text for details.
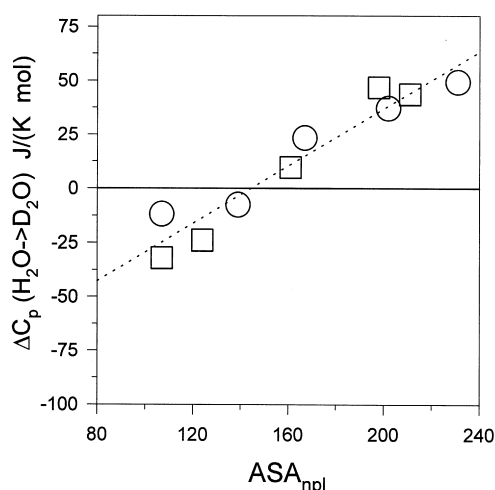
Fig. 2. Dependence of the heat capacity of transfer of the studied compound (circles, alcohols, squares, amides) at 25°C on the amount of the non-polar surface area in these compounds. The solid line (linear fit of all data points) is shown to guide the eye. See text for details.

tively, ASA_{pol} and ASA_{npl} are the water accessible polar and non-polar surface areas, respectively. In Eq. (4) two parameters are unknown, $\Delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{pol}$ and $\Delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{npl}$, but can be estimated from a non-linear fit of the data. The fit gave the following estimates for these parameters: $\Delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{pol} = -(0.88 \pm 0.13)$ J/(K mol Å^2) and $\Delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{npl} = (0.40 \pm 0.05)$ J/(K mol Å^2). It appears that the heat capacity change upon transfer from H_2O to D_2O follows a pattern similar to that observed for the hydration heat capacity changes [17,19–24], i.e. a positive effect for non-polar surfaces and negative effect for polar. In the case of the hydration heat capacity it was

shown that it is possible to estimate the heat capacity change upon protein unfolding from the changes in water accessible surface area using the hydration heat capacity contributions per unit of polar and non-polar surface areas [20,21]. Can we predict expected differences for the heat capacity change upon unfolding of protein in D_2O and in H_2O? By analogy with the hydration heat capacity we have estimated the solvent isotope effect of the heat capacity change for protein unfolding as:

$$\Delta\Delta_{H_2O}^{D_2O}C_p^{cal} = \Delta C_p^{D_2O} - \Delta C_p^{H_2O} = \Delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{pol}$$
$$\cdot \Delta ASA_{pol} + \Delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{npl} \cdot \Delta ASA_{npl}$$
$$(5)$$

where $\Delta\Delta_{H_2O}^{D_2O}C_p^{cal}$ is the difference in the heat capacity change upon protein unfolding in D_2O, $\Delta C_p^{D_2O}$, and in H_2O, $\Delta C_p^{H_2O}$, and ΔASA_{pol} and ΔASA_{npl} are the changes in the polar and non-polar surface areas upon protein unfolding. Using $\Delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{pol}$ and $\Delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{npl}$ derived above (Eq. (4)) we calculated $\Delta\Delta_{H_2O}^{D_2O}C_p^{cal}$ values for three different proteins: lysozyme, ribonuclease A, and cytochrome c. These proteins were selected because experimental data on the heat capacity change upon unfolding of these proteins in H_2O and D_2O have been measured by us previously [14]. Comparison of the calculated $\Delta\Delta_{H_2O}^{D_2O}C_p^{cal}$, according to Eq. (5), and experimental $\Delta\Delta_{H_2O}^{D_2O}C_p^{exp}$ values are presented in Table 3. It shows that simple additivity (Eq. (5)) predicts the experimentally observed solvent isotope effect of the heat capacity change upon unfolding.

It appears from the data presented in this and

Table 3
Changes in polar, ΔASA_{pol}, and non-polar, ΔASA_{npl}, water accessible surface areas upon unfolding; heat capacity change upon unfolding in H_2O, $\Delta C_p^{H_2O}$, in D_2O, $\Delta C_p^{D_2O}$; experimental, $\Delta\Delta_{H_2O}^{D_2O}C_p^{exp}$, and calculated, $\Delta\Delta_{H_2O}^{D_2O}C_p^{cal}$, solvent isotope on the heat capacity change upon protein unfolding, for lysozyme, ribonuclease A, and cytochrome c

Protein	ΔASA_{pol}[a] (Å^2)	ΔASA_{npl}[a] (Å^2)	$\Delta C_p^{H_2O}$[a] (kJ/mol)	$\Delta C_p^{D_2O}$[a] (kJ/mol)	$\Delta\Delta_{H_2O}^{D_2O}C_p^{exp}$ kJ/mol	$\Delta\Delta_{H_2O}^{D_2O}C_p^{cal}$[b] (kJ mol^{-1})
Lysozyme	4231	6365	6.7 ± 0.2	6.7 ± 0.3	0 ± 0.5	-1.2 ± 0.9
Ribonuclease A	4141	5273	5.2 ± 0.3	4.8 ± 0.4	-0.4 ± 0.7	-1.5 ± 0.8
Cytochrome c	3358	4942	5.5 ± 0.3	4.7 ± 0.2	-0.8 ± 0.5	-1.0 ± 0.7

[a] Makhatadze [14], ΔASA_{npl} includes both aromatic and aliphatic surface areas, ΔASA_{pol} includes all types of polar surface areas.
[b] Eq. (5), uncertainties calculated as $\delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{pol} \cdot \Delta ASA_{pol} + \delta\Delta_{H_2O}^{D_2O}\hat{C}_p^{npl} \cdot \Delta ASA_{npl}$.

the previous paper [14] that the solvent isotope effect will manifest itself in all thermodynamic parameters of protein unfolding: Gibbs energy, enthalpy, entropy, and heat capacity change. So far detailed analysis of two of these, the enthalpy [14] and heat capacity change (this work), have been performed. Further experimental studies on the model compounds will be required for the detailed understanding of the solvent isotope effects on the Gibbs energy and entropy functions. Such studies will also be important for a clearer understanding of the hydrophobic effect. Results presented in this paper show that the transfer of non-polar compounds from H_2O to D_2O is accompanied by a positive heat capacity change. A positive heat capacity change upon transfer of a non-polar compound from phase A to phase B means that phase A is more hydrophobic. In our case this implies that D_2O is less 'hydrophobic' than H_2O. However, the transfer process between two phases of a non-polar molecule that is accompanied by a positive heat capacity change has another characteristic thermodynamic property. The Gibbs energy for such a transfer is positive as well. This is not true for the transfer from H_2O to D_2O (Table 4). The Gibbs energy of transfer of three gaseous alkanes, methane, ethane, propane and butane, from H_2O to D_2O is negative, indicating that D_2O is more 'hydrophobic' than H_2O. Moreover, the absolute value of the Gibbs energy increases (becomes less negative) with the increase of the size of the non-polar molecule. The negative enthalpy change upon transfer exceeds the positive entropic contribution $(-T \cdot \Delta S)$ to the Gibbs energy of transfer. A large enthalpy of

transfer from H_2O to D_2O is consistent with the notion of stronger hydrogen bonding in D_2O [25–27], but does not seem to explain the positive heat capacity change. Such inconsistency between our current definitions of hydrophobic interactions and the thermodynamics of transfer of non-polar molecules from H_2O to D_2O certainly requires more experimental and theoretical effort to solve this puzzle.
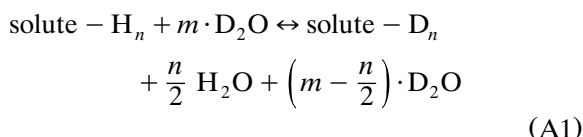
Acknowledgements

Appendix A

All studied solutes (methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-propionamide, *N*-methylformamide, *N*-methylacetamide, *N*-methylpropionamide, *N*-ethylacetamide) contain exchangeable hydrogens. Upon dissolving them in aqueous D_2O these hydrogens will exchange on deuterium and form H_2O. This reaction schematically can be represented as:

Table 4
Thermodynamics of transfer of gaseous alkanes from H_2O to D_2O[a]

Compound	$\Delta_{H_2O}^{D_2O} G$ (25°C) (J/mol)	$\Delta_{H_2O}^{D_2O} S$ (25°C) J/(mol K)	$\Delta_{H_2O}^{D_2O} H$ (25°C) (J/mol)	$\Delta_{H_2O}^{D_2O} C_p$ (25°C) J/(mol K)
Methane	−138	−3.18	−1088	36
Ethane	−113	−4.14	−1347	66
n-Propane	−96	−4.94	−1569	78
n-Butane	−84	−1.00	−385	90

[a]$\Delta_{H_2O}^{D_2O}G$(25°C), $\Delta_{H_2O}^{D_2O}H$(25°C), $\Delta_{H_2O}^{D_2O}S$(25°C) are taken from Ben-Naim and Marcus [27]. $\Delta_{H_2O}^{D_2O}C_p$(25°C) was calculated from the surface areas of methane (90 Å^2), ethane (165 Å^2), propane (195 Å^2) and butane (225 Å^2), and the heat capacity change upon transfer of a unit of non-polar surface from H_2O to D_2O $\Delta_{H_2O}^{D_2O}\hat{C}_p^{npl} = 0.40$ J/(K mol Å^2) (see text for details).

$$\text{solute} - H_n + m \cdot D_2O \leftrightarrow \text{solute} - D_n$$
$$+ \frac{n}{2} H_2O + \left(m - \frac{n}{2}\right) \cdot D_2O \tag{A1}$$

Under conditions when the concentration of solute is low, i.e. $m \gg n$, we can assume that all solute molecules will be in the deuterated form and all hydrogens form H_2O. This assumption was used to derive Eqs. (1) and (2).

Derivation of Eq. (1)

Heat capacity of the solutions in reference, C_p^I, and sample, C_p^{II}, cells, are

$$C_p^I = C_p^D \cdot V \cdot \rho_D \tag{A2}$$

$$C_p^{II} = C_p^D \cdot V \cdot \rho_D - C_p^D \cdot (\nu_H + \nu_s) \cdot \rho_D$$
$$+ C_p^H \cdot m_H + C_{p,s}^D \cdot m_s \tag{A3}$$

where C_p^D and C_p^H are the heat capacities of pure D_2O and H_2O, respectively, $C_{p,s}^D$ is the apparent partial heat capacity of solute, ρ_D is the density of pure D_2O, ν_s, is the volume occupied by the solute, ν_H, is the volume occupied by H_2O, and m_s and m_H are the partial masses in solution of the solute and H_2O, respectively. DSC records apparent differences in the heat capacities between the solvent and the solution, ΔC_p^{app}, defined as:

$$\Delta C_p^{app} - C_p^I - C_p^{II} = C_p^D \cdot (\nu_H + \nu_s) \cdot \rho_D$$
$$- C_p^H \cdot m_H - C_{p,s}^D \cdot m_s \tag{A4}$$

Apparent partial heat capacity of solvent, $C_{p,s}^D$, thus can be expressed as:

$$C_{p,s}^D = \frac{C_p^D \cdot \nu_H \cdot \rho_D + C_p^D \cdot \nu_s \cdot \rho_D - C_p^H \cdot m_H - \Delta C_p^{app}}{m_s} \tag{A5}$$

ν_s and ν_H are related to the partial volumes of the solute, $\bar{\nu}_s$, and of H_2O, $\bar{\nu}_H$, as:

$$\nu_s = \bar{\nu}_s \cdot m_s \tag{A6}$$

and

$$\nu_H = \bar{\nu}_H \cdot m_H \tag{A7}$$

substituting Eqs. (A6) and (A7) into Eq. (A5) gives:

$$C_{p,s}^D = \frac{C_p^D \cdot \bar{\nu}_s}{\bar{\nu}_D} - \frac{\Delta C_p^{app}}{m_s} + \left[\frac{C_p^D \cdot \nu_H \cdot \rho_D}{m_s} - C_p^H \frac{m_H}{m_s}\right] \tag{A8}$$

In this equation the first two terms represent the regular expression for the apparent partial heat capacity of the solute in a two-component system [17]. The term in parenthesis represents a correction term for hydrogen/deuterium exchange. This correction can be further simplified. The m_s and m_H are related to the concentration of the solute, c, molecular mass of solute, M_W^s, molecular mass of H_2O, M_W^H, and number of exchangeable protons in the solute, n, as:

$$m_s = c \cdot V \tag{A9}$$

$$m_H = \frac{n}{2} \frac{M_W^H}{M_W^s} m_s = \frac{n}{2} \frac{M_W^H}{M_W^s} \cdot c \cdot V \tag{A10}$$

using (A8.)−(A9.) we will get for the correction term:

$$\left[\frac{C_p^D \cdot \nu_H \cdot \rho_D}{m_s} - - C_p^H \frac{m_H}{m_s}\right]$$
$$= \frac{n}{2} \frac{M_W^H}{M_W^s} \left(C_p^D \cdot \bar{\nu}_H \cdot \rho_D - C_p^H\right) \tag{A11}$$

At 25°C parameters in Eq. (A11) have the following values: $\bar{\nu}_H = 1.003$ cm^3/g, $\rho_D = 1.1044$ g/cm^3, $C_p^D = 4.212$ J/(g K), $C_p^H = 4.178$ J/(g K), which gives the following expression for the correction term:

$$\frac{n}{2} \frac{M_W^H}{M_W^s} \left(C_p^D \cdot \bar{\nu}_H \cdot \rho_D - C_p^H\right) = 4.384 \cdot \frac{n}{M_W^S} J/K \tag{A12}$$

Derivation of Eq. (2)

The density of solution, ρ_1, is related to the volume of the cell, V, partial mass of the solute, m_s, partial mass of H_2O, m_H, and partial mass of D_2O, m_D, as:

$$\rho_1 = \frac{m_D + m_H + m_s}{V} \tag{A13}$$

The partial mass of D_2O in volume, V, is expressed as:

$$m_D = \rho_D \cdot (V - v) \tag{A14}$$

where v, is the volume occupied by the solute, v_s, and by H_2O, v_H, i.e. $v = v_H + v_s$. Combining Eqs. (A6)–(A10), Eq. (A14) and Eq. (A13) we will obtain:

$$\rho_1 = \frac{\rho_D \cdot \left(V - \bar{\nu}_s \cdot c \cdot V - \bar{\nu}_H \cdot c \cdot V \cdot \dfrac{n}{2} \dfrac{M_W^H}{M_W^s} \right) + c \cdot V + c \cdot V \cdot \dfrac{n}{2} \dfrac{M_W^H}{M_W^s}}{V} \tag{A15}$$

or in a simplified form:

$$\rho_1 = \rho_D \cdot \left(1 - \bar{\nu}_s \cdot c - \bar{\nu}_H \cdot c \cdot \frac{n}{2} \frac{M_W^H}{M_W^s} \right) + c + c \cdot \frac{n}{2} \frac{M_W^H}{M_W^s} \tag{A16}$$

The apparent partial volume of the solute, $\bar{\nu}_s$, then can be expressed as:

$$\bar{\nu}_s = \frac{\rho_D - \rho_1 + c - \rho_D \cdot \bar{\nu}_H \cdot c \cdot \dfrac{n}{2} \dfrac{M_W^H}{M_W^s} + c \cdot \dfrac{n}{2} \dfrac{M_W^H}{M_W^s}}{\rho_D \cdot c} \tag{A17}$$

rearrangement gives:

$$\bar{\nu}_s = \frac{\rho_D - \rho_1 + c}{\rho_D \cdot c} - \left[\bar{\nu}_H \cdot \frac{n}{2} \frac{M_W^H}{M_W^s} - \frac{n}{2} \frac{M_W^H}{M_W^s} \frac{1}{\rho_D} \right] \tag{A18}$$

in which the first term represents the regular expression for the calculation of the apparent partial volume in a two-component system [1], and the term in square parenthesis is the correction for the hydrogen/deuterium exchange. The latter can be further simplified to give a final form at 25°C:

$$\left[\bar{\nu}_H \cdot \frac{n}{2} \frac{M_W^H}{M_W^s} - \frac{n}{2} \frac{M_W^H}{M_W^s} \frac{1}{\rho_D} \right] = \frac{n}{2} \frac{M_W^H}{M_W^s} (\bar{\nu}_H - \bar{\nu}_D)$$

$$= 0.878 \cdot \frac{n}{2 \cdot M_W^s} \tag{A19}$$

using partial volumes of H_2O, $\bar{\nu}_H = 1.0031$ cm^3/g, and D_2O, $\bar{\nu}_D = 0.9579$ cm^3/g.

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