H/D Isotope Effects on Protein Hydration and Interaction in Solution

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Abstract. An approach has been suggested to study the H/D isotope effect on protein-water and protein-protein intermolecular interactions by determining the content of non-freezing water using low-temperature ^{1}H NMR in mixed (H_2O/D_2O) water solutions. Direct data are obtained on the amount of H_2O adsorbed (absolute hydration) in presence of the heavy isotope (deuterium D), and isothermals of H_2O/D_2O fractionation at protein surface groups are presented for temperatures between -10 ℃ and -35 ℃ and solutions of varying composition. The fractionation factor, $\phi = [x/(1-x)]/[x_0/(1-x_0)]$, where x and x_0 are the fractions of deuterons in hydration and bulk water, respectively, appeared to be extremely high: $\phi \gg 1$ at $0.03 < x_0 < 0.10$. The high values of ϕ indicate a decrease in apparent hydration of protein molecules. A probable reason of the effect can be an inter-protein molecular solvent-mediated interaction induced by D_2O. The excess of ϕ over 1 appears to provide a quantitative estimate of the fraction of hydration water affected by such interaction.

Key words: Isotope effect — Protein hydration — ^{1}H NMR — Deuterium oxide

Introduction

Pronounced effects of deuterium oxide (D_2O) at molecular to cellular levels of biological organization have been widely recognized. D_2O strongly stabilizes protein structure and functional activity (Mrevlishvili et al. 1980; Loh and Markley 1994; Makhatadze et al. 1995; Dong et al. 1997; Madern and Zaccai 1997; Kuhlman and Raleigh 1998), affects protein folding-unfolding kinetics (Parker and Clarke 1997; Perez and Griebenow 2001), promotes protein assemblies, stimulates formation of spindle microtubules, inhibits mitosis (Chakrabarti et al. 1999; Panda et al. 2000), enhances membrane and cytoskeleton resistivity to heat (Ho and Lin 1991).

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Isotopic phenomena play a specific role in hydration effects, particularly in intermolecular biological and colloidal interactions driven by different types of hydration forces (Rupley and Careri 1991; Loh and Markley 1994; Swint-Kruse and Robertson 1995; Israelachvili and Wennerstrom 1996; Glantz et al. 1999). The mechanism of isotopic effects still remains unclear, although they are attributed primarily to enhancement of hydrophobic interactions. An important step in understanding the effect of isotopes is to analyze quantitatively the hydration of proteins in presence of D_2O. So far, water binding to proteins in H_2O/D_2O mixtures has been studied mainly using moistened powders of globular proteins, protein crystals, and films using adsorption techniques (Mrevlishvili et al. 1980; Rupley and Careri 1991; Careri and Peyrard 2001). However, it is important to analyze protein hydration in solution and at different isotope fractions. We therefore used a low-temperature ^1H NMR method to determine protein hydration in the mixture of H_2O and D_2O. The approach is based on the fact that, in contrast to solutions of low-molecular substances, eutectic mixture, i.e. a mixture of ice crystals and concentrated solution, is not characteristic of macromolecules in water.

Materials and Methods

The amount of non-freezing water protons in protein solution at temperatures in the $-10\,℃$ to $-35\,℃$ range was measured by the intensity of the most narrow NMR water proton signal using low-temperature ^1H NMR. This quantity taken at $-35\,℃$ is accepted as protein hydration (Kuntz et al. 1969). Temperature dependences of integral intensity of the resonant water protons absorption band was registered using a Tesla BS-467 (60 MHz) ^1H NMR spectrometer equipped with a standard thermostatic unit allowing temperature maintenance accuracy of $\pm 1\,℃$. Superposition of five successive spectra was used for signal averaging procedure. The spectrum of buffer solution was taken as a baseline. In this record, no buffer water proton signal was revealed in the temperature range under study. Protein solution samples were routinely flash-cooled in liquid nitrogen at 77 K, followed by transfer into a cold nitrogen-gas stream (approximately 223 K). Temperature dependences of the amount of non-freezing water protons were registered by raising temperature from the starting point at 223 K ($-50\,℃$). The samples were held at $-50\,℃$ for 30 min and at other temperatures (up to $-10\,℃$) for 15 min. The duration of measurements in each sample was <3 h. Absolute hydration values were calculated using a signal of the standard (24% LiCl, 0.10% $MnCl_2$ aqueous solution), which stands non-freezing down to $-50\,℃$ and has water concentration of 48.5 mol/l at $-35\,℃$ (Hays and Fennema 1982). Increase in D_2O content leads to lowering of NMR water proton signal intensity. The number of hydration water hydrogens replaced by deuterons (D) on adding D_2O into protein solution is determined by the decrease in the integral intensity of the envelope resonant non-freezing water protons line, since the corresponding hydrogen fraction no longer contributes to the ^1H NMR signal. The comparison of the amount of non-freezing water hydro-

gens in mixtures of ordinary and heavy water was carried out at pH equaled to pH measured to guarantee that macromolecules were in identical electrostatic states. Diluted heavy water solutions were prepared by addition of concentrated protein solution into buffer solutions of different heavy water concentrations. Due to the high rate of $H_2O + D_2O \leftrightarrow 2HOD$ equilibration, no pre-incubation of samples was necessary. The amount of non-freezing water (taken as a value of hydration) can be used to determine the fraction x of D atoms in hydration water as a function of the fraction x_0 of D atoms in solution water:

$$x = \frac{H_{0,t} - H_{\%,t}}{H_{0,t}} = \frac{D_{0,t}}{H_{0,t}} \tag{1}$$

where $H_{0,t}$ and $H_{\%,t}$ are the protein hydration in absence (0) and in presence (%) of D_2O in the solution at temperature t, respectively. Isotope substitution is evaluated by the fractionation index (factor), which is the extent to which a hydrogen at a particular site becomes enriched in D over H relative to the solvent (Schowen and Schowen 1982; Loh and Markley 1994):

$$\phi = \frac{x(1 - x_0)}{x_0(1 - x)} \tag{2}$$

where x and x_0 are the D fractions in hydration water and bulk solvent, respectively.

Sample protein solutions were prepared using immunoglobulin G (IgG) and adult human haemoglobin (HbA) of human donor blood (fresh), and lyophilized equine haemoglobin (met form, met HbH) (Sigma) by addition of concentrated protein solutions to buffer solutions of adjusted D_2O concentration.

IgG was isolated by gradient elution on diethylaminoethyl cellulose. In order to separate probable admixture of cryoglobulins and aggregates, the samples were centrifuged at -1 ℃ for 1 h. Oxy form of haemoglobin (oxy HbA) was isolated according to a routine procedure (Drabkin 1946). Oxy HbA is a structural form of haemoglobin regardless of species with O_2 molecule as a ligand of haeme prosthetic group. There are H_2O or OH^- ligands in the met form of haemoglobin. Potassium ferricyanide $K_3[Fe(CN)_6]$ was used to convert HbA oxy form to its met form (met HbA). Protein solutions were prepared on the basis of the 0.01 mol/l phosphate buffer (pH 7.3). Protein concentration was 20 mg/ml for IgG and 50 mg/ml for oxy HbA and met HbH. All chemicals were reagent grade. 99.8 atomic percent D_2O was used.

Results and Discussion

Figure 1 presents data on the amount of H_2O adsorbed by the proteins on addition of the heavy isotope, i.e. the values of absolute hydration $H_{\%,t}$ at different temperatures. It can be seen (Fig. 1a,c) that hydration $H_{0,t}$ of IgG is 0.09 ± 0.02 (standard mean error in regression), oxy HbA is 0.33 ± 0.03, met HbA is 0.26 ± 0.03, and

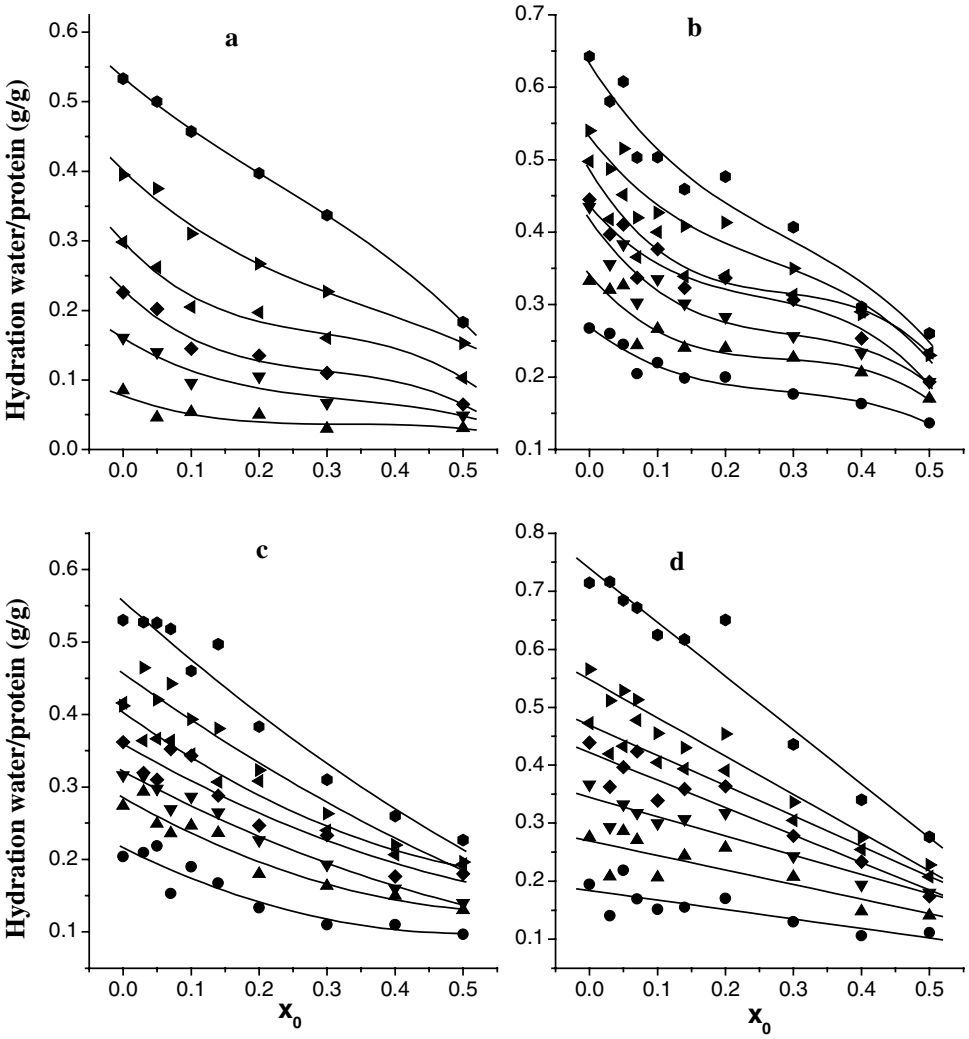

Figure 1. Isothermal plots of protein hydration *vs.* D fraction x_0 (% D_2O) in the bulk solvent of protein solution (Eq. 1): (a) immunoglobulin G (IgG); (b) human oxy haemoglobin (oxy HbA); (c) human met haemoglobin (met HbA); (d) equine met haemoglobin (met HbH). ⬢ −40 °C, ▲ −35 °C, ▼ −30 °C, ◆ −25 °C, ◀ −20 °C, ▶ −15, ⬢ −10 °C. The data were taken at each x_0 value at fixed temperature points by raising temperature in the spectrometer thermostatic unit and then plotted as isothermals of hydration on x_0 to show the effect of D_2O on absolute protein hydration.

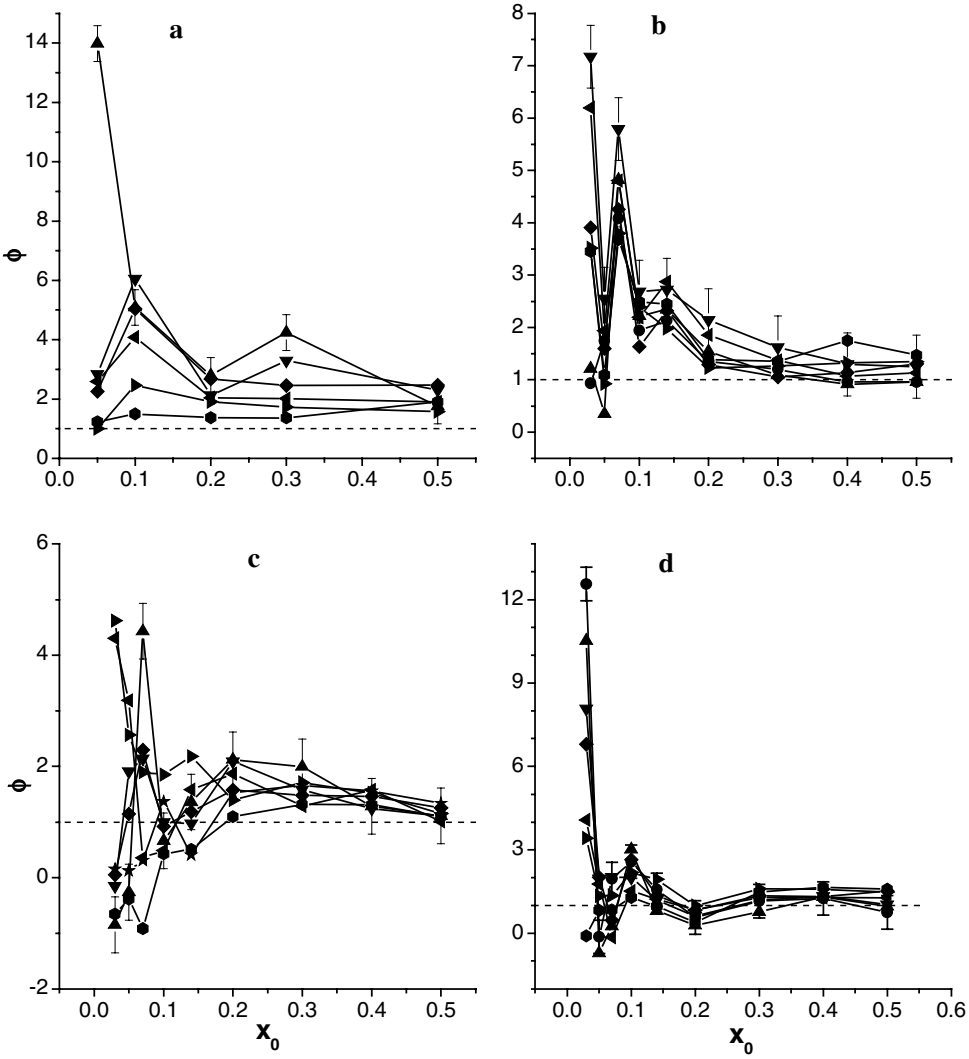

Figure 2. Isothermal plots of H/D fractionation factor ϕ (Eq. 2) *vs.* D fraction x_0 (% D_2O) in the bulk solvent of protein solution: (a) immunoglobulin G (IgG); (b) human oxy haemoglobin (oxy HbA); (c) human met haemoglobin (met HbA); (d) equine met haemoglobin (met HbH). ✹ −40 ℃, ▲ −35 ℃, ▼ −30 ℃, ◆ −25 ℃, ◄ −20 ℃, ► −15, ⬢ −10 ℃. Solid lines connecting the data points correspond to different temperatures of the range studied. Dashed lines indicate levels of the fractionation factor $\phi = 1$ (no H/D fractionation).

met HbH is 0.28 ± 0.03 water/protein (g/g) at $-35\,^{\circ}$C. This is in good agreement with the data obtained for haemoglobin previously by various techniques (Rupley and Careri 1991), and the values for IgG look reasonable for a protein with low solubility and pronounced tendency to form aggregates (Khristoforov et al. 1990).

Figure 2 shows the fractionation index ϕ calculated from the data of Figure 1. The values of ϕ (Figures 1b,d and 2b,d) mostly exceed unity. This is an evidence of preferential adsorption of the heavy isotope by the proteins and isotope enrichment of hydration water. The excess is most pronounced within the range of x_0 values 0–0.1 (i.e. 0 to 10 %) D_2O. This shows the isotope effect of low D_2O concentrations. Moreover, the ϕ values appeared to be very high. For example, $\phi \approx 14$ for IgG at $-35\,^{\circ}$C and $x_0 = 0.05$; $\phi \approx 12$ for met HbH at $-40\,^{\circ}$C and $x_0 = 0.03$; $\phi \approx 7$ for oxy HbA at $-30\,^{\circ}$C and $x_0 = 0.03$; $\phi \approx 4$ for met HbA at $-25\,^{\circ}$C and $x_0 = 0.03$. The jumps on the plots of ϕ *vs.* x_0, including those giving negative values of ϕ, apparently arise from the use of unsmoothed results of Figure 1.

Any deviation from $\phi = 1$ must stem from the effect of isotope H/D substitution on the value of zero vibrational (and librational) contributions to the hydrogen bond energy in two microenvironments – hydration shell and bulk water. The water at protein surface is generally accepted to differ from bulk water only to a minimum extent (Halle 2004). Hence the fractionation index for hydration water is to be closer to unity than the value $\phi = 1.021 \pm 0.001$ obtained for H/D fractionation between ice and ordinary water (Arnason 1969). ϕ does not considerably differ from unity even for water molecules directly coordinated by Co^{2+} in carbonic anhydrase (Venkatasubban and Silverman 1980). A more pronounced fractionation effect could be anticipated for hydrogenic sites of OH^-, $COOH^-$ and NH_2-groups due to the influence of covalent bonds, but even in that case $\phi = 1.0 \pm 0.1$ (Schowen and Schowen 1982). Nevertheless, high values of ϕ were reported for both living nature and inorganic substances (Lobyshev and Kalinichenko 1978 and references therein).

Hydration of macromolecules in solution is known to be affected considerably by their interactions, formation of different complexes, aggregates, clusters and protein-rich phases (Rupley and Careri 1991). On the other hand, there is extensive experimental evidence of the effect of D_2O on both intra- and intermolecular protein interactions in solution (Dong et al. 1977; Lobyshev and Kalinichenko 1978; Chakrabarti et al. 1999; Panda et al. 2000). Aggregation is retarded when D_2O stabilizes the conformational step that involves the disruption of hydrophobic forces necessary for subsequent aggregation. Accordingly, D_2O can promote aggregation when hydrophobic groups involved in aggregation are exposed to the solvent stably. And this is the case for most proteins, as considerable part of their surface is hydrophobic. Moreover, hydration forces originating from hydration of polar and charged protein surface groups can also be affected in H_2O/D_2O mixtures.

This suggests that the high values of ϕ observed in our experiment arise from a decrease in apparent hydration of protein molecules due to intensification of their interaction by adding the heavy isotope D. The apparent fractionation effect must be most pronounced in IgG and oxy HbA, as IgG tends to form aggregates in solu-

tion and hydration of oxy HbA is the highest of the proteins studied. The relative magnitudes of the effects seen from Figures 1 and 2 are apparently consistent with this expectation. Namely, IgG and oxy HbA have the highest values of ϕ when considering not only the values at lowest D_2O contents (0.03 and 0.05), but the entire range of D_2O concentrations.

Thus, the excess of ϕ over the reasonable values of 1 or 1.1, being a result of an inter-protein interaction, can be used as an estimate of that interaction and a direct quantitative estimate of the hydration water fraction affected due to interaction induced by D_2O. Calculating x from Eq. 2 for extremely high values of ϕ given above and taking into account Eq. 1, we have $H_{\%}= 0.58\ H_0$ for IgG at -35℃ and $x_0 = 0.05$. This means that adding 5% D_2O to the protein solution lowers protein hydration observed at -35℃ by at least 0.42, and the contribution of interaction induced by D_2O is $0.42 - 0.05 = 0.37$. Likewise, $H_{\%} = 0.73\ H_0$ and the interaction contribution is 0.24 for met HbH at -40℃ and $x_0 = 0.03$; for oxy HbA, we have $H_{\%} = 0.82\ H_0$ and 0.15; for met HbA, $H_{\%} = 0.89\ H_0$ and 0.08.

The highest values of ϕ within the -30 to -40℃ temperature range are indicative of the fact that the relative amount of hydration water affected by protein interaction and responsible for the NMR signal is greatest at these temperatures in particular. This can be water at polar and charged surface groups, as these surface groups are known to attach water that remains unfrozen and thus mobile enough to give observable NMR signal in this temperature interval. This does not necessarily mean that interaction occurs primarily due to contacts between such groups, but the effect of interaction over the water at polar and charged groups is most visible in the approach employed.

There is an apparent common pattern in the dependences of ϕ on x_0. The decrease of ϕ with increasing D_2O concentrations suggests that the protein interaction induced by D_2O is D_2O concentration dependent, i.e., the most remarkable effects are observed below the saturation level of the interaction, which is reached at D_2O concentrations of ~ 0.03. This result is consistent with the early data and theory of anomalous effects of low D_2O concentrations in biological systems (Lobyshev and Kalinichenko 1978 and references therein; Lobyshev and Vishnevskaia 1984; Kremlev and Lobyshev 1986). A further increase in D_2O content just leads to an increase of the factor $x_0/(1 - x_0)$ in Eq. 2 and a decrease of ϕ down to about 1.

The results presented above indicate that adding heavy water D_2O to a protein solution leads to a shift of the equilibrium between monomers and aggregates towards formation of protein aggregates due to intensification of intermolecular interactions, the highest effect being observed at low D_2O concentrations. The interaction strongly affects protein hydration causing it to decrease by 10 to 40%. The most pronounced effect could be attributed to water at polar and charged protein surface sites, although the interaction is likely to be due to strengthening of contacts between surface hydrophobic groups. This shows a definite value of hydration forces in the inter-protein interactions.

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