

D₂O as an Imperfect Replacement for H₂O: Problem or Opportunity for Protein Research?

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Cite This: *J. Phys. Chem. B* 2023, 127, 8086–8094

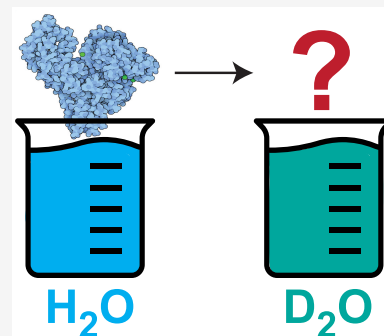
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ABSTRACT: D₂O is commonly used as a solvent instead of H₂O in spectroscopic studies of proteins, in particular, in infrared and nuclear-magnetic-resonance spectroscopy. D₂O is chemically equivalent to H₂O, and the differences, particularly in hydrogen-bond strength, are often ignored. However, replacing solvent water with D₂O can affect not only the kinetics but also the structure and stability of biomolecules. Recent experiments have shown that even the mesoscopic structures and the elastic properties of biomolecular assemblies, such as amyloids and protein networks, can be very different in D₂O and H₂O. We discuss these findings, which probably are just the tip of the iceberg, and which seem to call for obtaining a better understanding of the H₂O/D₂O-isotope effect on water–water and water–protein interactions. Such improved understanding may change the differences between H₂O and D₂O as biomolecular solvents from an elephant in the room to an opportunity for protein research.



INTRODUCTION

D₂O, or heavy water, is a stable isotopomer of H₂O, containing deuterium instead of the most common hydrogen isotope protium. Deuterium was discovered in 1931 by H. Urey,¹ who was awarded the Nobel Prize for this finding in 1934. The chemical and physical properties of D₂O were first studied by G. Lewis and co-workers in the early 1930s^{2,3} and are very similar to those of H₂O (Table 1). For this reason, D₂O is

Table 1. Selected Physical and Chemical Properties of H₂O and D₂O^{8–11}

property	H ₂ O	D ₂ O
molecular weight (g/mol)	18.02	20.03
melting point (°C)	0	3.82
boiling point (°C)	100	101.4
molar density (mol/L, 25.0 °C, 1 atm)	55.35	55.14
molecular polarizability (Å ³)	1.45	1.26
viscosity (25 °C)	0.891	1.095
pH/pD (25 °C)	6.9976	7.43
dielectric constant (25 °C)	78.37	78.06

often used as a solvent instead of H₂O in experiments where the H atoms of water form a problem, such as in nuclear magnetic resonance, neutron scattering, and infrared spectroscopy and imaging. This holds in particular for studies of biomolecules: in both protein NMR and infrared spectroscopy and imaging,^{4–6} it is standard practice to use D₂O as a solvent. In the case of infrared spectroscopy, this is done because the vibrational modes of the amide groups, which carry crucial information on the protein structure,⁷ have spectral overlap

with the bending mode of H₂O (both are in the 1600–1700 cm⁻¹ frequency range). The D₂O-bending frequency is 1250 cm⁻¹, eliminating the overlap problem and making D₂O the seemingly perfect replacement of H₂O.

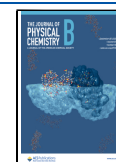
The effect of H/D substitution on the kinetics of chemical reactions is well-known, and has been extensively studied and applied, for instance to study reaction mechanisms¹² and to monitor protein folding.¹³ Interestingly, recent work shows that the kinetic effects induced by substituting D₂O for H₂O might also be useful for biomedical purposes:¹⁴ epithelial cells grown in a medium containing 45% D₂O show significantly reduced migration and proliferation rates (Figure 1), and a similar slowdown in dynamics was observed in other cells,^{15–17} an effect that might find use for the storage of biological materials such as organs, or for anticancer treatment.¹⁵

While the effect of H/D substitution on kinetics is well established, it is often (implicitly) assumed that the effect of H₂O/D₂O substitution on the structure of biomolecules and biomolecular assemblies is small. However, although the use of isotopic substitution in spectroscopic experiments has been mostly successful, there is ample evidence that replacing H₂O with D₂O can alter the thermodynamic and structural properties of proteins^{18–35} and even the formation process

Received: June 29, 2023

Revised: August 28, 2023

Published: September 18, 2023



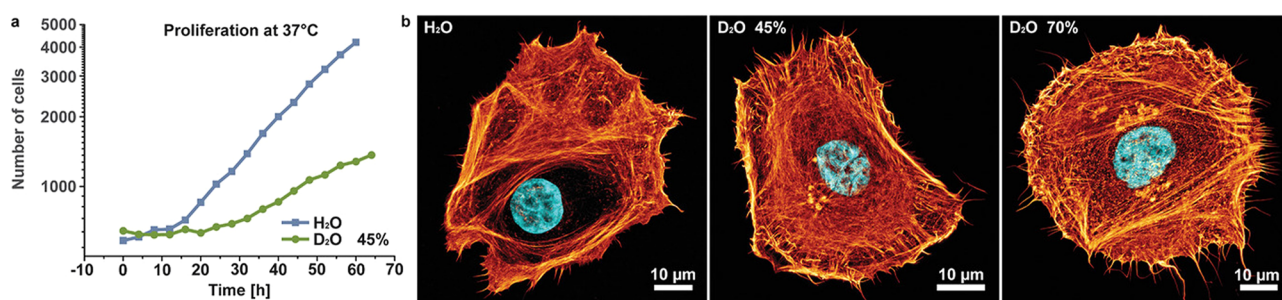


Figure 1. “Cells in slow motion.”¹⁴ (a) Epithelial cell proliferation is much slower in a D₂O-rich medium. The duration of the cell cycle is roughly 17 h under normal (H₂O) conditions and 44 h in water containing 45% D₂O. (b) Independent of the culturing conditions, the actin cytoskeleton (orange) and the nucleus (blue) remain intact. Shown are typical pictures, which suggest that structures are comparable for the different H₂O and D₂O concentrations. Figure adapted from ref 14, Copyright 2021 Schnauß et al. (licensed under CC BY-NC 4.0).

and structure of protein assemblies.^{14,25,33,36–41} The fact that H₂O/D₂O replacement can change the structure of biopolymers and their assemblies should not come as a complete surprise: the hydrogen-bond^a structures of H₂O and D₂O are known to be different,⁴² and hydration and the hydrophobic effect are essential for all biomacromolecules, ranging from polysaccharides to proteins. Hydrating water molecules create a water network around solutes that not only acts as structure stabilizer but also mediates intra- and intermolecular interactions. As stated recently by Fischer et al.,⁴³ hydration represents “an additional evolutionary constraint upon protein sequence to maintain ligand binding and modulate the affinity of those interactions”, to which we might add that since evolution has optimized protein structure and dynamics in H₂O rather than D₂O, and since the hydrogen-bond structures of these two liquids are different, differences in structure and dynamics are to be expected when replacing one with the other.

The H₂O/D₂O-induced changes in biomolecular structure seem to call for more detailed studies of the difference between liquid D₂O and H₂O, but they also suggest fascinating new research opportunities. In this Perspective, we first briefly describe the differences between H₂O and D₂O; then we summarize and discuss the existing experimental evidence for isotope-induced structural changes in biomolecules and biomolecular assemblies; finally, we discuss the current challenges and perspectives, in particular the possibility of using D₂O to investigate the role of hydration in protein stability and interactions.

■ H₂O VERSUS D₂O

The interplay of nuclear quantum effects (NQEs) underlying the physical and chemical differences between liquid D₂O and H₂O is quite subtle. Simply put, the low mass of the hydrogen atom makes it behave more as a delocalized quantum particle than the heavier deuterium. This delocalization can have a substantial effect on the hydrogen bond strength.¹⁰ Specifically, for an O–H···O hydrogen bond, the hydrogen-bond strength is a function of the O···O distance (the shorter, the stronger) and the O–H···O bond angle (the straighter, the stronger). The larger distance spread for H vs D leads to a strengthening of the H-bond, while the larger angular spread leads to a weakening. Hence, these two nuclear quantum effects have contrary consequences for the H-bond strength. Depending on the details of the H-bond, one or the other effect may dominate, resulting in a weakening or strengthening of H-bonds upon isotopic substitution. Short hydrogen bonds are

typically strengthened due to NQEs, whereas long ones are weakened.¹⁰ Here we summarize the most important differences that are generally agreed upon in the literature, focusing on the points that are relevant for understanding how replacing H₂O with D₂O can change the structures of biomolecules and biomolecular assemblies.

The structure of liquid D₂O and water has been investigated using different methods, in particular X-ray, γ -ray, and neutron scattering. By combining X-ray measurements with molecular simulations, it was found that the covalent bond between oxygen and protium (O–H) is 3% longer with respect to the one between oxygen and deuterium (O–D), see Figure 2

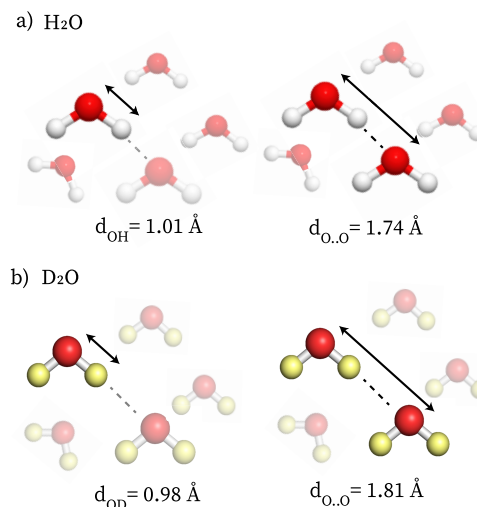


Figure 2. Average lengths of the covalent and hydrogen bonds in liquid H₂O (a) and D₂O (b).

(neutron scattering studies indicate a somewhat smaller isotope effect on the covalent bond length¹⁰). In D₂O, the hydrogen-bond network is more tetrahedral than that in H₂O and the hydrogen-bond coordination number is higher,⁴² both effects indicating stronger hydrogen bonds and a more structured hydrogen-bond network. The average hydrogen-bond distance (the O···O distance of two hydrogen-bonded water molecules) is 4% longer in D₂O, as is also reflected in its lower molar density compared to H₂O (cf. the situation in ice, where the hydrogen bonds are also stronger than in liquid water). In *ab initio* calculations on hydrogen-bonded oligomers, it was also found that the hydrogen-bond strength is 0.2–0.3 kcal/mol larger in D₂O than in H₂O.⁴⁴ Finally, the

Table 2. Effects of D₂O on the Properties of Proteins and Other Biomolecules^a

biomolecule	method	effect
bovine serum albumin ¹⁸	DSC	enhanced stability of the native state, $T_d^{D_2O} - T_d^{H_2O} \approx 2-3$ °C
bovine serum albumin ¹⁹	CD	enhanced stability of the native state Irr. $T_d^{D_2O} - Irr. T_d^{H_2O} \approx 8$ °C
bovine serum albumin ²⁰	DLS, FI, UV-vis, SE-HPLC	enhanced stability of the native state monomer % at 65 °C: 85% in D ₂ O, 75% in H ₂ O
lysozyme ¹⁸	DSC	enhanced stability of the native state, $T_d^{D_2O} - T_d^{H_2O} \approx 2-3$ °C
tubulin ²¹	CD, DSC, FI	enhanced stability of the native state, $T_d^{D_2O} - T_d^{H_2O} \approx 3$ °C
acyl carrier proteins ²²	NMR	enhanced stability of the native state, $\Delta G_{N \rightarrow U}^{D_2O} = 2.3$ kcal/mol; $\Delta G_{N \rightarrow U}^{H_2O} = 1.8$ kcal/mol
collagen peptides ²⁴	CD, DSC	enhanced stability of the folded state, $T_m^{D_2O} - T_m^{H_2O} \approx 4$ °C
ribonuclease A ²⁷	DSC	enhanced stability of the native state, $T_m^{D_2O} - T_m^{H_2O} \approx 4$ °C
<i>Drosophila</i> signal transduction protein ²⁶	NMR	enhanced stability of the folded state, $T_m^{D_2O} - T_m^{H_2O} \approx 12$ °C
κ -carrageenan ²⁵	DSC	enhanced stability of the folded state, $T_{gel \rightarrow liq}^{D_2O} - T_{gel \rightarrow liq}^{H_2O} \approx 3$ °C
elastin-like peptides ²⁸	DSC, CD, IR	enhanced stability of the collapsed state, Propensity to form β -turn/ β -aggregate, $LCST^{H_2O} - LCST^{D_2O} \approx 2-5$ °C
peptides containing alanine ²⁹	CD	propensity for PPII structure: 5–200% higher PPII signal in D ₂ O
plastocyanin ³²	MD	altered solvent–protein interactions: 10–30% reduction of protein–water H-bonds
test polypeptides ³⁴	MD	altered solvent–protein interactions
agarose (Ag2) ³³	NMR	lower solvent–polysaccharide affinity, $N_w^{H_2O}/N_w^{D_2O} \approx 3.8$
ribonuclease T1 ³¹	luminescence	increased protein rigidity, $IPL^{D_2O} = 36$ ms, $IPL^{H_2O} = 28$ ms
β -lactoglobulin ³¹	luminescence	increased protein rigidity, $IPL^{D_2O} = 44$ ms, $IPL^{H_2O} = 30$ ms
liver alcohol dehydrogenase ³¹	luminescence	increased protein rigidity $IPL^{D_2O} = 819$ ms, $IPL^{H_2O} = 630$ ms
alkaline phosphatase ³¹	luminescence	increased protein rigidity, $IPL^{D_2O} = 2142$ ms, $IPL^{H_2O} = 2060$ ms
apo-azurin ³¹	luminescence	increased protein rigidity $IPL^{D_2O} = 603$ ms, $IPL^{H_2O} = 564$ ms
TAS1R2/TAS1R3 receptor ³⁰	MD	smaller radius of gyration $R_g^{D_2O}$ is $\approx 3\%$ smaller than $R_g^{H_2O}$
azurin, ³⁵ lactoglobulin, ribonuclease	MD	smaller radius of gyration $R_g^{D_2O}$ is $\approx 1\%$ smaller than $R_g^{H_2O}$

Part of this table is taken from ref 26. ^aAbbreviations: T_d = denaturation temperature; Irr. T_d = irreversible denaturation temperature; T_m = melting temperature of the native state; T_0 = transition temperature from folded-to-unfolded; R_g = radius of gyration; IPL = intrinsic Trp phosphorescence lifetime; $\Delta G_{N \rightarrow U}$ = Gibbs energy of unfolding; $T_{gel \rightarrow liq}$ = gel-to-liquid transition temperature; LCST = lower critical solution temperature; N_w = number of hydration waters per mass unit of agarose; DSC = differential scanning calorimetry; SE-HPLC = size exclusion high-performance liquid chromatography; CD = circular dichroism; DLS = dynamic light scattering; FI = fluorescence measurements; NMR = nuclear magnetic resonance; MD = molecular dynamics simulations.

macroscopic thermodynamical properties (such as the specific heat and the melting point) of H₂O and D₂O also indicate stronger hydrogen bonding between D₂O molecules, with a difference in hydrogen-bond energy similar to that found in the *ab initio* calculations.^{10,45}

■ ISOTOPE-INDUCED EFFECTS ON BIOMOLECULAR STRUCTURE

We will now discuss examples of how the stronger hydrogen bonding in D₂O can influence biomolecular structure and stability. First, we discuss the effects on individual biomolecules and then the more recently discovered D₂O-induced effects on protein assemblies.

Effects of Replacing H₂O with D₂O on Protein Stability, Structure, and Hydration. D₂O-induced changes in protein stability depend in a complicated manner on changes in the (local) hydration, with both enthalpic and entropic contributions. Yet, the simple argument that the stronger hydrogen bonding between D₂O molecules suppresses protein unfolding, favoring compact, folded proteins with minimal hydration seems to be sound. In Table 2, we give an overview of experimental results demonstrating the effect of D₂O on biomolecular stability, structure, and rigidity, based on (and somewhat extending) the excellent overview given in ref 26. Most studies focus on the conformational stability in H₂O and D₂O. This is motivated by the potential use of D₂O as a way to slow down thermal degradation, especially in pharmaceutical applications. Several studies have shown that

the native or folded states of globular proteins such as bovine serum albumin (BSA), lysozyme, and tubulin are more stable in D₂O than in H₂O.^{18–21} For instance, using differential scanning calorimetry (DSC), it was found that the denaturation temperature of lysozyme and BSA is 2–3 °C higher in heavy water than in water.¹⁸ Circular dichroism (CD) experiments, which are more structure-sensitive than DSC measurements, showed that the onset temperature of the irreversible thermal denaturation (i.e., the temperature of the irreversible change of the secondary structure) of BSA is 58 °C in D₂O while it is 50 °C in H₂O.¹⁹ Upon heat-treatment at 65 °C, BSA also retains a larger percentage of monomers in heavy water than in water (85% versus 75%, respectively), again indicating that the BSA monomeric form is more stable in D₂O.²⁰ Similar results have been found for other, nonglobular proteins, such as acyl carrier proteins,²² collagen,²⁴ ribonuclease A²⁷ and *Drosophila* signal-transduction protein Drk.²⁶ Similar enhanced stability of the folded state was also observed for κ -carrageenan, which undergoes to a liquid-to-gel transition by forming double helices, that are stabilized significantly more in D₂O.²⁵

The increased stability of folded and native structures in D₂O indicates a stronger tendency to adopt a more compact, less solvent-exposed conformation in this solvent. For instance, a D₂O-induced tightening of the helical structure has been proposed for actin, based on combined rheological and fluorescence experiments.¹⁴ Similarly, Cremer et al. have shown that elastin-like polypeptides (ELPs) undergo a hydrophobic collapse that is accompanied by the formation

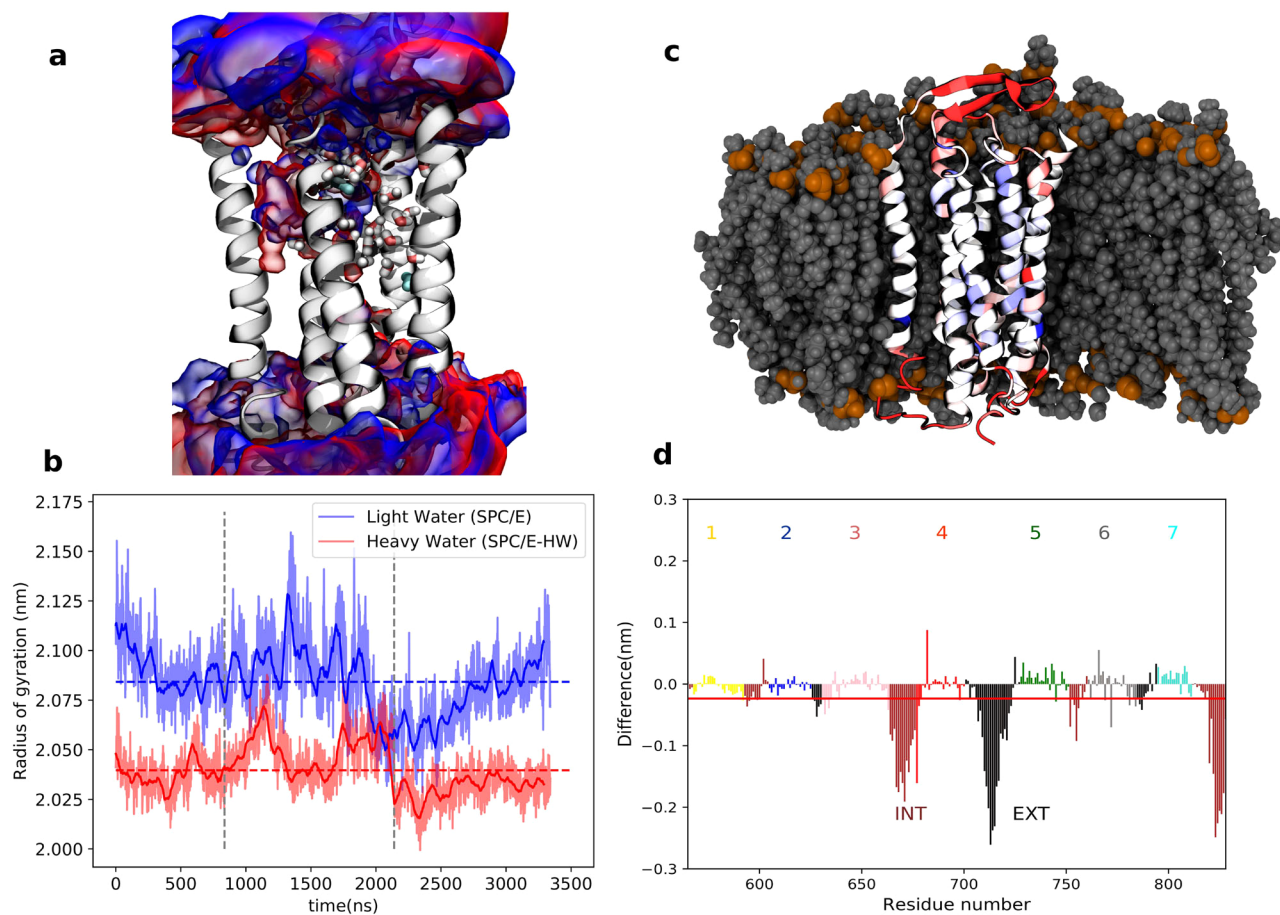


Figure 3. Differences between the behavior of the transmembrane part of the human sweet taste receptor in H₂O vs D₂O. (a) Structure of the TMD of the TAS1R2/TAS1R3 receptor with the probability density (volumetric map) of H₂O (blue) or D₂O (red) molecules within 10 Å of the protein. The conserved water molecules in the X-ray templates are shown in cyan. Water molecules predicted with the software OpenEyeS2 are shown in licorice representation. (b) Time evolution of the radii of gyration in H₂O (blue) and D₂O (red) from three microsecond time scale simulations (separated by vertical dashed lines) with total mean values as dashed lines, showing that the protein is more compact in D₂O. (c) Snapshot of the transmembrane part of the human sweet taste receptor color-coded that red/blue represents parts more/less rigid in D₂O vs H₂O. The embedding lipid membrane is represented in gray. (d) Difference in root-mean-square fluctuations in MD trajectories. Negative/positive values mean that structures are more/less rigid in D₂O than in H₂O. The red line represents the sum of all residues. INT, intracellular; EXT, extracellular. Adapted from ref 30, copyright 2021 Ben Abu et al. (licensed under CC BY).

of β -turn structures, which are significantly more stable in D₂O.²⁸ Increased stability of intermolecular β -sheet structures in D₂O has been suggested for insulin dimers because of the 2-fold slower assembly kinetics in heavy water with respect to water, as observed with infrared and two-dimensional infrared spectroscopy, and because of a larger fraction of dimer in D₂O than H₂O in the initial structures as revealed by molecular simulations based on solution-phase small-angle X-ray scattering experiments.³⁹ This again suggests a general preference for a more compact conformation in D₂O. Moreover, specific secondary structures can be enhanced when proteins are dissolved in D₂O. Circular-dichroism studies by Chellgren et al. have demonstrated that peptides containing alanine have a stronger propensity to form polyproline II (PP II) structure in D₂O than in H₂O.²⁹ Since it is believed that the PP II conformation perturbs the bulk hydrogen-bond network of the surrounding water less strongly than does an α -helical conformation, this effect was attributed to the increased energetic cost of protein solvation in D₂O.

The difference in protein stability and the preference for PP II structure suggest that interactions between solvent and protein might be modified in D₂O compared to H₂O, leading

to changes in the intraprotein hydrogen-bond network. This possibility has been investigated mostly by means of molecular dynamics simulations of various biomolecules, such as plastocyanin,³² RNA hairpins,²³ and peptides.³⁴ Interestingly, in ref 32, it was observed that a reduction of the number of hydrogen bonds between solvent and protein occurs mostly when polar and positively charged side groups are involved, while the opposite is observed for negatively charged side groups. Overall, however, a 10–30% reduction in the number of water molecules engaged in hydrogen bonds with the protein was observed in D₂O compared to H₂O, which was correlated to the enhancement of intramolecular interactions in this solvent.³² A lower affinity between D₂O and solute was also observed in NMR studies on agarose.³³ The increased rigidity which Cioni et al. have observed for different proteins (see Table 2) also supports the idea that protein–solvent interactions are altered in D₂O:³¹ using luminescence methods it was found for 5 proteins out of the 7 analyzed that D₂O increases protein rigidity, with a protein-dependent rigidity enhancement. In this respect it is interesting to note that some proteins crystallize more efficiently in D₂O than in H₂O,⁴⁶ a phenomenon that in the case of ref 46 was even accompanied

Table 3. Effects of D₂O on Biomolecular Self-Assembly^a

protein	method	effect
<i>Escherichia coli</i> protein BirA ⁵⁰	SE	increased binding energy, $K_{\text{dim}}^{\text{H}_2\text{O}}/K_{\text{dim}}^{\text{D}_2\text{O}} \approx 10$
androgen receptor ³⁶	NMR, DLS, microscopy	enhanced condensation, larger condensates 25 °C shift of cloud point at a H ₂ O/D ₂ O fraction of 1:1
κ -carrageenan ²⁵	rheology	faster assembly, higher elastic modulus, $G^{\text{D}_2\text{O}}/G^{\text{H}_2\text{O}} \approx 1.1\text{--}1.2$
gelatin ³⁷	U-tube, rheology	faster assembly, higher shear modulus, $r^{\text{D}_2\text{O}}/r^{\text{H}_2\text{O}} \approx 2.5$, $G^{\text{D}_2\text{O}}/G^{\text{H}_2\text{O}} \approx 3$
casein ^{38b}	rheology	faster assembly, higher elastic modulus: Gel.On. _{RG} ^{D₂O} = 9.1 ± 0.1 min; Gel.On. _{RG} ^{H₂O} = 14.6 ± 0.1 min; Gel.On. _{TG} ^{D₂O} = 1.3 ± 0.4 min; Gel.On. _{TG} ^{H₂O} = 11.3 ± 1.1 min; $G_{\text{RG}}^{\text{D}_2\text{O}} = 1636.7 \pm 75.7$ Pa; $G_{\text{RG}}^{\text{H}_2\text{O}} = 1183 \pm 55.1$ Pa; $G_{\text{TG}}^{\text{D}_2\text{O}} = 504 \pm 27.7$ Pa; $G_{\text{TG}}^{\text{H}_2\text{O}} = 210 \pm 26$ Pa
insulin ³⁹	2DIR, IR, FI	slower assembly, $\tau_{\text{lag}}^{\text{H}_2\text{O}} \approx 16$ h; $\tau_{\text{lag}}^{\text{D}_2\text{O}} \approx 20$ h
α -synuclein ⁴⁰	FI, NMR, SANS	faster assembly, $\tau_{\text{lag}}^{\text{H}_2\text{O}} \approx 34$ h; $\tau_{\text{lag}}^{\text{D}_2\text{O}} \approx 23$ h (0.150 M NaCl)
actin ⁵²	static light scattering	formation of multifilament bundles in D ₂ O, DCR ^{D₂O(70%)} /DCR ^{H₂O} ≈ 2.5
agarose ³³	turbidity	change in the network, $\tau^{\text{D}_2\text{O}}/\tau^{\text{H}_2\text{O}} \approx 1.1\text{--}1.3$
pectin ⁴¹	SAXS	change in network fractal dimension

^aAbbreviations: K_{dim} = equilibrium dissociation constant for dimerization; τ_{lag} = lag time; G' = elastic modulus at a frequency of 1 Hz; r = rate of initial gelation; G = shear modulus; DCR = derived count rate (light-scattering intensity); Gel.On. = gelation onset; τ = initial turbidity; SE = sedimentation equilibrium measurements; 2DIR = two-dimensional infrared spectroscopy; SAXS = small angle X-ray scattering; SANS = small-angle neutron scattering. ^bTwo methods were used to induce gelation, referred to as RG and TG.

by a difference in crystal symmetry and structure (whereas in general protein crystal structures seem to be independent of whether H₂O or D₂O is used^{47–49}). The D₂O-induced damping of conformational fluctuations can be attributed to stronger solvent–solvent interactions,³¹ which reduce protein hydration and promote intramolecular interactions (as was observed in ref 32). The reduction in structural fluctuations in D₂O may thus be explained by the fact that water–protein interactions can destabilize proteins by lowering the free-energy barriers between different conformations.

We conclude our list of proteins with the well-known and intriguing fact that D₂O tastes sweet. A recent molecular-dynamics study of this isotope effect by the Jungwirth group³⁰ has shown that the transmembrane part of the human sweet-taste sensor protein is more compact, stiffer, and subject to less structural fluctuations in D₂O than in H₂O (Figure 3). This study again supports the idea of a reduction in protein hydration in D₂O compared to H₂O. Indeed, in a more recent study the same group has found that in D₂O, water has a stronger propensity to form water/water hydrogen bonds than water/amino-acid hydrogen bonds (interestingly, this behavior does not follow the hydrophobicity scale of the amino acids).³⁵ It was also found that globular proteins (azurin, lactoglobulin, and ribonuclease) are significantly more compact in D₂O than in H₂O. Jungwirth et al. conclude that “D₂O is a somewhat worse solvent for biomolecules than H₂O. This also implies that association between proteins or between a protein and a biomembrane may be positively affected by water deuteration”. In the next section, we will see experimental results that support this idea.

D₂O-Induced Changes in Protein Assemblies and Networks. We have seen that D₂O increases the stability of the folded state of proteins, in particular promoting the formation of secondary structures that least disrupt the hydrogen-bond network of water, and that protein hydration is reduced in D₂O. More recently, it has become clear that these changes at the molecular level can affect the propensity and mechanisms of aggregation/assembly of biopolymers into larger supramolecular structures, leading to different mechan-

ical and thermodynamic properties of the final aggregate/assembly (Table 3). In particular, Salvatella et al. have found that androgen receptors have a stronger tendency to form biomolecular condensates by liquid–liquid phase separation (LLPS) in D₂O than in H₂O.³⁶ Interestingly, in this study, it was shown that replacing less than 10% water (as is common in NMR) with D₂O can already significantly affect the phase equilibrium of the condensation, with a decrease of the cloud point by 0.5 °C for each added percent of D₂O, and that the size of the condensates becomes larger with increasing amount of added D₂O. These changes were attributed to the enhancement in D₂O of the intermolecular interactions that drive the initial oligomerization. Similarly, an elegant study by Beckett et al. has shown that the dimerization of the *Escherichia coli* protein BirA is more favorable in D₂O than in H₂O, with a dimer dissociation constant that is 10 times smaller in the former.⁵⁰ A similar D₂O-induced alteration of the aggregation propensity (and possibly the final aggregate size) has been proposed for BSA aggregates, based on thioflavin fluorescence, turbidity, and circular dichroism experiments.^{19,20,51}

Several studies have shown a significant difference in protein assembly rates in water and D₂O, with assembly occurring faster in the latter. For instance, the aggregation and simultaneous double-helix formation of κ -carrageenan occurs faster in D₂O than in H₂O.²⁵ Faster aggregation in D₂O was also observed for gelatin,³⁷ casein,³⁸ and bovine serum albumin.¹⁹ These examples all show faster assembly in D₂O, but self-assembly processes can also become slower in D₂O. Recently, a ground-breaking study by Cho et al. has shown that amyloid formation of insulin occurs slower in D₂O than in H₂O (Figure 4).³⁹ This effect was attributed to the presence of intermediates that adopt intermolecular beta-sheet structures, which are more favored in D₂O than in H₂O. Using D₂O as a solvent instead of H₂O increases the free-energy barrier for unfolding these intermediates, which is a necessary step for the final fibril formation. A similar enhancement of oligomer stability in heavy water was suggested for transthyretin tetramer.⁴⁹ Interestingly, it was recently found that the fibrillization of alpha-synuclein (the protein responsible for

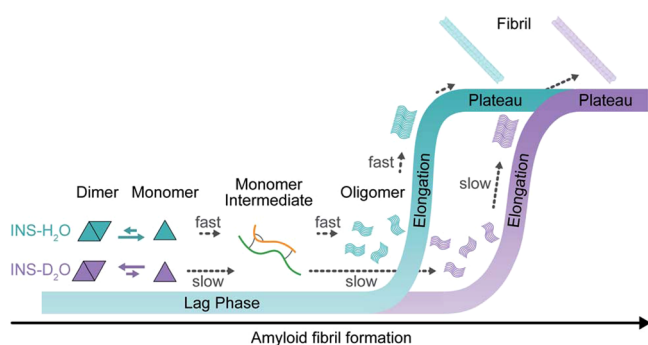


Figure 4. Insulin (INS) fibrillization kinetics in H_2O and D_2O and proposed fibrillization mechanism explaining the slower assembly in D_2O . Reproduced from ref 39, Chun et al., published by the Royal Society of Chemistry (licensed under CC BY-NC 3.0).

Parkinson's disease) proceeds *faster* in D_2O than in water.⁴⁰ This acceleration was attributed to enhanced protein–protein interactions in D_2O that facilitate the refolding of alpha-synuclein, which is required for initiating its fibrillization.

Surprisingly, not only protein-assembly kinetics but even the viscoelastic properties of biopolymer networks can be different in D_2O and H_2O . The mechanical properties of reconstituted actin networks are affected by using D_2O instead of H_2O : in D_2O the actin filaments behave as a transiently cross-linked network rather than the typical behavior of an entangled network (as is observed in H_2O). This peculiar behavior in D_2O was recently explained by the finding that D_2O induces the formation of multifilament bundles, leading to a structural reorganization of the actin network and different mechanical properties.⁵² The difference in the network structure was attributed to a larger stickiness between actin filaments in D_2O because of enhanced intermolecular interactions in this solvent.^{14,52} Similarly, the elastic modulus of gels formed by the aggregation of κ -carrageenan is ~ 10 – 20% higher in D_2O than in H_2O because of the larger number of cross-links formed between the chains.²⁵ Such increased network rigidity has also been observed in gelatin and casein gels.^{37,38} In contrast, Brenner et al. found that in agarose gel the mechanical properties are the same in D_2O and H_2O , even though D_2O does enhance the stability of the helical structure and gives rise to gels with larger heterogeneity on the micrometer scale (and not the nanometer scale).³³ Finally, an intriguing topological difference in biopolymer-network structure has been found in the case of pectin, for which recent experiments have shown that the fractal dimension of the gel network formed is higher in D_2O than in H_2O (indicating that in D_2O the gel is more clustered),⁴¹ an observation that again “highlights the need to be mindful of changes induced when substituting D_2O in systems with significant hydrogen bonding”.⁴¹

The Origin of D_2O -Induced Changes in Stability and Structure. In D_2O , biopolymers are exposed to a more strongly hydrogen-bonded water network,⁴² and therefore creating a solvation cavity to accommodate the protein (or increasing the solvent-exposed surface area of a protein) is energetically less favorable in D_2O because of the additional enthalpic cost required to break the water hydrogen bonds. This energetic loss is enhanced when the solvent needs to reorganize around nonpolar groups, and hence hydrophobic patches have a stronger tendency to cluster in D_2O than in H_2O , an effect we may refer to as isotopically enhanced

hydrophobic effect. However, a theoretical analysis by Graziano and Pica has shown that the $\text{H}_2\text{O}/\text{D}_2\text{O}$ effect on the hydrogen-bond structure may not be sufficient to explain D_2O -enhanced protein stability.¹¹ Due to the lower molecular polarizability of D_2O , van der Waals attractive interactions are less favorable in D_2O , and thus fewer interactions take place between protein and water. Reduced van der Waals interactions affect the binding affinity of D_2O to biomacromolecules, which may lead to changes in the hydration shell surrounding the biomolecules.^{23,35} The combination of reduced van der Waals interaction and the higher enthalpic cost of water–water hydrogen-bond breaking will likely change the hydration capability of D_2O with respect to H_2O in a synergistic way. Since contacts between water and protein can reduce the free energy barrier between the different protein conformations, the lower number of water–protein interactions in D_2O will lead to structurally more stable and less fluctuating proteins, as reported in the literature (Table 2). This proposed stabilization mechanism is also suggested in a recent study by Haidar et al.⁵³ From collision-induced unfolding and ion-mobility mass spectrometry, it was found that the stability of lysozyme, cytochrome c, and bovine ubiquitin in the gas phase is independent of whether the protein is hydrogenated or fully deuterated, in contrast with the increased stability of these proteins in D_2O solution, again indicating that the changes in protein properties are due to solvent effects. This idea seems to be further confirmed by the general absence of significant differences between the crystal structures of hydrogenated and perdeuterated proteins.^{47,49} A decrease in water–protein interaction in D_2O compared to H_2O is also consistent with the enhanced rigidity observed, for instance, in collagen peptides, where intramolecular hydrophobic interactions are minimal and thus enhanced hydrophobic effect alone cannot explain the increased rigidity.²⁴

We have seen that biomolecular assembly can occur at different rates in D_2O and H_2O (Table 3 and Figure 4). If the aggregation is driven by hydrophobic or hydrophilic interactions, the kinetics are expected to be different in D_2O . As discussed before, D_2O enhances the hydrophobic interactions (enhancing the aggregation) and has reduced protein hydration compared to H_2O . This latter effect implies that the desolvation enthalpy, i.e., the energy required to break the hydrogen bonds between water and hydrophilic groups to allow the formation of bonds between hydrophilic groups, is lower in D_2O than in H_2O . This is consistent with the faster assembly rate reported for several systems.^{25,37,38} However, if the aggregation process involves the formation of intermediates stabilized by hydrophobic interactions, the assembly might be slower in D_2O , as observed in the case of amyloid formation.³⁹ To form fibrils, intermediates have to undergo partial unfolding, a process that is energetically more unfavorable in D_2O since the intermediates are more stable due to the enhanced hydrophobic effect.

■ FROM ELEPHANT IN THE ROOM TO OPPORTUNITY FOR PROTEIN RESEARCH

Although in general replacing H_2O with D_2O has a limited effect on protein structure (as is demonstrated by the large number of successful studies in which this procedure was used), the experiments and simulations discussed above show that replacing H_2O with D_2O can in some cases significantly change the structure and stability of proteins and protein assemblies. On the one hand, this means that experiments on

proteins in which H_2O has been replaced with D_2O should be interpreted with caution. On the other hand, the possibility of “tuning” the hydration strength by varying the isotopic composition provides a unique tool to investigate protein hydration, and might be useful for gaining a better understanding of the role of water in defining protein structure. Water strongly influences the properties of proteins and is also believed to regulate and mediate protein–protein/ligand interactions in many biopolymers, such as collagen or silk fibroin, and water is also believed to play a crucial role in determining collagen interactions with minerals in bone tissue.⁵⁴ Experiments designed to investigate protein hydration usually measure how the protein properties change upon varying the solvent, for instance, by replacing or mixing water with an organic solvent. This clearly changes the protein hydration but unfortunately also modifies many other solvent properties, such as the dielectric constant and the molecular size, which might affect protein intra- and intermolecular interactions. Replacing water with D_2O is a unique method to specifically modify the water hydrogen bonding without changing the other solvent properties. Comparing protein behavior in H_2O and D_2O and their mixtures thus constitutes an elegant way to determine specifically the contribution of water hydrogen bonding to the physical and chemical properties of proteins without having to resort to changes in the solvent that alter more than the protein hydration. Such D_2O vs H_2O experiments may not always be easy to realize, but for instance two-dimensional infrared spectroscopy on proteins in H_2O has already been reported.^{39,55–57} This recent advancement enables researchers to study proteins in more natural systems, such as in cells or in blood serum.^{58,59} Since the protein amide-I frequencies and line shapes may change upon H/D exchange, extracting structural information from such 2D-IR spectra in H_2O will require adaptation of the currently existing theoretical and modeling framework, which was developed mainly for interpreting 2D-IR spectra of proteins in D_2O ; see ref. 58 for an excellent future perspective on this topic.

Since D_2O enhances the hydrophobic effect, a comparison of protein secondary structure in H_2O and D_2O can reveal the role of hydrophobic interactions in the stabilization of the proteins or in promoting their collapse. Similarly, comparing self-assembly kinetics in water and D_2O can be a valuable method to gain a better understanding of the aggregation process, in particular in the case of fibril formation. Fibril formation can occur spontaneously via a nucleation-and-growth mechanism (1-step-nucleation or 1SN) or in two steps via the formation of intermediate aggregates (2SN) stabilized by hydrophobic effects. Intermediates subsequently need to undergo structural transformations to attain the fibrillar conformation, representing the rate-limiting step for fibrillization. Since D_2O stabilizes hydrophobic interactions, the aggregation rate in D_2O with respect to H_2O is reduced if the mechanism involves intermediates, because their unfolding is energetically more unfavorable in D_2O . Comparing the fibrillization rate in water and D_2O can therefore reveal whether intermediates are present and hence if the amyloid formation occurs by a 2SN or 1SN mechanism. On the same note, the ability of D_2O to slow the aggregation and stabilize the intermediates can be used to study the intermediate species. Intermediates are transient and metastable aggregates, which are quite challenging to detect and characterize

structurally. By using D_2O , we can follow the protein self-assembly in “slow motion”.

Thus, we believe that the difference in biopolymer hydration in H_2O and D_2O can be exploited to gain a better understanding of biopolymers, in particular, of biopolymer–solvent interactions and their role in defining the structure and dynamics of proteins and protein assemblies. This constitutes an interesting next challenge for the scientific community working on proteins and protein assemblies.

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Notes

The authors declare no competing financial interest.

Biographies



Giulia Giubertoni is a research fellow at the University of Amsterdam in the group of Sander Woutersen, which she joined in 2020 after obtaining her Ph.D. at the FOM-Institute for Atomic and Molecular Physics in Amsterdam, some 20 years after her two co-authors obtained their Ph.D. there. She is also a guest researcher at the Max Planck Institute for Polymer Research under the supervision of Mischa Bonn. Giulia studies the role of hydration in the self-assembly of proteins that form the building blocks of biomaterials, using physical methods that range from multidimensional infrared spectroscopy to rheology. In 2021, she was granted an NWO-VENI grant to investigate the molecular origin of osteogenesis imperfecta.



Mischa Bonn is a Director at the Max Planck Institute for Polymer Research since 2011, heading the Molecular Spectroscopy Department. Mischa completed his M.Sc. degree in physical chemistry in 1993 at the University of Amsterdam and performed his Ph.D. research at the FOM-Institute for Atomic and Molecular Physics in Amsterdam, where he shared an office with (and had to listen to the music of) Sander Woutersen. After postdoctoral stays at the Fritz Haber Institute and Columbia University, he went to Leiden University and returned to the Institute for Atomic and Molecular Physics as group leader in 2004. His scientific interests focus on the development and application of ultrafast spectroscopies to study natural phenomena, specifically at interfaces and often involving Mischa's favorite molecule: water.



Sander Woutersen obtained his M.Sc. in physical chemistry at the University of Amsterdam (1995) and did his Ph.D. research at the FOM-Institute for Atomic and Molecular Physics in Amsterdam, where he shared an office with (and was regularly made fun of by) Mischa Bonn. After a postdoctoral fellowship with Peter Hamm at the Max Born Institute in Berlin, he returned to Amsterdam, where he eventually became professor in physical chemistry. Sander's research is at the interface between spectroscopy and soft matter.

ACKNOWLEDGMENTS

We thank Ioana Ilie for fruitful discussions. This publication is part of the project (with Project Number VI.Veni.212.240) of the NWO Talent Programme Veni 2021, which is financed by the Dutch Research Council (NWO).

ADDITIONAL NOTE

“We will use the term “hydrogen bond” to denote both hydrogen bonds and deuterium bonds.

REFERENCES

- (1) Urey, H. C.; Brickwedde, F. G.; Murphy, G. M. A Hydrogen Isotope of Mass 2. *Phys. Rev.* **1932**, *39*, 164–165.
- (2) Lewis, G. N.; Macdonald, R. T. The viscosity of $\text{H}^2\text{H}^2\text{O}$. *J. Am. Chem. Soc.* **1933**, *55*, 4730–4731.
- (3) Heavy Hydrogen and Heavy Water. *Nature* **1934**, *134*, 388–389.
- (4) Yang, H.; Yang, S.; Kong, J.; Dong, A.; Yu, S. Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy. *Nat. Protoc.* **2015**, *10*, 382–396.
- (5) Pleitez, M. A.; Khan, A. A.; Soldà, A.; Chmyrov, A.; Reber, J.; Gasparin, F.; Seeger, M. R.; Schätz, B.; Herzig, S.; Scheideler, M.; et al. Label-free metabolic imaging by mid-infrared optoacoustic microscopy in living cells. *Nat. Biotechnol.* **2020**, *38*, 293–296.
- (6) Alperstein, A. M.; Ostrander, J. S.; Zhang, T. O.; Zanni, M. T. Amyloid found in human cataracts with two-dimensional infrared spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116*, 6602–6607.
- (7) Barth, A.; Zscherp, C. What vibrations tell us about proteins. *Q. Rev. Biophys.* **2002**, *35*, 369–430.
- (8) Yang, J. *Deuterium: Discovery and Applications in Organic Chemistry*; Elsevier, 2016.
- (9) Li, L.; Jakowski, J.; Do, C.; Hong, K. Deuteration and Polymers: Rich History with Great Potential. *Macromolecules* **2021**, *54*, 3555–3584.
- (10) Ceriotti, M.; Fang, W.; Kusalik, P. G.; McKenzie, R. H.; Michaelides, A.; Morales, M. A.; Markland, T. E. Nuclear Quantum Effects in Water and Aqueous Systems: Experiment, Theory, and Current Challenges. *Chem. Rev.* **2016**, *116*, 7529–7550.
- (11) Pica, A.; Graziano, G. Effect of heavy water on the conformational stability of globular proteins. *Biopolymers* **2018**, *109*, No. e23076.
- (12) Bell, R. P. *The proton in chemistry*; Cornell University Press: Cornell, 1973.
- (13) Kaltashov, I. A.; Eyles, S. J. Studies of biomolecular conformations and conformational dynamics by mass spectrometry. *Mass Spectrom. Rev.* **2002**, *21*, 37–71.
- (14) Schnauß, J.; Kunschmann, T.; Grosser, S.; Mollenkopf, P.; Zech, T.; Freitag, J. S.; Prascevic, D.; Stange, R.; Röttger, L. S.; Röncke, S.; et al. Cells in Slow Motion: Apparent Undercooling Increases Glassy Behavior at Physiological Temperatures. *Adv. Mater.* **2021**, *33*, 2101840.
- (15) Jandova, J.; Hua, A. B.; Fimbres, J.; Wondrak, G. T. Deuterium Oxide (D_2O) Induces Early Stress Response Gene Expression and Impairs Growth and Metastasis of Experimental Malignant Melanoma. *Cancers* **2021**, *13*, 605.
- (16) Pant, I.; Shashidhar, R. Understanding the influence of heavy water stress on the physiology of *Salmonella Typhimurium*. *Applied Radiation and Isotopes* **2020**, *159*, 108990.
- (17) Kampmeyer, C.; Johansen, J.; Holmberg, C.; Karlson, M.; Gersing, S.; Bordallo, H.; Kragelund, B. B.; Lerche, M.; Jourdain, I.; Winther, J.; et al. Mutations in a Single Signaling Pathway Allow Cell Growth in Heavy Water. *ACS Synth. Biol.* **2020**, *9*, 733–748.
- (18) Efimova, Y. M.; Haemers, S.; Wierczinski, B.; Norde, W.; van Well, A. A. Stability of globular proteins in H_2O and D_2O . *Biopolymers* **2007**, *85*, 264–273.
- (19) Fu, L.; Villette, S.; Petoud, S.; Fernandez-Alonso, F.; Saboungi, M.-L. H/D Isotope Effects in Protein Thermal Denaturation: The Case of Bovine Serum Albumin. *J. Phys. Chem. B* **2011**, *115*, 1881–1888.
- (20) Reslan, M.; Kayser, V. The effect of deuterium oxide on the conformational stability and aggregation of bovine serum albumin. *Pharm. Dev. Technol.* **2018**, *23*, 1030–1036.
- (21) Das, A.; Sinha, S.; Acharya, B. R.; Paul, P.; Bhattacharyya, B.; Chakrabarti, G. Deuterium oxide stabilizes conformation of tubulin: a biophysical and biochemical study. *BMB reports* **2008**, *41*, 62–67.
- (22) Zhou, Y.; Yang, D. Equilibrium folding dynamics of meACP in water, heavy water, and low concentration of urea. *Sci. Rep.* **2017**, *7*, 16156.

- (23) Pathak, A. K.; Bandyopadhyay, T. Water isotope effect on the thermostability of a polio viral RNA hairpin: A metadynamics study. *J. Chem. Phys.* **2017**, *146*, 16S104.
- (24) Mizuno, K.; Bächinger, H. P. The effect of deuterium oxide on the stability of the collagen model peptides H-(Pro-Pro-Gly)₁₀-OH, H-(Gly-Pro-4(R)Hyp)₉-OH, and Type I collagen. *Biopolymers* **2010**, *93*, 93–101.
- (25) Cardoso, M. V. C.; Sabadini, E. The gelling of κ -carrageenan in light and heavy water. *Carbohydr. Res.* **2010**, *345*, 2368–2373.
- (26) Stadtmiller, S. S.; Pielak, G. J. Enthalpic stabilization of an SH3 domain by D₂O. *Protein Sci.* **2018**, *27*, 1710–1716.
- (27) Sasisanker, P.; Oleinikova, A.; Weingärtner, H.; Ravindra, R.; Winter, R. Solvation properties and stability of ribonuclease A in normal and deuterated water studied by dielectric relaxation and differential scanning/pressure perturbation calorimetry. *Phys. Chem. Chem. Phys.* **2004**, *6*, 1899–1905.
- (28) Cho, Y.; Sagle, L. B.; Iimura, S.; Zhang, Y.; Kherb, J.; Chilkoti, A.; Scholtz, J. M.; Cremer, P. S. Hydrogen Bonding of β -Turn Structure Is Stabilized in D₂O. *J. Am. Chem. Soc.* **2009**, *131*, 15188–15193.
- (29) Chellgren, B. W.; Creamer, T. P. Effects of H₂O and D₂O on Polyproline II Helical Structure. *J. Am. Chem. Soc.* **2004**, *126*, 14734–14735.
- (30) Ben Abu, N.; Mason, P. E.; Klein, H.; Dubovski, N.; Ben Shoshan-Galeczki, Y.; Malach, E.; Pražienková, V.; Maletínská, L.; Tempra, C.; Chamorro, V. C.; et al. Sweet taste of heavy water. *Communications Biology* **2021**, *4*, 440.
- (31) Cioni, P.; Strambini, G. B. Effect of Heavy Water on Protein Flexibility. *Biophys. J.* **2002**, *82*, 3246–3253.
- (32) Guzzi, R.; Arcangeli, C.; Bizzarri, A. R. A molecular dynamics simulation study of the solvent isotope effect on copper plastocyanin. *Biophys. Chem.* **1999**, *82*, 9–22.
- (33) Brenner, T.; Tuvikene, R.; Cao, Y.; Fang, Y.; Rikukawa, M.; Price, W. S.; Matsukawa, S. Hydrogen isotope replacement changes hydration and large scale structure, but not small scale structure, of agarose hydrogel networks. *Eur. Phys. J. E* **2019**, *42*, 53.
- (34) Sheu, S.-Y.; Schlag, E. W.; Selzle, H. L.; Yang, D.-Y. Molecular Dynamics of Hydrogen Bonds in Protein-D₂O: The Solvent Isotope Effect. *J. Phys. Chem. A* **2008**, *112*, 797–802.
- (35) Tempra, C.; Chamorro, V. C.; Jungwirth, P. Effects of Water Deuteration on Thermodynamic and Structural Properties of Proteins and Biomembranes. *J. Phys. Chem. B* **2023**, *127*, 1138–1143.
- (36) Bielskutė, S.; Garcia-Cabau, C.; Frigolé-Vivas, M.; Szulc, E.; De Mol, E.; Pesarrodon, M.; García, J.; Salvatella, X. Low amounts of heavy water increase the phase separation propensity of a fragment of the androgen receptor activation domain. *Protein Sci.* **2021**, *30*, 1427–1437.
- (37) Oakenfull, D.; Scott, A. Gelatin gels in deuterium oxide. *Food Hydrocolloids* **2003**, *17*, 207–210.
- (38) Bayrak, M.; Mata, J.; Raynes, J. K.; Greaves, M.; White, J.; Conn, C. E.; Flourey, J.; Logan, A. Investigating casein gel structure during gastric digestion using ultra-small and small-angle neutron scattering. *J. Colloid Interface Sci.* **2021**, *594*, 561–574.
- (39) Chun, S. Y.; Son, M. K.; Park, C. R.; Lim, C.; Kim, H. I.; Kwak, K.; Cho, M. Direct observation of protein structural transitions through entire amyloid aggregation processes in water using 2D-IR spectroscopy. *Chemical Science* **2022**, *13*, 4482–4489.
- (40) Stephens, A. D.; Kölbl, J.; Moons, R.; Chung, C. W.; Ruggiero, M. T.; Mahmoudi, N.; Shmool, T. A.; McCoy, T. M.; Nietlispach, D.; Routh, A. F.; et al. Decreased Water Mobility Contributes To Increased α -Synuclein Aggregation. *Angew. Chem., Int. Ed.* **2023**, *62*, e202212063.
- (41) Mansel, B. W.; Chu, C. Y.; Leis, A.; Hemar, Y.; Chen, H. L.; Lundin, L.; Williams, M. A. Zooming in: Structural Investigations of Rheologically Characterized Hydrogen-Bonded Low-Methoxyl Pectin Networks. *Biomacromolecules* **2015**, *16*, 3209–3216.
- (42) Soper, A. K.; Benmore, C. J. Quantum Differences between Heavy and Light Water. *Phys. Rev. Lett.* **2008**, *101*, 065502.
- (43) Darby, J. F.; Hopkins, A. P.; Shimizu, S.; Roberts, S. M.; Brannigan, J. A.; Turkenburg, J. P.; Thomas, G. H.; Hubbard, R. E.; Fischer, M. Water Networks Can Determine the Affinity of Ligand Binding to Proteins. *J. Am. Chem. Soc.* **2019**, *141*, 15818–15826.
- (44) Scheiner, S.; Cuma, M. Relative Stability of Hydrogen and Deuterium Bonds. *J. Am. Chem. Soc.* **1996**, *118*, 1511–1521.
- (45) Némethy, G.; Scheraga, H. A. Structure of Water and Hydrophobic Bonding in Proteins. IV. The Thermodynamic Properties of Liquid Deuterium Oxide. *J. Chem. Phys.* **1964**, *41*, 680–689.
- (46) Liu, B.; Garnett, J. A.; Lee, W.-c.; Lin, J.; Salgado, P.; Taylor, J.; Xu, Y.; Lambert, S.; Cota, E.; Matthews, S. Promoting crystallisation of the Salmonella enteritidis fimbriae 14 pilin SeFD using deuterium oxide. *Biochemical and biophysical research communications* **2012**, *421*, 208–213.
- (47) Artero, J.-B.; et al. A comparison of refined X-ray structures of hydrogenated and perdeuterated rat γ E-Crystallin in H₂O and D₂O. *Acta Crystallographica Section D: Biological Crystallography* **2005**, *61*, 1541–1549.
- (48) Chatake, T.; Ishikawa, T.; Yanagisawa, Y.; Yamada, T.; Tanaka, I.; Fujiwara, S.; Morimoro, Y. High-resolution X-ray study of the effects of deuteration on crystal growth and the crystal structure of proteinase K. *Acta Crystallographica Section F* **2011**, *67*, 1334–1338.
- (49) Yee, A. W.; Moulin, M.; Breteau, N.; Haertlein, M.; Mitchell, E. P.; Cooper, J. B.; Boeri Erba, E.; Forsyth, V. T. Impact of deuteration on the assembly kinetics of transthyretin monitored by native mass spectrometry and implications for amyloidosis. *Angew. Chem., Int. Ed.* **2016**, *55*, 9292–9296.
- (50) Eginton, C.; Beckett, D. A. Large Solvent Isotope Effect on Protein Association Thermodynamics. *Biochemistry* **2013**, *52*, 6595–6600.
- (51) Braun, M. K.; Wolf, M.; Matsarskaia, O.; Da Vela, S.; Roosen-Runge, F.; Sztucki, M.; Roth, R.; Zhang, F.; Schreiber, F. Strong isotope effects on effective interactions and phase behavior in protein solutions in the presence of multivalent ions. *J. Phys. Chem. B* **2017**, *121*, 1731–1739.
- (52) Mollenkopf, P.; Prascevic, D.; Bayerl, T. M.; Käs, J. A.; Schnauß, J. Heavy water induces bundling in entangled actin networks. *RSC Adv.* **2023**, *13*, 24795–24800.
- (53) Haidar, Y.; Konermann, L. Effects of Hydrogen/Deuterium Exchange on Protein Stability in Solution and in the Gas Phase. *J. Am. Soc. Mass Spectrom.* **2023**, *34*, 1447.
- (54) Nyman, J. S.; Ni, Q.; Nicoletta, D. P.; Wang, X. Measurements of mobile and bound water by nuclear magnetic resonance correlate with mechanical properties of bone. *Bone* **2008**, *42*, 193–199.
- (55) Hume, S.; Greetham, G. M.; Donaldson, P. M.; Towrie, M.; Parker, A. W.; Baker, M. J.; Hunt, N. T. 2D-IR Spectroscopy of Proteins in Water: Using the Solvent Thermal Response as an Internal Standard. *Anal. Chem.* **2020**, *92*, 3463–3469.
- (56) Rutherford, S. H.; Greetham, G. M.; Parker, A. W.; Nordon, A.; Baker, M. J.; Hunt, N. T. Measuring proteins in H₂O using 2D-IR spectroscopy: pre-processing steps and applications toward a protein library. *J. Chem. Phys.* **2022**, *157*, 205102.
- (57) Hume, S.; Hithell, G.; Greetham, G. M.; Donaldson, P. M.; Towrie, M.; Parker, A. W.; Baker, M. J.; Hunt, N. T. Measuring proteins in H₂O with 2D-IR spectroscopy. *Chemical science* **2019**, *10*, 6448–6456.
- (58) Rutherford, S. H.; Baker, M. J.; Hunt, N. T. 2D-IR spectroscopy of proteins in H₂O—A Perspective. *J. Chem. Phys.* **2023**, 030901.
- (59) Rutherford, S. H.; Greetham, G. M.; Donaldson, P. M.; Towrie, M.; Parker, A. W.; Baker, M. J.; Hunt, N. T. Detection of glycine as a model protein in blood serum using 2D-IR spectroscopy. *Anal. Chem.* **2021**, *93*, 920–927.